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RESEARCH ARTICLE

Effect of Endurance Training Program on BMI and Lipid Profiles among College Boys

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ABSTRACT

The risk factors associated with major non communicable diseases often develop during the adolescent and teenage period. Identifying those factors which could be modified through effective life style modifications including physical training is most vital. The effects of an endurance training program on body composition and lipid profiles were examined among Forty male under graduate students (Age: 19.63 ± 1.27; Height: 1.70±0.06, Weight: 66.20± 13.35, BMI: 22.99 ± 4.38), who were randomly assigned either to an experimental group (N =20) or a Control Group (N = 20). The experimental group underwent an endurance training program for twelve weeks while the control group maintained their regular routine. The experimental group trained three to five sessions in a week and 25 to 50 min per session with a gradual increase in the number of sessions and duration as the program progresses. Height, Weight, and BMI were measured before (Pre) and after (Post) the training program. Fasting blood samples were collected 24 hours before, and 48 hours after the training period and analyzed for lipid levels. The obtained data were statistically analyzed using ANACOVA to find out significant difference if any. We

observed a significant reduction in Body Weight, BMI. In conclusion the endurance training brings about positive changes in Lipid profiles among college boys.

Key Words: Endurance Training, HDL Cholesterol, LDL Cholesterol, Total Cholesterol, Triglycerides.

INTRODUCTION

Non communicable diseases are on the rise in the developing countries where four-fifths of the world people lives. The prevalence of coronary artery disease (CAD) is rising rapidly in urban as well as rural India. Marked increases in both CHD prevalence and risk factors is observed in urban India compared with rural settings. Sedentary lifestyle is identified as the major risk factor for the development of coronary heart disease in adults (1-3). Conscious effort in lifestyle modification along with control of risk factors are needed to prevent the development of coronary heart disease among Indian population. In adults, overweight and low levels of aerobic fitness are independently associated with immature mortality caused by coronary heart disease(4) and research findings suggests that the atherosclerotic process begins during childhood and carried on to adulthood(5,6). In addition, children of parents diagnosed with CVD are known to exhibit a higher probability of developing CVD at a much earlier age compared to others(7). Fatty streaks in the aorta associated with atherosclerotic process can form even at three years of age and during adolescence. In response to the rising incidence of CHD in adults, the American Heart Association and other governing bodies have continued to emphasize the importance of exercise in childhood as a means of preventing CHD later in life(8).

Since previous studies have illustrated that a considerable number of school-age children have risk factors that are predictive of CHD in adults(9), the early years of life may be a critical time period to reduce the risk of CHD(10,11). Several longitudinal studies, beginning from childhood, show that serum lipids and lipoproteins are important indicators of CHD development in adulthood (12,13). Low prevalence of risk factors associated with cardiovascular disease like smoking, hypertension, lipid abnormalities and diabetes was noticed among adolescents in an Indian urban population but an increase was noted on these risk factors during the period of 20–29 years with an exponential increase in age group 30–39 years (14) while others reported high prevalence of these risk factors for the young aged population (15). Previous studies have identified increased concentrations of LDL-C, decreased concentrations of HDL-cholesterol (HDL-C), hypertension, and hyperglycemia among other factors as the modifiabable risk factors to prevent cardiovascular disease in the general population (16) which is applicable to young population as well.

Physical activity and aerobic capacity are significantly related to the risk of cardio vascular disease and individuals with low physical fitness or aerobic capacity has an elevated chance of being at high risk of coronary heart disease. The cardiovascular benefits and physiological reactions to physical activity with a direct relationship to coronary heart disease risk factors reduction are similar among diverse population subgroups including youth and adolescents. Physical Exercise especially endurance type of activities enhances lipid metabolism leading to alteration of plasma lipase activity and hepatic lipase resulting in decrease cholesterol concentrations and has been shown to increase the intravenous fat clearance. One of the outcomes of exercise and physical training is to cause changes in the body composition and regular physical exercise has a favorable effect on body composition for individuals of all ages. Unfortunately, unlike studies involving adults, the role regular exercise has on CHD risk factors in younger population remains unclear especially among Indian population. Although the specific cardio protective benefits of aerobic training are well known there is very little literature available on these effects among young Indian population. In view of this the present study was taken up to investigate the effect of endurance training program on body composition, and lipid profiles among college boys.

MATERIALS AND METHODS

The experimental design adopted in the study was similar to a random group design involving forty male students out of 812 students from Sreekrishnapuram VT Bhattathiripad College doing under graduate course in arts and science who volunteered for the study (Age: 19.63 ± 1.27; Height: 1.70±0.06, Weight: 66.20± 13.35, BMI: 22.99 ± 4.38). A written explanation of the experimental procedure and potential risk factors were given to each member. The age of the subjects were ranging from 18 to 20. The 40 subjects were randomly assigned to either Control group ('CON', No: 20) or Experimental group, ('EXP', No: 20). Physical examination and medical check up at the initiation of the study yielded normal results in all the subjects and none of the subjects received any medication during the period of the study. The selected variables were tested 24 hours prior (Pre) and 48 hours after (Post) the training program. The experimental group underwent endurance training for a period of 12 weeks, whereas the control group maintained their regular routine activities. The general baseline characteristics of the 40 subjects who agreed to participate in the study are shown in Table 1.

Training Program

Endurance training program included 15 minutes warm-up with stretching motions, walking, running, and then continuous running at a constant self set pace for 25 to 40 minutes with three to five sessions per week for twelve weeks (Average training duration per Session (Mean ± SD)= 35.04 ± 5.40) and a warm down for 10 minutes. The initial load was fixed at 60% of maximum heart rate (220-age) and their after they were instructed to complete the set duration of the sessions with their own set pace. All participants of the experimental group completed 41 sessions (Total sessions: 46) in accordance to the progressive load applied.Body weight was measured with a platform beam balance (accuracy of 0.01 kg) and standing height was measured with a stadiometer (accuracy of 0.1 cm). BMI was estimated using the formula of body weight (kgs)/height in meter^2.(Table 2.)

Blood Sampling and Assessment

Blood tests were performed by certified medical personnel at a reputed clinical lab. Blood samples (5 mL), taken by a venepuncture, were then analysed for concentrations of total cholesterol and sub fractions. Blood sampling was done 24 hours before the exercise and 48 hours after the last session in all three studied groups. For the first time of blood sampling, the subjects were asked to avoid any strenuous activity from two days before the trial. Ten ml of blood was taken from the right brachial artery after at least 10 hours of fasting (Pre test). The blood sampling for the Post test was done 48 hours after the last training session with the same conditions. The resulting serums were kept in a freezer at a temperature of -80°C. Plasma triglyceride was measured by enzymatic colorimetric method using a kit made by Dia Sys Diagnostic Systems GmbH Alte Strasse 965558 Holzheim, Germany (Sensitivity: 1 mg / dl, Intra-assay CV%: 1.6). Total cholesterol was also measured by enzymatic colorimetric method using reagent kit (DiaSys Diagnostic Systems GmbH Alte Strasse 965558 Holzheim, Germany;Sensitivity: 3 mg/dl, Intra-assay CV%: 1.4). To measure HDL-C, enzymatic photometric method using reagent kit (Dia Sys Diagnostic Systems GmbH Alte Strasse 965558 Holzheim, Germany: Sensitivity: 1 mg/dl, Intra-assay CV%: 1.5) were used. LDL-C was determined by the equation proposed by Friedewald, Levy, and Fredrickson (1972)(17) as follows: LDL = TC - HDL - TG/0.5

The data collected from the experimental and control groups prior to and after completion of the training period on selected variables were statistically examined for significant differences if any, by applying analysis of covariance (ANCOVA). The pre test and posttest means of experimental and control groups were tested for significance by applying ANOVA. As both the groups (RT and CON) were selected from the same population and no attempt was made to equate the groups on the selected dependent variables or any other common variables, initial differences may exist, and there is a possibility of affecting the post test mean. For eliminating any possible influence of pre test means the adjusted posttest means of experimental and control group were tested for significance by using

ANCOVA. All the data were analyzed using SPSS statistical package. The level of confidence was fixed at 0.05 level of significance as the number of subjects was limited and also as the selected variables might fluctuate due to various extraneous factors.

RESULTS AND DISCUSSION

BMI

No significant difference was observed among the pre test and post test values of BMI of the Experimental Group and the Control Group, whereas the adjusted post test means of the Experimental Group and the Control Group shows a significant difference ($P \leq 0.05$). For BMI in the experimental group, the pre test and post test means (24.17±1.63vs 23.14±1.48) shows a reduction of 1.03 (4.26%) whereas the pre test and post test means of control group (21.80±5.82vs 21.99±5.83) shows an increase of 0.19 (0.87%). Table 3, Table 4 and Figure 1

Total Cholesterol (TC)

No significant difference was observed among the pre test and post test values of TC of the Experimental Group and the Control Group, whereas the adjusted post test means of the Experimental Group and the Control Group shows a significant difference in TC ($P \leq 0.05$). The pre test and post test means of TC for the experimental group (147.70 ± 17.04 vs139.60 ± 13.37) shows a reduction of 8.10 (5.48%) whereas the pre test and post test means of control group (145.90 ± 19.80 vs 147.15 ± 18.92) shows an increase of 1.25 (0.86 %). Although the mechanism of exercise-induced lipid changes is unclear, exercise itself may increase blood lipid consumption hence to decrease lipids levels (18). Previous studies have found that moderately active subjects had lower TC and increased HDL-C. These findings are similar to this study where BMI had reduced, and HDL increased after moderate intensity training (19,20). Figure 2.

High Density Lipoprotein Cholesterol (HDL-C)

No significant difference was observed among the pre test and post test values of HDL-C of the Experimental Group and the Control Group, whereas the adjusted post test means of the Experimental Group and the Control Group shows a significant difference in TC ($P \leq 0.05$). The pre test and post test means of HDL-C for the experimental group (48.81 ± 2.50 vs 51.00 ± 3.47) shows an increase of 2.19 (4.49%) whereas the pre test and post test means of control group (49.09 ± 4.41 vs 49.26 ± 5.05) shows an increase of 0.12 (0.35%). Studies has demonstrated that endurance athletes have 40–50% higher level of serum high-density lipoprotein cholesterol (HDL-C) concentrations when compared with their sedentary counterparts (21,22) and it is suggested that regular endurance exercise training may be particularly helpful in men with low HDL cholesterol, elevated TGs, and abdominal obesity (23,24). This increase has varied from 4 to 43% in various studies (25-28). These findings are similar to this study where BMI had reduced, and HDL increased after moderate intensity training. There are several possible reasons why HDL improved indicating a positive effect of short-term aerobic training, firstly improvement could be due to a reduction in BMI, which has shown a high correlation with increase HDL and secondly due to a reduction in TGs. In another study, HDL did not change significantly at the different levels of activity as compared to sedentary phase during 4 weeks of training (29) where as the subjects of our study underwent an endurance training program for twelve weeks.Figure 3.

Low Density Lipoprotein Cholesterol (LDL-C)

No significant difference was observed among the pre test and post test values of LDL-C of the Experimental Group and the Control Group, whereas the adjusted post test means of the Experimental Group and the Control Group shows a significant difference in TC ($P \leq 0.05$). The pre test and post test means of LDL-C for the experimental group (72.09 ± 10.51 vs 68.65 ± 10.42) shows a reduction of 3.44 (4.77%) whereas the pre test and post test means of control

group (70.42 ± 10.92 vs 70.56 ± 10.53) shows an increase of 0.14 (0.20%). Studies has demonstrated that LDL-C may also acutely decrease 5–8% in hypercholesterolemic men with exercise (26,30,31) though some studies did not show any reduction in TG level even after 4 weeks of training at different level of physical (32). Hepatic lipase enzyme plays a major role in the conversion of LDL-C to HDL-C. The amount of this enzyme is less in active individuals and exercise can decrease it more and maintain HDL-C concentration in larger amounts. These enzymatic changes, which are caused by exercise, improve the lipid profile (33). Previous studies have shown a similar reduction in LDL-C (34,35) and the noticed post training level of LDL-C could be due to a reduction in body weight and BMI, which has shown a high correlation with increase HDL and also due to the reduction in triglycerides. Figure 4.

Tri glycerines (TG)

No significant difference was observed among the pre test and post test values of TG of the Experimental Group and the Control Group, whereas the adjusted post test means of the Experimental Group and the Control Group shows a significant difference in TG ($P \leq 0.05$). The pre test and post test means of body weight for the experimental group (132.20 ± 58-48 vs 99.78 ± 43.63) shows a reduction of 34.42 (25.65%) whereas the pre test and post test means of control group (131.66 ± 66.40 vs 134.93±65.33) shows an increase of 3.27(2.48%). As far as triglycerides are concerned previous results shows that the reduction in TG after endurance training, consistent with the induction of metabolic changes, persists for up to 72 h (25-27,31,36), and is greatest in those with higher pre exercise TG values. The reduction in TG noted in this study was in conformation of earlier findings (31,37,38). Figure 5.

CONCLUSIONS

In our study we have found a small but significant positive alterations in lipid profiles and body composition due to the endurance training of twelve weeks duration. The findings of this study suggests that the twelve weeks of moderate intensity aerobic training has a beneficial effect namely reduced TC, improved HDL, reduced LDL, reduced TG and improved body composition. The effects of exercise training on the blood lipid and lipoprotein levels of normolipidemic younger population are equivocal. Previous studies on adults reveal that exercise training programs lasting longer than four months, are needed to see a positive alteration in blood lipids and lipoprotein levels [39]. In spite of the positive outcomes, one of the possible flaws in our study is that the exercise intervention length is probably not long enough to bring about notable changes even though a systematic training schedule is adopted and a control group is in place. To overcome these shot comings it is suggested that future studies should be taken with larger sample size and with uniform diet code to avoid effect of variable diet on lipid profile. More research with well defined samples and controlled training programmes of longer duration intensity is necessary to clarify the effects of exercise training on body composition and lipid profiles among college boys.

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Table 1.Baseline Characteristics of the Experimental and Control Groups

	Control group (N=20)		**Experimental group (N=20)**		**Total (N=40)**	
Age	19.1 ±1.21		20.15 ± 1.14		19.63 ± 1.27	
Mean ± SD	Minimum 18	Maximum 22	Minimum 18	Maximum 22	Minimum 18	Maximum 22
Height	1.70 ± 0.06		1.69 ± 0.07		1.70 ± 0.06	
Mean ± SD	Minimum 1.60	Maximum 1.80	Minimum 1.58	Maximum 1.84	Minimum 1.58	Maximum 1.84
Weight	62.95 ± 16.92		69.45 ± 7.56		66.20 ± 13.35	
Mean ± SD	Minimum 44.00	Maximum 110.00	Minimum 52.00	Maximum 84.00	Minimum 44	Maximum 110
BMI	21.80 ± 5.82		24.17 ±1.63		22.99 ± 4.38	
Mean ± SD	Minimum 15.37	Maximum 35.92	Minimum 17.99	Maximum 25.93	Minimum 15.37	Maximum 35.92

Table 2.Training Schedule with Weekly Load of Training

Week	No. of Training Sessions	Training duration of the Sessions(min.)	Weekly Load of Training (WLT)(min.)
1	3	25	75
2	3	25	75
3	3	30	90
4	3	30	90
5	3	35	105
6	4	35	140
7	4	35	140
8	4	40	160
9	4	40	160
10	5	40	200
11	5	40	200
12	5	40	200
Total	46		1535 min.
Average training duration per Session (Mean ± SD) = 35.04 ± 5.40			

Table 3.Analysis of Covariance for the Selected Variables among Experimental Group & Control Groups.

		Experimental Group	Control Group	F-Ratio
BODYWEIGHT Mean (SD)	**PRE TEST**	69.45(7.56)	62.95(16.92)	2.46
	POST TEST	66.50(7.20)	63.50(16.93)	0.53
	AD PO TEST	63.28	66.72	131.21*
BMI Mean (SD)	**PRE TEST**	24.17(1.63)	21.80(5.82)	3.08
	POST TEST	23.14(1.48)	21.99(5.83)	0.726
	AD PO TEST	21.97	23.17	136.68*
TOTAL CHOLESTEROL Mean (SD)	**PRE TEST**	147.70(17.04)	145.90(19.80)	0.095
	POST TEST	139.60(13.37)	147.15(18.92)	2.124
	AD PO TEST	138.81	147.94	99.84*
HDL CHOLESTEROL Mean (SD)	**PRE TEST**	48.81(2.54)	49.09(4.41)	0.064
	POST TEST	51.00(3.47)	49.26(5.05)	1.606
	AD PO TEST	51.15	49.11	9.087*
LDL CHOLESTEROL Mean (SD)	**PRE TEST**	72.09(10.51)	70.42(10.92)	0.243
	POST TEST	68.65(10.42)	70.56(10.53)	0.332
	AD PO TEST	67.84	71.36	53.97*
TRIGLYCERIDES Mean (SD)	**PRE TEST**	134.20(58.48)	131.66(66.41)	0.016
	POST TEST	99.78(43.63)	134.93(65.33)	4.004*
	AD PO TEST	98.69	136.01	56.95*

Table. 4: The Pre and Post Test Means of Endurance Training (Exp) and Control (Con) Groups with Percentage of Gain

Variable	Group	Pre Test	Post Test	Gain	Percentage of Gain
BMI Mean (SD)	Experimental	24.17(1.63)	23.14(1.48)	1.03	4.26%↓
	Control	21.80(5.82)	21.99(5.83)	0.19	0.87%↑
Total Cholesterol Mean (SD)	Experimental	147.70(17.04)	139.60(13.37)	8.1	5.48%↓
	Control	145.90(19.80)	147.15(18.92)	1.25	0.86%↑
HDL-Cholesterol Mean (SD)	Experimental	48.81(2.54)	51.00(3.47)	2.19	4.49%↑
	Control	49.09(4.41)	49.26(5.05)	0.17	0.35%↑
LDL-Cholesterol Mean (SD)	Experimental	72.09(10.51)	68.65(10.42)	3.44	4.77%↓
	Control	70.42(10.9 2)	70.56(10.53)	0.14	0.20%↑
Triglycerides Mean (SD)	Experimental	134.20(58.48)	99.78(43.63)	34.42	25.65%↓
	Control	131.66(66.41)	134.93(65.33)	3.27	2.48%↑

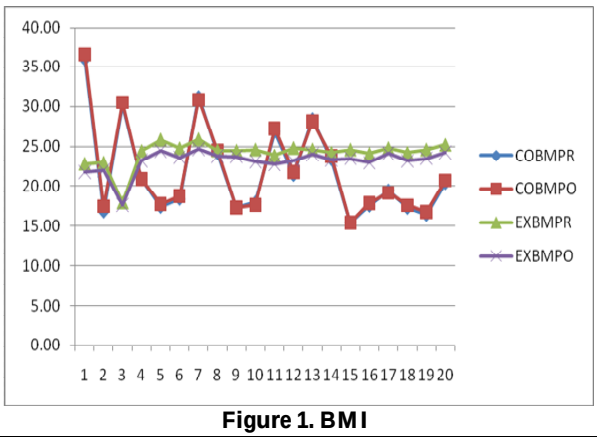

Figure 1. BMI

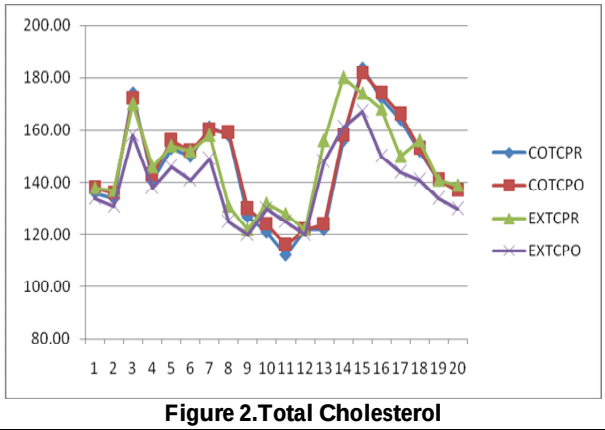

Figure 2.Total Cholesterol

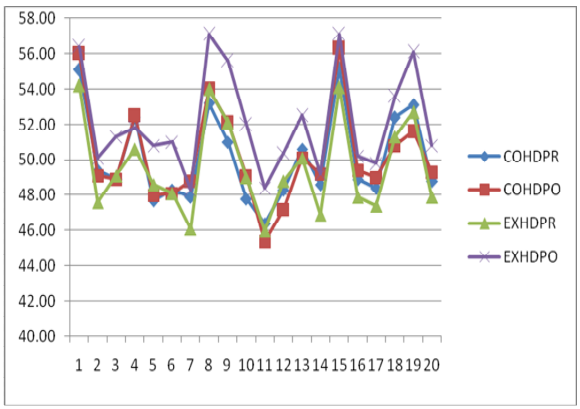

Figure 3. HDL-Cholesterol

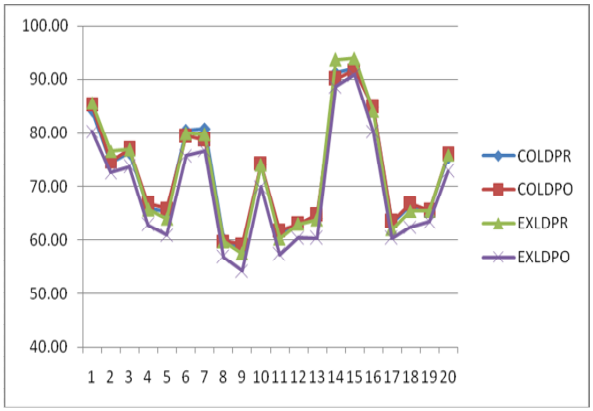

Figure 4. LDL-Cholesterol

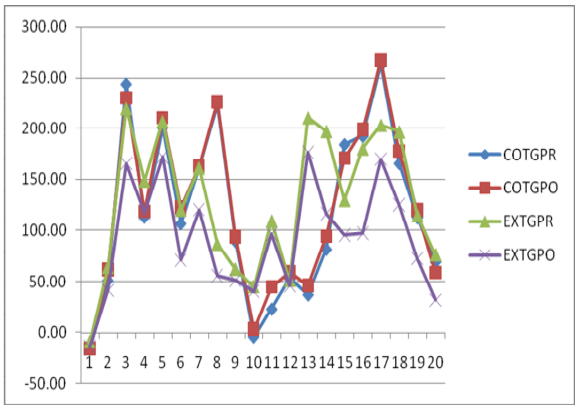

Figure5. Triglycerides

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RESEARCH ARTICLE

Screening of Rice (*Oryza sativa* L.) Genotypes for cold Tolerance at the Seedling and Reproductive Stages based on Morpho-Physiological Markers and Genetic Diversity Analysis

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ABSTRACT

Cold stress is an emerging threat for rice production in the northern region of Bangladesh particularly in boro season at the seedling stage of rice. In this regard, development of cold tolerant high yielding rice varieties could be an important approach to meet up the demand of bursting population in our country. In the present study, total 13 genotypes were employed to find out the cold tolerant rice genotypes. The first experiment was performed in the growth chamber at the seedling stage of rice following completely randomized design (CRD) with three treatments (control, 10^0C and 14^0C) and three replications. The germination percentage was reduced with the departure of time almost in all the genotypes and however, the maximum germination (%) was observed in *lekali dhan-1, lekali dhan-3* and *chandannath-3* under cold stress. Afterwards, most of the genotypes showed high sensitivity to cold at the seedling stage in the growth chamber, on the contrast's, only three genotypes namely *lekali dhan-1, lekali dhan-3* and *chandannath-3*, were displayed normal growth and development and consequently, identified as cold tolerant among 13 rice genotypes under cold stress conditions. The seedlings of identified cold tolerant genotypes at the seedling stage were transplanted directly in the field level from the growth chamber following randomized completely block design (RCBD) to evaluate the performance of rice genotypes at the reproductive stage (2^{nd} experiment). Among the morpho-physiological traits, number of unfilled grain per panicle and number of effective tiller exhibited the highest GCV (%) and PCV (%), heritability and genetic advance(GA%). Besides, number of filled grain per panicle and number of effective tiller showed positive correlation as well as positive direct effect with grain yield reflecting their importance for the selection of cold tolerant rice genotypes. Among the three cold treated genotypes, the higher value of plant height, total number of tiller, number of effective tiller, panicle length, number of filled grains per panicle, number of unfilled grains per panicle, days to maturity, 1000-seed weight and yield per plant was recorded in *lekali dhan-3* indicating it's suitability for the cultivation in the cold pron northern region of Bangladesh. In addition, the

identified cold tolerant genotypes could be used further in the breeding program for the development of cold tolerant high yielding rice genotypes and for isolating cold associated QTL.

Keywords: Rice, cold stress, completely randomized design, growth chamber, and seedling stage.

INTRODUCTION

Rice is the most important food crop that feeds over half of the world population and more than 400 million people in rice producing areas around the globe particularly from Asia, Africa and South America are still getting a major portion of their energy requirement from rice and its derived products with the demand for food (1). Rice is also a staple cereal crop in Bangladesh which plays a significant role in socio-economy of the country (2, 3). Rice production in Bangladesh has been increased from 10.79 million tons in 1970 to about 34.86 million tons in 2014-15 (4). Considering food security issue, breeding efforts for developing high-yield potential varieties have been continuing in the country, which has resulted in several high-yielding rice varieties (5). However, extreme climate change such as cold stress has already raised a radical change in northern region in Bangladesh and threatened the productivity of the existing rice varieties (6). Therefore, the development of suitable cold tolerant high-yielding rice cultivars could be the best approach to utilize these areas for rice cultivation (7).

Low temperature is a common problem in rice cultivation in temperate zones and high-elevation environments in tropical and subtropical areas as well as in irrigated areas which rely on the use of cold water (8). Rice is a temperature responsive crop and its production is greatly reduced by low temperature (9). In general, the mean critical temperature for rice is 4.7°C, and once the ambient temperature (AT) is below 5–10°C, irreversible low temperature -induced damage to seedling growth and development can occur (10). Rice is vulnerable to damage by temperatures below 15°C and low temperature can reduce up to 25% of the final yield in rice genotypes (11). Low temperature can damage the plant during any development stage such as germination, seedling, vegetative, reproductive and maturity (12, 13). During the early growth stages of rice, the occurrence of low temperature stress inhibits seedling establishment and eventually leads to non-uniform crop maturation (14). Even though temperature does not prevent rice germination, it delays its beginning and consequently, plant emergence (15). Under low temperature, delayed germination and seedling growth may result in non-uniform seedling growth and weak seedlings, which may affect final grain yield (16). Therefore, germination speed, in turn, relates to seedling vigor, and can be a significant determinant of good field performance under cold stress (17). A wide range of genetic variability has been observed in rice, in relation to the effects of low temperature on germination (8).

Rice is also highly susceptible to low temperature at reproductive stage (18, 19). Low temperature at reproductive stage causes abnormalities at anthesis such as cessation of anther development, non-ripening of pollen grains, non-emergence of anthers from spikelet's, partial or no anther dehiscence, little or no pollen shedding and failure of pollen to germinate after reaching stigmas (20). Cold stress also causes many types of phenotypic damage, such as delayed heading, incomplete panicle exertion and degeneration of spikelet (21). Spikelet sterility is the most common symptom of injury when rice plants experience cold temperature at the reproductive stage (22, 23). Selection and development of high cold-tolerant varieties is one of the most effective ways to prevent the damage of low temperature (9). Some agronomic characters, such as shoot length under controlled conditions, have the potential to be used as relatively good indicators for evaluating the level of seedling vigor in rice (24). However, specific agronomic characters suited to evaluating the cold tolerance of rice seedlings have not been well established (25). The screening of rice genotypes for cold tolerance can be done through field evaluation, as well as laboratory phenotypic screening climate chambers are efficient instruments and suitable for screening of genotypes because it's not depend on the natural weather condition (26). Therefore, the present study has been conducted to screen out the of cold tolerant rice genotypes based on morphological markers at the seedling stage. Furthermore, the yield and yield attributing traits of selected cold tolerant rice genotypes at the reproductive stage were evaluated.

MATERIALS AND METHODS

Experimental site and plant materials

The experiment was conducted in the plant growth chamber and field laboratory of Bangladesh Institute of Nuclear Agriculture (BINA) started on the 16th September 2018 and continued till 14th June 2019. Total 13 rice genotypes were employed as plant material in this experiment. Among them, 10 genotypes were collected from Nepal and others 3 from Bangladesh Institute of Nuclear Agriculture (BINA). The description of the rice genotypes are presented in the Table 1.

Seed germination and data collection

Approximate one hundred and twenty seeds of each genotypes were placed into brown bags and kept in the oven at 50°C for 2 days for breaking dormancy. Afterwards, seeds were sterilized by treating with 0.1% $HgCl_2$ and 70% ethanol and washed with distilled water. Then, 20 sterilized seeds of each genotypes were separately placed in three petridishes having moist filter paper and kept at three different temperature, 25°C as control, 10°C as first cold treatment, 14°C as second cold treatment for seed sprouting. In case of control treatment, the final counting of germination (%) were taken on 4th and 10th day and for cold treatments, the germination (%) was taken on 20th day of seed setting for germination.

$$\text{Germination (\%)} = \frac{\text{number of normal seedlings}}{\text{Total number seed set for germination}} \times 100$$

$$\text{Percent reduction of germination(\%)} = \frac{\text{germination at control - germination at treatment}}{\text{germination at control}} \times 100$$

Plant cultures and cold treatments at the seedling stage

After germination, seedling was raised in the seed bed of BINA field laboratory for 21 days. The twenty seedlings of each genotypes with normal growth and with no pest and disease infestation were transplanted in rows into trays and kept for seven days. Afterwards, trays with healthy seedlings were transferred into the growth chamber for cold stress imposition. The growth chamber had the light intensity of 3500Lux, 60% humidity and the ratio of day to night was 10:14. Two cold treatments *viz.,* 14°C and 10°C were employed on rice genotypes at the seedling stage in growth chamber (VS-91G09M-1300C). In case of cold treatments induction, the twenty-seven days old rice seedlings genotypes were kept at 14°C temperature (first cold treatment) and at 10°C temperature (second cold treatment) separately in trays containing soil for next 20 days. The soil was maintained in saturated condition and for that irrigation was provided twice a week. The injury scoring was determined in the rice seedlings considering leaf discoloration (LD) and survival rate for 12 days at 10°C and for 20 days at 14°C using a scale of 1 (highly cold tolerant) to 9 (highly cold sensitive) (27) standard evaluation system (28) (Table 2).

Plant cultivation procedure at the reproductive stage

The genotypes which showed comparatively normal growth and development under cold stress in the growth chamber at the seedling stage were selected as plant materials for further cultivation at reproductive stage. In that case, the seedlings of three rice genotypes namely *lekali dhan-1, lekali dhan-3 and chandannath-3* were directly transferred from the growth chamber to field level with their respective control to evaluate their yield performance. The soil of the plot was silt loam having the following characteristics: pH of 6.18, electrical conductivity (EC) of 0.15 dSm^{-1}, total nitrogen of 0.13%, organic carbon of 0.70%, and organic matter of 1.20%. The experimental plot was

prepared by ploughing with power tiller followed by laddering. The soil of 80 m^2 plot had been prepared by adding the following nutrients: urea (3kg), triple super phosphate (2.5kg), muriate of potash (1.25kg), gypsum (750g) and zinc sulfate (50g). All the fertilizers except urea were applied during the land preparation just before of the final tillage followed by laddering. After land preparation, the seedlings of rice genotypes were transplanted in the plots maintaining 30 cm distance between the plots, 20 cm distance between rows and 15 cm distance between plants. Urea was applied in three installments, first installment was applied at 8 days after transplanting (DAS), the second installment was applied at 18 DAS and the final installment was applied at 35 DAS. Different intercultural operations such as irrigation, drainage and weeding were done as and when required. The experiment at reproductive stage laid out by following randomized completely block design with two treatments (control seedling and pretreated cold stressed seedling) and three replications (each replication had 5 plants).

Measurements of morpho-physiological traits at the reproductive stage

The data of different parameters were taken from five plants for each replication and then, the average value was recorded. Plant height was taken from the base of the plant at 90 DAS using a centimeter scale. The total number of tiller was observed by close visual observation. The summation of all the tillers coming out from each rice plant was calculated as total number of tiller. The tillers which bear panicle and contribute in the yield were considered as number of effective tiller recorded under close observation. Panicle length was measured before harvesting using a centimeter scale. In the case of number of filled grain measurements, all the grains from each panicle was observed by pressing by hand for whether it is filled or not and by deducting number of filled grain from total number of grains in the panicle, number of unfilled grain was measured. 1000-seed weight was measured by weight machine. Days to maturity was calculated from sowing date in the seed bed to the date of harvesting. In case of yield measurement, all the rice plants from each plot were harvested and grains were shaded. Then weight of the grains from each plot was measured as yield (kg) per plot.

Estimation of genetic diversity related parameters in morpho-physiological traits Genotypic and phenotypic variances

Genotypic and phenotypic variances were estimated according to the formula given by Johnson et al (29).

$$\text{Genotypic variance}, \sigma_g^2 = \frac{\text{GMS} - \text{EMS}}{\text{r}}$$

Where,GMS = Genotypic mean square, EMS = Error mean, and r = Number of replication $\text{Phenotypic variance}, \sigma^2_p = \sigma^2_g + \text{EMS}$, Where, σ^2_g = Genotypic variance, and EMS = Error mean square

Genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV)

Genotypic and phenotypic coefficient of variations were estimated according to Burton (30) and Singh and Chaudhury (31).

$$\text{Genotypic coefficient of variations, GCV} = \frac{\sqrt{\sigma_g^2}}{\bar{X}} \times 100$$

Where,σ^2_g= Genotypic variance; and $\bar{X}$= Population mean

$$\text{Phenotypic coefficient of variations, PCV} = \frac{\sqrt{\sigma_p^2}}{\bar{X}} \times 100$$

Where,σ^2_p = Phenotypic variance; and $\bar{X}$= Population mean

Heritability

Heritability in broad sense (h^2b) was estimated according to the formula suggested by Johnson et al (29) and Hanson et al (32).

Heritability, $h^2_b \frac{\sigma^2 g}{\sigma^2 p} \times 100$

Where, h^2_b=Heritability in broad sense, $\delta^2 g$ = Genotypic variance; and $\delta^2 p$ = Phenotypic variance

Genetic advance

Estimation of genetic advance was done following formula given by Johnson et al (29) and Allard (33).

Genetic advance, $GA = h^2_b.K.\sigma_p$

Where, h^2_b = Heritability in broad sense, K= Selection differential, the value of which is 2.06 at 5% selection intensity, and σ_p = Phenotypic standard deviation

Estimation of genetic advance in percentage of mean, GA (%)

Genetic advance in percent of mean was calculated by the formula of Comstock and Robinson (34) as follows.

$$\text{Genetic advance in percentage of mean, GA (\%)} = \frac{\text{GA}}{\overline{X}} \times 100$$

Where, GA = Genetic advance, and $\overline{X}$= Population mean

Statistical analysis of data

Data were subjected to two-way analysis of variance using Minitab 18 software package (35) and least significant difference (LSD) test at $P < 0.05$ indicates significant differences among the treatments and genotypes according by different alphabetical letters in the same column. The correlation and path coefficient were analyzed by using Minitab 18 and BASICA software package respectively (36).

RESULTS

Initially, 13 rice genotypes were used in screening for cold tolerance based on their phenotypic characterization at the seedling stage and the seedlings of the genotypes which demonstrated superior growth performance under cold stress in the growth chamber, were transplanted directly in the field level to evaluate their performance at the reproductive stage with their respective control.

Germination (%) of rice genotypes under cold stress

The germination (%) of different rice genotypes was remarkably reduced with the advancement of days almost in all genotypes when cold stress was induced in compared to the control treated plants (Table 3). When the seeds were kept under 10°C for germination, the seeds of all the genotype were decayed and no seeds were germinated and therefore, the germination (%) was not measured at 10°C. However, the seeds were germinated well when the temperature raised to 14°C. Among the 13 genotypes, *khumal-7* (85%) and *khumal-2* (70.5%) demonstrated the maximum reduction of germination (%) after 10 days of cold stress exposure whereas the minimum reduction was displayed by *lekali dhan-3* (7.14%) followed by *chandannath-3* (7.14%) in control conditions. After 20 days of cold stress exposure, *khumal-7* (45%) and *khumal-5* (33.3%) demonstrated the maximum reduction of germination (%) whereas the minimum reduction was also displayed by *lekalidhan 3* (0%) and *chandannath-3* (0%) (Table 3).

Categorizing of rice genotypes based on injury scoring at the seedling stages

Rice seedlings exhibited considerable variation for cold tolerance at 14^0C and 10^0C cold stress exposure, consequently the genotypes were categorized into tolerant, moderately tolerant and susceptible genotypes considering injury score based on the visual appearance in the leaves (Table 4 & 5). After 20 days of cold treatment at 14^0C, three genotypes namely *lekali dhan-3, lekali dhan-1* and *chandannath-3* were identified as highly cold tolerant (Injury score 0-1), four genotypes *viz., begunipata, khumal-9, monjushri-2* and *datpahari* were considered as tolerant (Injury score 2-3) and the rest four genotypes *viz., khumal-6*, *BRRI dhan-55, khumal-7* and *khumal-2* were found as moderately tolerant (Injury score 4-5) (Table 4). However, when the temperature reduced to 10^0C, the rice seedlings showed more sensitivity to cold stress (Table 4). At 10^0C of cold treatment, three genotypes namely *lekali dhan-3, lekali dhan-1* and *chandannath-3* were identified as cold tolerant after 12 days exposure of cold treatment (Injury score 2-3), nine genotypes were found as cold sensitive (*begunipata, khumal-6, BRRI dhan 55, khumal-9, khumal-7, monjushri-2, datpahari, khumal-2* and *khumal-4*) (Injury score 6-7) and one genotype namely *khumal-5* was considered as highly cold sensitive (Injury score 8-9)(Table 5).

Analysis of variance of morpho-physiological traits in rice genotypes at the reproductive stage

The results of the study demonstrated that the variation among the genotypes considering the different morpho-physiological traits *viz.,* plant height, total number of tiller, effective tiller, panicle length, number of filled grain per panicle, number of unfilled grain per panicle, days to maturity, 1000-seed weight, and grain yield were highly significant (Table 6).

Susceptibility index (SI) of rice genotype's considering morpho-physiological traits at the reproductive stage

The result of the present study demonstrated that the pre-treated cold tolerant genotypes showed almost similar value in all treatments (control and cold) at the reproductive stage, therefore, the SI for all morpho-physiological traits viz., plant height susceptibility index (PHSI), total number of tiller susceptibility index (TNTSI), number of effective tiller susceptibility index (NETSI), panicle length susceptibility index (PLSI), number of filled grains per panicle susceptibility index (NFGSI), number of unfilled grains per panicle susceptibility index (NUFGSI), days to maturity susceptibility index (DMSI), thousand seed weight susceptibility index (THWSI), and yield per plot susceptibility index (YPSI) were lower in all genotypes (Table 7). In the present study, *lekali dhan-1* showed the lowest SI for different traits such as PHSI (2.98%), PLSI (2.68%), FGSI (2.06%), UFGSI (-22.5%) and TSWSI (0.31%) where as *lekali dhan-3* had minimum value for TNTSI (10.36%) and YPPSI (1.86%). Besides, *chandannath-3* also displayed the comparatively lower SI for number of effective tiller -2.88% and days to maturity 2.58% (Table 7).

Effect of cold stress on the morpho-physiological traits in three rice genotypes at the reproductive stage

As ten genotypes *viz., khumal-2, khumal-4, khumal-5, khumal-6, khumal-7, khumal-9, monjushri-2, begunipata, datpahari,* and *BRRI dhan-55* were completely died after 12 days of cold imposition at 10°C (Table 5), those genotypes were not considered for the further evaluation of their reproductive performance. On the contrast, only three genotypes *viz., lekali dhan-1, lekali dhan*-3 and *chandannath-3* showed almost normal growth and development under both cold treatments (10°C & 14°C) in compared to the control treated seedlings (Table 4 & 5) and therefore, those genotypes were used further in the field level to examine their performance for different yield and yield attributing traits with respective control.

Plant height

In case of control treated seedlings, the highest plant height was recorded in *lekali dhan-3* (109.57 cm) and the lowest value was found in *lekali dhan-1* (107.27cm). However, the cold treated seedlings showed the lower plant height in compared to the control condition plant. In case of cold treatments, cold treated *chandannath-3* (104.27cm) displayed the maximum plant height whereas lowest plant height was exhibited by previously cold treated *lekali dhan-3* (103.06cm) (Figure 1A).

Total number of tiller

In the present investigation, the highest total number of tiller was recorded in *lekali dhan -3* (11.7) in case of control treated seedlings at the reproductive stage. Among three genotypes, cold treated *lekali dhan-3* (10.01) demonstrated the highest total number of tiller where as lowest value was observed in cold treated *lekali dhan-1* (7.37) at the reproductive stage (Figure 1B).

Number of effective tiller

Considering number of effective tiller, the highest mean value of number of effective tiller was recorded in *lekali dhan-3*(10.67) and the lowest value was found in *lekali dhan-1* (8.17) in case of control treatment. On the other side, cold treated *lekali dhan-3* (9.73) also displayed the maximum mean value of number of effective tiller whereas lowest plant height was exhibited by previously cold treated *lekali dhan-1* (7.11) which is approximately similar to the control condition plants (Figure1C).

Panicle length

All cold treated genotypes experienced the reduction of panicle length in compared to the control. In the present study, control treated *lekali dhan-3* (22.73 cm) showed the highest panicle length and the lowest value was found in untreated *lekali dhan-1* (21.77 cm). The cold treated *lekali dhan-3* (22.04 cm) displayed the maximum mean value of panicle length whereas lowest panicle length was exhibited by previously cold treated *lekali dhan-1* (21.18 cm) (Figure 1D).

Filled grains per panicle

Cold stress affected the number of filled grains per panicle in all the genotypes. In case of control treatment, the maximum value of number of filled grains per panicle was recorded in *lekali dhan-3* (120.02) and the lowest value was found in *lekali dhan-1* (108.07). The cold treated seedlings showed the lower mean value of number of filled grains per panicle in the level of control. Among the three genotypes, cold treated *lekali dhan-3* (115.98) exhibited the maximum value of number of filled grains per panicle whereas lowest value was recorded in previously cold treated *lekali dhan-1* (105.84) (Figure 1E).

Unfilled grains per panicle

The result of the present study demonstrated that the significant difference between control treated and cold treated plants considering number of unfilled grains per panicle. The highest value of number of unfilled grains per panicle was recorded in cold treated *chandannath-3* (28.83) whereas lowest plant height was exhibited by previously cold treated *lekali dhan-1* (16.33) in case of cold treated plants (Figure 1F).

Days to maturity

Cold stress delayed the maturity time in all the genotypes in the level of control. In the present study, the maturity time of *lekali dhan-1* was 129 days in control condition whereas cold treated *lekali dhan-1* had matured two days later (131 days) however, it was the early matured genotype compared to the others cold treated genotypes. In the case of cold treated genotypes, *lekali dhan-3* (132.67 days) displayed the maximum mean value of days to maturity (Figure 1G).

1000-Seed Weight

The 1000-seed weight among the genotypes was statistically same in all treatments. In case of control treated plants, the highest value of 1000-seed weight was found in *chandannath-3* (29.57g) and the lowest value was found in *lekali dhan-1* (27.89g). However, the cold treated genotypes exhibited slightly lower value of 1000-seed weight in compared to the control condition plant. In the case of cold treatments, cold treated *chandannath-3* (29.00g) displayed the maximum mean value of 1000-seed weight whereas lowest plant height was exhibited by previously cold treated *lekali dhan-1* (27.81g) (Figure 1H).

Yield per plot

In the present investigation, cold treated genotypes displayed the similar performance like control treated plants considering grain yield. The genotypes namely *chandannath -3* (2.33kg) showed the maximum value in control situation and similarly, *chandannath -3* (2.28 kg) was also the highest yielder in case of cold treatment. Among the three genotypes, *lekali dhan-3* demonstrated the lower grain yield in both control and cold treatments in compared to the other genotypes (Figure 1I).

Evaluation of genetic parameters considering morpho-phyiological traits at the reproductive stage

Different genetic parameters *viz.,* genotypic variances, phenotypic variances, phenotypic co-efficient of variation (PCV), genotypic co-efficient of variation (GCV), heritability, genetic advance and genetic advance in percentage (GA %) were estimated for morpho-physiological traits in three rice genotypes at the reproductive stage (Table 8).

Variability parameters

The presence of genetic variability in breeding materials is essential for a successful breeding program (37). In the present investigation, all the studied traits demonstrated significant genotypic and phenotypic variance among the rice genotypes and notably, the PCV were higher than GCV for all the traits except number of filled grains per panicle (Table 7). Among all traits, number of unfilled grains per panicle showed maximum value of % PCV and % GCV (44.1% & 43.54%) followed by number of effective tiller (20.91%& 20.89%) and total number of tiller (18.10% & 18.01%) where as plant height (0.83% & 0.79%) had the lowest % PCV and %GCV followed by days to maturity (0.94% and 0.89%) and yield (3.71% & 3.57%) (Table 8).

Heritability and genetic advance

The results of the study indicated the high heritability in all the traits ranging from 89.02% to 99.99%. Among all the traits, number of filled grains per panicle (99.99%) displayed the highest value of heritability followed by number of effective tiller (99.73%), panicle length (99.73%), total number of tiller (99.00%), number of unfilled grains per panicle (97.67%), 1000-seed weight (96.36%), yield (92.72%), plant height (91.03%), and days to maturity (89.02%) (Table 8).

Only estimation of heritability is not enough to provide clear indication and screening of desirable genotypes and hence, knowledge about genetic advance is essential along with heritability. In case of genetic advance, the maximum value was exhibited by the number of unfilled grains per panicle (17.45) followed by number of filled grains per panicle (16.85) whereas the lowest genetic advance was found in grain yield (0.16) followed by plant height (1.64). Similarly, the maximum genetic advance as percent of mean was found for number of unfilled grains per panicle (88.73%) followed by number of effective tiller (42.97%) and the lowest was found in plant height (1.55%) followed by days to maturity (1.73%) (Table 8).

Correlation among yield and yield attributing in rice genotypes

Pearson's correlations among different yield and yield attributing traits of rice genotypes are presented in Table 9. In the present study, almost all the traits *viz.,* plant height (r=0.57*, P<0.05), total number of tiller (r=0.91***, P<0.001), number of effective tiller (r=0.82***, P<0.001), panicle length (r=0.82***, P<0.001), and number of filled grains per panicle (r=0.77***, P<0.001) had positive and significant relationship with grain yield whereas 1000-seed weight ($r=0.24^{NS}$) showed positive and non-significant correlation with grain yield of rice. The results of the study also revealed the positive significant relation between number of filled grain per panicle and panicle length (r=0.79**, P<0.01) in rice at the reproductive stage. However, in case of unfilled grain per panicle ($r= -0.21^{NS}$) and days to maturity ($r= -0.04^{NS}$), a negative and none significant correlation were recorded with grain yield (Table 9).

Path co-efficient among yield and yield attributing in rice genotypes

Partitioning of phenotypic correlation coefficients into direct and indirect effects on important traits of three rice genotypes by path analysis is shown in Table 10. In the estimation path-coefficient considering grain yield as dependent variable and other traits as independent variables, filled grains per panicle exhibited the maximum direct effect on grain yield (0.987) followed by number of effective tiller (0.841), 1000-seed weight (0.441), plant height (1.702), and total number of tiller (1.327). However, some plant characters *viz.,* panicle length (-0.395), number of unfilled grains per panicle (-0.204), and days to maturity (-0.811) showed the direct negative effects on grain yield in rice genotypes (Table 10).

DISCUSSION

Cold stress is very common at both the germination and seedling stages of rice cultivation in the northern region of Bangladesh during the winter periods. To understand the basis of acclimation stability under cold, some rice genotypes were examined to evaluate their cold tolerance at the seedling and reproductive stages. Seed germination ability is essential for optimum stand establishment and for increasing weed competitive ability in direct seeding rice areas (38). Temperature had significant effect on coleoptile and redicle length, fresh coleoptile and radicle weight, dry coleoptile and radicle length, seed damage percentage and germination rate in a period of being germination in stress condition (39). Low temperature stress at the germination stage of rice caused the reduction in final germination rate (40, 41, 42). Germination (%) seems to be the most appropriate trait to evaluate cold tolerance during seed germination period (17, 43). In the present investigation, cold stress significantly affect %germination almost in all rice genotypes (Table 3), however, some genotypes namely *lekali dhan-3* and *chandannath-3* showed higher germination percentage (Table 3) when exposed to cold stress. The low germination under cold may be due to less metabolic activity and inactive enzymes that play key role in germination (44). Disturbances to the germination process under stress conditions lead to excessive production of reactive oxygen species (ROS), in the absence of effective protection mechanisms (enzymatic or not), and metabolic changes that result in oxidative damage (45). The results of this study was in agreement with Farzin et al (46) who reported that cold stress heavily affects the germination percentage of rice though cold tolerant genotypes least hampered under stress situations (47). Cruz et al. (20) also reported that cold stress is one of the inhibitor for seed germination in rice.

When ambient temperature deviates from optimal, many physiological, biochemical, metabolic and molecular changes occur within plants which to maximize growth and developmental processes and to maintain cellular homeostasis during such adverse conditions (48). The injury scoring of rice genotypes for cold tolerance is one of the important criteria for the section of cold tolerant genotypes. Rice seedlings shows various cold injury symptoms when exposed to low temperatures (49). In the present investigation, rice genotypes showed significant variation for cold tolerance under cold treatment in the growth chamber. The results of the study revealed that, ten genotypes namely *khumal-2, khumal-7, khumal-9, khumal-6, BRRI dhan-55, datpahari, begunipata, monjushri-2, khumal-5, and khumal-4* were completely died showing high injury score under cold stress (10^0C) and only three genotypes namely *lekali dhan-1 lekali dhan-3*, and *chandannath-3* displayed maximum cold tolerance level with low injury score having normal plant growth at both 14^0C and 10^0C in the growth chamber (Table 4&5). The cold tolerant genotypes probably modify the cell metabolism in response to cold stress which are mainly linked to enhanced tolerance mechanisms. Nahar et al (50) reported that the cold tolerant varieties able to maintain their greenness might have the ability to maintain their photosynthetic pigments under cold stress conditions. Zhang et al (51) used the injury scoring for the screening of cold tolerant rice genotypes and suggested that the genotypes having low injury score in cold stress situation is cold tolerant. In addition, another researcher Rasel et al (52) and Siddique et al (53) successfully identified the stress tolerant genotypes through the using of injury scoring system in other crops. Another commonly used screening approach is susceptibility index (SI) for the selection of stress tolerant genotypes. In the present study, cold treated *lekali dhan-3* showed the lowest SI for all the morpho-physiological traits indicating its priority as cold tolerant rice genotypes (Table 7). Bevilacqua et al (54) and Soleymani and Shahrajabian (39) previously used the SI for the identification of cold tolerant rice genotypes.

Cold stress has pronounced effect on reproductive stage in rice (55). Rice plants have a lower threshold temperature (10–13°C) for cold damage during the early stages of development (germination and vegetative), what makes them less sensitive to cold than during the reproductive stage, which has a higher threshold temperature for damage (18–20°C) (20). In the present study, previously identified cold tolerant genotype *lekali dhan-3* showed almost similar performance in respective of yield and yield attributing traits in compared to the control treated genotypes at the reproductive stage. It indicates that tolerant genotypes could survived well after the imposition of cold stress period at the early seedling stage and consequently less affected in reproductive performance.This result is similar with Soleymaniand Shahrajabian (39).

However, Dubey et al (56) also reported that rice plants might suffer from low temperature at the seedling stage and plant height might be reduced during harvesting time.In the present study, plant height of *lekali dhan-1, lekali dhan-3* and *chandannath-3* at reproductive stage was reduced compared to their control genotypes (Figure 1A) however cold tolerant *lekali dhan-3* exhibited the maximum plant height like control treated plants. The growth retardation or stunting of plant can be occurred due to minimum low temperature (57). Besides,the reduction of plant height might be occurred because of disruption in photosynthesis, metabolic activities and hormonal activities at the seedling stage resulting in growth inhibition, plasma membrane disintegration, dehydration, solute leakage, metabolite imbalance, metabolic dysfunction etc.(58).

Low temperature profoundly affect yield attributing traits such as number of panicles, length of panicle, and number of full, empty and total grains in rice genotypes reducing less grain yield (59). In addition, low temperature effects on seedlings can be manifested as reduced tillering and stunted growth during reproductive growth (57). In this study, total number of tiller and number of effective tiller is decreased slightly in cold treated genotypes, however, number of effective tiller showed least reduction in all three genotypes in the level of control (Figure 1B) which indicated that cold treated seedlings produces less number of tiller compared to their control treated genotypes. Number of effective tiller had not been decreased much due to less competition for light, nutrients etc and less late tillering (60). Cold stress significantly reduce panicle emergence and panicle length which is one of the most important criterion of cold tolerance (61). The panicle length of the cold treated genotypes was reduced compared to control but the

reduction is not significant (Figure 1D) and number of unfilled grains per panicle is higher in cold treated genotypes in comparable to the control treated genotypes. Banik (62) found the similar result in rice at reproductive stage to our findings.

The developmental stages from pollen formation to fertilization are the most vulnerable to low temperature throughout the life cycle of rice plant (63). Cold stress increase malformed or deformed spikelet and grain like deformation in the shape of kernels, presence of double lemmas, paleae and empty glumes etc. (64). Jena et al (65) also reported that low temperature affect sterility. In the present study, cold tolerant *lekali dhan-1* and *chandannath-3* demonstrated the lower reduction of filled grains per panicle at the reproductive stage (Figure 1E). Similar result was found by Cruz et al (20). Nishiyama (66) reported that exposure of rice plants at the tetrad stage to a moderately low temperature (12°C) for 4 days resulted in male sterility in 80% of spikelet's. The genotypes with lower number of thousand-grain weight were due to lower percentage of fertile grain which was occurred by cold stress (67). The results of the study showed the less difference between cold treated and control genotypes in the case of 1000-seed weight (Figure 1H). The interaction between temperature and varieties showed that most cold tolerant variety in relation to temperature stress had least percentage yield decrease. In the present study, *lekali dhan-3* was showed less reduction in yield per plot compared to *lekali dhan-1* and *chandannath-3* (Figure 1I). Shinada et al (68) and Suzuki et al (69) also reported similar results of yield reduction under cold stress at the reproductive stages. Ye *et al*. (2009a) also reported that yield losses due to cold temperature are a result of incomplete pollen formation and subsequent floret sterility. Some of the genotypes having an exciting level of yield indicated their all-round tolerance to the cold (8).

The success on plant breeding activities entirely depends on the existence of genetic variability with respect to desired traits and selection skill of plant breeder (70). All the studied traits demonstrated wide range of variation was found for all rice genotypes which reflecting the large genetic differences among the genotypes. The higher value of PCV than GCV was recorded for most of the traits (Table 8) indicating that the environmental factors profoundly affect the phenotypic expression of the characters in that genotypes(37). The results of this study were in agreement with Malimar et al (71); Rashid et al (72); Gyawali et al (73). Besides, the recorded higher values of PCV and GCV for number of filled grain per panicle and number of effective tiller demonstrating their importance in selection for the improvement of that genotypes Adhikari et al. (70). However, very low GCV was found in case of plant height followed by days to maturity indicating lack of inherent variability and limited scope for improvement through selection for these traits (74). Das et al. (75) also reported similar findings for the screening of rice genotypes for salinity tolerance.

Heritability is an important parameter in plant breeding to select the high heritable plant trait (76). Plant breeders may select a genotype based on phenotypic expression for the desired characters of individual plant through simple selection method (Rasel et al. 2018). The results from the present study reported that all the traits exhibited high heritability (Table 8). Singh et al (77) also found the similar result among several morpho-physiological characters of eighty-three rice varieties. High heritability values indicates that the characters are less influenced by the environment for their phenotypic expression and selection can be applied by using these traits to improve rice genotypes for cold tolerance (78). High level heritability for different traits was also reported by Gyawali et al (73), Bandhi et al (79), Abebe et al (80). High heritability along with high genetic advance was noticed for number of filled grains per panicle (Table 8) suggested that high heritability combined with high genetic advance could be an indication of additive gene action and selection based on these parameters would be more reliable (81). Hosseini et al (82) also found high genetic advance for plant height, root dry weight and shoot length. High heritability coupled with high genetic advance as percent of mean was observed for number of filled grains per panicle, number of effective tiller and total number of tiller (Table 8) indicating the role of additive gene expression for these traits and would facilitate better scope for improvement of these traits through direct selection. Saha et al (83) also reported similar result for rice genotypes.

Correlation coefficient is a measure of the degree association and relationship between two variables and it is important in plant breeding as it can be used for indirect selection (84). In the present study, the highly significant and positive correlations were reported in between yield and total number of tiller, number of effective tiller, panicle length and number of filled grains per panicle (Table 9) indicating the importance of these parameters as useful selection criteria for the screening of cold tolerant genotypes. Positive correlation of panicle length, and thousand grain weight with grain yield was also reported by Kumar et al (85). However, negative and non-significant co-relation was found for number of unfilled grains per panicle and days to maturity with grain yield (Table 9) reflecting less importance of these parameters screening of cold tolerant genotypes. Similar findings were reported by Gyawali et al (73) which support the present findings.

A measurement of correlation would not provide the true contribution of the characters towards the yield and therefore, the simple correlation could be partitioned into direct and indirect effects through path coefficient analysis (86). The path coefficient analysis showed that plant height, total number of tiller, number of filled grains per panicle, days to maturity had direct positive effect on grain yield at phenotypic level (Table 10) indicating their importance for the selection of cold tolerant rice genotypes. Similar findings have also been reported by Oladosuet al (87). The residual effect at phenotypic level is 0.13542 indicated that the characters which are selected in this study contributed 86% of variability on yield (Table 10).

CONCLUSION

The present study was undertaken to screen out the suitable cold tolerant genotypes for the cultivation in the northern region of Bangladesh. In the present investigation, the genotypes namely *lekali dhan-3* and *chandannath-3* showed least germination reduction (%) under cold stress. In the growth chamber, the genotypes viz., *lekali dhan-1, lekali dhan-3, chandannath-3* also demonstrated the higher cold tolerance capacity at both 10^{o}C and 14^{o}C having lower cold injury, consequently, those genotypes were regarded as cold tolerant genotypes at the seedling stage. Afterwards, the cold tolerant genotypes were transplanted in the field with their respective control to evaluate their performance at the reproductive stage. At the reproductive stage, the cold treated genotype namely, *lekali dhan-3* compared to *lekali dhan-1, and chandannath-3* showed the good performance in terms of grain yield similar to control treated plants, however, *chandannath-3* displayed the maximum grain yield indicating its suitability for the cultivation in the cold prone northern regions and north-eastern haor regions of Bangladesh. Among the morpho-physiological traits, number of filled grain per panicle and number of effective tiller could get the most priority as selection traits for the screening of cold tolerant rice genotypes.

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Table 1. List of rice genotypes used in the experiment

Sl. No.	Name of the genotypes	Source of collection	Cultivating Areas
1	*Lekali dhan-1*	Nepal	High hills
2	*Lekali dhan-3*	Nepal	High hills

3	*Chandannath-3*	Nepal	High hills
4	*Manjushree-2*	Nepal	Kathmandu valleys
5	*BRRI Dhan-55*	Bangladesh	Plain lands
6	*Begunipata*	Bangladesh	Plain lands
7	*Datpahari*	Bangladesh	Mid hills
8	*Khumal-2*	Nepal	Mid hills
9	*Khumal-4*	Nepal	Mid hills
10	*Khumal-5*	Nepal	High hills
11	*Khumal-6*	Nepal	Kathmandu valleys
12	*Khumal-7*	Nepal	High hills
13	*Khumal-9*	Nepal	Mid hills

Table 2. Leaf discoloration (LD scale) scale scoring of rice genotypes cold tolerance at the seedling stage

LD scale	Condition of leaves and plants	Tolerance level
0-1	No damage to Leaves, normal leaf color	Strongly tolerant
2-3	Tip of leaves slightly dried, folded and light green	Tolerant
4-5	Some seedlings moderately folded and wilted, 30%-50% seedling dried, pale green to yellowish leaves	Moderately tolerant
6-7	Seedlings severely rolled and dried, reddish-brown leaves	Sensitive
8-9	Most seedlings dead and dying	High sensitive

Source: IRRI (1996)

Table 3. Germination percentage of 13 rice genotypes at different days under cold treatment (14 0 C)

Genotypes	Control (25^{0}C)			Treatment (14^{0}C)		Treatment (10^{0}C)
	%G_1 at 4th day	%G_2 at 10th day	%G reduction from G_1 to G_2	%G_3 at 20th day	%G reduction from G_1 to G_3	
Begunipata	95	65	31.5	80	15.7	The seeds of all genotypes were dried and therefore, no seeds were germinated.
Khumal-6	90	70	22.2	75	16. 6	
BRRI Dhan-55	100	45	55.0	75	25.0	
Khumal-9	65	50	23.0	55	15.3	
Khumal-7	100	15	85.0	55	45.0	
Monjushri-2	100	75	25.0	93	7.00	
DatPahari	100	70	30.0	90	10.0	
Khumal-2	85	25	70.5	65	23.5	
Khumal-4	95	60	36.8	75	21.0	
Lekali Dhan-3	70	65	7.14	70	0.00	
Lekali Dhan-1	75	70	6.67	70	6.67	
Chandannath-3	70	65	7.14	70	0.00	
Khumal-5	90	55	38. 8	60	33.3	

Here, %G denotes percentage of germination which is counted as the number of seeds germinated out of 100 seeds and %G reduction was calculated as follows- [(control value−salt treatment value)/control value × 100].

Table 4. Injury scoring of 13 rice genotypes at 14^{0}C temperature at the seedling stage

Genotyeps	Days of scoring										Final injury score	Tolerance level
	2th	4th	6th	8th	10th	12th	14th	16th	18th	20th		
Begunipata	0	0	1	1	1	1	2	2	2	3	3	Tolerant
Khumal-6	0	0	2	2	2	3	3	4	4	4	4	Moderately tolerant
BRRI Dhan-55	0	0	2	3	4	4	4	5	5	5	5	Moderately tolerant
Khumal-9	0	0	0	1	1	1	1	2	2	3	3	Tolerant
Khumal-7	0	0	1	1	1	3	3	3	4	4	4	Moderately tolerant
Monjushri-2	0	0	1	1	1	2	2	2	2	2	2	Tolerant
DatPahari	0	0	0	0	1	1	1	1	2	2	2	Tolerant
Khumal-2	0	2	3	3	4	4	4	5	5	5	5	Moderately tolerant
Khumal-4	0	2	3	3	4	5	5	6	6	6	6	Sensitive
Lekali Dhan-3	0	0	0	0	0	0	0	0	0	1	1	Strongly tolerant
Lekali Dhan-1	0	0	0	0	0	1	1	1	1	1	1	Strongly tolerant
Chandannath-3	0	0	0	0	0	0	0	1	1	1	1	Strongly tolerant
Khumal-5	0	1	4	5	5	5	6	6	7	7	7	Sensitive

Final injury score was counted on 20th day of experimental setting in growth chamber

Injury scoring was given according to IRRI standard protocol (1996), where score 0-1 denotes strongly tolerant (ST), score 2-3 denotes tolerant (T), score 4-5 denotes moderately tolerant (MT), score 6-7 denotes sensitive (S), and score 8-9 denotes highly sensitive (HS).

Table 5. Injury scoring of 13 rice genotypes at 10^{0}C temperature at the seedling stage

Genotypes	**Days of scoring**												**Final injury score**	**Tolerance level**
	1th	2th	3th	4th	5th	6th	7th	8th	9th	10th	11th	12th		
Begunipata	0	0	0	0	1	2	2	3	3	4	5	6	6	Sensitive
Khumal-6	0	0	2	3	4	5	5	6	6	6	7	7	7	Sensitive
BRRI Dhan-55	0	0	1	2	4	4	5	6	6	6	7	7	7	Highly
Khumal-9	0	0	1	1	2	4	5	6	6	6	7	7	7	Sensitive
Khumal-7	0	0	1	2	4	5	5	6	6	6	7	7	7	Sensitive
Monjushri-2	0	0	0	1	1	2	3	3	4	5	5	6	6	Sensitive
DatPahari	0	0	0	2	2	2	2	3	4	4	5	6	6	Sensitive
Khumal-2	0	2	2	3	4	4	5	7	7	7	7	7	7	Sensitive
Khumal-4	1	2	3	4	5	5	6	7	7	7	7	7	7	Sensitive
Lekali Dhan-3	0	0	0	0	0	0	0	0	1	1	2	2	2	Tolerant

Lekali Dhan-1	0	0	0	0	0	1	1	1	2	2	3	3	3	Tolerant
Chandannath-3	0	0	0	0	1	1	1	2	2	2	3	3	3	Tolerant
Khumal-5	1	5	6	6	6	7	7	8	8	8	9	9	9	Highly sensitive

Final injury score was counted on 12th day of experimental setting in growth chamber
Injury scoring was estimated according to IRRI standard protocol (1996), where score 0-1 denotes strongly tolerant (ST), score 2-3 denotes tolerant (T), score 4-5 denotes moderately tolerant (MT), score 6-7 denotes sensitive (S), and score 8-9 denotes highly sensitive (HS).

Table 6. Analysis of variance for different morpho-physiological traits of rice genotypes at the reproductive stage

Source of variation	df	PH	TNT	NET	PL	FGP	UFGP	DM	TSW	YPP
Treatment (A)	1	110.9***	7.96***	2.50***	2.50***	64.71***	46.7***	26.88***	0.317**	0.0382***
Variety (B)	2	2.17***	8.85***	10.12***	10.12***	200.7***	222.1***	4.22***	3.362***	0.019***
AxB	2	4.16***	0.127*	0.28***	0.28***	3.1***	2.72	0.88*	0.103*	0.0055**
Error	12	0.069	0.029	0.009	0.0091	0.008	1.75	0.166	0.04184	0.0005

*** indicates significant at 0.1% probability level, ** indicates significant at 1% probability level and * indicates significant at 5% probability level *df* denotes degrees of freedom, *PH* denotes plant height (cm), *TNT* denotes total number of tiller, *NET* denotes number of effective tiller, *PL* denotes panicle length (cm), *FGP* denotes number of filled grains per panicle, *UFGP* denotes number of unfilled grains per panicle, *DMT* denotes days to maturity, *TSW* denotes 1000-seed weight (g) and *YPP* denotes yield per plot (kg)

Table 7. Susceptibility index (SI) of different morpho-physiological parameters in rice genotypes at the reproductive stage

Genotype	PHSI	TNTSI	NETSI	PLSI	FGPSI	UFGPSI	DMSI	TSWSI	YPPSI
LekaliDhan 1	2.98	18.45	12.94	2.68	2.06	-22.50	-1.55	0.31	7.05
LekaliDhan 3	5.94	10.36	8.75	3.05	3.37	-12.00	-1.53	0.51	1.86
Chandannath 3	4.74	11.25	2.88	3.25	4.55	-19.31	-2.58	1.92	3.21

PHSI indicates plant height susceptibility index, *TNTSI* number of tiller susceptibility index, *NETSI* number of effective tiller susceptibility index, *PLSI* panicle length susceptibility index, *FGPSI* number of filled grains per panicle susceptibility index, *UFGPSI* number of unfilled grain per panicle susceptibility index, *DMSI* days to maturity susceptibility index, *TSWSI* thousand seed weight susceptibility index, *YPPSI* yield per plot susceptibility index

Table 8. Estimation of genetic parameters for different morpho-physiological traits in three rice genotypes

Characters	PH	TNT	NET	PL	FGP	UFGP	DM	TSW	YPP
GV (δ^2g)	0.70	2.94	3.37	3.37	66.9	73.4	1.35	1.11	0.01
PV (δ^2p)	0.77	2.97	3.38	3.38	66.9	75.2	1.52	1.15	0.01
Heritability (%)	91.0	99.0	99.7	99.7	99.9	97.6	89.0	96.3	92.7
GA	1.64	3.52	3.78	3.78	16.8	17.4	2.26	2.13	0.16

GA%	1.55	36.9	42.9	17.1	15.1	88.7	1.73	7.48	7.08
PCV%	0.83	18.1	20.9	8.36	7.34	44.1	0.94	3.77	3.71
GCV%	0.79	18.0	20.8	8.35	7.34	43.5	0.89	3.70	3.57

These are GV means genotypic variance and PV means phenotypic variance *GCV* means genotypic variance co-efficient of Variance, *PCV* means phenotypic co-efficient of variance, *GA* means genetic advance, *GA* (%) means genetic advance as percent of mean *df* denotes degrees of freedom, *PH* denotes plant height (cm), *TNT* denotes total number of tiller, *NET* denotes number of effective tiller, *PL* denotes panicle length (cm), *FGP* denotes number of filled grains per panicle, *UFGP* denotes number of unfilled grains per panicle, *DMT* denotes days to maturity, *TSW* denotes 1000 seed weight (g) and *YPP* denotes yield per plot (kg)

Table 9. Correlation coefficients among different morpho-physiological traits at phenotypic level in rice genotypes

Characters	PH	TNT	NET	PL	FGP	UFGP	DM	TSW
TNT	0.60**							
NET	0.37	0.91***						
PL	0.69**	0.90***	0.81***					
FGP	0.42	0.86***	0.96***	0.79***				
UFGP	-0.12	0.14	-0.03	0.10	-0.18			
DM	-0.76***	-0.07	0.20	-0.18	0.10	0.32		
TSW	0.36	0.43	0.09	0.40	0.03	0.79***	-0.15	
YPP	0.57*	0.91***	0.82***	0.82***	0.77***	-0.04	-0.21	0.24

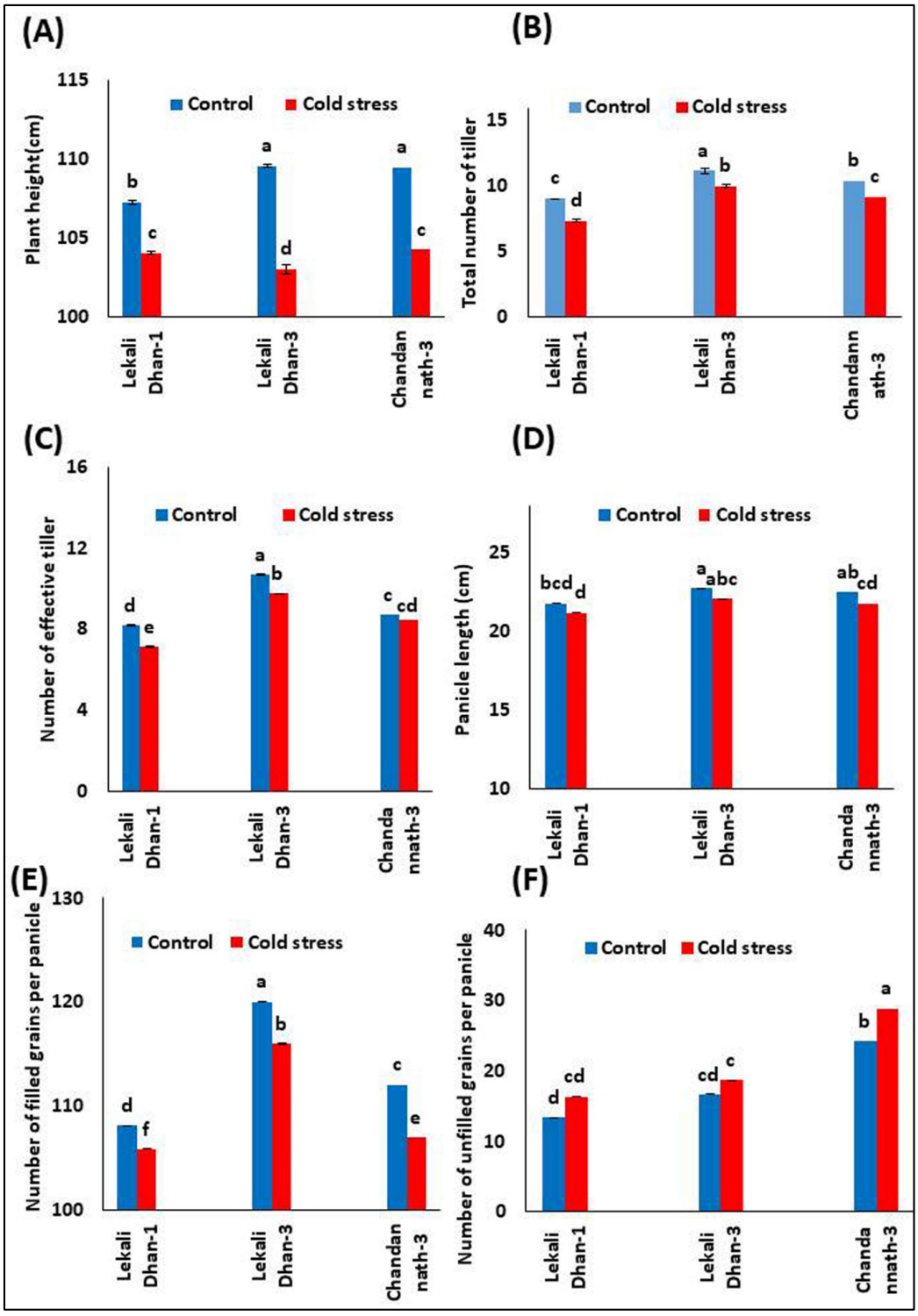
Here, *** indicates significant at 0.1% probability level, ** indicates significant at 1% probability level and * indicates significant at 5% probability level *PH* denotes plant height (cm), *TNT* denotes total number of tiller, *NET* denotes number of effective tiller, *PL* denotes panicle length (cm), *FGP* denotes number of filled grains per panicle, *UFGP* denotes number of unfilled grains per panicle, *DMT* denotes days to maturity, *TSW* denotes 1000 seed weight (g) and *YPP* denotes yield per plot (kg)

Table 10. Partitioning of direct and indirect effects of morpho-physiological characters of rice genotypes at phenotypic level by path coefficient analysis

Characters	PH	TNT	NET	PL	FGP	UFGP	DM	TSW	YPP
PH	**1.702**	0.796	-0.311	-0.273	-0.415	-0.024	-0.616	-0.159	0.700
TNT	1.021	**1.327**	-0.766	-0.356	-0.849	0.029	-0.057	-0.190	0.160
NET	0.630	1.208	**0.841**	-0.320	-0.948	-0.006	0.162	-0.044	-0.160
PL	1.174	1.195	-0.681	**-0.395**	-0.780	0.020	-0.146	-0.176	0.210
FGP	0.715	1.142	-0.808	-0.312	**0.987**	-0.037	0.081	-0.013	-0.220
UFGP	-0.204	0.186	0.025	-0.040	0.178	**-0.204**	0.259	-0.348	0.260
DM	-1.293	-0.093	-0.168	0.071	-0.099	0.065	**-0.811**	0.066	-0.640
TSW	0.613	0.571	-0.084	-0.158	-0.030	0.161	-0.122	**0.441**	0.510

Residual effect: 0.135421859

PH denotes plant height (cm), *TNT* denotes total number of tiller, *NET* denotes number of effective tiller, *PL* denotes panicle length (cm), *FGP* denotes number of filled grains per panicle, *UFGP* denotes number of unfilled grains per panicle, *DMT* denotes days to maturity, *TSW* denotes 1000 seed weight (g) and *YPP* denotes yield per plot (kg)


(A)
Plant height(cm)
Control
Cold stress
Lekali Dhan-1
Lekali Dhan-3
Chandan nath-3
(B)
Total number of tiller
Chandann ath-3
(C)
Number of effective tiller
Chanda nnath-3
(D)
Panicle length (cm)
(E)
Number of filled grains per panicle
(F)
Number of unfilled grains per panicle

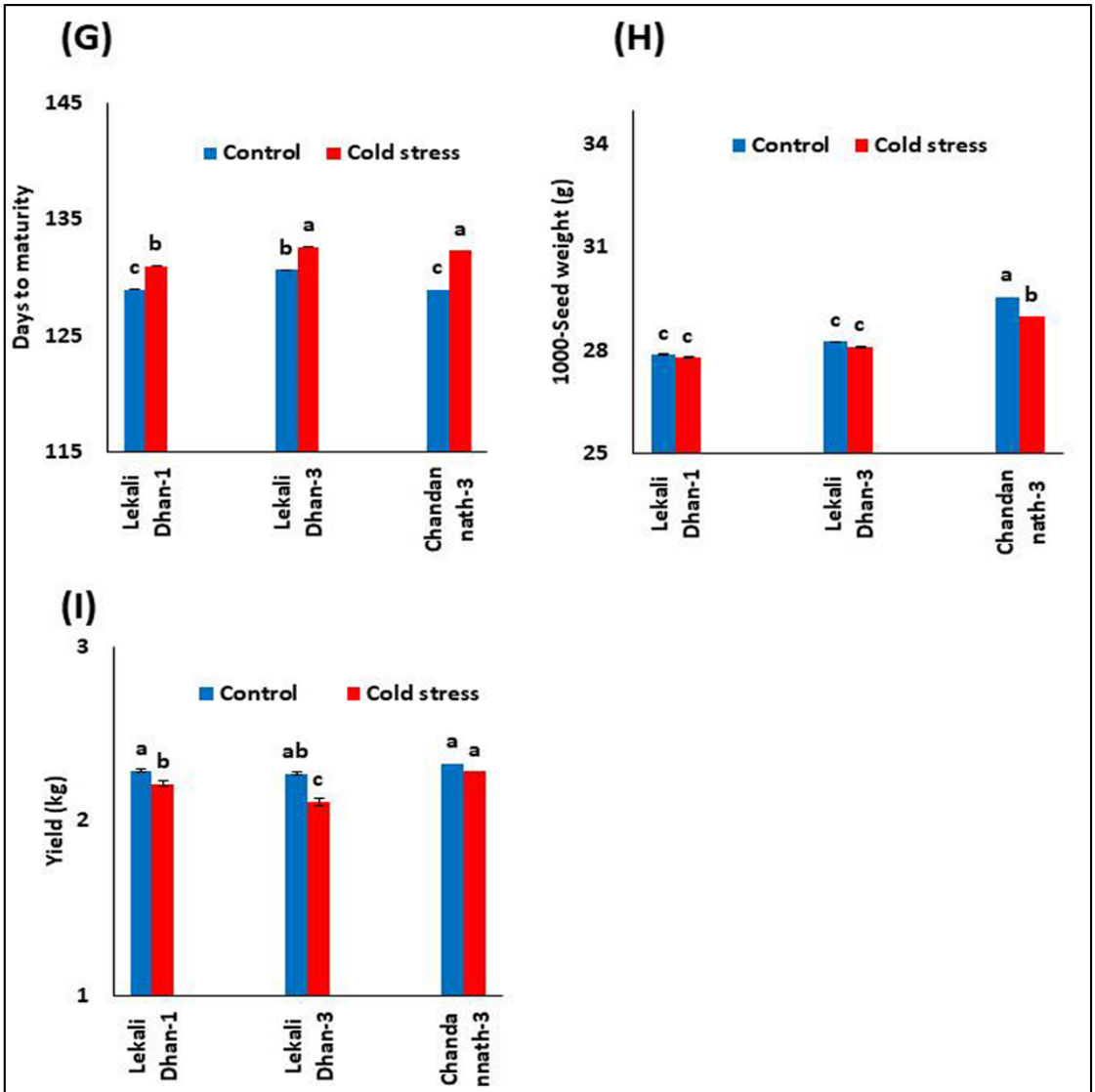


Figure 1. Effect of cold stress on A. plant height, B. total number of tiller, C. number of effective tiller, D. panicle length, E. number of filled grains per panicle, F. number of unfilled grains per panicle, G. days to maturity, H. 1000 Seed weight and I. yield per plot in rice genotypes. Vertical bars indicate standard error of mean against each variable. Different letter indicates significant difference among the genotype × treatment interactions at 1% and 0.1% level of significance.

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RESEARCH ARTICLE

Frequency of Menstrual Irregularities after Tubal Ligation in Women of Reproductive Age

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ABSTRACT

To find frequency of menstrual irregularities after tubal ligation in women of reproductive age.Case series. The study was conducted from 1st June 2017 to 30th November 2017 at Department of Obstetrics and Gynaecology/Population Welfare Department of Shaikh Zayed Hospital Rahim Yar Khan. A total of 96 women with parity more than 3 having tubal ligation ≥ 6 months were included. Patients with history of leiomyoma, diabetes, hypertension, uterine size of > 9 cm, hemoglobin < 9 mg/dL and pelvic inflammatory disease were excluded. All the patients were asked to note the menstrual interval on a paper to control recall bias. Women were asked to come after three menstrual cycles. All patients were interviewed by the researcher herself and asked to describe their three most recent menstrual cycles. A menstrual interval shorter than 21 days and longer than 35 days was defined as menstrual irregularities. Data was recorded and noted on especially designed proforma. In present study, age range in this study was from 28 to 39 years with mean age of 33±2.3 years, mean weight 71±13 kg, mean height 1.5±0.09 meters, mean BMI 30±5.1 Kg/m^2 and mean duration of tubal ligation 12.7±4.02 months. Menstrual irregularities were seen in 35.42% patients. Our study results revealed that more than one third of the women had menstrual irregularities after tubal ligation.

Keywords: Menstruation Disturbances; Sterilization, Tubal, Parity, Parturition

INTRODUCTION

Tubal ligation is the most commonly used method of family planning. It is a highly effective (1,2) and safe (3,4) procedure, but questions remain as to whether it causes menstrual abnormalities. Menstrual disorders are one of the problematic effects of tubal ligation, although the results of related studies have been inconsistent and inconclusive. (4,5) Several studies about the side-effects of tubal ligation on menstrual function have been conducted, (6,7) yet the

existence of a post tubal ligation syndrome has been debated. Although based on the conjecture, it has been hypothesized that tubal ligation may result in low blood flow to the ovaries, leading to impairment of follicular growth and altered gonadotropin signal and ovarian hormone levels, resulting in menstrual disorders.(8) Abnormalities reports associated with tubal ligation surgery include the entire spectrum of menstrual disorders, such as: more frequent menstrual periods, irregular menstrual cycles, menorrhagia, metrorrhagia, spotting, dysmenorrhea and oligomenorrhea.(9). The corresponding percentage of married women in reproductive age who used sterilization was 22% in developing countries and the corresponding percentage in developed countries was 11%.(5) These women represented 44% and 18% of all contraceptive users in developing and developed countries, respectively.(5) Questions regarding the existence of a post tubal ligation syndrome of menstrual abnormalities continue.Naqvi SSB and colleagues have found in a recent study that frequency of menstrual irregularities was 28% in women after tubal ligation.(10)

In another study, Umber F and colleagues have found that frequency of menstrual irregularities was 81% in women after tubal ligation. (11). There is variation in results on this topic in our general population. (10, 11) Resolving the debate about menstrual disorders after tubal ligation is important for safeguarding women's health. Therefore, we planned to determine the frequency of menstrual irregularities after tubal ligation in women of our area. Our study results will play an important role to establish the safety of tubal ligation in our general population where increasing population is a major issue due to low resources.

METHODOLOGY

This case series was conducted from 1st June 2017 to 30th November 2017 at Department of Obstetrics and Gynaecology/Population Welfare Department of Shaikh Zayed Hospital Rahim Yar Khan. A total of 96 women with parity more than 3 having tubal ligation ≥ 6 months were included. Patients with history of leiomyoma, diabetes, hypertension, uterine size of > 9 cm, hemoglobin < 9 mg/dL and pelvic inflammatory disease were excluded. All the patients were asked to note the menstrual interval on a paper to control recall bias. Women were asked to come after three menstrual cycles. All patients were interviewed by the researcher herself and asked to describe their three most recent menstrual cycles. A menstrual interval shorter than 21 days and longer than 35 days was defined as menstrual irregularities. Data was recorded and noted on especially designed proforma.

Statistical Methods

Frequencies and percentages were calculated for qualitative variables like age, parity, family history of menstrual irregularities, poor economic status and menstrual irregularities. Mean±SD was calculated for quantitative variables like weight, height, BMI, duration of tubal ligation and age. Stratification was done with regard to age, duration of menstrual irregularities, duration of tubal ligation, BMI, family history of menstrual irregularities, poor economic status and parity to see the effect of these variables on outcome. Post stratification chi square test was applied taking $p \leq 0.05$ as level of significance.

RESULTS

Age range in this study was from 28 to 39 years with mean age of 33±2.3 years, mean weight 71±13 kg, mean height 1.5±0.09 meters, mean BMI 30± 5.1 Kg/m^2 and mean duration of tubal ligation 12.7±4.02 months as shown in Table-I. Regarding demography of patients, majority of the patients (77.08%) belonged to 30-35 years age group. Most of the patients were para 4-5 (64.6%). Family history of menstrual irregularities was present in 24(25%) patients whereas majority of patients having low socioeconomic status (80.2%) as shown in Table-II. Menstrual irregularities were seen in 35.4% patients as shown in Figure-I. Stratification of patients having tubale ligation regarding age, parity, BMI, family history and socioeconomic status with respect to menstrual irregularities are shown in Table-III.

DISCUSSION

Tubal sterilization is a permanent method of contraception. There has been increasing concern that tubal ligation may increase the risk of menstrual dysfunction.(12) Endocrine and psychological problems have also been reported in women subjected to sterilization.(13). In our study menstrual irregularities were seen in 35.4% patients. Results of present study were comparable with Naqvi SSB and colleagues who found in a recent study that frequency of menstrual irregularities was 28% in women after tubal ligation.10Umber F and colleagues has found in another study that frequency of menstrual irregularities was 81% in women after tubal ligation.(11). Herbert B Peterson et al(6) risk of menstrual abnormalities after tubal sterilization and found that the women who had undergone sterilization were no more likely than those who had not undergone the procedure to report persistent changes in intermenstrual bleeding or the length of the menstrual cycle. They were more likely to have decreases in the number of days of bleeding (odds ratio, 2.4; 95 percent confidence interval, 1.1 to 5.2), the amount of bleeding (odds ratio, 1.5; 95 percent confidence interval, 1.1 to 2.0), and menstrual pain (odds ratio, 1.3; 95 percent confidence interval, 1.0 to 1.8) and to have an increase in cycle irregularity (odds ratio, 1.6; 95 percent confidence interval, 1.1 to 2.3). Among women who had had very heavy bleeding at base line, women who had undergone sterilization were more likely than women who had not undergone the procedure to report decreased bleeding (45 percent vs. 33 percent, P=0.03).

Mehri Jafari shobeiri et al (14) in a case-control study evaluated that menstrual abnormalities were not significantly different between the case and control groups (p = 0.824). The highest frequency of the menstrual abnormalities in the case group was 54.3% for the group aged between 30–39 while in the control group this value was 65% for those aged 40–45. There was significant difference in the menstrual abnormalities' frequency of two groups by different age groups (p=0.0176). Shahideh Jahanian Sadatmahalleh et al (15) in a Cohort study, found that women with tubal ligation had more menstrual irregularities than those without tubal ligation (24.3 vs. 10%, P=0.002). Women with tubal ligation had more polymenorrhea (9.3 vs. 1.4%, P=0.006), hypermenorrhea (12.1 vs. 2.1%, P=0.002), menorrhagia (62.9 vs. 22.1%, P<0.0001) and menometrorrhagia (15.7 vs. 3.6%, P=0.001) than those without tubal ligation. There was a significant difference in the pictorial blood loss assessment chart score between women with and without TL (P<0.0001). They suggested women should be informed by the health providers regarding the advantages and disadvantages of tubal ligation before the procedures. Kasonde and Bonnar did not find any increased menstrual blood loss up to 6–12 months after sterilization (16). Harlow et al[7] and Moradan et al (17) concluded that menstrual irregularity, length of menstruation, length of cycle and flow volume are similar in women with and without tubal ligation, but women with a history of tubal ligation experienced an increase in volume of menstrual flow compared with women who did not undergo tubal ligation.Wilcox et al[18] reported heavy menstrual flow (41%) after 5 years following tubal ligation.Chhabra and Mishra[19] found that women who had undergone tubal sterilization were not found to have major menstrual abnormalities except more women had irregularity in cycles after sterilization. In addition, more women complained of pain during menstruation. Whether this has something to do pelvic congestion needs evaluation.

CONCLUSION

In conclusion, in view of the foregoing we found conflicting results from literature. Yet our study results revealed more than one third of the women had menstrual irregularities after tubal ligation.

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Conflict of Interest

Dr. Ammara reports non-financial support from Getz Pharma, during the conduct of the study.

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Table –I Descriptive Statistics of Patients having Tubal Ligation

Demographics	Mean	S.D.
Age (years)	32.937	2.27
Weight (Kg)	71.083	12.88
Height (m)	1.543	0.095
BMI (Kg/m^2)	29.93	5.098
Duration of tubal ligation(months)	12.718	4.02

Table –II Demography Distribution of Patients having Tubal Ligation

Variable	No. of Patients	%age
Age (years)		
25 – 30	10	10.42
30 – 35	74	77.08
35 – 40	12	12.50
Parity		
4 – 5	62	64.58%
> 5	34	35.42%
Family History of Menstrual Irregularities		
Yes	24	25.0
No	72	75.0
Low Socioeconomic Status		
Yes	77	80.21
No	19	19.79

Table –III Age, Parity, BMI, Family History and Socio-economic Status Distribution of Patients having Tubal Ligation with respect to Outcome

Variables	With Menstrual Irregularities (n=34)	Without Menstrual Irregularities (n=62)	p-value
Age (in Years)			
25 – 30	5.9%	12.9%	0.286
30 – 35	85.3%	72.6%	
35 – 40	8.8%	14.5%	
Para			
4 – 5	61.8%	66.1%	0.669
> 5	38.2%	33.9%	
BMI (Kg/m^2)			
20 – 24	17.6%	29.0%	0.218
25 – 29.9	14.7%	9.7%	
≥ 30	67.7%	61.3%	
Family History of Tubal Ligation			
Yes	61.8%	4.8%	<0.001
No	38.2%	95.2%	
Low Socio-economic Status			
Yes	79.4%	80.6%	0.885
No	20.6%	19.4%	

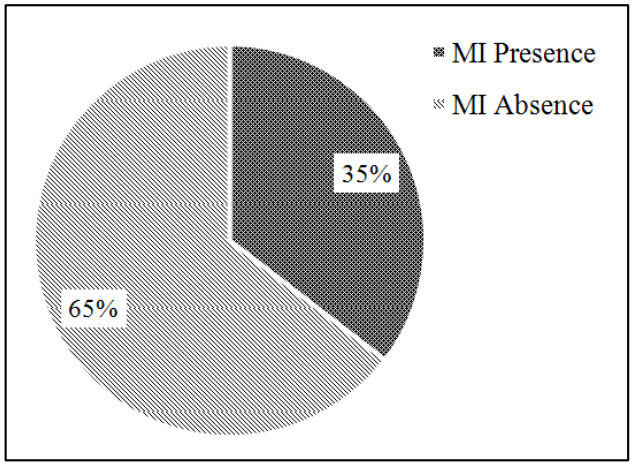

Figure – I. Distribution of Patients having Tubal Ligation with respect to Menstrual Irregularities (n=96)
MI=Menstrual Irregularities

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RESEARCH ARTICLE

Ocean Acidification Impacts on Hatching Success and Reproductive Tissue Damage in Anemonefish

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ABSTRACT

Oceans have continuously absorbed anthropogenic carbon dioxide from the atmosphere. The early life stages are likely vulnerable to low pH conditions. The present study aimed to assess the anemonefish (*Amphiprionsebae*), egg hatch rate and gonadal tissue condition in different pH levels in 6 weeks. Seawater pH was manually manipulated by bubbling known concentrations of CO_2 to achieve three pH treatments 8.1, 7.7 and 7.3. Egg hatch rate decreased with declining pH (ambient pH - 93 %, pH 7.7 - 92 %, pH 7.3 - 88 %) and a slight time delay was observed between the ambient and lower pH treatments. The condition of testicular and ovarian tissue was not affected by low pH levels. Overall, present experiment found that the slight negative effects on egg hatching success in low pH condition. The experimental results suggested that early life stages expected to vulnerable to near future ocean acidification.

Key words: *Amphiprionsebae*, carbon dioxide, low pH, egg hatch rate, tissue damage, ovary, testis.

INTRODUCTION

The atmospheric CO_2 levels are increased from last 20 decades, due to the anthropogenic activities and nearly 30 percent of elevated CO_2 are observed by ocean's surface water (IPCC, 2014). These absorbed CO_2 dissolves in seawater and it form carbonic acid, reduces the ocean's pH which the process known as ocean acidification (Calderia andWickett, 2003).The surface seawater pH has dropped around 0.1 units from 18th century to a current day, and is projected by fall additional 0.3 to 0.4 pH units by end of the 21st century. (Raven et al., 2005). Numerous marine individuals can control their acid-base stability by intra- and extra cellular bicarbonate buffering and dynamic ion transport in high level of CO_2 and low pH condition (Portneret al., 2005).However, higher CO_2 concentrations can interrupt the acid-base action, blood circulation and respiration in addition to the nervous system of marine

organisms and importantprolong effects of reduced growth rates and reproduction (Portneret al., 2004), and further physiological processes (Michaelidiset al., 2005, 2007), some of the organism's cellular efficiency process also affected by the acid-base imbalance (Portneret al., 2005).The fresh water fish showed the less pH compensation while compare to the marine fish species due to the higher concentration of bicarbonate (Heisler, 1993) and sodium chloride (Iwama andHeisler, 1991).Normally, fishes are accepting the slight changes in pH and increase in CO_2 (Ishimatsuet al., 2005, 2008) due to the strong acid-base controlling system, however, the premature stages like a eggs and larvae affected by low pH because of undeveloped acid-base regulatory process (Sayer et al., 1993). The fish larvae and embryos are more susceptible than adults while changing the environmental parameter (Bencic et al., 2001). The pelagic larval period is an important ontogenetic phase for many marine species (Cowen andSponaugle, 2009). The predicted near future CO_2 concentration levels affects the fish larval behavior, yolk size and inability to respond to predator odors. Further, the behavior changes are enhanced the higher mortality rate during an early life-history stages (Mundayet al., 2010).

Histology

Histology is the branch of scientific study used to examine the animal's tissue from the body under microscope to evaluate the tissue damage or infected parts (Yevich andBarszcz, 1983). Aquatic animal and their microanatomy of cells and organs health are diagnosed by the common histology method (Klontz, 1985). Fishes are excreting excess Hydrogen (H^+) ions into the seawater to compensate the body pH and the excretion process across different epithelia like gills, kidney and intestine (Gilmour and Perry 2009; Perry and Gilmour 2006). The changing ambient seawater pH is likely to affect the adult teleost fish.

Anemonefish

Anemonefish belong to the widely diverse and well distributed family Pomacentridae (damselfishes). There are 28 known species, they are distinguished and taxonomically separated from other damselfish (Hoff, 1996). Anemonefish are distributed in tropical and subtropical region of the Indian and Pacific oceans, their highest diversity found adjacent areas of Papua New Guinea, and the Great Barrier Reef (Society, National Geographic).

Clownfish and ocean acidification research

In recent days, most of the studies are related to theeffectsof ocean acidification on the calcifying organisms (Hall-Spencer et al., 2008; Kuffneret al., 2008). However, impact of ocean acidification on other marine organisms have been extended in recent years (Rosa and Seibel, 2008) like fishes, where acidification damagesthe sensory mechanisms (Mundayet al., 2009a). Generally, clownfish larvae have an innate capability to sensethe predators using olfactory signals and to differentiate between the chemical signals of predatory and non-predatory species, whereas, the ability of sensing the predatory cues arenot recognized by larvae when it exposed to low pH seawater (Dixson et al.,2010). The higher concentration of CO_2 is projected to take place in ocean by the end of the 21st century may have conspicuousvariations to the fish larval activities with extremely significant imports for population replenishment and sustainability (Mundayet al., 2010). Acidified seawater changed the clownfish larval behavior and probably increased the higher mortality rate during anearly life history development (Mundayet al., 2010). Last eight years, ocean acidification research work has received more attention in marine science community than other research work. Perturbation experiments are more convincing method used to examine the biological reactions to higherpCO_2. Overall, early developmental stages like gametes, larvae and juveniles are projected to be more sensitive to ocean acidification than adults. Experimental research are required to examine the pH thresholds for growth, reproduction and physiological responses for marine organisms. Ocean acidification research works are infancy in India, except few reports, therefore, the objective of the present experiment is to determine the ocean acidification impacts on the marine anemonefish egg hatching success and reproductive tissue damage.

MATERIALS AND METHODS

Experimental species

The live anemonefish were collected by scuba divers in the shallow region of gulf of manner, the depth range between 3 to 5 meters. The study species were sacrificed at the end of the 6 weeks experiment. The experimental fishes were dissected and their gonads - testis and ovary were removed and fixed in 10 % neutral buffered formaldehyde solution for further uses. The fishes were not severely distress in 6 week's experiment. There was no procedures done to cause harm to anemonefish and also there was no sensitive procedure involved in the experiment as well as there was no chemical agents used for muscle relaxant. The Anemonefish *A. sebae* is used for the experimental purpose, described by Bleeker in 1853. They are protandric hermaphrodite, beginning life as male and later changing to a female and most of them long term monogamous pairs (Yasir and Qin, 2007). They feed on benthic plants and weeds as well as zooplankton and detritus.

The nine pairs of adult anemonefish *A. sebae* having ~ 75 to 106 mm (presumptive females) (Figure 1) size and ~ 47 to 73 mm (presumptive males) in size and sea anemone *Stichodactylahaddoni* (9 no's) were procured from the ornamental fish traders (Mandapam) during 2016 and transported to the laboratory and acclimatized for two weeks and reared three months in one-ton FRB (Fiber-reinforced Plastic) tank for pair formation. After the pair formation each pair were separated to 250 L culture tank with anemone *S. haddoni* to mimic the natural environment. The Anemonefish were fed with different boiled feeds such as green mussels, clam, oyster, polychaetas and Acetes etc., twice in a day. Water quality parameters such as, temperature, salinity, dissolved oxygen, pH and photoperiod (12L: 12D) were optimally maintained during the experiment.

Carbonate chemistry manipulation system

The experimental system was setup by the three experimental tanks, seawater pH was physically controlled by foaming known concentrations of CO_2 (~400, 1000 and > 2000 ppm) as for three diverse pH levels 8.1, 7.7 and 7.3. The CO_2 treatment roughly 400 ppm represented current ambient CO_2 conditions and therefore acted as a control, similarly, other two treatments with lower pH represented predictions for future climate scenarios for the years of 2100 and 2300 respectively (Caldeira andWickett, 2003; IPCC, 2014).

To avoid acquit acidity shock, the experimental time frame was preceded by an acclimation time of 10 days, in which the CO_2 was progressively decreased until the target pCO_2/pH level was reached. The experimental system water quality parameter such as temperature, pH and salinity were estimated and observed by using pH meter and refractometer. During the experiment period, seawater samples were collected weekly to determine the total alkalinity and samples were analyzed by alkalinity titration unit (Metrohm, Switzerland). The measurements of temperature, salinity, pH and alkalinity were used to calculate the values of dissolved inorganic carbon (DIC), pCO_2, HCO_3^-,CO_3^{2-}, ΩCalcite, andΩAragonite. These calculations were made by program CO_2sys.xls (Pierrotet al., 2006).

Breeding and sampling of embryos

The spawning was observed during 3rd month after a brief courtship. Femalelaid clutches of eggs near by the anemone male fish takes the parental care until egg hatching. Parental care includes fanning and mouthing (Fautinand Allen 1992). The 10 egg sample were obtained from nine pairs of each treatment to examinethe different embryonic development stages. The developmental stages were observed from 0 to 172 hours of post fertilization (hpf).

Histology

For histological studies, a piece of testis and ovary tissues were obtained from three different treatments (ambient pH 8.1, pH 7.7 and pH 7.3), the tissues were fixed in 10 % neutral buffered formaldehyde solution for 24 h, then washed numerous times in water before being dehydrated through a graded series of ethanol, washed in xylene and embedded in paraffin wax. Thin pieces (3 µm) were cut with a rotary microtome, stained with Harris haemotoxylin and counter-stained with eosin (Coolidge and Howard, 1979). The stained tissues were examined under a compound microscope.

RESULTS

Seawater Carbon parameter in Anemonefish experiment

The room temperature 27 ± 2.5℃ was maintained at three different pH treatments. The salinity was constantly maintained with mean of 33. 2 ppt during throughout the experiment. The total alkalinity values were ranged between pH 8.1 and 7.3 (Table 1).

Description of male reproductive system of Anemonefish

The male reproductive system of anemonefish comprises a bilobed stomach, with whitish creamy testis, attached to the dorsal part of the abdominal cavity. They are equal in size whereas, some of them are unequal. The milt was slightly viscous and whitish in colour.

Eggs hatch rate

Three pairs of anemonefish egg exposed to the ambient pH (pH 8.1) condition and the egg hatch rate ranged between 89. 9 to 95.8 % (mean= 93.84 ± 1.9 %). Followed by the second group of anemonefish exposed to the pH 7.7 showed the similar egg hatch rate 87.2 to 95.7 % (mean = 92.96 ± 2.4 %). Whereas slight variation was observed in pH 7.3, which showed 86.5 and 90.1 % of egg hatch rate (mean = 88.65 ± 1.3 %) (Figure 2).

Egg hatching time delay stage

The embryo began to hatch by vigorous wriggling movement to breakdown the egg capsule, and tail had wrapped completely around the egg, the breaking point at the caudal half of the capsule was near to the stalk and the hatching emerged tail first. The egg hatches out was found in 172. 00 ± 1.0 hpf in ambient pH (pH 8.1) condition followed by treatment pH 7.7 (174.01 ± 2.0 hpf) and pH 7.3 (178.05 ±2.0 hpf). The six hours' time delay was observed at low pH treatment.

Anemonefish testis

The Ovotestis histological images of *A. sebae* shows the tissue morphology in three different pH conditions (Figure 3). The mature male anemonefish exposed to the ambient pH 8.1 conditions showed all the developmental stages such as spermatocytes, spermatogonia, spermatids and spermatozoa (Figure 3a). Similarly, treatment pH 7.7 (Figure 3b) and pH 7.3 also showed all the developmental stages and they were clearly visible (Figure 3c).

Anemonefish Ovary

The histological images of *A. sebae* ovary tissue assessed in the different pH conditions. The matured female fishes exposed to the different pH experiment contained the post vitellogenic oocytes. The female anemonefish exposed to the ambient pH 8.1 condition exhibited all the significant developmental stages such as post vitellogenic oocytes, vitellogenic oocytes and pre vitellogenic oocytes and they were clearly observed (Figure 4 a). The treatment pH 7.7 and lower pH 7.3 showed the similar developmental stages (Figure 4 b and c).

DISCUSSION

The laboratory experiment scrutinized the impact of pH (7.3 to 8.1) and pCO_2 (325-2570 µatm) condition on anemonefish eggs hatch rate and reproductive tissue damage.The changed pHis adequate for understanding the existing and future (to the year 2300) changes of carbonate chemistry across the anemonefish spawning environment in marine ecosystem.

Egg hatch rate

The experimental results showed (Fig. 2) that the mean egg hatch rate gradually decreased with decreasing pH at end of six weeks experiment. The egg hatch rate reduce slightly in higher pCO_2 treatment while compared to ambient (present day pCO_2) conditions. It seems pH had a substantial effect on the egg hatch rate in anemonefish. Correspondingly the elevated pCO_2 1573 µatm delays the eggs hatching time and young larval size significantly reduced at elevated pCO_2 2108 µatm in yellowfin tuna *Thunnusalbacares* (Bromhead, et al., 2014) Comparable to our observation the increased pCO_2 and elevated temperature reduces the hatching success and slow ontogenetic development in Antarctic dragonfish (Flynn et al., 2015).

The near future ocean acidification slightly impacts the *A. sebae* egg development. These findings are similar to the results of many others studies (Findlayet al., 2009; Eglisdottiret al., 2009). In contrast, the stone crab embryos are tolerant to the low pH condition (Gravinese, 2017) but, noticeable variation found in eggs hatch time delay (24 %). Similar reported also shows that the low pH conditions directly affect the egg hatching time in decapod crustacean (Miller et al.,2016). Gametes, embryos and larvae of vertebrates such as fishes are vulnerable to changes in ocean water chemistry. In lower vertebrate,) observed that the reduced pH levels are not significantly affect the egg hatching success and egg hatch timing in copepod *Calanusglacialis*,but the higher CO_2 concentration showed impact on the egg production rate of copepods(Thor et al.,2018).There is a significant negative effect on the egg hatching success of *Acartiaspinicauda* and *Centropagestenuiremis* in the pCO_2 2000 × 10^{-6} and 10,000 × 10^{-6} groups (Zhanget al., 2011).Their experimental concentration of CO_2 is much greater than those expected to found as a result of anthropogenic inputs of atmospheric CO_2.Earlier studies also shows that the clownfish eggs size impacted by the higher pCO_2treatment (Mundayet al.,2009).

The present study clearly showed that the egg hatching rate was slightly differed between the ambient pH and low pH conditions since the variation is very minimal (5.19 %) whereas invertebrates, egg hatching success of stone crab reduced by 28 % in low pH conditions than control (Gravinese, 2017), in barnacle *S. balanoides* egg hatch rate is highly (60 %) declined in low pH condition (Findlay et al., 2009) and even more declined found in lower invertebrate copepod *Calanusfinmarchicus* (86 %; Mayor et al., 2007). The variation of egg survival in higher CO_2 concentration between vertebrate and invertebrate, probably fishes have a better capacity to regulate the acid-base balance than most invertebrates (Portneret al., 2004; Widdicombe and Spicer, 2008).

Histology

The ocean acidification experiment study was conducted to assess the histology effects on testis, ovary of *A. sebae.* The experimental results demonstrated that developmental stages such as spermatocytes, spermatogonia, Spermatids and spermatozoa of ovotestis were similar between the ambient pH and low pH conditions. Similarly, Ovary developmental stages were also not affected by the lowest pH treatment. However, the higher CO_2 treatment did not affect the growth and survival rate of fresh water rainbow trout *Oncorhynchus mykiss* and also fish skin and gill morphology are not affected by the higher CO_2 treatments (Good et al., 2009) Whereas, the elevated pCO_2 treatments severely impact the cod larvae liver, pancreas, kidney, eye and the gut. These effects are found at 32 days of post hatch of the larvae and the levels of damage are increased with increasing the pCO_2 concentration. In higher pCO_2 treatment liver lipid vacuoles are enlarged in size compare to the control pH. Earlier findings conducted on commercially important fish early life stages showed significant impact by near future ocean acidification condition (Frommel et al., 2012). The present study clearly confirms that CO_2 induced ocean acidification experiment indicate minimal impact on the gills, because, the adult fishes have a very strong acid-base regulation (Portneret al., 2004; Widdicombe and Spicer, 2008), whereas, early life developmental stages are most vulnerable to the near future ocean acidification (Frommel et al., 2012; Findlay et al., 2009).

CONCLUSION

The present experimental study provides a detailed understanding of the slight negative effects on the egg hatch rate and slow ontogenetic development in low pH experiment. If continuously reduced the pH in seawater will have similar effects to the laboratory experiment. Whereas slight tolerance were observed at histology of testis and ovary, therefore experimental study conclude that early development stages are expected to more vulnerable to near future ocean acidification.

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Table 1. Seawater carbonate measured and calculated parameters in clownfish experiment

Measured parameter				
Treatment	**pH (Total)**	**T (°C)**	**Salinity (ppt)**	**AT (µmol Kg^{-1})**
pH 8.1	8.14 ± 0.21	27 ± 2.4	33± 2.0	2495.05 ± 8.95
pH 7.7	7.73 ±0.14	27 ± 2.3	33± 2.0	2473.19 ± 15.48
pH 7.3	7.35 ± 0.11	27± 2.5	33± 3.0	2466.25 ±18.98

Calculated parameter						
Treatment	**DIC (µmol Kg^{-1})**	**pCO2 (µatm)**	**HCO_3 (µmol Kg^{-1})**	**CO_3^2 (µmol Kg^{-1})**	**Ω Calcite**	**Ω Aragonite**
pH 8.1	2105. 14±24.04	325.07 ±21.12	1812.33 ±25.57	283.96 ± 14.19	6.97 ± 1.56	4.60 ±0.98
pH 7.7	2316.09 ±28.12	994.69 ±19.58	2157.51 ± 15.48	131.51 ±22.76	3.23 ±1.05	2.13 ± 0.75
pH 7.3	2453.63 ±19.86	2570.90 ±10.57	2324.60 ±18.64	59.07 ±16.95	1.45 ± 0.72	0.95 ± 0.05

Figure. 1 *Amphiprionsebae*

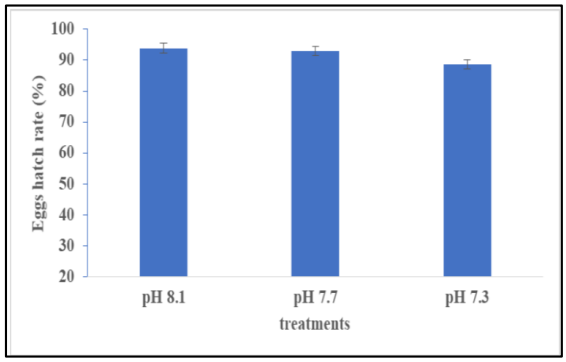

Figure 2. Eggs hatch rate of *A.sebae* in pH 8.1, 7.7 and 7.3 treatment

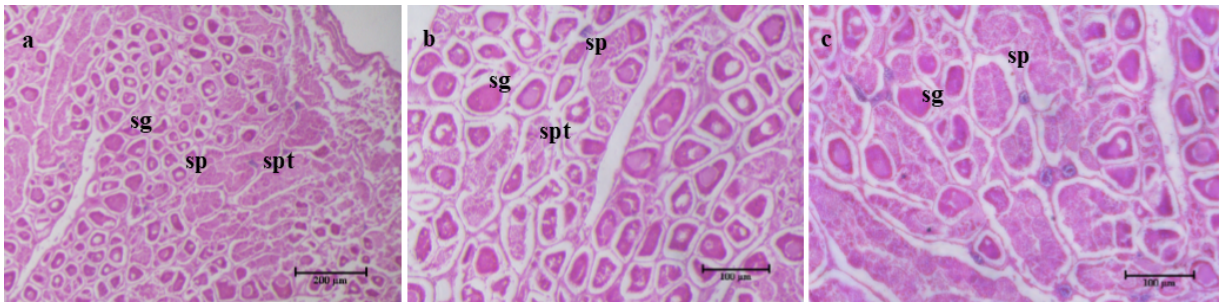

Figure 3. Ovotestis transverse section of *A. sebae* in three different pH treatments: pH 8.1 (a), pH 7.7 (b) and pH 7.3 (c).sp: spermatocytes; spt: spermatids; sg: Spermatogonia s: spermatozoa

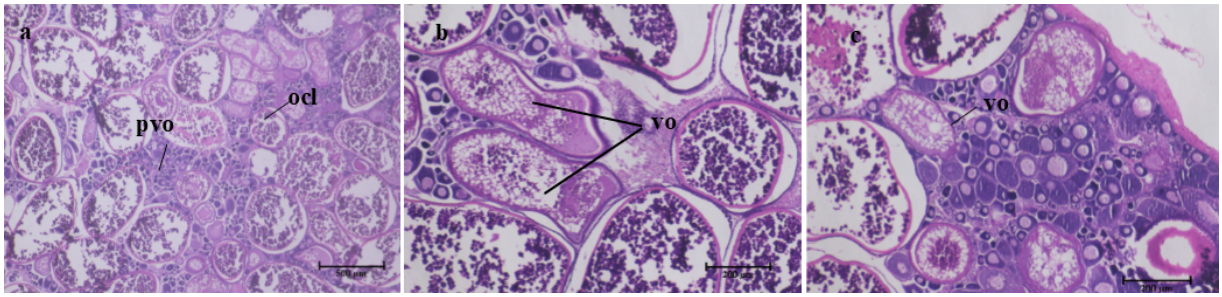

Figure 4. Ovary transverse section of *A. sebae* in three different pH treatments: pH 8.1 (A), pH 7.7 (B) and pH 7.3 (C). pvo: previtellogenic oocyte, vo: vitellogenic oocyte, ocl: ovary central lumen

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RESEARCH ARTICLE

Chemical Composition and Antioxidant Activity of Essential Oil of *Achillea fragrantissima*

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ABSTRACT

The aim of this study was to evaluate the composition of *Achillea fragrantissima* (*A. fragrantissima*) essential oil and to test its antioxidant activity. Gas chromatography–mass spectometry (GC-MS) was used to investigate essential oil composition, the evaluation of the antioxidant activity of essential oil of *A. fragrantissima* was done using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, ferrous radical scavenging activity and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) ferrous radical scavenging activity methods. Sixty four components accounting for 98.23% of the oil were identified. The major identified compounds were α-thujone 18.37%, Santolina alcohol 15.64%, Artemisia ketone 8.11%, β-thujone 7.59%, yomogi alcohol 5.09%, Artemisia alcohol 4.4%, β-sesquiphellandrene 3.08%, Artemisylacetate 2.04%, iso-3-thujanol acetate 1.53%, methyl chavicol 1.57%, α-terpinene 1.47%, 3-methyl-3-butenyl isovalerate 1.32%, α-Terpineol 1.29%, bicyclogermacrene 1.21%, cis-hexenylbutyrate 1.07%. The major oil components were oxygenated monoterpene 70.39%, oxygenated sesquiterpene 11.41%, sesquiterpene hydrocarbon 5.72%, monoterpene hydrocarbon 3.05%, and ester 5.72%. The essential oil of *A. fragrantissima* showed significant antioxidant activity in all three tests.

Key words: *Achillea fragrantissima*, antioxidant, essential oil, GCMS.

INTRODUCTION

Essential oil, the plant secondary metabolites, is a complex mixture of aromatic, volatile, lipid soluble organic compounds having antimicrobial and antioxidant activities through their ability to overcome resistance mechanisms

shown by many pathogens (Predoi et al 2018). Essential oils are widely used in cosmetics, perfumes in foods flavoring and preservation (Bhavaniramya et al., 2019). The genus, *Achillea* L., belongs to Asteraceae (Compositae), the largest family of vascular plants. In Jordan Astreaceae have 91 genera and about 238 species. Several species of this genus are used in traditional medicine in the treatment of many different diseases (Al-Jaber et al.,2018). *A.fragrantissima* which grows in Jordan's desert and dry areas is an aromatic medicinal plant, normally used for the treatment of diabetes, gastrointestinal disturbances including intestinal colic, respiratory diseases, hypercholesterolemia, high blood pressure, parasitic worms, kidney stones, arthritis and various infections (Al-Jaber et al., 2018; Bakr et al., 2014; Hatem et al., 2018, Eissa et al., 2014; Hammad et al 2013).

The aerobic organisms use oxygen to produce ATP through oxidation and reduction reactions. These reactions cause the production of reactive oxygen species (ROS) which seek to be stabilized by stealing electrons from neighboring biomolecules (proteins, carbohydrates, lipids and DNA) causing a chain reactions that may lead to cancer, parkinson, Alzheimer, diabetes and other diseases (Magder, 2006; Finkel and Holbrook, 2000). Antioxidants are substances that inhibit or slow down production of ROS resulting from oxidation of cellular components (Adwas et al., 2019).Antioxidants could be enzymatic or nonenzymatic, natural or synthetic molecules. The natural antioxidants including minerals and vitamins are present in fruits, vegetables and spices (Sindhi et al., 2013). The aim of the present study was to explore the chemical composition of the essential oil from the aerial parts of *A.fragrantissima* collected from Mutah, Alkarak, south Jordan and to evaluate its antioxidant activity.

MATERIALS AND METHODS

Collection and Authentication of Plants

An amount of fresh wild *A. fragrantissima* was collected from Mutah, Alkarak Province, South Jordan, during the flowering period and the vegetative phase. The plant materials were taxonomically identified and authenticated by Professor Saleh Al-Quran, Botanical Survey, Department of Biology, Mutah University.

Isolation of Essential Oil

The collected *A. fragrantissima* was finely chopped and subjected to hydrodistillation for 4 h using a Clevenger-type apparatus, yielding 0.19% (v/wt), pale yellowish oil. Subsequently, the oil was dried over anhydrous sodium sulfate and immediately stored in GC-grade hexane at 4ºC till the time of the analysis by gas chromatography/mass spectrometry (GC/MS) was done (Al-Sarayreh et al., 2015).

Essential Oil Composition
GC–FID analysis

The oil was analyzed in an Agilent (Palo Alto, USA) 6890N gas chromatograph fitted with a 5% phenyl–95% methylsilicone (HP5, 30 m × 0.25 mm × 0.25 μm) fused silica capillary column. The oven temperature was programmed to run from 60°C to 240°C at 3°C/min with hydrogen being used as the carrier gas (1.4 ml/min). 1.0 μl of a 1% solution of the oils in hexane was injected in split mode (1:50). The injector and the flame ionization detector (FID) were kept at 250°C and 280°C, respectively. Concentrations (% contents) of the oil ingredients for *A. fragrantissima* were determined using their relative area percentages obtained from GC chromatogram, assuming a unity response by all components (Al-Sarayreh et al., 2015).

GC–MS analysis

Chemical analysis of the essential oil was carried out using gas chromatography–mass spectrometry (Agilent (Palo Alto, USA) 6890N gas chromatograph). The chromatographic conditions were as follows: column oven program, 60ºC (1 min, isothermal) to 246ºC (3 min, isothermal) at 3ºC/min, the injector and detector temperatures were 250ºC and 300ºC, respectively. Helium was the carrier gas (flow rate 0.90 ml/min) and the ionization voltage was maintained at 70eV. A HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thicknesses) was used. A hydrocarbon mixture of *n*-alkanes (C_8-C_{20}) was analyzed separately by GC-MS under same chromatographic conditions using the same HP-5 column. Kovats Retention Indexes (KRIs) were calculated by injection of a series of *n*-alkanes (C_8–C_{20}) in the same column and conditions as above for gas chromatography analyses. Identification of the oil components was based on computer search using the library of mass spectral data and comparison of calculated Kovats retention index (KRI) with those of the available authentic standards and literature data (Al-Sarayreh et al., 2015).

Antioxidant tests

DPPH· free radical scavenging activity

The total radical scavenging capacity of the obtained essential oil was determined and compared to those of the positive controls (ascorbic acid and α-tocopherol) according to the procedure described in Al-Qudah et al (2017). Briefly, a 1.0 ml sample of various concentrations (0.005 - 0.50 mg/ml) of the tested essential oil (dissolved in methanol) was added to 2 ml of 0.1 mM DPPH• methanolic solution. The solutions were allowed to stand at room temperature in the dark for 30 min, then, the absorbance of each solution was measured at 517 nm using a UV- visible spectrophotometer. All determinations were performed in triplicate. The ability to scavenge the DPPH• radical was calculated using the following equation:

DPPH• scavenging effect (%) = (A_c - A_s) /(A_c) *100 %

Where A_C is the absorbance of the blank and A_S is the absorbance of the tested solution. The IC_{50} was determined from the sigmoidal curve obtained by plotting the percentages of DPPH scavenging relative to the control versus logarithmic concentration of test compound using nonlinear regression analysis of GraphPad Prism 6 (GraphPad Software, San Diego, California, USA). Each concentration was tested three times in 3 independent experiments (Al-Qudah et al., 2017).

ABTS radical scavenging assay

The total antioxidant activity, measured by the radical cation 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) decolorization assay method, was evaluated according to the procedure described by Al-Qudah et al. (2017). The $ABTS^{\bullet+}$ cation radical solution was prepared by reacting similar quantities of 7 mM of ABTS and 2.4 mM of potassium persulfate (K2S2O8) solutions for 16 h at room temperature in the dark. Before use, this solution was diluted with methanol until an absorbance of 0.75 ± 0.02 at 734 nm was obtained. The reaction mixture comprised 3 ml of ABTS•+ solution and 1 ml of the essential oil solutions at various concentration (0.005 - 0.50 mg/ml). The absorbance's of all prepared solutions, including the blank sample, were measured at 734 nm using a UV- visible spectrophotometer and after at least 5 minutes of incubation. The ABTS scavenging capacity of the essential oils was compared with that observed for ascorbic acid and α-tocopherol (the positive controls). The percentage inhibition was calculated according to the equation:

ABTS radical scavenging activity (%) = (A_{blank} – A_{sample} /A_{blank}) × 100 %

Where A_{blank} is the absorbance of the blank solution and A_{sample} is the absorbance of the remaining $ABTS^{\bullet+}$ solutions in the presence of the scavenger. The IC_{50} was determined from the sigmoidal curve obtained by plotting the percentages of $ABTS^{\bullet+}$ scavenging relative to the control versus logarithmic concentration of test compound using non-linear regression analysis of GraphPad Prism 6 (GraphPad Software, San Diego, California, USA). Each concentration was tested three times in 3 independent experiments (Al-Qudah et al., 2017).

Ferrous radical scavenging assay

The ability of the essential oils and the control antioxidants to chelate ferrous ion from the formation of ferrozine-Fe^{2+} complex was determined as recently described in our publication Al-Qudah et al. (2017) with some modifications. Briefly, a 3 ml of methanol solution containing the different concentrations of the tested essential oil (0.005 - 0.50 mg/ml) was added to a 0.25 ml of 2 mM $FeCl_2$. Subsequently, a 0.2 ml of 5 mM ferrozine solution was added to the mixture and allowed to stand at room temperature for 10 min after vigorous shaking. The reduction in the absorbance of the red color was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation by each concentration of the oil was calculated relative to the control lacking the test material using the same equation above. The IC_{50} for chelating Fe^{2+} was determined from the sigmoidal curve obtained by plotting the percentages of chelating Fe^{2+} vs. the logarithmic concentration of the test compound (in g/ml) using the non-linear regression analysis of the GraphPad Prism 6 as described above. The chelating activity test was conducted in triplicate for each concentration of the essential oil in each of the three independent experiments (Al-Qudah et al., 2017).

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oil of *A. fragrantissima*

Table 1 shows the sixty four identified components of *A. fragrantissima* essential oil, their percentages and retention indices using GC-MS techniques. Al-jaber et al. (2018) reported that the essential oil content of *A. fragrantissima* from Jordan were α-thujone (25.88%), santolina alcohol (19.70%), β-thujone (17.20%), artemisia ketone (11.68), and β-sesquiphellandrene (9.70%). While the major components including monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons were 2.94%, 83.01% and 11.97%, respectively. Those results are close to the obtained results in the present study. Aqel et al. (2012) reported that the identified components of essential oil of *A.fragrantissima* from Jordan were 4-terpineol (15.65%), Linalool (11%), carvone (9.42%), β-phellandrene (6.2%), γ-terpinene (5.6%), β-pinene (4.55%), verbenone (4.42%), cedrol (3.0%) and ϱ-cymene (2.95%). Hatem et al. (2018) showed that the major contents of the essential oil of *A. fragrantissima* from Lebanon were Artemisia ketone (29.97%), α-Thujone (13.34%), Germacrene (11.5%) followed by α-Cubebene (6.25%), Spathulenol (3.63%),β-Sesquiphellandrene (3.52%), γ-Muurolene (3.27%), the oxygenated monoterpenes formed (56.66%), sesquiterpene hydrocarbons (30.58%), oxygenated sesquiterpenes 10.23%, and Monoterpene hydrocarbons 1.23%.

The major components of *A.fragrantissima* from Egypt were *Santolina triene* (1.97%), 2,5,5-trimethyl-3,6-heptadien-2-ol (8.23%) Eucalyptol 8.17, trans-2,7-Dimethyl-4,6octadien-2-ol (24.40%), 1,5-Heptadien-4-one-3,6-trimethyl (7.65%), Artemisia alcohol (3.49%), à Thujone (33.97%), Cissabinol (1.92%), Lavandulol (0.71%), 2-Octen-4-ol, 2-methyl (2.02%), 3-Cyclohexen1ol,4-methyl1 (1 methylethyl) (CAS) (2.15%), à terpineol (0.05%), Estragole (0.71%) Lavandulyl acetate (0.49%), Sabinyl acetate (2.12 %) and Germacrened (0.94%) (Zeedan et al. 2014). The chemical composition of the essential oil depends on plant genetics, nutritional status, climatic conditions, type of the plant organs beside time and harvesting season. Thus, these factors influence the chemical composition of essential oil through inducing the synthesis of certain molecules by the plant (Stevanovic et al., 2018).Table 1.

Antioxidant activity evaluation

DPPH, ABST and Ferrous radical scavenging assays were used for evaluating the antioxidant activity of essential oil of *A. fragrantissima*. The results of the three tests are shown in figures 1, 2 and 3. At concentration of 0.10 mg/ml *A. fragrantissima* essential oil exhibited a significant antioxidant activity where it is able to inhibit 83% of DPPH radical, 80% of ABTS radical and 78% of Ferrous radical. The antioxidant activity of essential oil relies on its composition. Terpenes and phenolic compounds play a major role in free radical scavenging activity (Wozniak et al., 2019). In the present study terpenes are the major constituents of *A. fragrantissima* essential oil. Plants produce these compounds and others as defense mechanisms against ultraviolet light and radiation (Torres-Martinez et al., 2017). Table 2 shows the results of the half maximal inhibitory concentration (IC50) for *A. fragrantissima* essential oil required to inhibit 50% of ROS. The calculated IC_{50} for *A. fragrantissima* essential oil for DPPH, ABTS and ferrous radical scavenging are less than those of the positive controls.Table 2. *A.fragrantissima* essential oil could be used as natural antioxidant to prevent the oxidative stress and as additives in the food supplements.

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Table 1. Constituents (%) of the essential oil of *A.fragrantissima* grown in South Jordan

Tr	KI	Compound	% A	Tr	KI	Compound	% A	Tr	KI	Compound	% A
4.072	902	Santolina triene	0.64	6.875	1220	trans-pulegol	0.24	9.4	1475	E-ethyl cinnamate	0.66
4.425	942	α-pinene	0.25	6.99	1231	3Z-Hexenyl-2-methylbutanoate	0.1	9.518	1487	6-nonyl-5,6-dihydro-2,4-pyran-2-one	0.27
4.775	980	Sabinene	0.69	7.047	1236	tetrahydro linalool acetate	0.14	9.598	1495	Germacrene D	0.65
4.929	998	yomogi alcohol	5.09	7.125	1244	methyl ether carvacrol	0.17	9.641	1499	bicyclogermacrene	1.21
5.046	1011	Isoamyl isobutyrate	0.42	7.32	1264	piperitone	0.65	9.742	1509	α-muurolene	0.27
5.166	1024	α-terpinene	1.47	7.43	1275	iso-3-thujanol acetate	1.53	9.792	1514	α-alaskene	0.34
5.297	1039	Santolina alcohol	15.64	7.546	1286	trans-sabinyl acetate	0.16	9.99	1534	β-sesquiphellandrene	3.08
5.509	1065	Artemisia ketone	8.11	7.827	1314	trans-2-tert-butyl cyclohexanol acetate	0.13	10.294	1564	Elemol	0.18
5.625	1079	Artemisia alcohol	4.4	7.883	1320	4-hydroxy-cryptone	0.08	11.147	1649	cubenol	1.58
5.816	1101	2-methylbutyl isovalerate	0.49	7.945	1326	myrtenyl acetate	0.09	11.217	1656	Z-methyl jasmonate	0.9
5.903	1111	3-methyl-3-butenyl isovalerate	1.32	8.144	1347	Benzyl butanoate	0.27	11.336	1668	7-epi-α-eudesmol	0.26
6.038	1127	α-thujone	18.37	8.24	1357	Eugenol	0.27	11.527	1687	cis-14-nor-muurol-5-en-4-one	2.75
6.113	1135	β-thujone	7.59	8.425	1377	Z-Ethyl cinnamate	0.22	11.717	1705	11-αH-himachal-4-en-1-β-ol	1.33
6.226	1148	iso-3-thyjanol	0.32	8.526	1387	thujic acid	0.12	11.767	1709	cis-thujopsenal	0.49
6.274	1154	Cis-Sabinol	0.24	8.643	1399	Z-Jasmone	0.96	11.817	1714	Z-Nuciferal	0.15
6.339	1161	Artemisylacetate	2.04	9.143	1449	Z-β-Farnesene	0.17	12.035	1734	cuparenal	0.3
6.379	1166	Cis-α-Necrodol	0.87	6.765	1209	methyl chavicol	1.57	12.311	1760	α-amyl cinnamyl acetate	0.14
6.416	1170	Terpinen-4-ol	0.89	10.543	1589	globulol	0.26	12.456	1773	squamulosone	0.23
6.525	1183	Iso-pinocampheol	0.52	10.68	1602	longiborenol	0.49	12.586	1785	γ-Eudesmol acetate	0.16
6.577	1189	cis-hexenylbutyrate	1.07	10.823	1616	β-oplopenone	0.97	12.728	1798	callitrin	0.6
6.64	1196	α-Terpineol	1.29	10.95	1629	1-epi-cubenol	0.87	14.204	1946	phytol	0.71
14.329	1958	11-acetoxyeudesman-4-α-ol	0.79								
Monoterpene hydrocarbon			3.05								
Sesquiterpene hydrocarbon			5.72								
Oxygenated Sesquiterpene			11.41								
Ester			5.72								
Other			1.23								

Table 2. The IC50 antioxidant activity of *A.fragrantissima* essential oil and the positive controls (ascorbic acid and α-tocopherol) determined by DPPH, ABST and metal ion chelating assays

	IC 50		
Name of compound	**DPPH**	**APTS**	**Ferrous**
EO of A. fragrantissima	0.00373 ± 1.12X10-4	0.01067 ± 0.00214	0.01522 ± 5.79X10-4
α-Tocopherol	0.0023 ± 1.70 * 10-5	0.00177 ± 4.71*10-5	0.00293 ± 2.02 * 10-5
Ascorbic acid	0.00178 ± 2.30 * 10-6	0.00155 ± 4.71 *10-5	0.00189 ± 4.72 * 10-5

The IC_{50} values were obtained from the generated sigmoidal curves of plotting the mean percentages of scavenging activity vs. logarithmic concentrations of *A. fragrantissima* essential oil (in g/ml) using non-linear regression analysis of GraphPad Prism 6 software. The results are expressed as the IC_{50} values (mg/ml) from three independent experiments performed in triplicates.

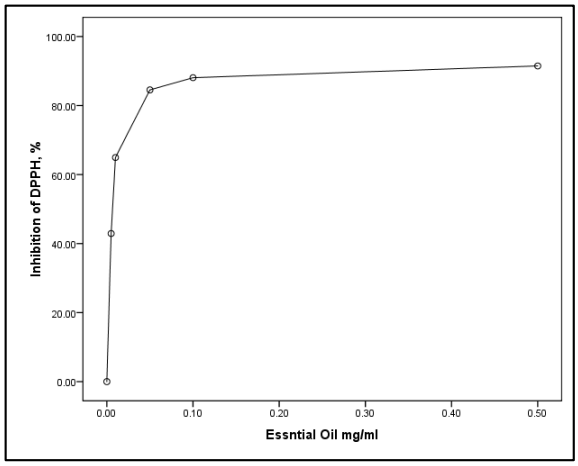

Figure 1. Antioxidant activity DPPH radical scavenging ability of *A. fragrantissima* essential oil

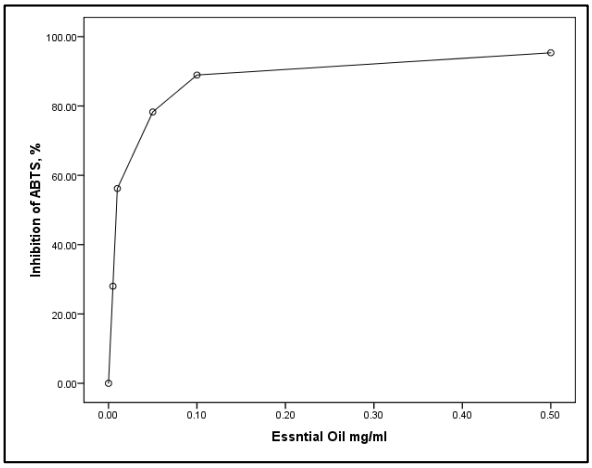

Figure 2. Antioxidant activity ABTS radical scavenging ability of *A. fragrantissima* essential oil

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REVIEW ARTICLE

Review on Microbial Conversion of Agrowastes to Value Added Products

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ABSTRACT

Lignocellulosic materials are the most promising feedstock as natural and renewable resource essential to the functioning of modern industrial societies. A considerable amount of such materials as waste byproducts are being generated through agricultural practices mainly from various agro based industries. Sadly, much of the lignocellulosic biomass is often disposed of by burning, which is not restricted to developing countries alone. Recently lignocellulosic biomasses have gained increasing research interests and special importance because of their renewable nature. Therefore, the huge amounts of lignocellulosic biomass can potentially be converted into different high value products including bio-fuels, value added fine chemicals, and cheap energy sources for microbial fermentation and enzyme production.

Keywords: Microbialconversion, agrowastes, value added products.

INTRODUCTION

Physico-chemical characteristics of lignocellulosic biomass

All plant materials are mostly composed of three major units i.e., cellulose, hemicellulose and lignin. Lignocellulosic materials including agricultural wastes, forestry residues, grasses and woody materials have great potential for bio-fuel production. Typically, most of the agricultural lignocellulosic biomass is comprised of about 10e25% lignin, 20e30% hemicellulose, and 40e50% cellulose (Iqbal, Ahmed, Zia, & Irfan,2011; Kumar, Barrett, Delwiche, &Stroeve, 2009; Malherbe &Cloete, 2002). Cellulose is a major structural component of plant cell walls, which is responsible for mechanical strength while, hemicellulose macromolecules are often repeated polymers of pentoses and hexoses. Lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl alcohol and pcoumaryl alcohol) produced through

a biosynthetic process and forms a protective seal around the other two components i.e., cellulose and hemicelluloses (Fig. 1) (Calvo-Flores &Dobado, 2010; Jiang, Nowakowski, &Bridgwater, 2010; Menon&Rao, 2012). In general the composition of lignocellulose highly depends on its source whether it is derived from the hardwood, softwood, or grasses.

Physical and structural properties of cellulose

Cellulose is a highly stable polymer consisting of glucose and attached with linear chains up to 12,000 residues. It is majorly composed of (1,4)-D-glucopyranose units, which are attached by b-1,4 linkages with an average molecular weight of around 100,000 (Himmel et al., 2007). Plant biomass contain 40e50% of cellulose molecules which are held together by intermolecular hydrogen bonds in native state, but they have a strong tendency to form intra-molecular and intermolecular hydrogen bonds and this tendency increases the rigidity of cellulose and make highly insoluble and highly resistant to most organic solvents. Naturally cellulose molecules are exists as bundles which aggregated together in the form of micro-fibrils order i.e., crystalline and amorphous regions (Iqbal et al., 2011; Taherzadeh&Karimi, 2008).

Physical and structural properties of hemicelluloses

Hemicellulose is the second most abundant heterogeneous polymers that mainly consist of glucuronoxylan, glucomannan and trace amounts of other polysaccharides. Grasses and straws contain arabinan, galactan and xylan, while mannan is a component of hardwood and softwood hemicellulose (Brigham, Adney, &Himmel, 1996). They are catalogued with sugar as a backbone, i.e., xylans, mannans and glucans, with xylans and mannans being the most common (Wyman et al., 2005). Galactans, arabinans and arabinogalactans are included in the hemicellulose group; however, they do notshare the equatorial b-1,4 linked backbone structure. In hardwoods, glucuronoxylan (O-acetyl-4-O-methyl-glucurono-b-Dxylan) is the predominant component. Xylospyranose is the backbone of the polymer and connected with b-1,4 linkages. Hemicellulosic biomass contains 25e35% of hemicellulose, with an average molecular weight of <30,000. Cellulose and hemicellulose binds tightly with non-covalent attractions to the surface of each cellulose micro-fibril. Hemicelluloses were originally believed to be intermediates in the biosynthesis of cellulose (Vercoe, Stack, Blackman, & Richardson, 2005).

Physical and structural properties of lignin

Lignin is generally the most complex and smallest fraction, representing about 10e25% of the biomass by weight. It has a long-chain, heterogeneous polymer composed largely of phenyl-propane units most commonly linked by ether bonds. Lignin acts like a glue by filling the gap between and around the cellulose and hemicellulose complexion with the polymers. It is present in all plant biomass; therefore, it is considered byproduct or as a residue in bio-ethanol production process. Lignin is comprised of complex and large polymer of phenyl-propane, methoxy groups and noncarbohydrate poly phenolic substance, which bind cell walls component together (Hamelinck, Hooijdonk, &Faaij, 2005). Phenyl-propanes (3 carbons attached with 6 carbon atom rings) are main block of lignin. These phenyl-propanes denoted as 0, I, II methoxyl groups attached to rings give special structure I, II and III. These groups depend on the plant source which they are obtained. Structure I exist in plants (grasses) and structure II found in the wood (conifers) while structure III present in deciduous wood.

Role of pre-treatment

Pre-treatment is an important step for the recovery of cellulosic content from lignin based biomass as compare to the starchy materials. While dealing with lignocellulosic biomasses, pre-treatment is also required to break down the lignin barrier to recover cellulose, which is further subjected to enzymatic hydrolysis to convert into fermentable

sugars. During the past few decades, several pre-treatmentapproaches have been developed for generating cost-effective fermentable sugar from most of the agricultural cellulose and hemicellulose containing lignocellulosic materials (Yang & Wyman, 2008). In this background, there are a number of reports on pre-treatment technologies for a variety of feedstocks. Some of the most promising pre-treatment categories have already been commercialized for the productions of bio-energy are summarized in the Table 2. An effective pretreatment is characterized by several criteria: preserving hemicellulose fractions, to yield maximum fermentable sugar contents, limiting the loss of carbohydrate, to minimize the formation of inhibitors due to degradation products,

Chemical pre-treatments

To date chemical pre-treatment is the most studied technique among various pre-treatment categories that was originally developed and therefore has extensively been used for delignification of cellulosic materials. Chemical hydrolysis is an important treatment method for recovery of sugar monomers from cellulose and hemicellulose polymers from lignocellulosic biomass by optimizing chemical reagents. The most commonly used chemical pre-treatments include: acid and alkali based hydrolysis approaches.

Acid based hydrolysis

Chemical treatment of cellulosic biomass with concentrated hydrochloric acid or sulphuric acid is conventional procedure. The entire process of pre-treatment can be operating at very low temperature as compared to dilute-acid pre-treatment. On the other hand one of the possible drawbacks of this process is that it's required in higher concentration (30e70%), therefore cause high level of corrosive reaction. In this background the whole process needs further expenditure in the form of specialized non-metallic or non-corrosive material such as ceramic or carbon-brick lining. In comparison to the other pre-treatment procedures particularly dilute-acid hydrolysis the environmental hazards and high operating cost involved in concentrated-acid hydrolysis reduce the interest on industrial scale (Katzen, Madson, &Monceaux, 1995; Wyman 1999). Dilute-acid pre-treatment has some advantages over concentrated-acid hydrolysis to solve the issues like acid recovery, toxicity, acid and special maintenance against corrosion materials (Sivers&Zacchi, 1995; Sun & Chen, 2007). Acid pre-treatment has been applied on several biomass feed-stocks like herbaceous material (grass), hardwoods and agricultural wastes. Most of the substrates give better results by solubilizing the hemicellulose (Liao, Liu, Wen, Frear, & Chen, 2007; Wyman et al., 2005). Other two factors including temperature and incubation time had also important impact on alteration the structure of biomass. Major disadvantage of this process is the formation of secondary Products which can lower the yield of sugars due to conversion of products in to furfural and hydroxyl-methyl furfural compounds and these compounds interfere in bioethanol Fermentation process.

Alkali based hydrolysis

Alkali based pre-treatment involves the use of bases, such as sodium and ammonium hydroxide, for the pre-treatment of agricultural lignocellulosic feed-stocks. Alkaline hydrolysis causes various structural alterations inside the lignocellulosic material during treatment process such as the depletion of lignin barrier, cellulose swelling, and partial decrystallization and solvation of cellulose and hemicelluloses, respectively (Cheng et al., 2010; Ibrahim, El-Zawawy, Abdel-Fattah, Soliman, &Agblevor, 2011; Sills & Gossett, 2011; Zhu, Wan, & Li,2010). Lignocellulosic feed-stocks that have been shown to benefit from the method of alkaline pre-treatment are corn stover, switch-grass, bagasse, wheat, and rice straw (Hu, Wang, & Wen, 2008; Zhao, Wang, Zhu, Ragauskas, & Deng, 2008; Zhu, Sheng, Yan, Qiao, &Lv, 2012). Zhao et al. (2008) had showed the effectiveness of sodium hydroxide pretreatment for hardwoods, wheat straw, switch-grass, and soft-woods with less than 26% lignin content.

Biological pre-treatment

Biological pre-treatment employs wood degrading microorganisms, including white rot fungi (WRF), brown or soft-rot fungi, and bacteria to modify the chemical composition and or structure of the lignocellulosic biomass. Bio-delignification is useful for pr treatment purposes because it replaces or supplements the chemical-based pre-treatments, which include mechanical treatment with acid, alkali, and steam explosion (Iqbal et al. 2013). In spite of this biological pretreatments are more effective, economical, eco-friendly and less health hazardous as compare to the physico-chemical or chemical-bas pre-treatment approaches. Therefore, from the last few years research scientists are directing their interests towards biological delignification. Recent advances in the characterization of ligninolytic enzymes involving the degradation of lignin have given new impetus to the research in this area, which has now become amenable to biotechnological exploitation (Asgher, Iqbal, &Asad, 2012).

Bioconversions of lignocellulosic materials to useful products normally require multi-step processes that include pretreatment, enzymatic hydrolysis, and fermentation (Xiao et al., 2012), so that the modified or pre-treated biomass is more amenable to enzyme digestion. Increasing understanding of termites and fungal systems has provided insights for developing more effective pre-treatment technologies to realize the above mentioned advantages or benefits of biological pre-treatment over some others. However, biological pre-treatment is a very slow process that also requires careful control of growth conditions and large amount of space to perform treatment. In addition to this most of the lignolytic microorganisms solubilize consume not only lignin but also hemicellulose and cellulose. Because of these drawbacks limitations the biological pre-treatment faces technoeconomic barriers and therefore is less attractive commercially (Eggeman&Elander, 2005).

Enzymes production

To date, the production of various ligninolytic enzymes including LiP, MnP, versatile peroxidise (VP), and laccases and other lignocellulolytic mainly endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and b-glucosidases (EC 3.2.1.21) have been widely studied in submerged and solid culture processes in the laboratory, ranging from flask shake to large scale (Elisashvili, Penninckx, Kachlishvili, Asatiani, &Kvestiadze, 2006; Moldes, Bustos, Torrado, & Dominguez, 2007; Xia & Len, 1999). There are large numbers of microorganisms capable for degrading cellulose (Jungebloud et al., 2007) Trichoderma, Aspergillus, Penicillium and Fusarium species are commonly used for cellulases production (Iqbal et al., 2011). Selections of desired fungal strains depend on several factors and selection of substrate for optimizing the cellulase producing conditions (Shazia, Bajwa, &Shafique, 2007). Ligninolytic, cellulases and hemicellulases are important industrial enzymes having numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Asgher, Ahmed, &Iqbal, 2011; Asgher&Iqbal, 2011; Eun, Beauchemin, Hong, & Bauer, 2006; Iqbal&Asgher, 2013; Iqbal et al., 2011; Irshad et al., 2013; Oberoi, Chavan, Bansal, &Dhillon, 2010; Papinutti&Forchiassin, 2007; Papinutti&Lechner, 2008; Stoilova, Krastanov, &Stanchev, 2010; Yoon, Ngoh, & Chua, 2012). A range of differentlignocellulosic materials that has successfully been adopted for the production of different enzymes having industrial importance are summarized in Table 1.

Enzymatic hydrolysis

Enzymatic hydrolysis is an effective and economical method to achieve fermentable sugars under mild and eco-friendly reaction conditions from the pre-treated cellulosic biomass (Wyman et al., 2005). The entire process of enzymatic hydrolysis critically depends on variety of factors viz., pH, time, temperature substrates and enzyme activities, etc. Enzymatic saccharification is done separately from fermentation known as separate hydrolysis and fermentation (SHF). When cellulose hydrolysis and fermentation are carried out simultaneously the phenomenon is known as simultaneous saccharification and fermentation (SSF). Now a days this process of simultaneous

saccharification of both cellulose and hemicellulose is achieved by co-fermentation of both hexoses and pentoses sugars (SSCF) with the help of genetically engineered microbes that ferment xylose and glucose in the same medium where both enzymes for cellulose and hemicelluloses are available. The major advantage of this technology is that SSF and SSCF can be performed in the same tank which makes the entire process cheap, feasible and costeffective (reduce the capital and operational investment). Biological, physical and chemical methods have been employed for detoxification (removal of inhibitory compounds in fermentation) of lignocellulosichydrolyzates (Olsson & Hahn-Hagerdal, 1996). Lignocellulosic materials have different degree of inhibition and tolerance levels vary according to different microbial strains. Degree of tolerance varies with different strains of *Saccharomyces cerevisiae*, so inhibitory compounds are detoxified by changing the substrate concentration and altering the pH of media (Linden, Peetre, & Hahn-Hagerdal, 1992; Palmqvist, Galbe, & Hahn- Hagerdal, 1998).

Fermentation strategy

Ethanol production from biomass is mainly categorized into three steps process (1) achieve a fermentable sugars (2) conversion of fermentable sugars into ethanol and (3) ethanol separation and purification through distillation (Asgher, Shahid, Kamal, &Iqbal, 2014; Demirbas, 2005). Difference between lignocelluloses or starch ethanol production is the step for obtaining sugars before fermentation. Sugar crops or starchy crops nee milling and grinding for recovery of sugars by extraction and fermentation becoming a relatively simple process that requires no hydrolysis or pre-treatment steps for obtaining sugars and transformation into ethanol (Icoz, Tugrul, Saral, &Icoz, 2009). Bio-ethanol production is mainly done by fed-batch process and low ethanol produce by multistage continuous fermentation. Basic steps for the conversion of lignocellulosic biomass are: (1) pre-treatment process which can reduce the lignin content and render cellulose and hemicellulose content for enzymatic hydrolysis (2) steps to convert enzymatic hydrolysis to break down polysaccharide to simple sugars (3) conversions of sugars (hexoses andpentoses) for ethanol production through microorganisms (4) production of ethanol from pentose sugars. Fig. 1 illustrating a step by step procedure to convert lignocellulosic biomass into ethanol. Several fungal species belonging with genera Fusarium, RhizopusMonilia, Neurospora and Paecilomyces have been found potential for fermenting glucose as well as xylose (Singh, Kumar, &Schugerl, 1992). Production of bio-ethanol from cellulose is mostly conducted by using fermentative organism, but the conversion rate is very low due to byproducts formation. Filamentous fungus Fusariumoxysporum is also known for the production of bio-ethanol through SSF by direct utilizing the cellulose, but their conversion rate is low due to production of acetic acid as a byproduct (Panagiotou, Villas- Boas, Christakopoulos, Nielsen, & Olsson, 2005).

CONCLUSION

Concluded remarks and future outlook

The energy and environmental crises which the modern world is experiencing is forcing to re-evaluate the efficient utilization or finding alternative uses for natural, renewable resources, using clean technologies. In this regard, lignocellulosic biomass holds considerable potential to meet the current energy demand of the modern world. This is also essential in order to overcome the excessive dependence on petroleum for liquid fuels. Further advanced biotechnologies are crucial for discovery, characterization of new enzymes, and production in homologous or heterologous systems and ultimately lead to low-cost conversion of lignocellulosic biomasses into bio-fuels and bio-chemicals. In current scenario future trends are being directed to lignocellulose biotechnology and genetic engineering for improved processes and products. To overcome the current energy problems it is envisaged that lignocellulosic biomass in addition of green biotechnology will be the main focus of the future research.

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Table 1. List of various lignocellulosic materials used for the production of different microbial enzymes(Iqbal et al.2013)

Lignocellulosic material	Pre-treatment type	Microbial culture	Enzymes produced	Reference
Sugar cane bagasse	Biological/chemical	*P. chrysosporium*; *T. versicolor*; *Trichodermaviride*; *P. Sanguineus*; *Trichodermaviride*	MnP, LiP, laccase, cellulasesxylanase	El-Nasser, Helmy, and El-Gammal, 1997;El-Gammal, Kamel, Adeeb, and Helmy, 1998;Kansoh, Essam, and Zeinat 1999;Irshad et al., 2012, Irshad et al., 2012, Yoon et al., 2012 and Irshad et al., 2013
Orange peel waste	Chemical	*Trichodermaviride*	Endoglucanase, exoglucanase, β-glucosidase	Irshad et al., 2012 and Irshad et al., 2013
Corn cobs	Biological	*Trametesversicolor*; *P. chrysosporium*; *Aspergillusniger*	MnP, LiP, laccase, protease, xylanase	El-Nasser et al., 1997; Ahmed et al., 2011, Iqbal et al., 2011, Asgher and Iqbal, 2011, Asgher et al., 2012 and Asgher et al., 2012
Corn stover	Biological/chemical	*P. chrysosporium*; *T. versicolor*; *Penicilliumdecumbens*	MnP, LiP, laccase, cellulasexylanase,	El-Nasser et al., 1997; Yang et al., 2001, Iqbal et al., 2011 and Asgher et al., 2011
Rice Straw	Biological/chemical	*P. chrysosporium*; *T. versicolor*; *Trichodermareesei*	MnP, LiP, laccase, cellulase	Eun et al., 2006, Iqbal et al., 2011 and Asgher et al., 2011
Penut shells	Biological	*G. leucidum*; *P. chrysosporium*	Laccase, xylanase	El-Nasser et al., 1997; Irshad, Bahadur, et al., 2012
Newspaper	Chemical	*Trichodermaviride*	Endoglucanase, exoglucanase, β-glucosidase	Irshad et al., 2013
Wheat straw	Biological/ chemical	*P. chrysosporium*; *T. versicolor*; *T. viride*; *F. trogii*; *L. edodes*; *P. dryinus*; *P. tuberregium*	MnP, LiP, laccase, cellulasesxylanase	El-Nasser et al., 1997; Kachlishvili, Penninckx, Tsiklauri, and Elisashvili, 2006; Elisashvili, Kachlishvili, and Penninckx, 2008; Iqbal et al., 2011, Asgher et al., 2011 and Irshad et al.,

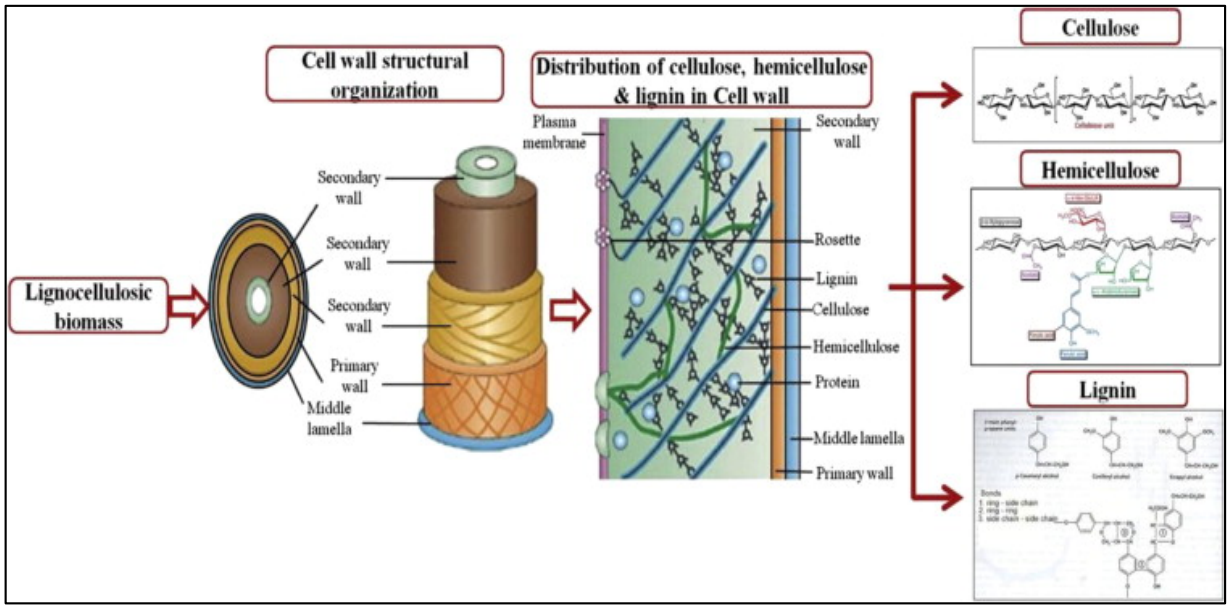

Figure. 1.Diagrammatic illustration of the framework of lignocellulose; cellulose; hemicellulose and lignin (Menon & Rao, 2012).

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RESEARCH ARTICLE

Molecular Diagnosis of Aminoglycoside Transferase Genes in Methicillin - Resistant *Staphylococcus aureus* (MRSA) Strains

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ABSTRACT

Resistance mechanism of aminoglycoside is the inactivation of enzyme action (aminoglycoside modifying enzymes (AMEs) for example. aminoglycoside phosphotransferase, acetyltransferase and nucleotidyltransferas) by the genes in plasmids or transposons. *aacA-aph D*coded by *aac(6')*gene, it is the most commonly encountered AMEs in *staphy.aureus*. Other AMEs are *aph(3")-IIIa* (phosphotransferases) coded by *aph(3')-III*agene. Fifty isolatesfrom clinical specimens collected from the major three hospitals in Najaf /Iraq. The isolates were examined and identified by as standard biochemical testand confirmed by anautomated Vitek system. Antimicrobial susceptibility test was done on 6 antibiotics by the disk diffusion method. All of the genes were investigated by polymerase chain reaction (PCR) methods. Fifty MRSA strains samples were determined by the polymerase chain reaction (PCR) methods, for the detection of genes (three types): *aacA-aphD*, *aadD* and *aph (3") - IIIa.* The obtained results were correlated genotyping with the susceptibility of the antibiotics (gentamicin, tobramicin, kanamicin, neomicin, amikacin and netilmicin). *aacA-aphD, aaadD* and *aph(3") – IIIa* genes (positive strains) were found in 34, 27 and 22strains respectably. A high rate of aminoglycoside resistance was determined in methicillin - resistant *staphylococci.* The *aacA-aphD*was the most commonly detected.

Keywords: MRSA, Aminoglycoside resistance, PCR and *Staphylococci.*

INTRODUCTION

Resistance to aminoglycoside antibiotics in bacteria can be the result of several different mechanisms (1) such as (1) Synthesis of enzymes from the group of transferases (acetyltransferases, phosphotransferases, nucleotidy ltransferases) modifying the antibiotic molecule (2). Mutations in genes coding for ribosomal proteins most often

involving S12 protein and associated with high levels of resistance to streptomycin (3). Lack of enzymes responsible for the active transport of aminoglycosides into the bacterial cell (small colony variant (SCV) in *S.aureus*, *Streptococcus* and *Enterococcus* types and all absolutely anaerobic bacteria) (4). Synthesis of 16S rRNA methylases (ArmA, RmtA-RtmD, NmpA) modifying the antibiotic target (mechanism described so far only in some *Enterobacteriaceae*)(5). Active removal of an antibiotic from a bacterial cell by mechanism of various membrane pumps (Gram-negative bacilli especially of the genus *Pseudomonas*) (6). Resistance mechanism to aminoglycosides in *Staphy. aureus* is the synthesis of transferase enzymes (7). These are: two-domain acetyltransferases/phosphotransferasesAAC(*6'*)-Ies/APH(2")-Ias, encoded by the *aacA-aph D* gene and conditioning resistance to gentamycin, tobramycin, amikacin,kanamycinand netilmicin, nucleotidyl transferase),ANT-Ia encoded by *aadD* gene and conditioning resistances to tobramycins, kanamycins, neomycins and phosphotransferase APH(3")-IIIasencoded by *aph(3")-IIIa*sgene (8). Vancomycin-resistant *Staphylococcus aureus* (VRSA) also describes APH (3') - III phosphotransferase (kanamycin and neomycin resistance) encoded by the *aphA-3(aph (3')-IIIas)* gene. Plasmids were transmitted to *S.aureus* from *Enterococcus granulosa* within those embedded in these plasmids transposons Tn3851, Tn4031 and Tn5404 (10). In addition, *S. aureus* can synthesize transferases, for example., ANT (6), encoded by gene*ant (6)* plasmid pS194, or *ant(6)-Ia(aadE*s*)*, deactivating streptomycins (9).streptomycin resistances of *S.aureus* may also be the results of a mutations in the coding gene ribosomal S12 proteins (10). Resistance or reduced sensitivity to aminoglycosides may be the result of interfering with the transport of aminoglycosides into the microbial cells.This category of resistances is most common in *S.aureus* SCV (smalls colony variants)caused by mutations in the transport enzymes genes (11). Aims of this study was to determines the frequency of transferase genes aminoglycosides and the determination of their resistance phenotype on various aminoglycoside antibiotics in a groups.

MATERIALS AND METHODS

The study were 50 MRSA strains cultured and designated for the species in the laboratory of microbiological diagnostics in the hospital and also all the isolate came from another patient hospitalized at (Al-Sadar,Al-Hakeem and Al-Zahraa) in Al-Najaf city. Strains were identified and their drug sensitivity determined using a system Vitek-2 and ATB Staph. Methicillin resistance was confirmed using Cefoxitin-disk diffusion test. Isolation DNA and genotype detection of *aac A- aph*D, *aad D*and *aph (3")-IIIa* by PCR. Test bacterial strains were propagated overnight in liquid medium BHI at 35°C. 1.5ml cultures were centrifuged for 3 min at 12,000 rpm and the pellet was resuspended in 0.6 ml TE buffer (50 mol^{-3} Tris, pH 8.0; 10 mol^{-3} EDTA, pH 8.0) and then centrifuged 3min at 12,000 rpm. The bacterial pellet was then suspended in 0.2 ml TE buffer and added 10 units of lysostafin solution and incubated for 30 min. at 37°C. After this time 0.3 ml DNazole (Thermo Fisher Scientific.UK) was added to the cell lysate to obtaintotal cell lysis. The released DNA was precipitated by the addition of 2.5 volumes of 96% ethanoland placed for 20 min at -20^{O} C, Samples were centrifuged for 3 min at 12,000 rpm athen the resulting DNA pellet was washed with 1ml70% ethanol. Alcohol was evaporated for 15 min. at 37^{o}C, and DNA was dissolved in 0.1 ml sterile deionized water.Samples were stored at - 20° C. All samples were examined of PCR methods included: 5ml from 10x Taq buffer with KCl; 1 mol Tris-HCl (pH 8.8), 0.5 ml of MgCl2, 0.5 mleach of four 25mol^{-3} dNTPS, 1 ml for each primer, 0.2 ml Taq polymerase (promega, korean) and 3ml of the tested DNA. The total volume of the sample after topping up with deionized water was 50 ml. The following parameters were used in the PCR reaction: initial denaturation of 94 °C, 5 min; 94^{o}C, 30s, denaturation 58^{o} C,30s 25 cycles, elongation 72^{o}C, 30s, final elongation 72^{o} C, 7 min. The primers were synthesized by the following order of nucleotides according to Ida *et al.*(12). The *aacA - aphD* gene: forward primer, 5'-CGA TGT GGA TTG CGA AAA CT-3 '; reverse primer,3'-CAC CGA AATAAC TAG AAC CC-5 '; reverse primer ,273 bp amplified fragment.The *aad D* gene: forward primer, 5'-ATG GCTCTC TTG GTC GTC AG-3 '; reverse primer,3'-TAA GCA CAC GTT CCT GGC TG-5 '; amplified fragment 367 bp. The *aph (3")-IIIa* gene: forward primer, 5'-CAT TAT ACA GAG CCT TGG GA-3 '; reverse primer,3 'AGG TCC TCG TTA TTC CCG TA-5 '; amplified fragment of 152 bp.

RESULTS

In 50 strains of MRSA were determined by PCR the presence of three genes *aph (3")-IIIa, aad D* and *aacA - aphD*. Selected for all strains MRSA determined sensitivity and resistant by diffusion - disk method. The results are shown in Table 2.

DISCUSSION

aacA-aphD genes coding for AAC(6')-Ie/APH(2")-Ia acetyltransferase / phosphor -transferase occur in transposons (example. Tn4001, Tn4001-like), located in large plasmids and chromosomes (example. SCC*mec* IVc) (13). AAC(6')-Ie/APH (2")-Ia in *S.aureus*, the only enzyme known to determine gentamicin resistance to date.Gentamicin MICs for strains that have this enzyme range from 8 mg / L to> 1024 mg/L (MIC_{50} is 128 mg / L and MIC_{90} 512 mg / L) (14). For sensitive strains on aminoglycosides and strains synthesizing ANT (4') - Ia or APH (3') - IIIa enzymes , gentamicin MICs are in the range of 0.25-1.0 mg / L. Therefore, some doubtsraises by *S.aureus* strains, synthesizing only ANT (4 '), for which the diameter of the inhibition zone the height around the disc with gentamicin was 17 mm. AAC (6 ')Ie / APH (2") – Ia synthesis - also most often determines resistance to tobramycin. MIC for tobramycin for strains synthesizing this enzyme, ranges from 8 mg / L to 256 mg / L (MIC_{50} is 32mg / L and MIC_{90} 64 mg / L) (15). In addition, strains synthesizing this enzyme are always resistant tokanamycin (MIC≥64 mg/L). The only enzyme in *S.aureus* that degrades netilmycin is AAC (6 ') - Ie / APH (2 '') - Ia. Netilmycin is a very weak inducer of the *aacA-aphD* gene, which may reason that *S.aureus* strains having an inductive mechanism for regulating this gene. Ida *et al*(12) described natural *S.aureus* strains in which the Tn4001-like elements containing the *aacA-aphD* gene have retained promoter of the β-lactamase operon (reduced *blaZ* gene), which can due to resistance to aminoglycosides by β-lactamase antibiotics and antagonism β- lactamase and aminoglycosides. MIC increased from 4 to 32 mg / L and gentamicin from 128 to1024 mg / L (17). Some point mutations in the *aacA-aphD* gene may extend the spectrum AAC (6') -Ie/APH (2")-Ia, example. for arbecacin (18).The *aadD* gene coding for ANT nucleotidyltransferase(4')-Ia is found in both small(pUB110) and in large plasmids, e.g. conjugates. Acopy of pUB110 exists in some SCCmec methicillin chromosomal cassettes (19). *[a]aph(3")-IIIa*gene encoding [a]APH (3")-IIIa phosphotransferase is most commonly found in plasmid. Our studies have shown that aminoglycoside transferase genes in MRSA strains we study most often occur together. The presence of the *aacA-aphD* gene in the MRSA strains we studied has always been a prerequisite resistance to gentamycin, kanamycin and tobramycin. Only strains possessing this gene were resistant to netilmycin, while resistant for amikacin. All strains having only *aacA-aphD* were sensitive to neomycin. Gentamicin-resistant *S.aureus* strains (having the *aacA-aphD* gene) are clinically resistant to all aminoglycoside antibiotics, distreptamine derivatives. The presence of the *aadD* gene determined the resistance of the tested strains to tobramycin, kanamycin and neomycin. These strains were sensitive to gentamicin and netilmycin. Occurrence of the *aph(3")*-IIIa gene determined the resistance of MRSA subjects to kanamycin and neomycin.Strains possessing this gene have also been shown to be resistant to amikacin.These strains remained sensitive to gentamycin, tobramycin and netilmycin.

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Table 1. Criteria for *S.aureus* sensitivity to aminoglycoside antibiotics

Aminoglycoside antibiotics	***aacA-aphD***				***aadD***				***aph(3")-IIIa***			
	MIC (mg/L)		**Method diffusion**		**MIC (mg/L)**		**Method diffusion**		**MIC (mg/L)**		**Method diffusion**	
	S	R	S	R	S	R	S	R	S	R	S	R
Gentamicin	≤2	≥14	≥13	≤10	≤1	≥1	≥16	≤15	≤1	≥1	≥18	≤17
Amikacin	≤14	≥62	≥15	≤12	≤6	≥14	≥16	≤12	≤6	≥30	≥15	≤12
Netilmycin	≤6	≥30	≥13	≤10	≤1	≥1	-	-	≤1	≥2	≥18	≤17
Tobramycin	≤2	≥14	≥13	≤10	≤1	≥1	≥17	≥16	≤1	≥2	≥18	≤17
Kanamycin	≤14	≥62	≥14	≤11	-	-	-	-	≤6	≥30	≥15	≤12
neomycin	-	-	-	-	-	-	-	-	≤6	≥30	≥15	≤12

S-sensitive, R-resistant

Table 2. Occurrence of aminoglycoside transferase genes and antibiotic resistance phenotypeaminoglycosides in MRSA strains

Aminoglycoside transferase genes	Number of strains (No.50)	GEN		AMI		NET		TOB		KAN		NEO	
		S	R	S	R	S	R	S	R	S	R	S	R
aacA-aphD	**34(68%)**	0	34	12	22	31	3	0	34	30	14	6	28
aadD	**27(54%)**	5	22	11	16	25	2	0	27	0	27	3	24
aph(3")-IIIa	**22(44%)**	11	11	15	7	20	2	10	12	0	22	0	22

GEN-gentamicin, TOB-tobramycin, KAN-kanamycin, NEO-neomycin, AMI-amikacin, NET-netilmicin,S-sensitive, R-resistant

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RESEARCH ARTICLE

Antecedents Percussion Instruments of Tamil Music (தமிழர் இசை முற்காலத் தோற்கருவிகள்)

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ABSTRACT

There would have been the possibility of hearing a variety of sounds, naturally and artificially, in the life of man. Sounds like that would have brought so much joy and so much suffering. Musical instruments may appear when the idea of artificially generating such sounds is prevalent. The sounds heard in life events are broken down into syllables and rhythms. It is well known in the Sangam literature that Tamils are capable of comparing the sounds of birds, animals, beetles, toads and waterfalls with the sound of musical instruments. Percussion instruments can be described as multifocal instruments, and rhythmic instruments are both types of instruments. With music-raising gates, they can be divided into neurosurgeons, piercers, losers, and cannibals. They are made in various forms, such as wood, bamboo, nerve, rope, and leather. The materials and textures are fine-tuned to their musicianship. Natural objects such as horn, bone, and conch are also used as instruments. The instruments also have the name of rhythmic instruments. Various types Percussion Instruments have been provided in the past. However, this article describes the earliest leather instruments Music has been an integral part of his life, ranging from the top of the human community to the first shopkeeper. This is especially the case with the Sangam period people. The art of music is also talked about. They were viewed as two sides of a coin. It is known that there was a singing power for the singers and the energy for the singing. It is the pride of the ancient people that created the music with a variety of techniques that created creative thinking as a result of the exploration of nature.

Keywords: Tamil Language, Tamil Literature, Tamil Music, Tamil Music Instruments, Tamil Music Percussion Instruments

INTRODUCTION

மனிதனுடைய வாழ்வில் இயற்கையாகவும், செயற்கையாகவும் பல்வேறு விதமான ஒலிகள் கேட்கும் வாய்ப்பு இருந்திருக்கும். அவ்வாறு கேட்ட ஒலிகள் பல இன்பத்தையும், பல துன்பத்தையும் தந்திருக்கும். அவ்வாறு ஏற்பட்ட ஒலிகளைச் செயற்கையாக உண்டுபண்ணும் எண்ணம் மேலோங்கிய போது இசைக்கருவிகள் தோன்றி இருக்கக்கூடும். வாழ்க்கை நிகழ்வுகளில் கேட்கப்படும் ஒலிகள் முறைப்படுத்தப்பெற்று பண்ணிசைகளாகவும், தாள இசைகளாகவும் பிரிக்கப்பெறுகின்றன. பறவை, விலங்கு, வண்டு, தேரை, அருவி ஆகியன எழுப்பும் ஒலிகளை இசைக்கருவிகளின் ஒலியுடன் ஒப்புமைப் படுத்திப் பார்க்கும் ஆற்றல் கொண்டவர்களாக தமிழர் இருந்துள்ளனர் என்பது சங்க இலக்கியம் வாயிலாக அறியலாகும் செய்தியாகும். பண்களை இசைக்கத் தகுந்தவற்றைப் பண்ணிசைக் கருவிகள் என்றும் தாளத்தை பொருத்தமாகக் குறித்துச் சுவையுடன் ஒலிக்கும் கருவிகளைத் தாளக் கருவிகள் என்றும் இரு வகைகளாகக் குறிப்பிடலாம். இசை எழுப்பும் வாயில்களைக் கொண்டு அவற்றை நரம்புக்கருவி, துளைக்கருவி, தோற்கருவி, கஞ்சக்கருவி எனப் பிரிக்கலாம். அவையாவன, மரம், மூங்கில், நரம்பு, கயிறு, தோல் முதலியனவற்றினால் பல்வேறு உருவங்களில் செய்யப்பெறுகின்றன. செய்பொருட்களும் உருவமைப்பும் அவற்றின் இசைப்பயனுக்குத் தக்கவாறு கலையழகுடன் காணப்பெறுகின்றன. இயற்கைப் பொருள்களான கொம்பு, எலும்பு, சங்கு போன்றவைகளும் இசைக்கருவிகளாகப் பயன்படுத்தப் பெற்றுள்ளன. தோற்கருவிகளுக்குத் தாள இசைக்கருவிகள் என்ற பெயரும் உண்டு. கால அளவினை (மாத்திரை) குறித்துக் காட்டும் தட்டொலி கொண்ட தோற்கருவிகள் பல்வேறு வகைகள், உருவங்களில் முற்காலத்தில் வழங்கப்பெற்றுள்ளன. முற்காலத்தில் வழக்கில் இருந்த தோல் இசைக் கருவிகளை இக்கட்டுரை விளக்குகிறது.

“பேரிகை படகம் இடக்கை உடுக்கை
சீர்மிகு மத்தளம் சல்லிகை கரடிகை
திமிலை குடமுழாத் தக்கை கணப்பறை
தமருகம் தண்ணுமை தாவில் தடாரி
அந்தரி முழவொடு சந்திர வளையம்
மொந்தை முரசே கண்விடு நும்பு
நிசாளம் துடுமை சிறுபறை அடக்கம்
மாசில் தருணிச்சம் விரலேறு பாகம்
தாக்க உபர்வகம் துடிப்பெரும் பறையென
மிக்க நூலோர் விரித்துரைத் தனரே” (சிலம்பு : 3 : 27)

இதில் 34 வகையான தோற்கருவிகளின் பெயர்கள் சுட்டப்பெற்றுள்ளன. இவற்றுள் பல பிற்காலத்தனவாகும்.

பறை

ஒவ்வொரு திணைக்கும் ஒவ்வொரு யாழ் இருப்பது போல பறையும் உண்டு. பறை என்பது செய்தி எனப்பொருள்படும். இது தோற்கருவிகளைக் குறிக்கும் பொதுவான சொல். தோற்கருவிகள் அனைத்தும் பறையிலிருந்து வளர்ச்சி பெற்றவையாகும். இவை போர்ப்பறை, வெருப்பறை, வெறியாட்டுப் பறை, தட்டைப்பறை, சாக்காட்டுப்பறை என்று ஓசை மற்றும் பயன்பாடு கருதி வெவ்வேறு பெயர்களில் அழைக்கப்பெற்று வந்துள்ளன. சங்ககாலப் பாடல்களில் பல்வேறு இடங்களில் பறை பற்றிய குறிப்புகள் கிடைக்கின்றன.

தண்ணுமை

தண்ணுமை இருமுகப்பறை என்று வழங்கப்பெறுகின்றது. நீண்டு குவிந்த இரண்டு தலைகளை உடையது. "ஒரு முகத்தைத் தாழச்செய்து மறுமுகத்தில் இரு தடிகளால் ஒலித்து முழக்கப்படும்"(1) மிகவும் அச்சுறுத்துகின்ற ஒலியை உடையது. நெற்கதிரை அரிக்கும் உழவர்கள் தண்ணுமையைப் பயன்படுத்தியுள்ளனர். மறம் பொருந்திய வீரர்களைப் போரிடுவதற்காக அழைக்கும் தன்மையை உடையது தண்ணுமை. ஓசையைக் கேட்ட வீரர்கள் வெற்றி பெறும் (வென்றி) வேட்கையுடையவராய், மன்றத்தினைக் கைப்பற்றும் பொருட்டு, பெரிய போரைச் செய்த அச்சம் பொருந்திய கண் உடையது இவ்விசைக்கருவி என்பதனைப்

"மறப்படை நுவலும் அரிக்குரல் தண்ணுமை
இன்னிசை கேட்ட துன்னரு மறவர்
வென்றிதரு வேட்கையர் மன்றம் கொண்மார்
பேரம குழந்த வெகுவரு பறந்தலை" (புறநா. 270 : 8-11)

என்னும் புறநானூற்றுப் பாடல் உணர்த்துகிறது.

தண்ணுமை வீரர்களைப் போரிடுவதற்கு அழைப்பதற்காக இசைக்கப் பெற்றது. இதன் இசையைக் கேட்ட வீரர்கள், வெற்றி ஒன்றையே குறிக்கோளாகக் கொண்டு கோட்டையைக் கைப்பற்றும் விதமாகப் போரிட்டவர் என்ற செய்தியை,

"கன்றுபெறு வல்சிப் பாணன் கையதை
வள்ளுயிர்த் தண்ணுமை போல" (நற். 310 : 9-10)

என்ற நற்றிணைப் பாடல் உணர்த்துகின்றது.

“தட்டை துண்றுமை பின்னர் இயவர்
தீங்குழல் ஆம்பலின் இனிய விமிரும்
புதன்மலர் மாலையும்” (ஐங்கு. 215 : 3-5)

“வெண்ணெல் அரியுநர் தண்ணுமை வெரீஇச்
செங்கண் எருமை இனம்பரி எருத்தல்” (மலை : 471 - 472)

“மடிவாய்த் தண்ணுமை தழங்குகுரல் கேட்ட” (நற். 298 : 3)

“... மாக்கண் தண்றுமை
கைவல் இளையர் கையலை அழுங்க” (பதிற். 51 : 33-34)

“விசியுறு தண்ணுமை” (புறநா. 89 : 7)

முழவு

தோற்கருவிகளில் பறை, முரசு இசைக்கருவிகளுக்கு அடுத்து அதிகப்பதிவுகளைப் பெறுவது முழவு ஆகும். “முழவு அகமுழவு, அகப்புற முழவு, புற முழவு, புறப்புற முழவு, பண்ணமை முழவு, நானி முழவு, கலை முழவு”(2) என ஏழு வகைகளாகப் பிரிக்கப்பெற்றுள்ளது. முழவு தற்காலத்தில் மத்தளம் என்ற பெயரில் வழங்கப்பெறுகிறது. இருப்பினும் முழவு சற்று பெரிய உருவமாக இருந்துள்ளது.

“முழவு முதலரையா தடவுநிலை பெண்ணை” (குறுந். 301 : 1)

‘முழாவாரைப் போந்தை யரவாய் மாமடல்” (புறநா. 375 : 4)

என்று பனைமரத்தின் அடிப்பாகம் முழவுடன் உவமைப்படுத்தப் பெற்றுள்ளது முழவும் முழாவும் ஒன்றா என்ற சிந்தனையும் எழுகின்றது. பலாப்பழத்துடன் முழவைப் பொருத்தி ஒப்புமை கண்டுள்ளனர்.

“முன்றில் நீடிய முழ்வுறழ் பலவின்” (அகநா. 72 : 11)
என்கிறது.

முழவு கையாலும், குறுங்கம்பு கொண்டு அடித்தும் தாளம் எழுப்பி சுவைக்கும் இசைக்கருவியாகும் அதன் ஒலி இனிமையாக இருப்பதினால் அனைவரும் இன்றளவும் விரும்புகின்றனர். “இது மண்ணமைந்த கண்ணையும் பருத்த உருவமும் கொண்டது”(3). உடல்பகுதி கூடாக அமைய அதன் இரு திறப்புப் பகுதிகளிலும் தோலால் மூடப்பட்டுக் காணப்படும் இருபக்கத் தோலும் வாராலும் இழுத்துக்கட்டப்பெற்றிருக்கும். இது போன்று பல்வேறு குறிப்புகள் முழவைப்பற்றி இடம் பெற்றுள்ளன.

முரசு

முரசு என்பது பெரிய உருவில் அமைந்த கொட்டாகும். குடைவுடைய மரத்தாலும் பரந்த கண்ணை மூடியுள்ள தோலாலும் இறுக்கிக் கட்டப்பெற்ற தோல் வாரால் முரசு செய்யப்பெற்றிருக்கும். இதன் தோல் கொல்லும் தன்மையுடைய காளையைக் கொன்று உரிக்கப்பெற்றது என்று சங்க இலக்கியங்களில் கூறப்பெற்றுள்ளது. முரசுகளில் சிறந்தது அவைமுரசாகும். இது பகை மன்னர்களின் காவல் மரத்தால் செய்யப்பெறும் என்று கூறப்பெறுகிறது. “வீரமுரசு, வெற்றிமுரசு, பொது முரசு, மணமுரசு என முரசு பலவகைப்படும்”[4]. மேலும் முரசு எவ்வாறு செய்யப்பட்டது என்பதை பதிற்றுப்பத்து கீழ்க்கண்டவாறு சுட்டுகிறது.

‘கடம்பறுத் தியற்றிய வலம்படு வியன்பனை
ஆடுநர் பெயர்த்துவந் தரும்பணி கூஉய்க்
கடிபுக் கண்ணூஉந் தொடித்தோளியவர்” (பதிற். 17 : 5-7)

கடம்பமாகிய காவல் மரத்தை வெட்டி அதைக்கொண்டு முரசு செய்து அதற்குப் பலியிட்டு பாராட்டிய செய்தியை மேற்கண்ட பாடல் சுட்டுகிறது. முரசு பற்றிய செய்திகள், குறிப்பாக, மதுரைக்காஞ்சி, புறநானூறு, குறிஞ்சிப்பாட்டு, பரிபாடல், ஐங்குறுநூறு, அகநானூறு, குறுந்தொகை, கலித்தொகை ஆகியவற்றில் அதிகமாகக் காணப்பெறுகிறது. தற்காலத்தில் கோயில்களில் முரசு ஒலிக்கப்பெறுகிறது. குறிப்பாக பூசை நேரங்களில் ஒலிக்கப்பெறும் முரசின் உச்ச வளர்ச்சியாக இன்று மின்சார மணிகளுடன் இணைந்த மின்சார முரசு ஒலிக்கப்பெறுகிறது.

சிறுமுழா

முழவின் அமைப்பைப் பனைமரத்துக்கு ஒப்புமையாகக் கூறுமிடத்து சிறு முழவிற்கும் அதனையே தந்துள்ளனர்.

"முழாவரைப் போந்தை" (புறநா. 375 : 4)

என்ற பாடலடியை நோக்குமிடத்து முழவிற்கும் சிறுமுழவிற்கு நெருங்கிய தொடர்பு இருப்பது புலனாகிறது. இருப்பினும் இரண்டையும் ஒன்றாகக் கருத இயலவில்லை.

'ஒருதலைப் பதலை தூங்க ஒருதலைத்
தூம்பகச் சிறுமுழாத் தூங்கத் தூங்கி" (புறநா. 103 : 1-2)

'பாடுவல் விறலியோர் வண்ண நீரும்
மணமுழா வமைமின் பண்யாழ் நிறுமின்" (புறநா. 152 : 13-14)

ஆகிய பாடலடிகள் சிறுமுழாவின் பயன்பாட்டையும் பெருமையினையும் உணர்த்துபவைகள் ஆகும்.

கிணை

கிணை பற்றிய பல குறிப்புகள் பாட்டிலும் தொகைகளிலும் கிடைத்துள்ளன. வாரினால் இழுத்துக் கட்டப்பட்ட ஒரு கண்ணை உடையது. சிறிய கோல் கொண்டு அடித்து ஒலி எழுப்பப்பெறுவது இப்பறையை இசைப்பவர்களை கிணையன், கிணைவன், கிணைமகன் அவர்களின் மனைவியரை கிணைமகள் என்று அழைத்தனர். பாடலுக்குத் தாளமாக கிணை இசைக்கப்பெற்றதை

"பாடின் தெண்கிணை கறங்க" (அகநா. 301 : 10)

என்ற பாடலடி உணர்த்துகின்றது.

துடி

பாட்டும் தொகையும் பல்வேறு இடங்களில் பேசக்கூடிய ஒரு பறை துடியாகும். மரத்தால் செய்யப்பெற்று தோல் வார்களால் இறுக வலித்து கட்டப்பெற்றிருக்கும்.வாரால் கட்டப்பெற்றது

போல நூலாலும் கட்டப் பெற்றிருந்தது.துடி இடி போன்ற ஓசையுடையது. இதனை இசைப்பவன் துடியன் என அழைக்கப்பட்டுள்ளனர். இதனை உடுக்கை எனவும் இடைச்சுருங்கு பறை எனவும் அழைப்பர்.

"தமிந்துடி தூங்கும் கனைகாற் பந்தர்" (பெரும்பா : 124)

என்ற அடிகளில் இருந்து துடியின் ஓசை கடுமையாக இருக்கும் என்பது புலப்படுகின்றது.

தடாரி

புறநானூற்றில் பல்வேறு இடங்களில் தடாரியைப் பற்றிய குறிப்புகள் காணக் கிடைக்கின்றன. இது வார்களால் பிணித்துக் கட்டப்பெற்றிருக்கும். அகன்ற கண்ணை உடைய பறை அரித்த ஓசையை ஏற்படுத்தக்கூடியது. 'தடாரி என்ற சொல் தெடாரி என்றும் வழங்கப்பட்டுள்ளது"(5). தடாரி என்றால் உடுக்கை, கிணைப்பறை என்று சென்னைப் பல்கலைக்கழகத் தமிழகராதி பொருள் கூறுகின்றது. அக்காலத்தில் பாடுவோர் தடாரியைத் தட்டி சுவை ஊட்டிப் பாடுவதைக் கேட்ட புரவலர்கள் பரிசில் அளித்தனர் என்ற செய்தியை,

"பாடுநர்க் கிருந்த பீடுடையாள
தேய்வை வெண்காழ் புரையும் விசிபிணி
வேய்வை காணா விருந்திற் போர்வை
அரிக்குரல் தடாரி யுருப்ப ஒற்றிப்
பாடி வந்திசின் பெரும் பாடான்று" (புறநா. 369 : 18-22)

என்ற அடிகள் உணர்த்துகின்றன.

பதலை

பதலை என்றும் தோற்கருவி ஒரு பக்கத்தில் அடித்து இசைக்கப்பெறுவது பாணர்கள் தாங்கள் செல்லும் இடங்களுக்கு எல்லாம் இக்கருவியினைக் கொண்டு சென்று பயன்படுத்தியுள்ளனர். இது ஒரு கண் மாக்கிணை என்று குறிக்கப்பெறுகிறது.

"புணர்புரி நரம்பின் தீந்தொடை பமுனிய
வணரமை நல்யா ழிளையர் பொறுப்ப
பண்ணமை முழவும் பதலையும் பிறவும்

கண்ணறிந் தியற்றிய தூம்பொரு சுருக்கிச்
சாவிற் றகைத்த துறை கூடு கலப்பையர்" (பதிற். 41 : 1-5)

பதலை பற்றிய விரிவான செய்திகளோ குறிப்புகளோ அதிகமாகக் கிடைக்கப்பெறவில்லை. முழவு போன்று இதுவும் ஒரு சிறப்பான இசைக்கருவியாக இருந்திருக்கூடும்.

எல்லரி

எல்லரி பல இசைக்கருவிகளுடன் இசைக்கவல்லது வலிமையுடையது.

"கடிவளர் பொலிக்கும் வல்வா யெல்லரி" (மலை : 10)

"எல்லரி தொடுமின்" (புறநா. 152 : 16)
என்ற இரண்டு குறிப்புகள் மட்டுமே எல்லரி பற்றிக்கிடைக்கின்றன. இது ஒலி மிகுந்த இசைக்கருவியாக இருந்திருக்கலாம் என கருதத்தக்கது.

ஆகுளி

ஆகுளி கைகளால் தட்டி இசைக்கப்பெறும் ஒரு கருவியாகும். இக்கருவி தனித்தும் சில சமயங்களில் தும்பு மண்முழா, எல்லரி, பதலை, ஒருகன் மாக்கிணை, யாழ், சங்கு, தாளம் ஆகிய இசைக்கருவிகளுடன் சேர்ந்து இசைக்கப்பெற்றுள்ளது.

"விரலூன்று படுகண் ஆகுளி கடுப்பக்
குடிஞை இரட்டும் நெடுமலை யடுக்கத்து" (மலை : 140 - 141)
என்று மலைபடுகடாம் குறிப்பிடுகின்றது.

மேலும் மதுரைக்காஞ்சி, புறநானூறு ஆகிய இலக்கியங்களில் ஆகுளி பற்றிய செய்திகள் இடம்பெற்றுள்ளன. இக்கருவி தனித்தும், தூம்பு, மண்முழா, எல்லரி, பதலை, ஒருகண் மாக்கினை, யாழ், சங்கு ஆகிய இசைக்கருவிகளுடன் சேர்த்தும் இசைக்கப்பெற்றுள்ளது.

மத்தரி

மத்தரி என்னும் கருவி தடாரி, தண்ணுமை போன்று அவற்றுடன் இணைந்து இயக்கப்படும் ஒரு தாளக் கருவியாகும்.

"மத்தரி தடாரி தண்ணுமை மகுளி
ஒத்தளந்து சீர்தூக்கி" (பரி. 12 : 41-42)

மத்தியில் அடித்து அரிக்கின்ற ஓசையை எழுப்புவதால் மத்தரி என்று அழைக்கப்பட்டிருக்கலாம். மத்தரி ஓர் இருமுகப் பறையாகும். இருபுறமும் தோலால் மூடப்பெற்றிருக்கும்.

மகுளி

மகுளி முழவு போன்று இசை வேறுபாடு இல்லாமல் பொருத்தமாக மற்றக் கருவிகளுடன் இணைந்து அமைந்துள்ளது. தாளத்தை நன்கு அமைத்துக் காட்டும் இசைக்கருவிகளுள் இதுவும் ஒன்று.

"உருள்துடி மகுளியின் பொருள் தெரிந்து இசைக்கும்
கடுங்குரற் குடிஞைய நெடும் பெருங் குன்றம்" (அகநா. 19 : 4-5)

மகுளியின் ஓசை கடுமையாக இருக்கும் என்பதும், ஆந்தையின் அலறல் போன்று இருக்கும் என்பதும் இப்பாடல் மூலம் அறிய முடிகின்றது.

சில்லரி

சில்லரி என்பது ஒருமுகம் அமைந்த தோலால் மூடப்பெற்ற பறைக் கருவியாகும். இதனை பிற்காலத்தில் சல்லரி எனவும் அழைத்தனர்.

"அரிக்கூடு இன்இயம் கறங்க நேர்நிறுத்து" (ம.கா : 612)

இதனை அரித்து எழும் ஓசையை உடைய சல்லிகை கரடிகை ஆகிய இரண்டும் என்கின்றனர் உரையாசிரியர்கள்.

"தேரை ஒலியின் மானச் சீரமைத்துச்

சில்லரி கறங்கும் சிறுபல் லியத்தோடு" (அகநா. 301 : 19-20)

என்று அகநானூறு கூறுகின்றது.

சில்லரி தேரையின் ஒலியை ஒத்த ஒலியை வெளிப்படுத்தும் கருவியாகும். தனியாகவும் சங்கு, தரை, முரசு போன்ற கருவிகளுடனும் இது இசைக்கப்பெறும் எனக்கூறப்பெறுகிறது.

கொட்டு

கொட்டப்படும் அனைத்து இசைக்கருவிகளும் கொட்டு எனப் பெயர்பெறும்.

'மள்ளர் கொட்டின் மஞ்ஞை ஆனும்" (ஐங்கு. 371 : 1)

வீரர்கள் அடிக்கும் கொட்டின் ஒலியைக் கேட்ட மயில்கள் ஆடுகின்றன.வீரர்கள் அடிப்பது போர்ப்பறை அந்தப்பறையே இங்கு கொட்டு என வழங்கப்பெறுகின்றது என்று ஐங்குறுநூறு சுட்டிச் செல்கின்றது.

தட்டை

தினைப்புனக் காவலில் இருப்பவர்கள் பறவைகளை விரட்டுவதற்காக அடித்து முழக்கப்பெறும் இசைக்கருவி தட்டை எனப்பெறும். தட்டி ஒலி எழுப்பப் பெறுவதால் தட்டை எனப் பெயர் பெற்றிருக்கக்கூடும்.

"...கொடிச்சி
அவ்வாய்த் தட்டையொடு அவனை யாகென" (நற். 134 : 4-5)

'... அவ்வாய்த்
தட்டையும் புடைக்க கவறும் தொடுக்கென" (நற். 206 : 3-4)

மேற்கண்ட பாடல்கள் பறவை விரட்டப் பயன்படும் கருவியாகவே தட்டையைக் குறிப்பிடுகின்றன.

குளிர்

தட்டையைப் போல தினைப்புனத்தில் உள்ள பறவைகளை விரட்டப் பயன்பட்ட கருவி குளிர் ஆகும்.

“படுகிளி கடியுங் கொடிச்சிகைக் குளிரே
இசையின் இசையா இன்பாணித்தே” (குறுந். 291 : 2-3)

குளிர் பற்றி இதுதவிர விரிவான தகவல்கள் கிடைக்கப்பெறவில்லை. “தமிழர் இசையானது வாய்ப்பாட்டு, கருயிசை என வளர்ந்தது. ஆதிகாலத்தில் மனிதன் விலங்குகளோடும் வேட்டைத் தொழில்களோடும் வாழ்ந்த போது இசை ஒலிக்குறியாக மட்டும் இயங்கியது”(3). அவர்களுடைய உழைப்போடு அது நேரடித் தொடர்பு கொண்டதாய் இருந்தது. குறிப்பிட்ட ஒலியை இசைத்தால் வேட்டைத் தொழில் பெருகும் என்ற நம்பிக்கை அம்மக்களிடம் இருந்தது. வேட்டையில் கிடைத்த உணவுப் பொருட்களைப் பகுத்துண்டு அவர்கள் வாழ்ந்தனர்.

முற்காலத்தில் தமிழர்கள் நிரம்பிய இசையறிவினைப் பெற்றிருந்தனர் என்பதற்குச் சங்கப்பாடல்கள் அதிகமான சான்றுகளைப் பகர்கின்றன. “குறிப்பாக பரிபாடல் பண்ணமைக்கப்பெற்ற பாடல்களைக் கொண்டுள்ளது குறிக்கத்தக்கது. குரலிசை, கருவியிசை ஆகியவற்றில் முற்காலத் தமிழர்கள் வல்லவர்களாக இருந்துள்ளனர்”(7) வாழ்க்கையின் ஒவ்வொரு நிகழ்வினும் இசைக்கு இன்றியமையா இடம் கொடுத்துள்ளனர். பிறப்பு முதல் இறப்பு வரை இது தொடர்ந்து வந்துள்ளது. “இயற்கை ஒலிகளையும், இசையொலிகளையும் ஒப்பிட்டுப் பார்த்து அதிலிருக்கும் நுட்பங்களை அறிந்து பயன்படுத்தியுள்ளனர்” (8) என்பதன் மூலம் பண்டைத்தமிழரின் இசையாற்றல் புலனாகிறது. இசைக்கருவிகளை இயற்கை ஒலிகளை ஒப்புமைப்படுத்திக் கண்டுபிடித்தனர். ஒவ்வொரு இசைக்கருவியைத் தனித்தனியாக இசைத்தும், கூட்டாக இணைத்தும் இசைத்து மகிழ்ந்துள்ளனர். இசைக்கருவிகளை, தோற்கருவிகள், துணைக்கருவிகள், யாழ்கள் என வகைப்படுத்தி அவற்றுக்குள்ளும் பல்வேறு வகைகளில் அவற்றை உருவாக்கிய ஆற்றல் கொண்டவர்கள். மனித சமூகத்தில் உயர்ந்த இடத்தில் இருப்பவர்கள் முதல் கடைக் கோடியில் இருப்பவர் வரை அவரது வாழ்வின் ஓர் அங்கமாக இசை இருந்துள்ளது. குறிப்பாக இதனை இயல்பாகவே சங்ககால மக்கள் பெற்றுள்ளனர். இசையோடு ஆடல் கலையும் பேசப்பெறுகிறது. இரண்டையும் ஒரு நாணயத்தின் இரு பக்கங்கள் போலக் கருதினர். ஆடுவோர்க்கு பாடும் ஆற்றலும், பாடுவோர்க்கு ஆடும் ஆற்றலும் இருந்ததை அறிய முடிகிறது. இயற்கையை உற்றுநோக்கியதன் விளைவாகப் படைப்பாற்றல் சிந்தனையை

உருவாக்கிப் பல்வேறு நுட்பங்களுடன் இசையை வடிவமைத்த பெருமை பண்டைக்கால மக்களைச் சாரும்.

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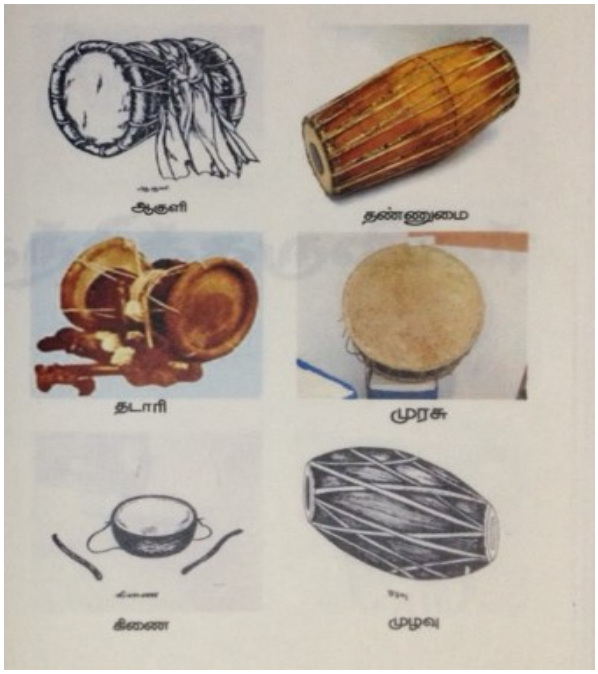

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RESEARCH ARTICLE

Protective effect of Alphalipoic acid (ALA) on Some Oxidants and Antioxidant on Heart Damage Induced by Monosodium Glutamate (MSG) in Male Rabbits

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ABSTRACT

Monosodium Glutamate is one of the most world's most widely used food additives. Its toxic effect have been shown in numerous animal studies, in which study MSG effects on cardiovascular system, therefore, This study was aimed to investigate the adverse effect of Mono sodium glutamate (MSG) on the ECG alteration and damage large blood vessels . Thirty two male rabbits was divided equally and randomly into four groups as following. Control group in which rabbits where fed with normal diet without supplementation, second group was gived MSG orally (8mg/kg.BW), While animals of third group were given ALA orally (60mg/kg.BW) while animal in fourth group were given orally (MSG 8mg/kg.bw and ALA 60mg/kg.bw), all treatment are extended to 10 weeks. there is a significant increase ($p<0.05$) in peroxynitrate (ONOO) andmalondialdehyde(MDA) a significant decrease ($p<0.05$) in glutathione(GSH) in second group in comparison with control group and group received ALA.

Key Words: ALA, MSG, oxidant, antioxidant

INTRODUCTION

Monosodium glutamate (MSG), one of the most common food additive used all over the world. The sodium salt of naturally occurring (non-essential) L form glutamic acid is a well-known food flavor enhancer. Its palatable and favorite flavor is a must in almost all Chinese and South-Asian dishes. L-glutamate is the molecule responsible for the umami taste (the 5thbasic taste in addition to saltiness, sweetness, bitterness and sourness(Amira ,*et al*.,2016).It is added to the food either as a purified monosodium salt or as a component of a mixture of amino acids and small peptides resulting from the acid or enzymatic hydrolysis of proteins (Uneyama,*et al*., 2009). Study recorded, the consumption MSG has increased all over the world (Beyreuther, *et al*., 2007).ALA is a specific antioxidant; it can

easily quench radicals, has an amphiphilic character, and does not exhibit any serious side effects (Gora, *et al*., 2011). ALA a compound that contains sulfur in the form of two thiol groups .ALA is also called thioctic acid and chemically symbol (C8H14O2S2) with an oxidized (disulfide, LA) and with a reduced (di-thiol: dihydro-lipoic acid, DHLA) form of LA(Luc Rochette,*et al* .,2015) .

ALA acts as a cofactor for several mitochondrial enzymes by ability to directly scavenge ROS, its metal chelating activity, and its potential to react with, and regenerate, other antioxidants such as glutathione and vitamins E and C (Singh,*et al*.,2008).Peroxynitrite is able to traverse cell membranes in part through anion channels (Denicola .,1998).Malondialdehyde (MDA,) which is one of the end products of the lipid peroxidation process . Lipid peroxidation is associated with aging and a variety of chronic health diseases, such as cancer and atherosclerosis (Marnett .,2000).Reduced glutathione (GSH) is synthesized by two sequential adenosinetriphosphate (ATP)-dependent reactions .The key function of GSH molecule is the maintenance of protein structure and function, the regulation of protein synthesis and degradation, the maintenance of immune function, protection against oxidative damage, and detoxification of reactive chemicals. GSH molecule also plays role in immune function (Wang. 1998).Aimed of research discerning role and effective ALA, ONOO, MDA and GSH of the amiss in the heart by MSG.

MATERIALS AND METHODS

The experiment protocol

The study is perform during the period from November, 2018 to April 2019.Rabbits kept in animal house for acclimation to the laboratory condition for two weeks. Thirty two healthy adult male rabbits aged (7-9) months, weighting 1300 -1500 gm. Were obtained from the animal house of Science College in Baghdad University.

1. GI rabbits were given feed without supplementation (as control) for 10 weeks
2. GII rabbits were given oral intubation daily 8mg/kg B.W of MSG dissolved by water for 10 weeks according to method reported by(Rogres ,*et al*., 1990)
3. GIII rabbits were given oral intuation daily 60mg /kg BW of ALA suspended with DMSO for 10 weeks according to method reported by (Al-Ali., 2018)
4. GIV rabbits were given8mg/kg B.W oral intubation daily of MSG dissolved by water and given 60mg/kg. B.W of ALA.

Blood collection

Animals were anesthetized by injection of (90mg/kg) Ketamine and (40mg/kg) Xylazine .Blood samples were collected at10weeks of study of experiment via heart puncture technique was done by using a 10 ml disposable syringe and 10 ml of blood was drawn slowly and gently. 10 ml of blood collected in gel test tubes (for serum preparation) which leaves for 30 minutes in room temperature and then used for getting serum by centrifugation at 3000 rpm for 15 minutes to separate serum and put in Eppendorf tubes which kept at freezer in -20C . Eppendorf tube which kept at freezer in -20C (Amin, *et al*., 2008).

Estimation of serum peroxynitrate (ONOO)

µM/L.Peroxynitrate was measured by method of (Vanuffelen .,1998) ,

Estimation of Serum Malondialdehyde (MDA)

µM/l.Malondialdehyhe was estimated by Thiobarbituric acid (TBA) assay method of (Muslih,*et al* .,2002).

Estimation of serum reduced Glutathione (mg/dl)

Serum glutathione concentration has been measured by using the Ellmans reagent method previously used by (Alzamely .,2001) .

RESULTS

Protective role of ALA in oxidant (ONOO, MDA) concentration in MSG treated male rabbits and serum antioxidant (GSH).

ONOO (Peroxynitrate)

The effect of daily oral intubation of MSG for 10 weeks caused significant ($p<0.05$) increase the mean value of ONOO concentration in GII in comparing to the control GI,GIII, and GIV treated group .It appears also that ALA intubation caused reduction in mean value of ONOO after 10 weeks of experiment in compared with GI .

MDA(Malondialdehyde)

Showed a general trend for the MDA value to increase significantly ($p<0.0$) after 10weeks in MSG treated groups (8mg/kg B.W) GII as compared to control group and other treated groups (GI, GIII, GIV). The result also showed that oral intubation of (60mg/kg B.W) of ALA caused significant ($p<0.05$)decrease in serum MDA concentration of mean value in comparing to GI GII and another groups, on the other hand combined intubation of MSG with ALA caused non-significant in this parameter comparing to GII,GIII groups .

GSH (Glutathione)

Showed the mean value of serum GSH concentration a significantly decrease ($p<0.05$) after ten weeks in MSG treated group(8mg/kg B.W) GII as compared to GIII and GIV groups .The result also showed that oral intubation of (60mg/kg B.W) of ALA caused significant ($p<0.05$)increase in serum GSH comparing to GII .

DISCUSSION

Effect of ALA on some serum oxidant (ONOO,MDA) andantioxidant (GSH) on the damage induced by MSG in male rabbits.

Serum Peroxynitrite (ONOO)

The data in table (1) showed a significant increase ($p<0.05$) (ONOO) in GII received MSG compared with GI, GIII and GIV and a signifiacnt decrease in GIII received ALA compared with GII the present study agreement with (PACHER Pal *.et al*., 2007). MSG causes oxidative stress which appearances by animportant increase in rates of lipid peroxidation. Peroxynitrate itself is also strong oxidant and can react with electron rich groups Peroxynitrate can also be formed through superoxide react NO to formed peroxynitrate. Increased glutamate release is sustained activation of glutamate receptors, and increased accumulation of calcium (Ca^{+2}). There is direct evidence that activation of glutamate receptors and the Ca^{+2} influx induces the formation of reactive oxygen species, superoxide anion and hydrogen peroxide .Oxidative stress initiates lipid peroxidation cascades that leads to the damage of highly vulnerable cell membranes during the first few days after injury . ONOO has been shown to trigger apoptosis in the

cardiomyocytes (Arstall, *et al*., 1999) , as well as endothelial and the vascular smooth muscle cell, induce decrease in spontaneous contractions of the cardiomyocytes and cause irreversible inhibition of mitochondrial respiratory chain . Peroxynitrite also activates MMPs and nuclear enzyme PARP, which contribute which contributed to impaired cardiovascular functions in most cardiovascular disease and inflammatory disorders (Jagtap,*et al*.,2005).

Peroxynitrateactivate ERK, a MAPK has been linked with hypertrophic and antiapoptotic response in the heart andinhibit NF_B activation triggered via inflammatorystimuli in cardiac and endothelial cell lines. Furthermore, it inducesthe upregulation of adhesion molecule in endothelial cells, the disruption of endothelial glycocalyx and may enhance the adhesion of the neutrophils to endothelium, through complex interactions with various cell signaling pathway and depending on the environment can stimulate or inhibit platelet aggregation. Alpha lipoic acid has free radical scavenger properties and direct antioxidant effects on the recycling of other cellular antioxidants. Alpha lipoic acid has been also found to be effective in the treatment of various oxidative stress models, such as ischemia-reperfusion, diabetes, cataract formation, neurodegeneration, and radiation injury (Bilska., 2005). Therefore, ALA and other antioxidants may reduce harmful effects of oxidative stress (Emmez , *et al* .,2010).

Malonydialdehyde (MDA)

The data in table (1) showed a significant increase (P<0.05) MDA in group GII received MSG in compare with GIII group. The result of the current study agreewith (Farombi .,2006 and Ali ,*et al* .,2016 and Zeinab ,*et al* .,2014). Our study showed that treated MSG caused an increase in serum MDA , as a result of chronic oral intubation of MSG causes oxidative stress which is manifested by significant increase in levels of lipid peroxidation as evidenced by increased levels of MDA and by the decrease superoxide dismutase and reduced glutathione, glutathione peroxidase and glutathione S-transferase (Farombi .,2006). MDA is an evidence of lipid peroxidation promote by iron overload. Study revealed thatMSG supplementation generates free radicals and a depletion of anti-oxidants in the thymus and spleen a precursor to the pathogenesis of many diseases (Pavlovic , *et al*.,2007).They present study showed the chronic oral intubation of MSG causes oxidative stress which is demonstrated by important increase in the levels of lipid peroxidation as shown by increased levels of MDA in heart (Singh K,*et al* .,2005).MDA significantly decrease (p<0.05) in GIII received ALA group in comparison with (GI, GII and GIV)groups.The result of the study agreement with result of (Yasser ,*et al*.,2015).

ALA is a hydrophilic and hydrophobic characteristics, being extensively distributed in plants and animals tissues in cellular membranes and in the cytosol (Deng,*et al*.,2013).Metabolic therapy of the ALA is an under-utilised method for the treatment of different heart diseases. This form of therapy differs fundamentally from standard cardiovascular pharmacological therapy. It involves the administration of a substance normally occurring in the body in order to favourably influence metabolic reactions occurring within the cell, by improving cellular energy productionand suppressing free radical generation. Lipoic acid as antioxidant efficiently cross the blood brain barrier to accumulate in several brain regions(Pannerselvam,*et al*., 2002).It reduces the amount of the hydroxyl radical that was generated by Fenton reaction, and it also scavenges the peroxide and the superoxide radical . ALA can scavenge a number of free radicals in different environments .It is found to be capable of regenerating many endogenous antioxidants in the body (Bustamante, *et al*.,1998) The protective role of ALA on lipid peroxidation status .It may be attributed to the bioactivity of lipoic acid to directly react with oxidation ,as well as its ability to interfere with the oxidation processes in the lipid and aqueous cellular compartment(Packer, Roy, & Sen., 1997). On administration of lipoic acid the level of lipid peroxidation in plasma liver and brain was significantly decreased so after administration of lipoic acid the antioxidant was primarily accumulated in the liver, heart and skeletal muscle after which it efficiently crosses the blood brain barrier to accumulate in several brain regions (Arivazhagan ,*et al*., 2002).

Glutathione (GSH)

Our study showed a significant decrease ($p<0.05$) serum levels of GSH in GII received MSG compared to the control group. This resultagree with(Ali,*et al* ., 2016). Oral intubation of the MSG 8mg/kg BW daily in 10weeks induced ROS production in the body could be explained by the glutamate induce an increase in the activity of α Ketoglutarate dehydrogenase to potential ROS generator .Moreover , an increased intracellular calcium level via glutamate receptors can stimulate free radical generation and inhibition of cysteine uptake leading to decreased GSH levels that may increase ROS –induced renal cell damaged(Sharma .,2015).Researches have been recorded decreased GSH pool through cytotoxicity and oxidative stress state (Womgcharoen, *et al* .,2012).In a study involving isolated rabbits organ exogenous GSH shown to protect organ damage , furthermore, the GSH perfusion medium resulted in an increase in the intracellular level in both damaged and healthy organ by entering the directly or indirectly into the cells (Ali ,*et al* ., 2016).Glutathione , an endogen antioxidants reduced in the oxidative status as signalizing of oxidative stresses , reflecting the redox commensuration between oxidation andantioxidations .Various oxidants and antioxidants have been additive effect on oxidative status study appeared that administrated MSG lead to decreased cardiac reduced glutathione (GSH) level in the serum (Singh. *et al* .,2005).

GSH significant increase ($p<0.05$) in GIII received ALA group in compare with GII group the result of the study agree with the result of ALA supplementation resulted in increases in total antioxidant capacity GSH and reduced MDA (Şehirli, *et al*.,2008and Golbidi .,2011). ALA an essential co-factor for mitochondrial energy metabolism is a natural product that can be synthesized in mitochondria by lipoic acid synthase and also can be absorbed from the diet (10). It is both hydrophilic and hyrdophobic, and therefore it can easily be absorbed and can easily be distributed into all tissues of body. ALA displays its antioxidant activity especially through GSH activity. (Sarper,*et al*.,2018).Other study demonstrated the beneficial effects of ALA in reducing the age-associated alterations in GSH impossible delivery of exogenous GSH to tissues such as the brain and heart. (Suh,*et al*.,2004) The bioavailability of cysteine delivery agents (e.g., *N*-acetylcysteine) is low. However, ALA can modulate the age-related alteration in GSH levels as it is easily taken up into neural tissues.It was suggested that ALA provides resulted in increase in total antioxidant capacity and reduced GSH. Some in vivo and in vitro experimental trials showed that ALA administration increased the intracellular GSH level by 30–70 %(Kocaoglu , *et al*.,2017).

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Table 1.Effect of daily oral intubation of 60mg/kg B.W of ALA for 10 weeks on serum oxidant (MDA and ONOO) and antioxidant (GSH) concentration in 8mg/kg B.W of MSG treated male rabbits.

Group **Parameter**	**GI** **Control**	**GII** **(MSG)**	**GIII** **(ALA)**	**GIV** **(MSG+ALA)**
ONOO (µM/l)	4.05±0.83 B	6.44±0.39 A	3.20±0.69 C	4.008±0.59 AB
MDA(µM/l)	0.012±0.001 AB	0.017±0.002 A	0.007±0.0009 C	0.014±0.001 AB
GSH(mg/dl)	9.82±1.98 BC	5.70±1.21 C	17.76±2.68 A	13.49±2.47 AB

Value are expressed as mean ±SE n=8/group , significant differences (p<0.05) ,.The different letters refer to significant difference between difference groups . GII (Monosodium glutamate) ,GIII (Alpha lipoic acid), GIV received (Alpha lipoic acid +Monosodium glutamate) .MSG=8mg/kgB.W; ALA=60mg/kgB.W.

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RESEARCH ARTICLE

Evaluation of Water Quality Index from 2008 to 2010 of Euphrates River : Case Study, Upstream of Al-Hindiya Barrage, Iraq

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ABSTRACT

The water shortage in current years due to claiming change and reduce of water share of Euphrates river from source. The changing caused to increase in elements, salts and acidity index (PH). This study aim to compare the water quality of 2008 with 2009 and 2010.This study is done in order to measure water quality by using water quality index (WQI). The WQI is a tool utilizing to calculate more than parameters to evaluate water quality and it works mathematically. WQI gives water validity to drinking. The study carries out in upstream of Al-Hindiya barrage on Euphrates River. The results in study show the WQI by utilizing Sumitomo and Nemerow model in 2008, 2009 and 2010 are acceptable as they < 1. The second tested model by using Weighted arithmetic index method from Brown RM et al. and Dhirendra MJ et,al indicates that all tested samples were valid. According to these results of the three models, it can be said that the overall water quality in 2008, 2009 and 2010 is valid; however; some parameter needed to be treated in the Sumitomo and Nemerow model. Sumitomo and Nemerow model indicate that the year of 2009 that is less discharge than 2008 and 2010 has more invalid elements.

Key words: water quality index, Al-Hindiya barrage, Sumitomo and Nemerow.

INTRODUCTION

The problems of water shortage in many countries lead to reduce agriculture, industry and many other activities. Water shortage and rising of temperature lead to degradation of water quality therefore; it is necessary putting tool to test and monitoring the Euphrates river water in upstream of Al-Hindiya barrage. Fadhil and Abdulkider, 2013 used water quality index (WQI) to evaluate the ground water quality of Dibdiba aquifer for drinking purpose. They

tested twenty wells to calculate multi parameters. The results showed that the values of WQI ranged between 432.6 and 184.5. They proved that water for Dibdiba aquifer is not suitable for drinking of human use. Roşu Cristina et al., 2014 carried out the study of WQI in Mediaş town in faculity of environmental Sciences and Engineering Cluj-Napoca of Romania. The WQI utilizes the analyzing water quality of multi parameter. The WQI ranged between 76 was very poor to 375 was doing not suitable. Rafah Rasheed Ismail et al., 2018 used the remote sensing technology in verification of the pollution region in Diyala River, Tigris River, and study the influence of wastewater treatment station in AL-Rustamiyah in Baghdad capital that is site on Diyala River. From obtained results on four classes of WQI that are successful in evaluating and mapping water was polluted that confirmed which the water of River of Tigris is subjected to pollution by Diyala River that agree with the ecological tested of water samples took from five locations in the search region. Shahad and Khalid, 2018 studied development WQI method of drinking water of Iraq (IQS 417, 2009) to predict the validation in Euphrates River to drinking via classify the quality of the Euphrates water at variation stations.

To evaluate the water, the concentration of eight parameters were examined Cl, TDS, pH, Ca, Mg, Na, SO^{-4}, and NO^{-3}. The good water quality and decrease after it receives pollution from sources for example domestic sewage the water becomes Poor. The WQI utilizes the parameters witch weight value from one to five as to the significance of parameter. The equation which given was generated to estimate the end index that gathers the influence of all the (8) parameters. The area used of Al-Shanafiyah station until Al-Haritha station unfit as a source to drink water due to classificatory of the drink water at the stretch of the river classifying as Poor by WQI. Abbood et al., 2014 evaluated the WQI in Main Drain River of Iraq via Application the Canadian Council of Ministers of the Environment Water Quality Index. They studied fifteen water quality parameters measured for 10 stations through the length of main drain river from Baghdad to Babylon after that Qadiysiah then ThiQar and Al-Basrah. The work carried out through 2004 to 2011. The results got from WQI water river was between 26.6- 35.5 that show worst water quality because of influence of different pollutants sources. Adel Mashaan Rabee et al., 2011 utilized nine parameters in predicting the quality of Tigris River for public usage via taking five locations of sampling along the river in Baghdad region. The parameters that were tested are temperature, pH, BOD, NO_3, PO_4, fecal coliform, TU, and TDS. The results showed that the WQI in water of Tigris River was at medium class. Muthanna et al. 2018 studied the groundwater quality for dibdaba formation and to determining its water quality index and evaluating its use for irrigation. This study aims to assess the water quality in the Euphrates River at Al-Hindiya barrage from 2008 to 2010 for drinking validity by using WQI concept using Sumitomo- Nemerow and Weighted arithmetic index methods.

METHODOLOGY

New Al-Hindiya barrage is very important project in Iraq because of providing several rivers and canals from its upstream (Musib project, shat Al-Hila, Kifil project, Beni Hassan project, and Husinia project) that feed area of an approximately 500 hectar. It sited on Euphrates River, which located in Sadat Al-Hindiya town of Babylon governorate as shown in Fig.1. New Al-Hindiya barrage site in 44 43'41.82"N and 44 16'8.19"E. The length of the barrage was 115m and it has six arch gates in 16 m width in each. The barrage has a hydro power station with a length of 79.6m and lock of navigation 20 m length. The height of barrage was 10 m, bed level 24.90 m and top level 34.90 m.

The water samples for upstream of Al-Hindiya barrage was taking by Al-Hindiya barrage office (daily measurements note book of 2008, 2009 and 2010) . The tests consist of ten parameters: TDS (total dissolve solid), pH(acidity index), Calcium(Ca), Sodium(Na) , Magnesium(Mg), Sulfate(So_3), Electric conductivity of water (EC), Nitrate (No_3), Chloride (Cl), and total hardness (T.H.). Table.1 The Iraqi standard for drinking purpose as permitted value, 2009. Table.2. The status WQI on Mishra and Patel, 2001.

WATER QUALITY INDEX MODELS

The water quality indexes are calculating and predicting of Al-Hindiya barrage in Babylon city at Sadat Al-Hindiya town ship by two methods:

1. Sumitomo and Nemerow model,
2. Weighted arithmetic index method.

Sumitomo and Nemerow model

The water pollution index using Sumitomo and Nemerow model by following equations:

$$Pij = f\left(\frac{Ci}{Lij}\right) \quad \text{..... (1)}$$

where:
Pij= the pollution index for the use j
Ci= multi item of water qualities.
Lij= permissible level for use (standard level).
i= number of the i-item of water quality.
j= number of the j-item of water utilize (use)

The pollution index for the use j if equal 1 was critical value but if pollution index for the use j was more than one therefore the water required some treatment

$$Pij = f\left(\max of \left(\frac{ci}{Lij}\right) \text{ and mean of } \left(\frac{ci}{Lij}\right)\right) \quad \text{.....(2)}$$

$$Pij = m * \left(\max\left(\frac{ci}{Lij}\right)^2 + \text{mean}\left(\frac{Ci}{Lij}\right)^2\right)^{0.5} \quad \text{.....(3)}$$

where:
m = the proportionality constant
If pij =1 and max (ci/Li)=1 and mean (ci/Lij) =1 substitute in eq.(3)
m=constant equal to $1/\sqrt{2}$

$$Pij = \frac{1}{\sqrt{2}} * \left(\max\left(\frac{ci}{Lij}\right)^2 + \text{mean}\left(\frac{Ci}{Lij}\right)^2\right)^{0.5} \quad \text{.....(4)}$$

$$\frac{Ci}{Lij} \text{ of PH} = \frac{ci - Lij}{Lij\,max - Lij} \quad \text{.....(5)}$$

$$Lij = \frac{Lijmax + Lij\ min}{2} \quad \text{.....(6)}$$

The other test take Ci/Lij as show in Table (3)

Weighted arithmetic index method

From Brown RM et al., 1972 and Dhirendra MJ et,al., 2009. Using equation of constant of proportionality (K),

$$K = \frac{1}{\sum_{i=1}^{n} \frac{1}{si}} \quad \text{.....(7)}$$

where:
Si = permissible limit for i^{th} parameter
n = number of parameters

$$Wi = k/Si \quad \text{.....(8)}$$

where:
Wi= weightage of i^{th} parameter.

$$Qi = \frac{100Vi}{Si} \quad \text{.....(9)}$$

$$WQI = \frac{\sum_{i=1}^{n} Qi\ Wi}{\sum_{i=1}^{n} Wi} \quad \text{.....(10)}$$

where:
Vi= current reading for i^{th} parameter
WQI= water quality index.
Qi = sub index of i^{th} parameter Qi

RESULTS AND DISCUSSION

The physical and chemical parameter were concentrating on water sample of Euphrates River in Al-Hindiya barrage using three method to evaluate the water quality of river.

By using Sumitomo and Nemerow model

In this method using Sumitomo and Nemerow model from eq.1 to eq.6 to obtain on The pollution index for the use j was less than 1 (the water do not need treatment) or equal 1 was critical value but if pollution index for the use j was more than one therefore the water required some treatment. Table (3) shows water quality index by using Sumitomo and Nemerow model.

pH= MIN+MAX/2=8.5+6.5/2=7.5
Lij=(ci-Lij)/(Lmax-Lij)=(ci-7.5)/(8.5-7.5)

$$SAR = \frac{Na}{\sqrt{\frac{Ca+Mg}{2}}} \quad \text{.....(11)}$$

Sodium adsorption ratio (SAR)= Max. value of sodium dividing on root square of Max. value of calcium and magnesium(Larry W. Mays,2005). The result from table.3 and Eq.11 obtain on SAR = 23.5

$Plj - Sqr\left(\frac{aver+max}{2}\right) - 0.934 < 1\ ok$ but Max value of SO4=1.25>1 need treatment.

Tables (4) to (7) show the discharge (Q) and upstream water level (U/S W.L) of Al-Hindiya barrage during 2008, 2009 and 2010 to compare the discharge and U/S W.L with Max WQI.

Sodium adsorption ratio (SAR)= Max. value of sodium dividing on root square of Max. value of calcium and magnesium(Larry W. Mays,2005). The result from table.3 and Eq.11 obtain on SAR = 12.6

$$Plj = Sqr\left(\frac{aver+max}{2}\right) = 0.993 < 1\ ok$$

The Maximum values of WQI of T.H,Ca,SO4 and TDS equal to 1.3,1.17,1.14 and 1.2, respectively. these indices point to the water need to treatment because of WQI more than one.

Sodium adsorption ratio (SAR)= Max. value of sodium dividing on root square of Max. value of calcium and magnesium(Larry W. Mays,2005). The result from table.3 and Eq.11 obtain on SAR = 23.5

$Plj = Sqr\left(\frac{average+max}{2}\right) = 0.995 < 1\ ok$ but Max value of T.H=1.3>1 need treatment.

When Ci/Lij>1 use equation, Ci/Lij= 1+k * log(Ci/Lij) the K=5 from Sumitomo and Nemerow. Figures (2) to (4) show average and maximum WQI for (U/S W.L) of Al-Hindiya barrage during 2008, 2009 and 2010.

Weighted arithmetic index method from Brown RM et al., 1972 and Dhirendra MJ et,al., 2009. In 2008 depending on eq. (7), eq.(8) and (9). This method was utilized by Brown RM et al., 1972 and Dhirendra MJ et,al., 2009 which Using equation of constant of proportionality (K) as shown of equation 7 to 10. They used permissible limit for i^{th} parameter with weightage of i^{th} parameter. They using Vi= current reading for i^{th} parameter as shown in eq.9 and obtained on WQI= water quality index as explaind equation 10.

Table (9) to (11) show WQI by using weighted arithmetic index method from 2008 to 2010. The WQI average equal to 41.5 the index is good as shown in table (8) because of the good pointing was between 26-50.

The WQI average equal to 40.7 the index is good as shown in table (9) because of the good pointing was between 26-50. The WQI average equal to 31.7 the index is good as shown in table (10) because of the good pointing was between 26-50

CONCLUSIONS

The concluding which obtains from result will be as following: using the water quality index (WQI) by Sumitomo and Nemerow model,1970 will obtain on WQI of 2008 is 0.934 < 1 ok. But in maximum value of SO4 is 1.25. this value more than 1 therefore the increasing of parameter SO4 explains the water need to treatment to reduce the sulfate value. WQI of 2009 is 0.993 < 1 ok. But in maximum values of T.H, Ca, SO4 and TDS is1.3, 1.17,1.14 and 1.2, respectively. this index of four parameters points to the water need to treatment of T.H, Ca, SO4 and TDS more than 2008. WQI of 2010 is 0.995 <1 0k But in maximum values of T.H is 1.3. this value more than 1 therefore the increasing

of parameter T.H explains the water need to treatment to reduce the total hardness. using the water quality index (WQI) by Weighted arithmetic index method from Brown RM et al., 1972 and Dhirendra MJ et,al., 2009. WQI of 2008, 2009 and 2010 is 41.5, 40.7 and 31.7, respectively. These indices are good because of they was between 26-50. From conclusion shows the water need treatment to total hardness from 2008 to 2010 while in 2008 also need treatment to sulfate calcium, total dissolved solid. The Sumitomo and Nemerow model show that the year of 2009 which is less discharge than two are 2008 and 2010 has WQI more than 1 and the water needs to treatment. The decreasing of discharge at 2009 caused increasing of polluting parameters.

RECOMMENDATIONS

1. We recommended study of WQI of Al-Hindiya barrage water from 2011 to 2019.
2. Studying of biological, chemical and physical of Euphrates river through shortage time and summer season.

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Table.1 The Iraqi standard for drinking purpose as permitted value (Ministry of Environment, 2009)

parameters	PH	TDS	Ca^{+2}	Mg^{+2}	Na^{+1}	Cl^{-1}	EC	NO_3	$So4^{-2}$	T.H
Iraqi standards	6.5-8.5	1000	150	100	200	350	1563	50	400	500

Table 2. The status WQI on Mishra and Patel, 2001

WQI value	Water Quality
0-25	Excellent
26-50	Good
51-75	poor
76-100	Very poor
>100	Unfit for drinking purpose

Table 3. Water quality index by using Sumitomo and Nemerow model of 2008.

Date 2009		Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Average	Max
EC	Av.	1133	1169	1155	1125	1150	1262	1430	1203	1430
	limit	1563	1563	1563	1563	1563	1563	1563	1563	1563
	Ci/Lij	0.725	0.748	0.739	0.72	0.736	0.807	0.915	0.77	0.915
TDS	Av.	842	812	844	772	818.7	994	996	868.4	996
	limit	1000	1000	1000	1000	1000	1000	1000	1000	1000
	Ci/Lij	0.842	0.812	0.844	0.772	0.819	0.994	0.996	0.868	0.996
Ca (mg/L)	Av.	12.9	0.45	7	15.37	7.067	9.3	26.85	11.28	26.85
	limit	150	150	150	150	150	150	150	150	150
	Ci/Lij	0.09	0.003	0.05	0.07	0.05	0.06	0.18	0.07	0.18
Mg(mg/L)	Av.	46.5	44.6	48.7	15.4	49.3	58.5	72.7	47.2	72.7
	limit	100	100	100	100	100	100	100	100	100
	Ci/Lij	0.47	0.45	0.49	0.1	0.49	0.59	0.74	0.47	0.73
Na(mg/L)	Av.	-	-	-	151	95.3	157	166	128	166
	limit	200	200	200	200	200	200	200	200	200
	Ci/Lij	-	-	-	0.75	0.45	0.53	0.83	0.64	0.83
CL (mg/L)	Av.	101	117	117	117	120	132	174	136	174
	limit		350	350	350	350	350	350	350	350
	Ci/Lij	0.29	0.33	0.33	0.33	0.34	0.38	0.5	0.36	0.5
So4 (mg/L)	Av.	499	278	257	266	325	313	420	331	499
	limit		400	400	400	400	400	400	400	400

	Ci/Lij	1.25	0.7	0.64	0.67	0.81	0.78	1.05	0.84	1.25
NO3 (mg/L)	Av.	-	-	-	6.35	0.83	0.75	1.35	0.82	1.35
	limit	50	50	50	50	50	50	50	50	50
	Ci/Lij	-	-	-	0.01	0.02	0.02	0.03	0.02	0.03
T.H	Av.	223	184	182	188	210	250	365	229	365
	limit	500	500	500	500	500	500	500	500	500
	Ci/Lij	0.45	0.37	0.36	0.38	0.42	0.5	0.73	0.46	0.73
pH	Av.	8.1	7.7	7.8	7.9	8.1	8.1	8.2	7.99	8.2
	limit	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5
	Ci/Lij	0.6	0.2	0.34	0.35	0.63	0.64	0.65	0.49	0.65

pH= MIN+MAX/2=8.5+6.5/2=7.5

Lij=(ci-Lij)/(Lmax-Lij)=(ci-7.5)/(8.5-7.5)

Table 4. Discharge and upstream water level of Al Hindiya barrage from 2008 to 2010

Month	Average Q (m^3/s)2008	U/S W.L	Average Q (m^3/s)2009	U/S W.L	Average Q (m^3/s)2010	U/S W.L
January	344	31.7	258	31.6	246	31.8
February	361	31.7	216	31.5	331	31.9
March	422	31.7	247	31.6	302	31.8
April	383	31.7	226	31.6	281	31.8
May	336	31.6	213	31.8	236	31.7
June	476	31.9	309	31.9	384	31.8
July	586	31.9	370	31.8	487	31.7
August	491	31.9	329	31.8	429	31.7
September	503	31.9	312	31.7	417	31.8
October	459	31.9	270	31.6	386	31.7
November	347	31.8	289	31.6	273	31.6
December	283	31.8	259	31.7	278	31.6
Average	416	31.8	275	31.7	337.5	31.7

Table 5.Water quality index by using Sumitomo and Nemerow model of 2009.

Date 2009		Jan.	Feb.	Mar..	Apr..	Mayy	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.	Average
EC	Av.	1405	1320	1304	1179	1405	1375	1570	1520	1593	1603	1426	1319	1418
	limit	1563	1563	1563	1563	1563	1563	1563	1563	1563	1563	1563	1563	1563
	Ci/Lij	0.9	0.84	0.83	0.75	0.9	0.88	1	0.97	1.02	1.03	0.91	0.84	0.91
TDS	Av.	900	918	894	867	1024	1200	1156	1146	1122	632	1048	1002	992.4
	limit	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
	Ci/Lij	0.9	0.92	0.89	0.86	1.02	1.2	1.16	1.15	1.12	0.63	1.05	1.00	0.992
Ca mg/L	Av.	22.39	13.81	14.51	8.3	145.3	147.3	150	176	156	115	113.3	147	100.7
	limit	150	150	150	150	150	150	150	150	150	150	150	150	150
	Ci/Lij	0.15	0.09	0.1	0.06	0.97	0.98	1	1.17	1.04	0.77	0.76	0.98	0.67
Mg mg/L	Av.	68.3	59.6	63.1	49.7	54.6	48.8	65.6	50.3	65.9	40.6	57.3	62.5	57.2
	limit	100	100	100	100	100	100	100	100	100	100	100	100	100
	Ci/Lij	0.68	0.6	0.63	0.5	0.55	0.49	0.66	0.5	0.66	0.41	0.57	0.62	0.57
Na	Av.	134	123	122	103	114	93	131	121	-	-	-	-	112

mg/L	limit	200	200	200	200	200	200	200	200	200	200	200	200	200
	Ci/Lij	0.67	0.61	0.61	0.52	0.57	0.47	0.65	0.61	-	-	-	-	0.59
CL mg/L	Av.	152	147	334	142	155	153	165	138	145	159	144	138	164
	limit	350	350	350	350	350	350	350	350	350	350	350	350	350
	Ci/Lij	0.43	0.42	0.95	0.41	0.44	0.44	0.47	0.39	0.41	0.45	0.41	0.39	0.47
So4 mg/L	Av.	340	313	334	334	372	439	431	447	456	426	342	365	375
	limit	400	400	400	400	400	400	400	400	400	400	400	400	400
	Ci/Lij	0.85	0.78	0.84	0.59	0.93	1.1	1.08	1.12	1.14	1.07	0.86	0.91	0.94
NO3 mg/L	Av.	1.15	1.25	0.8	0.9	0.25	1.5	2.5	2.4	0.05	0.6	1.03	0.7	1.09
	limit	50	50	50	50	50	50	50	50	50	50	50	50	50
	Ci/Lij	0.02	0.03	0.02	0.02	0.01	0.03	0.05	0.05	0.001	0.01	0.02	0.01	0.02
T.H	Av.	336	279	295	210	587	569	624	647	662	456	519	625	484
	limit	500	500	500	500	500	500	500	500	500	500	500	500	500
	Ci/Lij	0.7	0.6	0.6	0.4	1.2	1.1	1.2	1.3	1.3	0.9	1	1.3	1
pH	Av.	8.1	8	7.9	8	8	7.9	7.5	7.8	8	7.6	7.9	7.9	7.9
	limit	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5	6.5-8.5
	Ci/Lij	0.56	0.46	0.45	0.48	0.54	0.39	0.04	0.3	0.46	0.06	0.4	0.4	0.38

Table 6. Water quality index by using Sumitomo and Nemerow model of 2010.

2010							positive Ion					
	Average	limit	ci/Lij	Average	limit	Ci/Lij	Average	limit	Ci/Lij	Average	limit	Ci/Lij
Date	EC			TDS			Ca(mg/l)			Mg(mg/l)		
Jan.	1224	1563	0.783	892	1000	0.892	144	150	0.96	45.4	100	0.45
Feb.	1259	1563	0.806	1004	1000	1.004	148	150	0.99	70.4	100	0.7
Average	1242	1563	0.794	948	1000	0.948	146	150	0.97	57.9	100	0.58
Max	1259	1563	0.806	1004	1000	1.004	148	150	0.99	70.4	100	0.7

Table 7.Water quality index by using Sumitomo and Nemerow model of 2010

Negative Ion														
Average	limit	Ci/Li	Average	limit	Ci/Li	Average	limit	Ci/Li	Average	limit	Ci/Li	Average	limit	Ci/Li
CL(mg/l)			So4(mg/l)			NO3(mg/l)			T.H			pH		
126	350	0.36	324	400	0.81	0.55	50	0.01	548	500	1.1	7.8	6.5-8.5	0.3
111	350	0.32	392	400	0.98	0.7	50	0.01	659	500	1.3	7.6	6.5-8.5	0.1
119	350	0.34	358	400	0.9	0.63	50	0.01	604	500	1.2	7.7	6.5-8.5	0.2
126	350	0.36	392	400	0.98	0.7	50	0.01	659	500	1.3	7.8	6.5-8.5	0.3

Table 8. Chemical parameters, highest permitted value for water(Si), 1/Si,k and Wi

chemical parameters	highest permitted value for water(Si)	1/Si	k	Wi
Ec	1563	6E-04	5.941	0.004
TDS	1000	0.001		0.006
Ca	150	0.007		0.04
mg	100	0.01		0.059
Na	200	0.005		0.03
Cl	350	0.003		0.017
So4	400	0.003		0.015
No3	50	0.02		0.119
T.H	500	0.002		0.012
PH	8.5	0.118		0.699
Sum		0.168		1

Table (9) .Q of parameters Qi==100vi/Si during 2008

2008	EC	TDS	Ca	Mg	Na	Cl	So4	No3	T.H	pH	Q1*	Q2*	Q3*	Q4*	Q5*	Q6*	Q7*	Q8*	Q9*	Q10*	
Date	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	WQI
June	72	84	9	47	-	30	120	-	44.6	60	0.29	0.5	0.36	2.77	-	0.5	1.8	-	0.5	41.94	48.7
July	75	81	0	45	-	30	70	-	36.8	20	0.3	0.49	0	2.66	-	0.5	1.1	-	0.4	13.98	19.4
Aug.	74	84	5	49	-	30	60	-	36.4	34	0.3	0.5	0.2	2.89	-	0.5	0.9	-	0.4	23.77	29.5
Sept.	72	77	7	10	75	30	70	0.7	37.6	35	0.29	0.46	0.28	0.59	2.25	0.5	1.1	0.1	0.5	24.47	30.4
Oct.	74	82	5	49	45	30	80	1.67	42	63	0.3	0.49	0.2	2.89	1.35	0.5	1.2	0.2	0.5	44.04	51.7
Nov.	81	99	6	59	53	40	80	1.5	50	64	0.32	0.59	0.24	3.48	1.59	0.7	1.2	0.2	0.6	44.74	53.6
Dec.	91	1	18	73	83	50	110	2.7	73	65	0.36	0.01	0.72	4.31	2.49	0.9	1.7	0.3	0.9	45.43	57
											if depending max 57 poor			Good 26-50				Average WQI			41.5

Table 10. Q of parameters during 2009

2009	EC	TDS	Ca	Mg	Na	Cl	So4	No3	T.H	PH	Q1*	Q2*	Q3*	Q4*	Q5*	Q6*	Q7*	Q8*	Q9*	Q10*	WQI
Date	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	
Jan.	90	90	15	68	67	43	85	2	67	56	0.36	0.54	0.6	4.01	2.01	0.7	1.3	0.2	0.8	39.14	49.7
Feb.	84	92	9	60	61	42	78	3	56	46	0.34	0.55	0.36	3.54	1.83	0.7	1.2	0.4	0.7	32.15	41.7
Mar.	83	89	10	63	61	95	84	2	59	45	0.33	0.53	0.4	3.72	1.83	1.6	1.3	0.2	0.7	31.45	42.1
Apr.	75	87	6	50	52	41	59	2	42	48	0.3	0.52	0.24	2.95	1.56	0.7	0.9	0.2	0.5	33.55	41.4
May	90	102	97	55	57	44	93	1	117	54	0.36	0.61	3.88	3.25	1.71	0.7	1.4	0.1	1.4	37.75	51.2
Jun.	88	120	98	49	47	44	110	3	114	39	0.35	0.72	3.92	2.89	1.41	0.7	1.7	0.4	1.4	27.26	40.7
July	100	116	100	66	65	47	108	5	125	4	0.4	0.7	4	3.89	1.95	0.8	1.6	0.6	1.5	2.80	18.3
Aug.	97	115	117	50	61	39	112	5	129	30	0.39	0.69	4.68	2.95	1.83	0.7	1.7	0.6	1.5	20.97	36
Sept.	102	112	104	66	-	41	114	0	132	46	0.41	0.67	4.16	3.89	-	0.7	1.7	0	1.6	32.15	45.3
Oct.	103	63	77	41	-	45	107	1	91	6	0.41	0.38	3.08	2.42	-	0.8	1.6	0.1	1.1	4.19	14.1
Nov.	91	105	76	57	-	41	86	2	104	40	0.36	0.63	3.04	3.36	-	0.7	1.3	0.2	1.2	27.96	38.8
Dec.	84	100	98	62	-	39	91	1	125	40	0.34	0.6	3.92	3.66	-	0.7	1.4	0.1	1.5	27.96	40.1
																Average WQI = 40.7					

Table 11. Q of parameters during 2010

2008	EC	TDS	Ca	Mg	Na	Cl	So4	No3	T.H	pH	Q1*	Q2*	Q3*	Q4*	Q5*	Q6*	Q7*	Q8*	Q9*	Q10*	WQI
Date	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	
Jan.	78	89	96	45	-	36	81	1	110	56	0.31	0.53	3.84	2.66	-	0.6	1.2	0.1	1.3	30	40.6
Feb.	81	100	99	70	-	32	98	1	130	10	0.32	0.6	3.96	4.13	-	0.5	1.5	0.1	1.6	10	22.7
											if depending max 40.6poor							Good 26-50		Av. WQI	31.7

Figure 1. The Google map of Al-Hindiya barrage

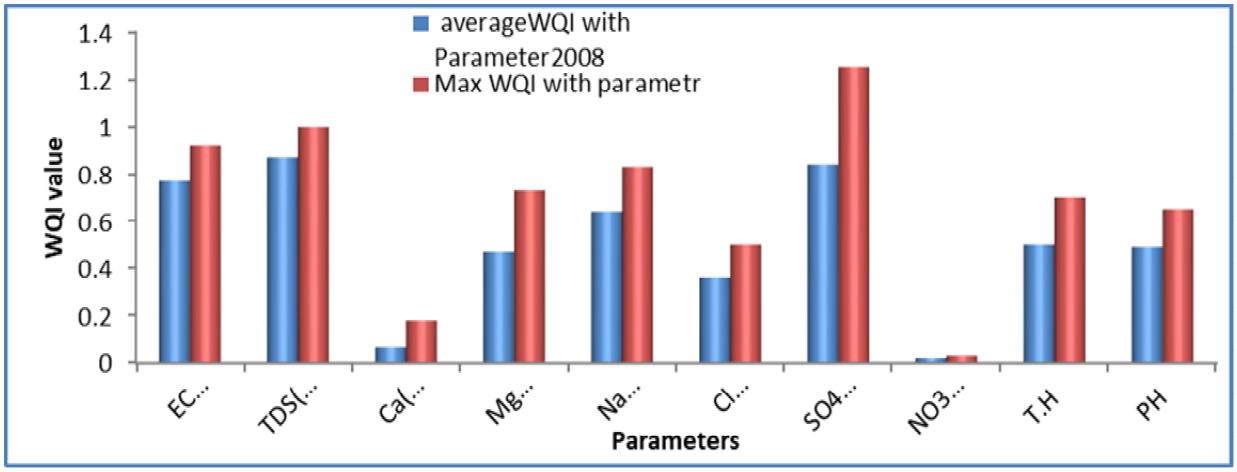

Figure 2. The average WQI and Max WQI in 2008

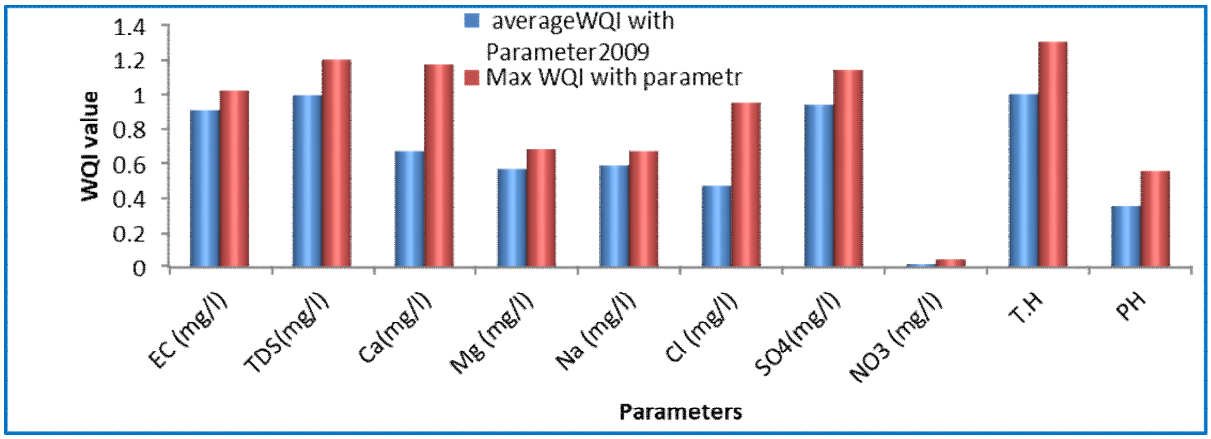

Figure 3. The average WQI and Max WQI in 2009

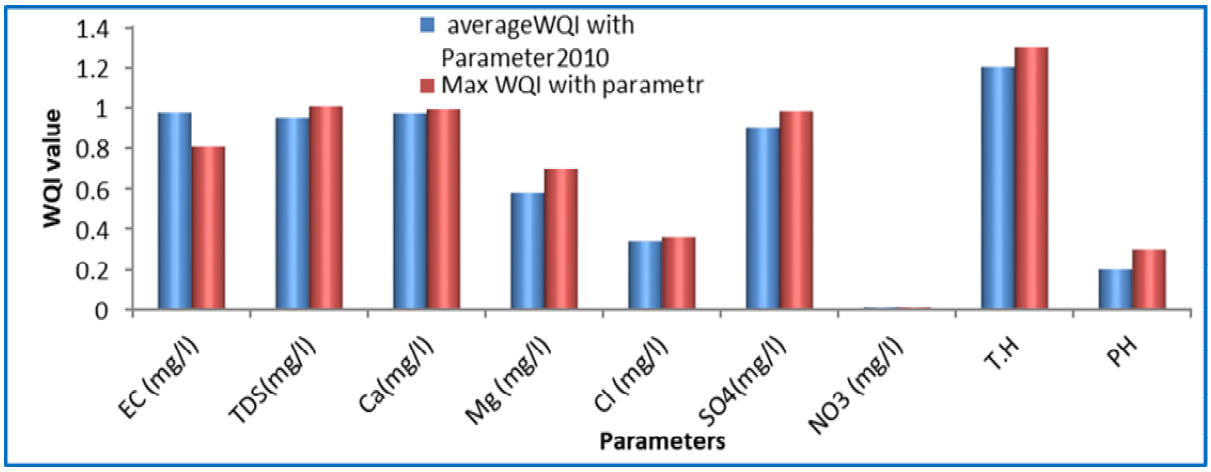

Figure 4. The average WQI and Max WQI in 2008

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RESEARCH ARTICLE

Assessment of Hypercapnia Induced Biomarker Response in Sea Star *Goniodiscaster scaber* (Moebius, 1859) – A Microcosm Approach for Climate Change Induced Ocean Acidification

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ABSTRACT

The on-going pH fall in seawater due to massive anthropogenic carbon dioxide mixing in seawater and subsequent change in seawater carbonate chemistry is termed as ocean acidification. Ocean pH was already reduced from pH 8.24 to 8.14 in regional oceans.Business-as-usual scenario prediction has been foreseen that seawater pH will further decrease to pH 7.8 by the end of this century.Today ocean acidification is considered as global issue and its impact is highly concerned on marine flora and fauna. The present study deals with the estimation of physiology related biomarkers towards the impact of decreasing pH on the sea star *Goniodiscaster scaber*. *G.scaber* were exposed to three different scenario levels of seawater pH (S1 – pH 8.2, S2 – pH 8.0 and S3 – pH 7.8) in microcosm hypercapnia using the method of CO_2 bubbling for 15 days. At the end of exposure, biomarkers such as acetylcholine esterase (AChE), catalase (CAT), glutathione-S-Transferase (GST), Lipid peroxidase (LPx) and reduced glutathione (GSH) were quantified with spectroscopic measurements.The concentration of malondialdehyde is highest (0.340 nanomoles of MDA/mg protein) in pH 7.8 exposed animals. The high-level MDA formation and the lowest catalase activity (0.350 µmoles H_2O_2 consumed/min/mg protein) indicate that sea star would not able to cope up the scenario 3 level of ocean acidification. Based on the hypercapnia exposure, biomarker levels moved towards the pathetic level and the changes in each biomarker were highly significant ($p < 0.01$).

Keywords: Ocean Acidification, reduced pH, hypercapnia, sea star and biomarkers.

INTRODUCTION

As Intergovernmental Panel on Climate Change mentioned in section 3.3.11 in IPCC special report, it is a time for giving importance to explore ocean acidification impact on marine ecosystem so as the global ocean research peers are intensively focusing on ocean acidification research [1]. Ocean acidification is the on-going decrease in the pH of global oceans which is happened by continuous uptake of carbon dioxide from the atmosphere [2, 3, 4]. About thirty percentages of CO_2 sources to ocean is from anthropogenic emission. The seawater carbonate system includes pCO2, carbonic acid, carbonate ions and bicarbonate ions are abruptly changed during CO_2 uptake process. Eventually the hydrogen ion concentration will increase in seawater during high CO_2 uptake thereby pH is reduced. pH of seawater has been reducing since the starting period of industrialization era so initially the ocean pH was above 8.25 and but at present, the ocean pH is reduced to 8.12. 'Business as usual scenario' levels of pH reduction were predicted by IPCC for the year 2050 and 2100 will be pH 8.0 and pH 7.8 respectively [5].

Sensitive marine animals cannot tolerate 0.1 pH variation to thrive. In recent report, Coral reefs were bleached due to combination of temperature and pH stressors [6]. Marine calcifiers are the sensitive responders to ocean acidification. Therefore, Ocean acidification has been known to cause a serious threat to the marine calcifiers which produce their shells or skeletons using calcium carbonate ($CaCO_3$) [7]. Many findings have been reported in view of calcification response in marine calcifiers towards ocean acidification. But biomarker response of marine calcifiers against ocean acidification was less studied. We chose sea star (phylum Echinodermata) to study the hypercapnia induced biomarker response towards the simulation of ocean acidification impacts. Hypercapnia is the physical condition of abnormally high level of carbon dioxide in the hydrology or circulation system. Sea star is considered as a key stone animal in marine ecosystem. Miniature stress to sea star would have serious pose to surrounding food chain. Biomarkers are useful to measure the early threatening signals of hypercapnia exposure and to measure the pre-adverse effects. This monitoring would also be used to assess the health of associated communities of the sea star [8]. Apart from hypercapnia, thermal stress associated responses of biomarker were reported frequently [9,10,11]. Theoretically, ocean acidification affects the maintenance of acid-base regulation in animal metabolism thereby alterations in antioxidant system and oxidative damage in cellular system. Therefore, we hypothesized that hypercapnic low pH exposure to sea star alters the acid base regulation therefore the physiology would be negatively affected. In the present study, the CO_2 perturbation experiment was carried out to manipulate the pH as per business as usual scenario levels of the year 2050 (pH 8.0) and 2100 (pH 7.8) predicted by IPCC and ambient level pH (pH 8.2) was considered as control. The effects of CO_2 hypercapnia condition have been investigated in the biscuit sea star *Goniodiscaster scaber* a bottom dwelling animal for the assessment of physiology related biomarker enzymes.

MATERIALS AND METHODS

Sea star collection & Laboratory acclimatization

Sea star *Goniodiscaster scaber* was collected by diving and hand picking by fisherman at Vedalai coast (Latitude 9.216943°N and Longitude 79.079000°E), Gulf of Mannar, India during April 2018. Totally 43 numbers of sea stars were collected and immediately brought to the Pudumadam marine field research station, Ramnad district, Tamil Nadu, India and acclimated into 100 litres tank with aerator. Size of the animals in average was 10.6±1.44 cm total length, 3.57±0.55 cm arm length and 4.67±0.57 cm circular length. The average weight of the animal was 83.07±52.49 gms. Pudumadam shore's sea water was filtered and used for whole experiment. *Sargassum* sp. was fed once per day up to acclimation period and stopped the day before start of experiment. Acclimation was done for five days.

Experimental hypercapnia set up

Three different scenario levels were selected as per the prediction reported by Intergovernmental Panel on Climate Change [12]. Scenario 1 (S1) is the current ocean pH level, today the ocean pH is 8.2. pH 8.2 is nominal pH but actual ocean pH in wild condition is between 8.1 and 8.2. Scenario 2 (S2) is the near future ocean acidification i.e. in the year 2050 the ocean pH is expected to become pH 8.0. Scenario 3 (S3) is the century end ocean acidification i.e. in the year 2100 the ocean pH is expected to become pH 7.8. These scenario levels of ocean acidification were simulated in laboratory using CO_2 gas bubbling method [13]. CO_2 gas cylinder connected with two storage tanks containing filtered seawater. CO_2 gas was released into the storage tank 1 & 2 and labelled as S2 and S3 respectively. CO_2 gas bubbling was stopped when S2 & S3 tank reach the pH 8.0 and pH 7.8 respectively. S1 tank was not bubbled with CO_2 because S1 was the ambient pH (pH 8.2) therefore S1 was considered as control. Three healthy *G. scaber* were introduced into S1, S2 and S3 microcosm chambers (7 litres) for different pH treatments viz., pH 8.2, pH 8.0 and pH 7.8 respectively. Every 2 hours the pH was monitored and maintained to stabilize the three-nominal pH. Continuous hypercapnia exposure of 3 level pH treatments to *G.scaber* was ended on 15th day.

Physicochemical parameters

During experiment, the sea water in all test chambers renewed with filtered seawater everyday using siphoning. The physicochemical parameters such as pH, salinity, temperature and total alkalinity were measured.Among these, pH and temperature was measured using pH ion meter (Eutech bench pH/Ion meter Model pH 2100). Salinity was measured using hand refractometer (Atago Master-S). Total alkalinity was measured by volumetric titration method [14]. The associated carbonate parameters such as carbonate, bicarbonate, pCO2, fCO2, TCO2, calcite and aragonite were derived using CO2CALC software [15].

Biomarker analysis
Tissue preparation

At the end of 15th day hypercapnia exposure, the digestive tissues of *G. scaber* exposed to S1 (pH 8.2), S2 (pH 8.0) and S3 (pH 7.8) were dissected out for tissue preparation. The outer skeleton of *G. scaber* was discarded and digestive tissues were gently removed then weighed. One gram of tissue was used for each of the following assay.

Protein

According to the method of Lowry *et al.* (1951), *G.scaber* exposed to S1 (pH 8.2), S2 (pH 8.0) and S3 (pH 7.8) were estimated for their protein concentration [16].

Acetylcholine esterase (AChE)

AChE were spectroscopically quantified using the method described by Ellman *et al.,* (1961) [17]. Animals exposed to S1 (pH 8.2), S2 (pH 8.0) and S3 (pH 7.8) digestive tissues were homogenized with 5 ml of Tris-HCL + sucrose buffer then centrifuged for 5000 rpm for 15 min at 4^{o}C. Enzymes were more prevalent in supernatant. 3.12 ml of buffered Dithionitrobensoic acid (DTNB) in S1, S2 and S3 labelled test tube. 0.8 ml of enzymes were added in all 3 tubes. OD was read at 0th and 5th min at 405 nm at 25^{o}C. To all the tubes, 0.08 ml of Acetyl Thiocholine Iodine (ACTI) was added and mixed well then OD was read at 0th and 5th min at 412 nm at 25^{o}C. To avoid non-enzyme reaction. Preincubation of DTNB + samples before the addition of ACTI was done.

Catalase (CAT)

Catalase activity was measured according to Sinha *et al.* (1972) [18], 1ml of 10mM phosphate buffer was added to blank. 0.4 ml of 10mM phosphate buffer was added to 3 sample test tubes. 0.1 ml of each enzyme sample added to S1,S2 and S3 tubes. 0.5 ml of 0.2 M Hydrogen peroxide (H_2O_2) was added to each tubes. 2ml of Dichromate Acetic Acid (DAA) was added to all tube at 0^{th} sec, 30^{th} sec and 60^{th} sec so that reaction will be arrested. After DAA addition blue color was formed. All tubes were boiled at water bath for 10 min at 100°C then Green color was developed. All tubes were cooled to room temperature and OD was measured at 570 nm.

Glutathione- S–transferase (GST)

GST enzyme was assayed with the method of Habig *et al*. (1974) [19]. 0.1 ml of 1mM Glutathione (GSH) was added to S1, S2 and S3 tubes. 1 ml of 50mM phosphate buffer was added to each tube. 0.5 ml of enzyme source was added to all tubes. 1.8 ml of distilled water and 1.3 ml of distilled water was added to blank and test respectively. All tubes were incubated for 2 min at room temperature. 0.1 ml of 1-chloro-2,4, dinitrobenzene (CDNB) was added to all tubes. OD was measure at 340 nm at 0^{th} and 3^{rd} minute.

Lipid Peroxidase (LPx)

LPx was assayed using the method followed by Ohkawa *et al.* (1979) [20]. 0.2 ml of enzyme sample was added to each test tube. 0.2 ml of SDS and 1.5 ml of 20% acetic acid was added and mixed well. 1.5 ml of 0.8 Thiobarbituric acid (TBA) was added and heated in water bath at 95°C for 60 sec. 0.1 ml of distilled water was added to all tubes. OD was measured at 532 nm. MDA standard prepared for 2 – 20 nm.

Reduced Glutathione (GSH)

GSH activity was measured using the method followed by Moron *et al.* (1979) [21]. 0.8 ml of samples were added to each test tube. 0.8 ml of 0.2 ml of phosphate buffer was added to control. 2 ml of 600 mM DTNB was added to all tubes. 0.2 ml of 5% trichloro acetic acid was added to blank and test. Absorbance was read at 412 nm. Standards were prepared using 2mM reduced glutathione.

Statistical analysis

Descriptive statistics were analysed for seawater physicochemical parameters and single factor one way analysis of variance was used to analyze the significance of the changes observed in biomarker analysis of the *G. scaber*.

RESULTS AND DISCUSSION

The seawater carbonate system of the oceans has been altered over the year and the effect to the living creatures is not still clear[22]. In the perspective of calcification, the increasing CO_2 level in ocean waters cause to have more corrosive conditions for marine calcifying organisms and more difficult for them to build and maintain their carbonate skeletons [23]. Experimental exposure to reduced pH, and the associated changes in seawater carbonate chemistry has been shown to reduce the calcification of various marine invertebrate species [24, 25, 26]. However, the physiology related biomarker response against ocean acidification has gaps to study.

Hypercapnia changes of physicochemical parameters

Physicochemical parameters in S1, S2 and S3 seawater chambers were adopted as per hypercapnic nominal pH 8.2, 8.0 and 7.8 respectively. The observed physicochemical parameters of each nominal chambers were summarised in Table 1. The mean pH of S1, S2 and S3 were 8.23±0.02, 8.03±0.03 and 7.82±0.01 respectively. In all chamber the effective pH was maintained against the nominal pH. The mean temperature of S1, S2 and S3 were 29.8±0.4ºC 29.8±0.3ºC and 29.7±0.2ºC respectively. The temperature level was maneuvered during the hypercapnic experiment. The mean salinity of S1, S2 and S3 were 31.8±0.7 PSU, 31.9±0.7 PSU and 32.4±0.8 PSU respectively. Salinity was not varied maximum during study period. The mean total alkalinity of S1, S2 and S3 were 2492±47 µmol/kgSW, 2462±46 µmol/kgSW and 2395±45 µmol/kgSW respectively. Alkalinity is varied slightly due to CO_2-air gas exchanges. These four parameters were taken as input in CO2CALC software and the related seawater carbonate parameters such as pCO_2, fCO_2, TCO_2, OH, Boron Alkalinity, carbonate ion concentration, bicarbonate ion concentration, Revelle factor, calcite and aragonite were derived and summarized in Table 1. These parameters were significantly changed according to pH, temperature, salinity and total alkalinity.

Biomarker analysis

Sea stars have key ecological role in its ecosystem and their detritus feeding habits. As they have the unique water vascular system for its feeding and metabolism, they are vulnerable to physiological stress against hypercapnic condition in surrounding waters. Therefore in the present study sea star *G.scaber* was assessed to analyze the hypercapnia stress induced by the microcosm simulated ocean acidification using a series of biomarker enzymes. Various biotic and abiotic stressors are well known to indicate through biomarker responses [27]. In this study biomarkers indicative of neurotoxicity (acetylcholinesterase, AChE), oxidative stress (lipid peroxidation, LPx) and phase II biotransformation of xenobiotics (glutathione S transferase, GST and reduced glutathione, GSH) were measured to assess the effects of reduced pH scenarios on the test animals.

Protein

The level of protein concentration had depleted towards hypercapnic condition. The Highest protein level (0.285 mg/g) was observed in pH 8.2 (control) whereas the lowest protein level (0.096 mg/g) was observed in pH 7.8 treated animals (Fig. 1a). Hypercapnia has been shown to cause metabolic suppression in protostome invertebrates through disruptions in internal acid/base balance, although it is also clear that differences could be seen and some species have a better tolerance of pCO_2-induced acidosis than others [28].

Acetylcholine esterase (AChE)

AChE values showed in present study had moderate positive values in S1 (control) animals whereas S2 and S3 treated animals showed the low level acetylcholine esterase which ensures the inhibitory function of ACTI by acid base regulation. Significant changes in ACTI activity was recorded across all pH treatments. Highest activity (1.808 µmol ACTI min/ mg protein) was observed in pH 8.2 (control) whereas lowest activity (1.298 µmol ACTI min/ mg protein) was seen in pH 7.8 treated animals (Fig. 1b). Acetylcholine is the neurotransmitter in the central nervous system whereas AChE is the serine hydrolase which comes under the enzyme family of carboxylesterase. Acetyl Choline esterase enzyme in nervous system is essential for the various functions of the neurotransmitter acetylcholine (ACh). The declination of Acetylcholine esterase results in the failure of neuronal activity. Therefore, presence of acetylcholine esterase is more important than the prevalence of acetylcholine or its impact on tissue [29]. After every signal process in nervous system, breaking down of acetylcholine into its two component parts, acetic acid and choline is occurred so that acetylcholine will not be accumulated in nervous system [30]. AChE reduction leads to accumulation of acetylcholine which tends animals to weakness, paralysis and death [31]. Echinoderms like

Thyone briareus (sea cucumber) and *Asterias forbesi* (sea star) reported for their presence of acetylcholine esterase level in which sea star had 0.384 µmol AChE min/ mg protein and sea cucumber had 0.820 µmol AChE min/ mg protein in their radial nerve cords.

Catalase (CAT)

Animal metabolism is highly impaired by extreme low or extreme high temperature with reference to its optimum temperature. As invertebrates are ectotherms, they respond strongly to the varying temperature [32]. However, studies of metabolism & physiology in varying pH are not clear. In present study, the highest activity (0.626 µmoles H_2O_2 consumed/min/mg protein) was observed in pH 8.2 (control) whereas lowest activity (0.350 µmoles H_2O_2 consumed/min/mg protein) expressed in pH 7.8 (Fig. 1c). As catalase is the essential antioxidant, its reduced state of catalase in low pH indicated that sea star under scenario of pH 7.8 would have diminished physiological activity and lose its defence systems against H_2O_2. Previous studies reported that the antioxidant system be activated or inhibited under chemical stress [33, 34]. These two kinds of responses are not contradictory and depend on the intensity and duration of the stress that the organisms exposed to the sensitivity of the species being considered. Activation can be considered as an adaptation to the environmental stress to prevent toxicity (protective response to reactive oxygen species), whereas inhibition suggests a later stage of oxidative stress, characterized by increased sensitivity to contaminant exposure [35].

Glutathione- S–transferase (GST)

The increasing sign of GST enzyme in animal cells indicated that they were rapidly involving cellular detoxification [36]. In present study, lowest activity (0.996 µmoles of GSH & CDNB conjugate formed/min/mg protein) was observed in pH 8.2 (control) whereas highest activity (1.223 µmoles of GSH & CDNB conjugate formed/min/mg protein) (Fig. 1d). These detoxification functions against hypercapnic exposure implying that glutathione conjugated with cellular stressors.

Lipid Peroxidase (LPx)

The concentration of malondialdehyde is highest (0.340 nm of MDA/mg protein) in pH 7.8. Where as lowest activity (0.117 nm of MDA/mg protein) was observed in pH 8.2 (control) (Fig. 1e). In the present study the hypercapnic responses were highly elevated on the activity of LPx which is implying that highest MDA formation in low pH treatment. Increasing malondialdehyde represent the more lipid damage during hypercapnic condition. Therefore, it would be hypothesized that the early life stages could affect the lipid biosynthesis and regulation. On the contrary early life stages of sea urchin did not affect lipid utilization under elevated pCO_2 levels [37]. However, the effect of hypercapnic response can be species specific. Compare to previous reports, our results agreed with increasing the level of malondialdehyde with hypercapnic exposure [38, 39].

Reduced Glutathione (GSH)

Reduced glutathione is highly found as intracellular thiol and has a role in antioxidant detoxification mechanism [40]. As reduced glutathione is considered as first line defence mechanism of cells, its depletion is known to indicative of stress and predisposing factor for highly adverse effects.Highest activity (0.073 µmoles of RG/min/mg protein) (control) whereas lowest activity (0.039 µmoles of RG/min/mg protein) was observed in pH 7.8 (Fig. 1f).

Statistical analysis

All quantitative variables of the physicochemical parameters have been expressed in mean ± SD (Table 1). All the biomarker assessed in the present study have significant impact based on the hypercapnia stress. In specific, the changes in each biomarker were highly significant ($p < 0.01$) according to the three scenario levels of hypercapnia exposure (Table 2).

CONCLUSION

In this present study the activities of AChE, CAT, GST, LPx and GSH was observed for the acute exposure to a series of reduced seawater pH.The CO_2 stressor was found to trigger neurotoxicity, oxidative and xenobiotic stress. Therefore, the decreased levels of protein, AChE, CAT, and reduced glutathione as well as the increased levels of GST and LPx indicates the alarming signs of the neurotoxicity and xenobiotic stress. The overall view in the present study enunciated that the *G.scaber* would lost the capability to tolerate to the hypercapnia conditions of S3 scenario expected in the year 2100. Therefore, it is mandatory to improvise the research in this area for better understanding of the genomic perspective of their mode of adaptation towards the future impact of ocean acidification.

Ethical approval and informed consent

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Authors' Contributions

K. Rangesh and M. Anand designed the work and carried out the manuscript writing, correction and discussion part. K. Rangesh, B. Rajeswari and R. Jeeva Priya executed work in microcosm experiment and biomarker assessment. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Physicochemical parameters of the seawater during microcosm hypercapnic treatment of three scenarios

Factors	Unit	S1	S2	S3
pH	--	8.23±0.02	8.03±0.03	7.82±0.01
Temp	(°C)	29.8±0.4	29.8±0.3	29.7±0.2
Salinty	(PSU)	31.8±0.7	31.9±0.7	32.4±0.8
Total Alkalinity	µmol/kgSW	2492±47	2462±46	2395±45
pCO_2	µatm	247±72	446±6	763±3
fCO_2	µatm	247±26	445±2	761±03
TCO_2	µmol/kgSW	2013±42	2135±8	2183±11
B Alk	µmol/kgSW	126±8	87±7	62±9

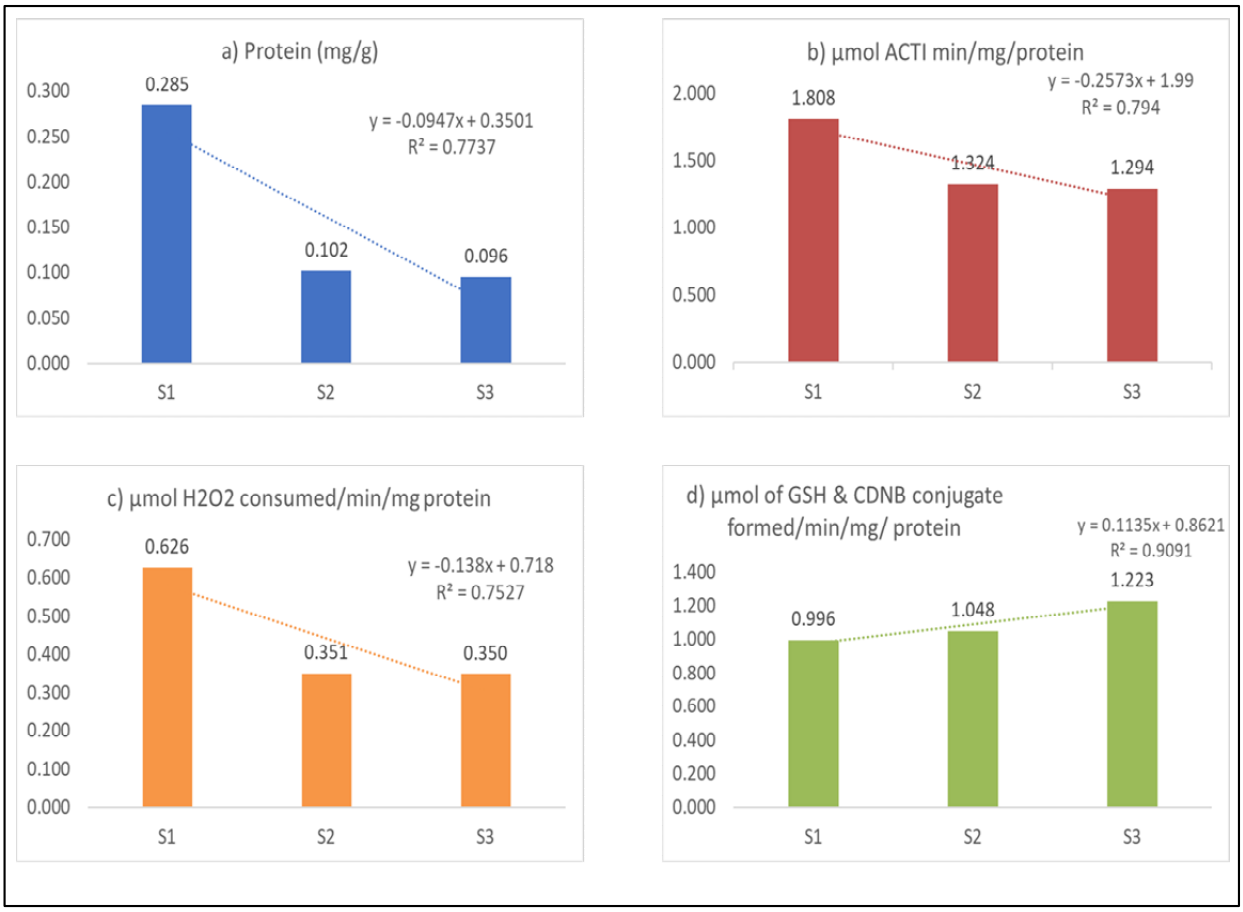
OH	µmol/kgSW	14±5	9±2	5±0.8
CO_3	µmol/kgSW	343±6	240±6	162±6
HCO_3	µmol/kgSW	1663±46	1883±7	2000±91
Revelle	--	7.9±0.6	9.5±0.9	11±0.6
Calcite	ΩCa	8.5±0.8	6.0±0.5	4.0±0.4
Aragonite	ΩAr	5.7±0.7	4.0±0.1	2.6±0.9

* Physiochemical parameters derived using CO2CALC software. S1, S2 & S3 – Scenario of seawater pH levels (S1 – pH 8.2; S2 – pH 8.0; and S3 – pH 7.8).

Table 2. One-way ANOVA of biomarker enzymes in response to different hypercapnic exposure

Biomarkers	p Value	Significance level
Protein	5.57E-06	0.000*
AChE	4.69E-07	0.000*
CAT	8.69E-07	0.000*
GST	8.55E-07	0.000*
LPx	5.31E-07	0.000*
RG	2.73E-07	0.000*

* Highly significant variations



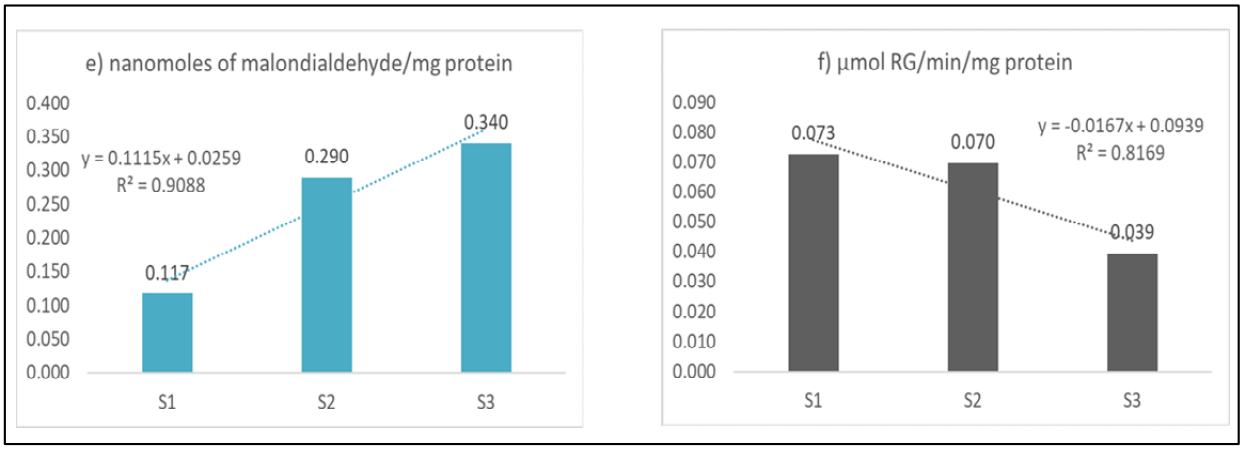


Fig. 1. Physiological responses in digestive tissues of *G. scaber* at different pH exposure; a) Protein level b) Acetylcholine esterase activity c) Catalase activity d) Glutathione S- transferase activity e) Lipid peroxidation activity and f) Reduced Glutathione activity. S1, S2 & S3 – Scenario of seawater pH levels (S1 – pH 8.2; S2 – pH 8.0; and S3 – pH 7.8).

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REVIEW ARTICLE

Accelerating India's Energy Sector to Sustainable Sources, Potentials and Prospects

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ABSTRACT

Renewable energy sources such as solar, wind, hydro, biomass and others are sustainable as they are stable, nonpolluting and abundantly available at free of cost. These sources have adequate capability to deliver solutions to the existed energy constraint being faced by the developed and developing countries. India, being a fast developing country, will need a secured renewable energy supply of two-three times more than that of the total energy consumed today. In 2018, India occupied the second position among the prominent economic countries to lead to progression of renewable energy. At present, the contribution of renewable power is approximately 35% of India's total installed electric power capacity and is aimed to share 40% by 2030. The present article explores an overview of renewable energy progression in terms of solar, wind, hydro and biomass in India. There are two different approaches which have been analyzed; first approach is the current status of renewable energy in terms of sources and technologies. Second approach is the representation of the future renewable energy potentials. Through this study, Indian electricity target can be achieved by better government policies, public awareness and affordable renewable energy systems in future. A moderate growth has been observed since 2010, however, a major shift is observed from 2018 onwards.

Keywords- Renewable Energy; Renewable Energy Sources (RES); Energy Progression; Wind Energy; Solar Energy.

INTRODUCTION

Energy is essential for all living organisms on the earth and is mostly derived from Sun, a primary source of energy. This provides sustainable live, essential services such as cooking, clinics, transportations and education, and is necessary for the generation of industry, business, jobs and better economy (1). The current energy generation and consumption system is unsustainable, mostly from fossil fuel. The environmental issues are because of the greenhouse gasses (GHGs) emission, which adds to the global warming (2-6). To keep the average global temperature rise below 1.5°C, people need to adopt renewable energy uses and should leave 80% of fossil fuel to be reserved in the ground. For transforming the global energy sector from fossil-based to zero-carbon based, the energy progression can be a pathway by 2050 (7). While there are substantial economic, world population and technological constraint to be resolved, social and structural hindrances may be more serious. These turns act as an initiative for enhancements in existing systems, instead of adopting new approaches, conventions and technologies (8-10). To enhance the general people's awareness towards renewable energy technologies instead of traditional energy consumption, efficient renewable energy systems (RESs) are being promoted to overcome the potential energy crunch in future. The renewable energy sectors have attracted more attention in the field of research, industries, and economy, hence, different countries and regions should take adequate initiatives through different policies to enhance their renewable energy potential.

The implementation of mechanized systems in place of manual labour by use of energy brought a revolution in the late 18th and early 19th centuries, which subsequently enhanced the economy through production. The burning of coal and wood biomass were the main energy sources in the world during the early 20th century and the percentage of share was around 60% and 40% respectively (11-13). As a global platform, UN Sustainable Energy for all (SEforALL) empowers to provide multiple opportunities to various sustainable energy utilizations, by improving energy efficient technology and enhancing the use of renewable energy to fulfill the energy requirement in different countries (14). International Energy Agency (IEA) market analysis and forecast from 2018 to 2023 on renewable energy and technologies provide a specific scenario based on trends and progresses for renewable energy in three major sectors namely; electricity, heat and transport sectors (15-18). The renewable power additions have grown by almost 12% in 2019, the fastest stride since 2015 to reach almost 200 GW through solar photovoltaic (SPV) power and wind power (15, 16, 19). The aims of Sustainable Development Goals (SDGs) – 17 of United Nations (UN) is to build more prosperous, more reliable, and more secure world with modern renewable energy services by 2030 (20-26). It needs to grow additionally by 300 GW on average each year with GHGs mitigation between 2018 and 2030 to acquire the goals of the Paris Agreement (19, 27-29). Renewable energy powers are comprised of small, medium and large scale solar, wind and hydro systems. Small and medium solar systems are used in residential power requirements. In the residential SPV systems, the surplus power is injected to the nearby grid during the day time and power is consumed from the grid at night. However, in rural areas hybrid renewable energy systems (HREs) are used in off-gird or micro grids (MGs) mode to power individual houses (30-35).

Around 239.2 million people in India out of an estimated 1.1 billion, 14% of the total global population, do not have access to electricity. According to Energy Access Outlook 2017 (36-39), many million people still suffer from unreliable supply (7). It creates health risk, poor development and improper quality of life. After China and U.S.A., the per capita use of energy in India is placed at the third rank in world (40, 41, 21). An estimated 70% of India's energy requirement is used by fossil fuel to lead 6.65% of carbon dioxide emission of the world (42, 43, 52). The Bhakra Nangal Dam in Himachal Pradesh, which was inaugurated by Jawaharlal Nehru, the first Prime Minister of India, named it as the "New Temple of Resurgent India". It has the potential to generate 1500 MW (44, 45, 48). The Ministry of non-conventional energy sources was set up in the year 1980 in India for the first time in world. Renewable energy in India is regulated under Ministry of New and Renewable Energy (MNRE) (45, 49-51). The primary aim is of MNRE to supplement the energy requirements of the country (46, 47). The Government of India has committed to achieve more than 40% of the generated electricity from renewable energy sources (RES) in its

declaration to the United Nations Framework Convention on Climate Change (UNFSCC) by 2030 (21, 27, 52-54). It has risen significantly in the past decade at a compound annual growth rate of more than 5%, but still energy deficiency is more in rural areas as comparison with urban areas (16, 17, 52, 55). Rural people use kerosene byproducts for lighting, which also contribute to climate changing through black carbon emissions (56-61). The government has developed many plans to overcome the power gap through very low carbon emitted renewable power sources (specifically power from hydro, wind, solar and biomass) (11, 21, 26, 45, 62).

Out of the already installed capacity of the renewable power of 80.47 GW, the individual contribution of wind, solar, biomass and small hydro are 36.37 GW, 29.55 GW, 9.81 GW and 4.6 GW respectively till 30 June 2019 (51, 52, 63-76). The new electrical power target needs renewable power capacity of 227 GW (earlier 175 GW) requiring additional $50 billion in investments by 2022 (77). It is being identified that the five predominant sources - solar, wind, biomass, ocean and geothermal of the renewable energy can easily fulfill the future energy requirement (14, 23, 52, 78-82). The use of modern renewable energy sources can ensure a better life standard of people. For example, use of LPG instead of firewood or kerosene for cooking can minimize air pollution. The use of electricity can facilitate better education, transportation and production to many more millions of overarching livelihood (83-89).

Energy Progression Pathways

The rapid pace of modernization in both developing and developed countries like India, China, U.S. Russia, German and Japan are embarking upon long-term structural deviations in the global energy system. Access to better energy is essential to decrease poverty in the world. At present approximately 70% of the global electricity requirement is managed by fossil fuels (1, 2, 7). In the last two decades, the world has skilled two main fundamental energy progressions: fuel and technologies. These progressions are very essential to maintain healthy environment, including zero carbon emission and economy development [3-6, 14]. Energy progression for the first time has been happened in 19th century during which the world moved from traditional wood to coal. It has been observed that wood makes a leading fuel role for more decades but later overtaken by coal. The next energy progression occurred when coal itself was overtaken by hydrocarbons (oil and natural gas) in the 20th century (8-10, 15, 23).

According to world energy transition timeline of WEO, the world is now concentrating on energy mix with low-carbon sources for sustainable energy progression to optimize customer processes. Renewable energy and technologies are now being an emerging area to hike today's global energy capacity levels to 50% by 2050, especially from SPV, wind, biomass and geothermal (16-20). The International Renewable Energy Agency (IRENA) published that the total cumulative renewable power capacity has reached 2,378 GW by the end of 2018 in data and statistic chapter. It is around third of total installed electric power capacity in the world (18, 21) as shown in figure 1. The top five countries which have installed renewable power capacity are China, followed by the United States, Brazil, India and Germany. In this year, Asia added 61% of renewable energy capacity and 70% of solar energy capacity to the world. Sustaining the progress, China, India, Japan and Korea accounted for most of this.

India's Geographic Sketch

India is the seventh-largest, the second-most populous country in the world, with three hundreds clear sunny days, over a dozen enduring rivers, a coastline of 7516.6 km and total area of 3.287x10^{6}km^2. The mainland of India extends between 8^{o}4' and 37^{o}6' N latitude and 68^{o}77' and 97^{o}25' E longitude. The country possesses a wide range of natural sources and largest young workforce for leading the industry revolution, renewable environment and economic development (9, 42, 45, 51). Most part of India receives high intensity of solar sources with an average of 5 kWh/ m^2/day (38, 51, 55, 63, 76) as presented in figure 2.

Current Status of Indian Electricity Sector

The electricity sector is contributing significantly to the country's economic growth. It needs a valuable rolein defining, designing, and implementing the different projects, which will contribute benefits to the customers (15, 18). This sector has one national grid with an installed capacity of 357.88 GW (including central, state and private sector) till 30 June 2019 to become third big producer and consumer in the world (43, 55, 56, 65). It is elaborately stated in table 1 and figure 3. The total electricity generation and consumption in India during 2018-19 fiscal year is 1,547 TWh and 1,181 kWh respectively (45, 48-52). The Parliament of India passed the electricity Act, 2003to transform the power sector in India (46). National Action Plan on Climate Change (NAPCC) made a policy which provides a roadmap for the share of renewable energy in the total electric generation capacity in the country. The electricity projection is given by Central Electricity Authority (CEA) for All-India level forecast, including energy consumption, energy requirement, peak demand etc.(47-50).

The compound annual growth rate (CAGR) of the electricity projections is given in table 2 (20).

The CAGR formula is defind as, CAGR = $\left(\frac{End\ value}{Start\ value}\right)^{\left(\frac{1}{years}\right)} - 1$ (1)

The Indian electricity market is a system; commodity capable of purchases, sales and trading of electricity, is given in table 3. It is design and developed to provide reliable electricity access to consumers, is given in figure 4 (power flow rightward and cash flow leftward) (20, 41, 45).

Discussion on renewable energy

India is the biggest emitter of GHGs after US and China in the world (4, 5). In Paris agreement, Indian government has committed to boost renewable energy capacity to 40% and to reduce emission of carbon by 33-35% from its 2005 levels by 2030 (7, 27, 28, 58, 78). According to the International Energy Agency (IEA) report, sustainable energy demand will propagate at the rate of 7.3% of cumulative average annual growth rate from 2007 to 2030 (18, 19, 24, 36, 37). About the international environmental gifts: Indian prime minister has donated 'Gandhi Solar Park' to the United Nations (UN) in Climate Action Summit on 24 September 2019, which will generate 50 kW/day of electricity. Another one is 'Gandhi Peace Garden' made up of 150 trees, which is located at Old Westbury's university campus (89). These actions say about India's commitment against fighting with climate change in Paris Agreement at Paris and International Solar Alliance (ISA) at New Delhi.

The renewable energy sector in India has become an important performer in the grid connected power generation capacity. The renewable energy installed capacity reached at 117.92 GW in the year 2018, whereas 52.26 GW in the year 2010 (21, 50-54). Recently in last few years, the Government of India has introduced the concept of solar parks, Green Energy Corridor of Rs. 38,000 crore investment, net-metering policy, launching of a massive grid connected rooftop solar programme with installation target of 40 GW (55, 65), solar pump scheme with target of 1000,000 installations, no charges for inter-state transmission, solar alliance, compulsory procurement of 100% power from waste to energy plants, training of 50,000 people as solar PV installer under Suryamitra Skill Development scheme etc. (51-54, 62, 65). Till June 30, 2019, renewable power capacity (including large hydro) had jumped to over 128 GW to become fifth global rank and making up 34.6% share of total installed power capacity in India (55). This is counted as doubling of capacity in one decade of journey. Installed grid interactive hydropower (excluding large hydropower), wind power, solar power, bio power and waste-to-power additions accounted for 4.60 GW (6% share), 36.37 GW (45% share), 29.55 GW (37% share), 9.13 GW (12%) and 0.14GW (0.04% share) respectively of this capacity. The wind and solar power capacity is ranked as fourth and fifth in the world (17, 19, 48-55) and the percentage share in the total renewable energy installation as recorded by MNRE is shown in figure 5 (51).

Government of India has taken enhanced target of renewable power capacity to 227 GW (earlier 175 GW) by 2022.It comprises 113 GW from solar, 66 GW from wind, 10 GW from bio-power, 5 GW from small hydro-power and 31 GW from floating solar and offshore wind power (51, 77, 87) as given in figure 6. The country also has an ambitious aim to establish 500 GW of renewable energy capacity by 2030. By achieving this, India will be one of the superior renewable energy producers in the world (13-18, 52,88, 90). Presently India offers tremendous structure and opportunities for achievement of future potentials. The road ahead will depend on which structure will be followed.

CONCLUSIONS

This article gives elaborate knowledge of renewable energy progression and electricity status in India. It has been found that India has enough natural resources for energy progression to become a surplus power producing country in the world. Due to lack of technology, 70% of India's energy requirement is being fulfilled through fossil fuels leading to 6.65% of carbon dioxide emission of the world. The Government agencies like MNRE have taken many optimistic actions to use renewable energy instead of fossil fuels to mitigate the environmental issues and energy crisis. During 2018-19 fiscal year, the total electricity produced and consumed in the country is 1,547 TWh and 1,181 kWh/year respectively. The biggest challenge is to achieve its target more than 40% renewable power of total electric power capacity by 2030 as compared to present 34% of renewable power contribution. Thus, India is far ahead of many developing countries with respect to the renewable energy targets. Development of technologies and efficient renewable energy systems in suitable geographical area is recommended for adequate power generation for the country, its people, and the world economy. The pace has elite up and needs to be sustained over time.

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Table 1. Installed electricity capacity (MW) region wise till 30 June 2019 in India

	Thermal					Nuclear	Hydro	RES	Grand Total
Region	Coal	Lignite	Gas	Diesel	Total				
Eastern	27463.64	0.00	100.00	0.00	27563.64	0.00	4942.12	1421.14	33926.90
Western	72853.62	1540.00	10806.49	0.00	85200.11	1840.00	7547.50	23579.85	118167.46
Northern	50360.20	1580.00	5781.26	0.00	577721.46	1620.00	19707.77	14636.45	93685.68
Southern	43042.02	3140.00	6473.66	561.58	53217.26	3320.00	11774.83	39383.64	107695.73
North-East	770.02	0.00	1775.81	36.00	2581.83	0.00	1427.00	333.12	4341.94
Islands	0.00	0.00	0.00	40.05	40.05	0.00	0.00	17.73	57.78
All India	1984489.50	6260.00	24937.22	637.63	226324.34	6780.00	45399.22	79371.92	357875.48

*The breakup of renewable energy sources (RES) in India till 31 May 2019 is mentioned below (MW):

Small Hydro Power	Wind Power	Solar Power	Bio-Power		Total
			Biomass Power	Waste to Energy	
4603.75	36089.12	29409.25	9131.50	138.30	79371.92

Table 2.CAGR of the projections for the period FY 2016-17 to FY 2021-22

CAGR (%)	FY 2010-11 to FY 2015-16	FY 2015-16 to FY 2016-17	FY 2016-17 to FY 2021-22
Electricity Consumption (MU)	—	8.00	7.15
Electricity Requirement (MU)	5.28	6.42	6.18
Peak Electricity Demand (MW)	4.63	9.32	6.88

Table 3.Indian electricity market (as per the Indian Electricity Act 2003) [41, 45]

	Centre	State
Policy	Ministry of Power (MoP)	State Government
Plan	Central Electricity Authority (CEA)	
Regulations	Central Electricity Regulatory Commission (CERC) and Central Government Appointed Committee (CAC)	State Electricity Regulatory Commission (SERC) and State Government Appointed Committee (SAC)
Generation	Central Generating Stations (CGS) and Mega Power Projects	Generation Companies (Gencos) and Independent Power Producers (IPP)
System Operations	National Load Dispatch Centre (NLDC) and Regional Load Dispatch Centre	State Load Dispatch Centre (SLDC)

	(RLDC)	
Transmission	Central Transmission Utilities (CTU) and Transmission Licensees	State Transmission Utilities (STU) and Transmission Licensees
Distribution	Distribution Licensees	
Trading	Power Exchange (i.e. Indian Energy Exchange (IEX) and Power Exchange India Limited (PXIL)) and Trading Licensees	Trading Licensees
Appeal	Appellate Tribunal	

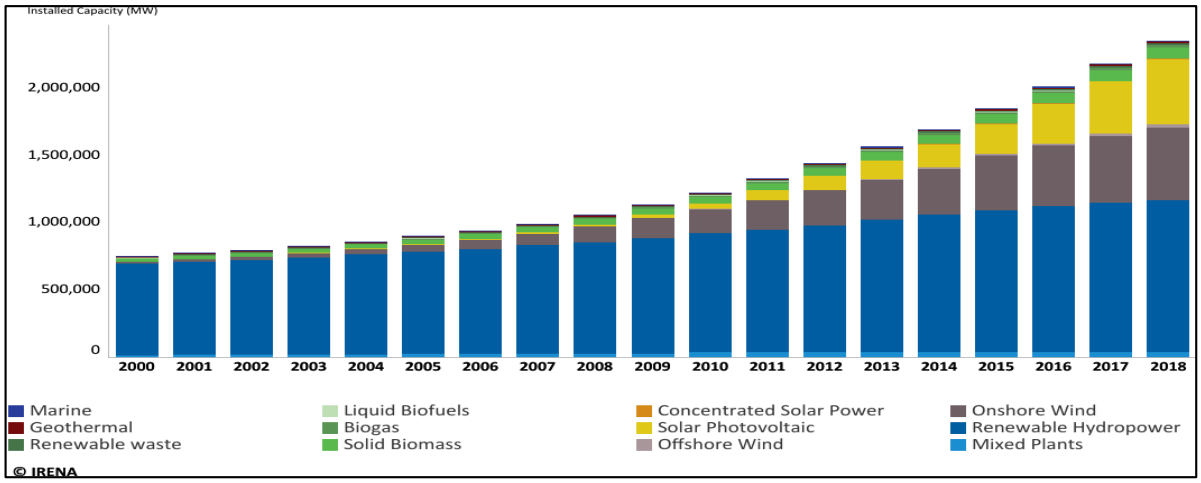

Figure 1.Renewable energy installed capacity trends in the world [7] [21].

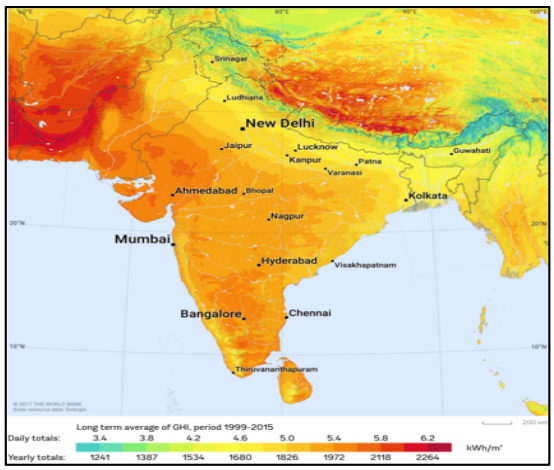

Figure 2.Global horizontal irradiance map in India [63].

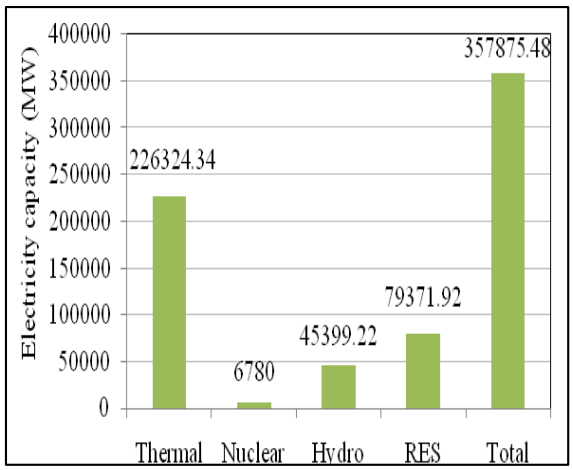

Figure 3. All India installed electricity capacity (MW) till 30 June 2019.

Figure 4. Electricity access market

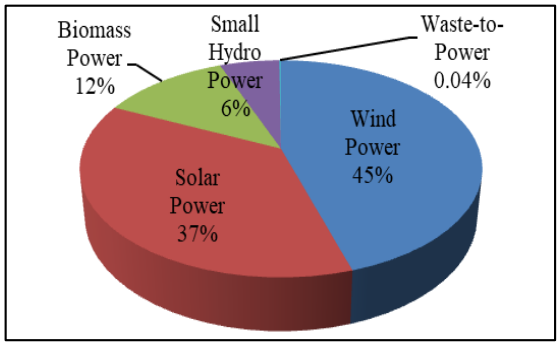

Figure 5. Installed grid interactive renewable power capacity in India till 30 June 2019.

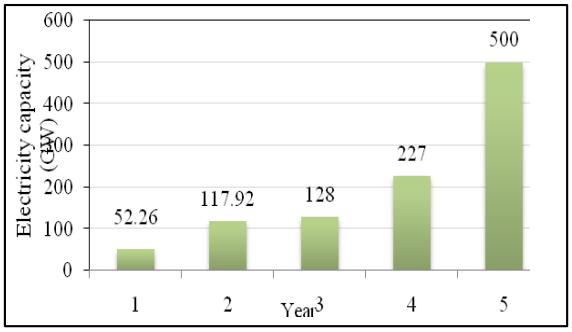

Figure 6.Renewable energy: present vs. future potential for years 2010, 2018, June 2019, 2022 and 2030.

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RESEARCH ARTICLE

Food and Feeding Habits of *Upeneus vittatus* (Forsskal, 1775) from Gulf of Mannar Coast (Mandapam, Tamil Nadu) of India

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ABSTRACT

The Goat fishes were considered as ecosystem engineers and fishery indicators. The baseline data of the biology of goat fishes is essential to monitor the overall health of the ecosystem. We investigated the food and feeding habits of *U. vittatus* collected from Mandapam landing centre. Assessment of the gut content of *U. vittatus* based on the frequency of occurrence showed that shrimps and molluscs present in 50.8%, crabs in 15.1% and also constitute the heavy diet with teleostean fishes (9.1%). The teleostean fishes were observed only in the *U.vittatus* measuring above 141mm in length. Percentage of volume of teleostean fishes was high in fishes with length above 161mm while shrimps and molluscs shell remnants were observed in the diet throughout the year. Crab also represented in the diet throughout the year. The study concluded that *U. vittatus* was mainly dependent on crustaceans.

Keywords: *Upeneus vittatus*, Gulf of Mannar, food and feeding, Mandapam landing centre.

INTRODUCTION

The family Mullidae is having high economical part in fisheries and considered as important demersal fish group which is widely dispersed throughout the world seas. Mullidae consists of fifteen genera in which only *Mullus*, *Upeneus* and *Pseudopeneus* are seen in Mediterranean Sea (1,2). *U. vittatus,* the striped goatfish, of Family Mullidae is an economically important demersal species living commonly in muddy sand bottoms or sandy bottoms ranging from five to hundred meters depth. Major fish catch is covered from bottom trawls. For the key identity, upenoids has a pair of barbells under the symphysis of lower jaw which is attached to the tip of ceratohyal and it indicated that upenoids fishes have the bottom feeding habits. The circumtropical family includes about 50 species belonging to six

genera. Most of the goatfishes are dwelling inshore regions and are economically important throughout their distributed regions. Based on its commercial value, goatfishes are the most studied fish group including intense biological and taxonomic studies (3,4,5,6,7,8,9). The goat fishes are widely distributed in the tropical and subtropical, Indo-Pacific and Western Atlantic regions. Nineteen species of *Upeneus* sp. have been recorded from the seas around India. Reports on the food habits of *U. indicus* and *U. cinnabarinus* from the Chennai coast have been also reported (10). Various aspects of studies including biological classification, osteology, and fishery of *Upeneus* sp from the Indian coasts have been reported in detail (11). Eighteen species of *Upeneus* sp. from western Indian Ocean were also reported (12). As the goat fishes having wide range of variation in its abundance, these fishes are common representative fishes in any ground fish fishery. Though many studies have been reported so far, the data on the specified growth, mortality and population dynamics of these species is limited (13,14,15).

Studies on food and feeding habits of goat fishes are very essential and important to understand the growth rate, gonadal maturity and other physiological activities (16, 17, 18, 19, 20, 21). Among the goatfishes, *U. vittatus* along with *U. moluccensis* occupies an important position in the trawl catches at South Indian fishing harbours (22, 23, 24, 25, 26, 27). Studies on various aspects of goatfish biology have been published from various regions of the world (28, 29).

MATERIALS AND METHODS

U.vittatus was collected from mechanized trawler landings at Mandapam fishing harbour. The study was intended for the observation of specimens collected during the study period from April 2012 to March 2013. The data on total length, weight, sex, stage of maturity of each fish were recorded immediately after collection of the sample. Guts were analyzed for the food contents (30). The dissection was carried out to study the stomach contents and the composition of food items was stored in 5% formalin. The classification of food organisms from stomach was based commonly on damaged shell remnants, partly digested prawns, crabs as well as teleostean fishes. The isolates from gut materials were classified as crabs, shrimps, teleostean fishes, mollusk remnants and unidentified food contents. A minimal number of food contents could not be able to identify due to its advanced stage in digestion and they were classified under unidentified food items. Depending on the amount of expansion of stomach and extent of food in the stomachs, they were classified as full, ¾ full, ½ full, ¼ full, trace amount and nil or empty and the points were allotted as hundred, seventy five, fifty, twenty five and zero respectively. The percentage occurrence of various food contents was determined from the total number of occurrence of all food items in each month. To assess the quantity of each food item, Index of Preponderance method was followed (31). The monthly averages got by volumetric and occurrence methodology were substituted in the given formula and the Index of Preponderance values were calculated as

I = (100 X V X O) / Sum of (V X O)

Where I is the Index of Preponderance of the food items, V and O are its percentage of volume and occurrence respectively. The representative samples in the present study with stomach were classified as full, ¾ full, ½ full were referred as active grazing ability and those under the category of ¼ full and empty were referred to have poor grazing ablility.

RESULTS

The preliminary separation of stomach items showed that the food which taken by fish was mainly constitute shrimp (27.8%), Molluscs remnants (23.0%), crabs (15.1%), teleostean fishes (9.1%) and the remaining (25.0%) constituted the unidentified food items. The shrimp identified in the stomachs were mostly *Penaeus* sp. The remanants of teleostean fish could not be find out since they were seen in later stage of digestion.

Monthly variations of feeding habits

Shrimps contributed to be the dominant food content over the year, crabs constituted in a considerable amount in the month of February. Bivalve mollusks shell remnants constituted in a considerable amount in the month of January. Fishes were consumed in a considerable amount in the month of April and were absent during July, August and September.

Variations in food and feeding habits with related to size

It was observed that the food contents have been less diverse in similar size fishes. Shrimps and crabs constituted the major food items in fishes below 140mm in length. Teleostean fishes were seen in the gut of fishes which were above 141mm. Bivalve mollusks remnants were also found in reasonable quantities in fishes below 140mm, but the incidence of teleostean fishes were high in fishes more than 161mm in total length.

Intensity of feeding

Towards the study of feeding intensity, the gut content of the fishes were grouped into various orders named as full, ¾ full, ½ full, ¼ full and empty depending on the degree of fullness. Percentage occurrence of *U.vittatus* gut in various degrees of fullness is given in Fig.1. Fishes having large percentage (50.0%) of completely full guts were seen mainly in the month of April. Therefore the fishes were found to be actively grazing in the month of April. Fishes with average feeding intensity (44.4%) were found during November of the study period. Fishes with empty guts were found to be high (50%) during the month of February as shown in Fig. 1.

Percentage frequency of various food items

The percentage frequency of various food items during the period of April 2012 to March 2013 is shown in Fig.2.

Index of Preponderance

Index of Preponderance of food components during April 2012 to March 2013 has given in Table 1.

Shrimps (*Penaeus* sp)

The percentage frequency of shrimps was highest with 61.6% during August 2012 and lowest with 26.4% during March 2013 as shown in Fig. 2. Shrimps were the most predominant diet with Index of preponderance value of 43.97% as given in Table 1.

Crabs

During February 2013 the percentage frequency of crabs was highest with 25.8% and lowest with 9.8% during in month of August 2012. The index of preponderance value of crabs was 5.36% and it is the third most fed food. Shrimps and Crabs together constituted the main food of this species in mostly all the months.

Teleostean fishes

Percentage frequency of teleostean fishes was very high level with 27.1% during April 2012 and lowest with 5.1% during January 2013. The index of preponderance value for teleostean fishes was 4.19% and it is the least fed food as far as the results were concerned.

Molluscs remnants

In the Percentage occurrence of molluscs remnants, there was higher percentage in January 2013 with 20.5% and lowest during October 2012 with 5.0%. Bivalve remnants were the second mostly fed food according to the index of preponderance values (15.56%) as given in Table 1.

Unidentified food items

Certain food items during the study period could not be able to identify because of the later stage of digestion. This was noticed in all months during the study period.

DISCUSSION

The results of the gut content analysis in present study confirmed that *U. vittatus* is a carnivorous fish species grazing mainly on crustaceans and occasionally on teleostean fishes whereas molluscs remnants are accidental entry. The various parts of the food items indicated that the species could mainly fed on sub-benthic and benthic organisms detected by chemoreceptor rich barbells found on the fish chin. Goatfishes fed on small benthic crustaceans, worms, molluscs and small fish was reported previously which agreed to the present study (24, 26, 28). The whole composition of the *U.vittatus* diet indicated that the adult fishes fully was carnivorous, which grazed on small crustaceans, bivalve molluscs. Teleostean fishes also contribute to the gut content during most of the months in the study period. Teleostean fishes were found in high frequency in fishes with length more than 161mm.

In Mangalore coast, *U. vittatus* is documented as a carnivore feeder which fed mainly on teleosts and prawns (22). In our investigation, *U. vittatus* species from Mandapam coast commonly grazed on shrimps, bivalve molluscs, crabs and teleosts. In two coasts in Mandapam *Penaeus* sp. is the major part among shrimps species. Megalaspis fry (teleosts) were seen in *U. vittatus* from Mandapam coast and also fry of Megalaspis were seen in *U. vittatus* from Visakhapatnam coast (32). The percentage of emptiness and fullness indices in stomach are very essential to assess food and feeding intensity. Most mullids fed commonly on crustaceans and polychaetes; nevertheless substantial differences were seen in the diets of various species (33). The crabs increased in importance for the mulled *U. lineatus (*26).The most important prey of *P. barberinus* was crustaceans in addition to that the bivalves became a highly important part of the diets of the larger size mullid *P. barberinus* from Gulf of Aqaba. Studies related to food and feeding habits of reef fish in north eastern New Zealand reported that *U. ineatus* fed commonly on small crustaceans (34).

The mullids primarily fed over muddy or sandy bottoms upon sub surface or surface living invertebrates. The long barbells could be swept over or acrossing the sediments and are receptive to contacts by touching on prey animals. The stomach contents of *P. maculates* included the crabs as well as shrimps in higher percentages whereas polychaetes were low percentage. In *Mullus martinicus,* pelecypods, shrimps, crabs, crab larvae, shrimp larvae and ophiuroids were reported (35). The present investigation finds out that the food and feeding habit of *U.vittatus* is size dependant. Larger fishes groups more often fed on crabs and teleosts in maximum quantities than those in smaller length groups. The presence of lively mobile benthic organisms such as teleosts, crabs and shrimps in stomach of bigger size fishes revealed that fishes with larger size feed more proximity to the bottom. Small difference in feeding behavior of mullids from many regions was noticed and that can be recognized to the environmental condition of substratum in each locality.

CONCLUSION

Result of this study inferred that the *U. vittatus* from Mandapam coast mainly graze on bivalve mollusks remnants, crustaceans and teleostean fishes. The percentage occurrence of various food items in the gut content showed that the fish chose its food in the bottom dwelling organisms and the primary preferable food item was the shrimp. Thus, the present investigation revealed that *U.vittatus* is a demersal carnivore, whose diet is mainly composed of crustaceans (shrimps and crabs), bivalve molluscs and teleostean fishes.

Authors' Contributions

A.R. Lakshmikanth and M. Anand designed and executed the work. A.R. Lakshmikanth, M. Anand and K. Rangesh contributed for the manuscript writing, revision and discussion part. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Index of Preponderance of food components during April 2012 to March 2013

Food Items	*Oi*	*Vi*	*Vi x Oi*	*ViOi/□ViOi x 100*
Fish	9.1	10.7	97.37	4.19(5)
Molluscs	23	15.7	361.1	15.56(3)
Shrimp	27.8	36.7	1020.26	43.97(1)
Crab	15.1	8.2	123.82	5.36(4)
U.I	25	28.7	717.5	30.92(2)
Total	100	100	2320.05	100

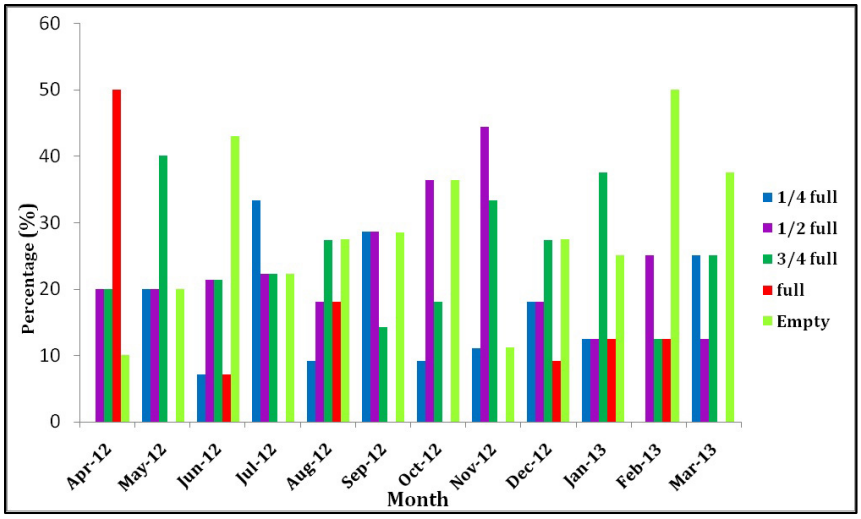

Fig. 1. Percentage occurrence of guts in degrees of fullness in *U.vittatus* from April 2012 to March 2013

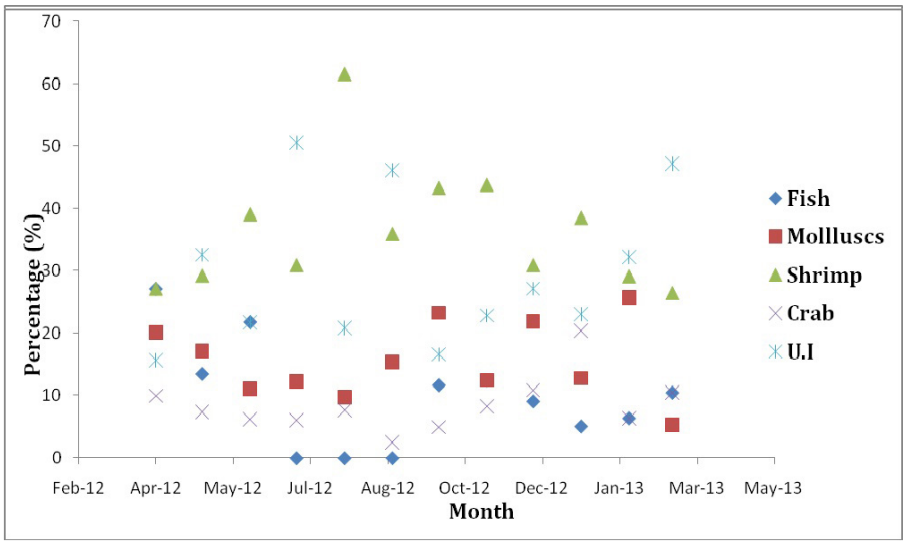

Fig. 2. Percent frequency of dominant food components from April 2012 to March 2013

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RESEARCH ARTICLE

Performance Evaluation of One MW On-Grid SPV Power Generation Plant in Odisha, India

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ABSTRACT

Solar energy is an abundant source of renewable energy radiating both light and heat from the sun. In the past few decades, it has more attention towards power generation due to gradual reduction of fossil fuels, energy crisis and environmental issues in India. Solar photovoltaic systems have become the leading substitute in medium and large scale of renewable energy technologies. The total solar power installed capacity reached about 32 GW in 2019 and aims to achieve 100 GW by 2022. The paper is aimed on the design, development and performance evaluation of one MW on-grid solar power generation plant at Khordha, Odisha. It is established since 2010 under guidelines of Ministry of New & Renewable Energy and policy of Odisha government. The lowest and highest power generation has been observed in the month of August and May about 97,600 kWh and 1,56,600 kWh respectively. The performance evaluation including performance ratio, capacity utilization factor and payback period of the plant is carried out, that will support in efficient designing and development of new grid interactive systems in future.

Keywords: Solar energy; Solar photovoltaic (SPV); On-grid; Solar power plant.

INTRODUCTION

Renewable energy generated from renewable resources is not depleted or exhausted when used. This includes solar, wind, hydro, rain, bio-mass, ocean and geothermal. This is a better substitute to non-renewable energy resources such as fossil fuels. Solar energy is a sustainable, inexhaustible and abundant resource for all forms of energy in the environment (1). The total amount of solar energy absorbed by earth is approximately 3.85 x 10^{24} J per year. It has

been found that the energy used by globally in one year is equal to the energy radiated from sun in one hour (2). It is harvested in the form of light and heat from the sun. The energy is harnessed using range of technologies such as solar photovoltaic (SPV), concentrated solar power (CSP), solar heating and cooling (SHC) (3). It does not release any greenhouse gases (GHGs) when generating electricity (4). Among these technologies, solar PV (SPV) is better suitable for the environment in terms of sustainable, affordable, adoptable, reliable, and efficient in power generation. Globally, Solar PV technology uses around 98% and CSP technology by 2% (5). Solar cells, also called PV cells contain mostly silicon material which converts sunlight directly into electricity through the PV phenomenon (6). Solar PV module is an assembly of solar cells; more than one solar module is constituted to from PV string and array of a SPV power plant. It generates required amount of power for commercial and residential applications through proper synchronization with nearby utility grid (7, 8).

India is a tropical country situated along the equatorial line of the earth with around 300 clear sunny days per year and an average solar radiation of 5 kWh/m^2/day. India is already a leader in both hydro and wind power generation (9, 10). The government has renewable energy capacity target is 175 GW under Ministry of New and Renewable Energy (MNRE), including 100 GW of solar, 60 GW of wind, 10 GW of bio mass and 5 GW of small hydro by 2022. Interim 100 GW of solar target with an investment of Rs. 7,156 billion includes 40 GW only from rooftop solar power. The country has the lowest capital cost per MW in the world for installation of the solar PV plants. The total solar power installed capacity in India is reached to 31.696 GW as of October 2019 (11-15). World's second largest (first one is Tengger Desert Solar Park- 1,547 MW, China) solar power plant is Pavagada solar park in Karnataka, India with a capacity of 1,400 MW. After total installation it will become world's top most solar PV power plant with a capacity of 2,050 MW (16). An efficient and economical electricity industry in the state Odisha, India is fulfilling by the Odisha Electricity Regulatory Commission (OERC), established under the Orissa Electricity Reform act, 1995 (17). The electricity consumers in the state are around 9.6 million and peak power demand is about 5,641 MW in fiscal year of 2018-19. The state Odisha achieves 394.73 MW of solar power capacity including studying one MW solar power plant as of March 2019, with leading agencies and stakeholders like GEDCOL, GRIDCO, NTPC, OPTCL, Sunark solar, MGM solar and others (18-22).

As India not being a member of International Energy agency (IEA), the studies and deliberations on SPV power plants as per IEC 61724 standard are not accessible (23-26). Hence, it is essential to evaluate the performance of the installed solar power plant in India. This paper systematically addresses the designing and development of one MW on-grid SPV power plant including the major components like solar modules, inverters, panel box and step-up transformer. The average energy produced by the plant is found to be 13-15 lakh units per year of its installed capacity.

MATERIALS AND METHODS

The empirical data used for this study were collected at site by observation, calculation and experimentation of operational one MW SPV power plant. The four major steps involved in this section are: planning, surveying, design and development, and operation.

Planning

Planning is a management process of organizing the essential activities to achieve a desired goal. It also consists of customer satisfaction, business model, conceptual skills, future forecasting, missions and resources to fulfil the targets. The proper planning process can increases the efficiency and reduces the risks of an organization.

Surveying

Site survey and assessment of the geographical area is very essential for preparing a single line diagram of a solar power plant. An energy audit is the first step for finalization of proposal by identifies better economic and environmental issues in the suitable geographical location. Khordha is an administrative division of the state of Odisha, India. The operational condition of one MW on-grid solar power plant occupied an area of 5.5 acres is given in "Figure 1", which is located at about 30 km from Khordha town with latitude 19.9585° N, longitude 85.4692° E and an altitude of 55 m. Ambient air temperature varies between 20 to 400C over the year. The annual average global horizontal irradiance (GHI) of this place is 5.31 kWh/m2/day (average of 5 kWh/m2/day in almost all the districts) (1-8) and average monthly data is given in "Figure 2". The power produced by a solar power plant depends on its sun's insolation (radiation), geographical location and efficient system design.

Design and Development

According to the MNRE, anyone can generate electricity through setup of solar power plant and surplus electricity can be exported over Net-metering system. The MNRE and State Nodal agencies are also providing 30%-70% subsidy benefits for residential, institutional and non-profit organizations (9-10). The scheme leads to design, development and installation of solar PV system in different location of the state Odisha, India. Solar PV systems are On-grid and Off-grid types. On-grid solar power system is an electricity generation system which includes solar PV modules, inverters, transformer and electrical appliances. The solar PV module is consists of solar cells which is fitted on the mounting structure. Each Si-cell is able to produce a maximum open-circuit voltage (Voc) between 0.5 to 0.6 volts, short-circuit current (Isc) between 28 to 40 mA/cm^2 and efficiency between 10 to 22% (11-17). A solar PV array is the total power-generating unit, including of any number of PV modules and strings. The transformer is synchronized with utility grid for further power utilization through transmission and distribution system. An off-grid system is not synchronized to the utility grid, therefore requires storage system for further power use by customer (18-19). A block diagram of a grid-connected SPV power plant is presented in "Figure 3". The observation, calculation and experimentation data of one MW on-grid SPV power plant is given in Table 1.

Operation

The SPV power plant consists of three main units, such as generation unit, control unit and transmission unit as given in "Fig. 3" (20). The function of generation unit is to generate electricity and it consists of Solar PV modules (each of 250 Wp, polycrystalline) laying on the mounting structure, array junction box, copper cables etc. The solar PV module is an assembly of solar cells and each Si-cell can able to produce a maximum Voc of approximately 0.6 volt. The main intermediate and operational part of the solar power plant is control unit. It consists of grid-tied inverter (ABB central inverter-PVS800), lightening arrestor, grounding, control panel board and monitoring section. The last one is transmission unit and function is to transmit the available power through step-up transformer into utility grid (21, 22).

RESULT AND DISCUSSION

Polycrystalline modules are commonly used in solar PV power plant, as it has medium cost, widely available, high generation, more efficient and high durability (23). The monthly power generation of one MW on-grid SPV power plant at Khordha is given in Table 2. As per their tariff agreement with government, the cost is Rs 18.72 per unit. In the Indian solar context, Performance Ratio (PR) and Capacity Utilization Factor (CUF) are used for performance evaluation of a plant. PR is an enhanced way than CUF to determine the quality of plants on yearly basis. It also helps to calculate the Payback Period (PB) of the total installed system (24-28). The PR, CUF and PB are evaluated using "equations (1), (2) and (3)".

$$PR = \frac{Energy\ Measured\ (kWh)}{Installed\ Plant\ Area * PV\ Module\ Efficiency * Irrediance\ Measured} = 80\% \quad \text{.....(1)}$$

$$CUF = \frac{Energy\ Measured\ (kWh)}{365 * 24 * Installed\ Plant\ Capacity} = 16.17\% \quad \text{.....(2)}$$

$$PB = \frac{Total\ Installed\ System\ Cost}{Annual\ Units\ Generated * Cost\ of\ one\ Unit} = 8\ \text{years} \quad \text{.....(3)}$$

The above calculated data indicates a huge demand on installation of MW level SPV power plant across the country and also in the world (29,30). The details financial analysis and feasibility plan model for installation, O&M of one MW SPV power plant is given in Table 3.

CONCLUSION

Based on the empirical study, the main observation, calculation and experimentation values are as follows:

- The power plant is installed in a suitable place with annual average global horizontal irradiance is 5.31 kWh/ m^2/day.
- Initially the tariff rate agreement was Rs. 18.52 per unit but after 10 years it would be renewed with new tariff rate up to 25 years.
- As per table-2, total power generation is about 14,16,200 kWh in 2019.
- The power plant has shown annually performance ratio of about 80%, CUF of about 16.17% and payback period of about 8 years.

The above data satisfies under global standard values and indicates that the plant condition is in better position still 2010. It could help on widely installation, O&M of MW level solar PV power plant across the country to mitigate energy crisis and climate issues in future.

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Table 1. Project details of installed one MW on-grid SPV power plant.

Type of Installation	Ground Mounted
Shading Consideration	Shadow free
Maximum Solar Insolation at the site	6.85 kWh/m^2/ day
SPV Power Plant Capacity	1 MWp
PV Module type	Polycrystalline
Efficiency	15%
Tilt angel	Approx. 25^0 N
Facing of Modules	South-East
No. of SPV Modules	1MWp/250Wp = 4,000
No. of SPV String	40
PV Modules Connection in each String	Series
Inverter	Two numbers of 500 kW, 3 phase & MPPT type
Transformer	1250 kVA
Circuit Breaker	Inverter to Panel box = 1000A 3p; Panel box to Transformer = 1600A 3p
Isolator	Vmax= 12 kV; Imax=1250 A
Cables (Copper)	DC Side= 10 mm^2; AC Side = LT: 15 mm^2 & HT: 180 mm^2
Grid Voltage	11 kV
Phase Connection	3-phase
Grid Frequency	50 Hz
Occupied Area	5.5 Acres

Table 2. Average monthly energy output.

Month, 2019	Generation (kWh)
January	120,350
February	1,32,600
March	1,47,350
April	1,54,200
May	1,56,600
June	1,00,400
July	99,550
August	97,600
September	98,200
October	1,00,100
November	1,08,600
December	1,00,650
Total	**14,16,200 kWh**

Table 3. Investment (Approx.) model for one MW Solar plant in India.

Components/services	Total cost (Rs.)
Solar PV Modules (Poly)	28,000,000
Solar Inverters	8,000,000
Transformer	1,000,000
Protective Devices	800,000
Electrical Cables	1,000,000
Software	1,200,000
Execution and Commissioning	1,000,000
Construction Works	4,500,000
Grid Permission	1,000,000
O&M Cost/year	1,000,000
Degradation: 1st 10years	0.05%
11-25years	0.67%
Equity Percentage	30%
Loan Interest rate	12%
Depreciation	5.28%
Corporate Tax	30.28%
Total Project Cost	4-5 Corers
Annual Units Generation	15,00,000
Solar Power tariff	Govt. policy
Pay-back period	6-7 years

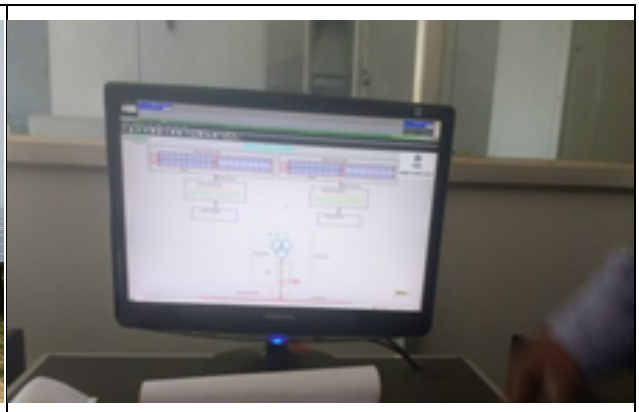

Figure 1. One MW on-grid SPV power plant at Khordha, Odisha.

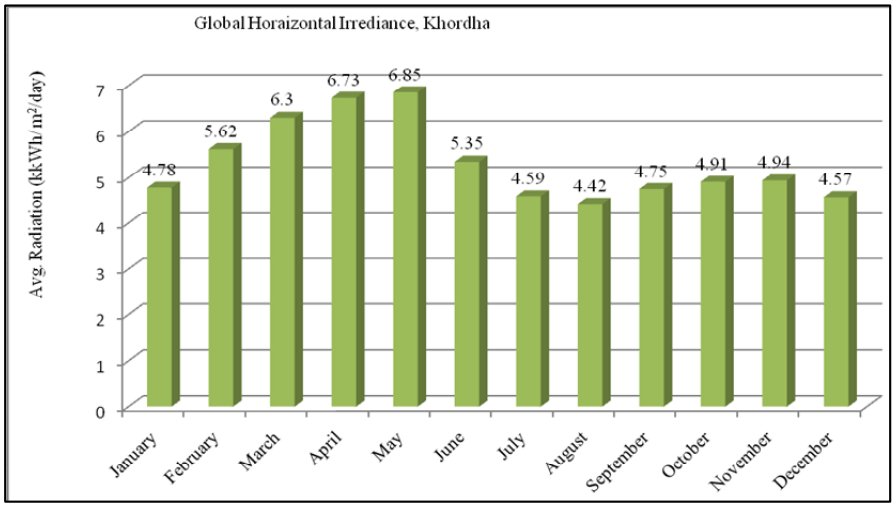

Figure 2. Global horizontal irradiance of Khordha, Odisha.

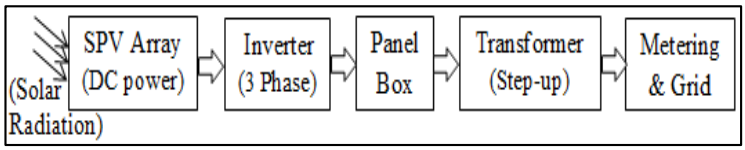

Figure 3. Block diagram of SPV power plant.

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RESEARCH ARTICLE

Antioxidant Activity, Total Phenolic, and Total Flavonoid Content of Various Extracts and Fractions of Aerial Parts of *Ipomoea horsfalliae*

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ABSTRACT

Numerous species of the genus *Ipomoea* are widely used in traditional medicine as powerful cathartics and are reported to have anti-inflommatory, anti-hypertensive,anti-diabetic and anticancer activities. The dried aerial parts of *Ipomoea horsfalliae* were extracted with petroleum ether, chloroform, ethyl acetate, ethanol and water by using soxhlet apparatus and the solvent was removed under pressure to obtain extracts. Percentage yield of extract in each solvent was recorded. All the extracts were subjected to preliminary phytochemical screening. The results showed that most of the phytoconstituents tested were present in ethyl acetate extract. Total phenolic and flavonoids content were tested by spectrophotometric method and results showed that phenolic content is more in ethyl acetate extract and fraction 3 (F3) and the values are 82.09±09 mg GAE/g and 73.11±2.13mg GAE/g respectively. The total phenolic content observed was 58.3±0.93 mg QE/g and 52.53±1.46 mg QE/g for ethyl acetate extract and fraction 3 (F3) respectively. The antioxidant activity of different concentrations of the extracts was evaluated using different antioxidant tests Namely DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and ABTS methods. DPPH result shows that ethyl acetate extract (IC_{50} 22.12±2.31 µg/ml) and fraction 3 (F3) (IC_{50} 13.42±1.27 µg/ml) showing good antioxidant activity.ABTS results also supporting that that ethyl acetate extracts (IC_{50} 19.25±2.85 µg/ml) and fraction 3 (F3) (IC_{50} 12.12±1.38 µg/ml) are good antioxidant. Fractionation of ethyl acetate extract was done by using different solvent system and repeated the antioxidant studies to get the most active fraction. GCMS analysis was performed by using fraction 3 (F3) which shows different compounds present.

Keywords: total phenolic content, total flavonoid content, antioxidant activity. GC-MS, *Ipomoea horsfolliae*

INTRODUCTION

The genus Ipomoea contributes about 500-600 species all over the world and honored as the largest genus of family Convolvulacea (Austin et al., 1996). This family is dominated by twining or climbing woody or herbaceous plants that often have heart-shaped leaves and funnel-shaped flowers (Austin et al., 1997). Concolvulaceae family characterized by widespread occurrence of flavonols,kaempferol,quercetin and their O-methylated derivatives(Mann et al.,1999)Genus Ipomoea belonging to this family is widely distributed in tropical and subtropical countries. These plants are used in traditional medicine as powerful cathartics,diuretics,ulcers,aphrodisiac and in the treatment of skin diseases,ulcers,bronchitis,inflammation,diabetes,fever and general weakness(Singh et al.,1988).The bioactive compounds found in the plants of this genus are ergoline alkaloids, phenolic compounds, coumarins, norisoprenoids, diterpenes, isocoumarins ,triterpenes ,nortropane alkaloids,benzenoids,anthocyanins,glycolipids,lignans and indolizidine alkaloids (Meira et al., 2012) . Plants are one of the important source of bioactive compounds with antioxidant capacity and during the last years, the interest for the use of natural products has significantly increased.

Antioxidant refers to a compound that can delay or inhibit the oxidation of biomolecules by inhibiting the initiation or propagation of oxidative chain reactions and which can thus prevent or repair damage done to the body's cells by reactive oxygen species.(Tachakittirungrod et al.,2007). Naturally, our body creates antioxidant compounds which consist of enzyme and non-enzyme. However, this compound is not able to resist the oxidants created in stress oxidative condition. Therefore, exogenous antioxidant compound is needed (Halliwel et al., 1995). The protective effects of plant secondary metabolites can be attributed to direct scavenging activities against reactive oxygen species (ROS), as well as to the induction of intracellular antioxidant effect [patel,milutinovi]. However, synthetic antioxidant like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and tertiary butyl-hydroquinone (TBHQ) are known to ameliorate oxidative damages but they have been restricted due to their carcinogenic and harmful effect on the lungs and liver (Zengin et al.,2015). Thus, in order to protect human beings against oxidative damage, recently, there have been great efforts to find safe and potent natural antioxidants from various plant sources .In fact, different epidemiological studies have shown as the decrease of premature death and mortality from cancer or other chronic diseases are associated with antioxidant-rich diet including fruit, vegetables, and other botanicals (pizarro et al.,2009 and scalbert et al.,2005). The objective the study was to evaluate antioxidant activity, total phenolic content and total flavonoid content by using different methods. GCMS analysis was performed to find out different compounds present in the most active extract/fraction.

MATERIALS AND METHODS

Materials

Quercetin, gallic acid, DPPH and 2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid diammonium salt (ABTS) was procured from Sigma Aldrich, India. Folin–Ciocalteu reagents and ascorbic acid were purchased from S. D. Fine Chem Limited, India. Aluminium chloride, trichloroacetic acid, ferric chloride, and potassium ferricyanide were purchased from Merck (Bangalore, India). All chemicals and solvents used were of analytical grade. Aerial parts of *Ipomoea horsafolliae* were obtained locally from Calicut district (Kerala, India).The plant materials were identified and authenticated by Dr. A.K. Pradeep., Assistant Professor -Department of Botany, Calicut University (Calicut, India). Voucher specimens were deposited in the same department herbarium as specimen No.148253.

Preparation of extracts

The aerial parts of *Ipomoea horsfolliae* was dried properly in shade for 3 weeks, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. About 1 kg of air-dried plant material was extracted in soxhlet assembly

successively with petroleum ether, chloroform, ethyl acetate, ethanol and water (order of increasing polarity). Each time before extracting with the next solvent, the powdered material was dried at room temperature. Each extract was concentrated by using rotary vacuum evaporator. The extract obtained with each solvent was weighed and the percentage yield was calculated in terms of dried weight of the plant material. The color and consistency of the extract were also noted. All the solvents used for this entire work were of analytical grade (Merck, Mumbai). The percentage yield was calculated for the extracts and major compounds with reference to the crude material taken using the formula given below.

$$\% \text{ yield of extract} = \frac{\text{weight in grams of extracts obtained}}{\text{weight in grams of plant material taken}} \times 100$$

Phytochemical analysis

Phytochemical tests were carried out using standard procedures to identify constituents, as described by Harborne (1984), Trease and Evans (1979), and kokate (1999)

Fractionation of ethyl acetate extract

The ethyl acetate extracts were subjected to column chromatography using silica gel (mesh size 60-120). Fifty grams of extract was submitted to flash chromatography using silica gel (mesh size 60-120) as stationary phase. The elution was done using increasing solvent polarity made of hexane; ethyl acetate and methanol mixtures. Fractions of each were collected and evaporated under vacuum. Similar fractions were pooled together to produce four fractions (F1-F4), evaporated to dryness and kept in the dark for subsequent analysis. Then the silica column was prepared using ethyl acetate by wet packing method and the column was washed using 100 ml of ethyl acetate. Then, the ethyl acetate extract was mixed with silica gel and made fine powder for easy distribution of sample. The powdered sample mass was placed on the top of the pre-packed silica column. Then, the elution was started using ethyl acetate and increasing concentration of methanol. Each 10 ml of fractions were collected in vials and further analyzed by thin layer chromatography.

Measurement of total phenolic content

Folin-Ciocalteau reagent method was used to determine the total phenolic content of extracts /fractions (Amma et al., 2010, Singleton and Rossi, 1965). 1.0 ml of Folin-Ciocalteau reagent was added to 0.2 ml of samples (extracts/ fractions) and then 0.8 ml of sodium carbonate (7.5 % w/v) was added. Allowed to stand for 30 min and absorption was measured at 765 nm in a spectrophotometer (UV-1650, Shimadzu Corporation, Kyoto, Japan). The results were expressed as Gallic acid equivalents (GAE) in milligrams per gram of sample.

Measurement of total flavonoids content

The total flavonoid content was estimated by two complimentary methods (Chang et al., 2002); including AlCl3 and 2,4-DNPH.

Aluminum chloride method

Samples were (2 mg/ml) prepared in 80% ethanol. 0.5 ml of samples were mixed with 1.5 ml of 95% ethanol, 0.1 ml of 1M potassium acetate, 0.1 ml of 10% aluminum chloride and 2.8 ml of distilled water. The mixture was incubated for 30 min at room temperature and absorbance was measured at 415 nm using a spectrophotometer (UV-1650,

Shimadzu Corporation, Kyoto, Japan). Quercetin was used as the reference standard and results were expressed as milligrams Quercetin equivalent per gram of the sample.

(2.4-dinitrophenylhydrazine) colorimetric method

Sample solutions (1 ml) were mixed with 2 ml of 1% 2, 4-dinitrophenylhydrazine reagent and 2 ml of methanol. The mixture was reacted at 50 °C for 50 min and cooled to room temperature. They were further mixed with 5 ml of 1% potassium hydroxide in 70% methanol and incubated at room temperature for 2 min. 1 ml of the reaction mixture was then added to 5 ml of methanol and the precipitate was removed by centrifugation. The supernatant was collected and adjusted to 25 ml and absorbance was measured at 495 nm. (±)Naringenin was used as the reference standard and results were expressed as milligrams naringenin equivalent per gram of the sample.

Measurement of DPPH radical scavenging capacity

Antioxidant potential of the extracts and fractions was determined by using DPPH assay (Ahmad et al., 2013, Brand-Williams et al., 1995). A 2.4% w/v solution of DPPH was prepared in methanol and was further diluted with methanol to obtain an absorbance of about 0.98 ± 0.02 at 517 nm. An aliquot of 100 µl of the samples at different concentrations of 10 to 100 µg/ ml was added to 1ml of the DPPH solution. The mixture was incubated for 15 min in dark and absorbance was measured at 517 nm. Ascorbic acid was used as the standard. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity
DPPH radical scavenging activity was determined using the following formula;

% inhibition = [(Absorbance of Control – Absorbance of sample)/ Absorbance of Control] × 100.

Measurement of ABTS radical scavenging capacity

ABTS radical scavenging activity of the extracts and fractions was performed according to previously reported method (Ahmad et al., 2013, Re et al., 1999). For the development of radicals, ABTS (7 mM) was mixed with potassium persulfate (2.45 mM) solution and incubated overnight in the dark. This solution of ABTS was diluted using PBS (pH 7.4) to give an absorbance of 0.70 ± 0.02 at 730 nm. 0.3 ml of sample (5 to 200 µg/ml concentrations) was mixed with 1 ml of ABTS solution and absorbance was determined after one minute. ABTS radical scavenging activity was determined using the formula as mentioned in DPPH method.

GC-MS analysis

The GC-MS analysis was carried out using a Clarus 500 Perkin- Elmer (Auto System XL). Gas Chromatograph equipped and coupled to a mass detector Turbo mass gold – Perking Elmer Turbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl ply siloxane), 300 m x 0.25 mm x 1 µm df capillary column. The instrument was set to an initial temperature of 110°C and maintained at this temperature for 2 min. At the end of this period, the oven temperature was raised up to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 (MHz). The chemical constituents were identified by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS). The percentage of each component was calculated from the relative peak area of each component in the chromatogram. Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The spectrum of the known component was compared with the spectrum of the known

components stored in the NIST library. The name, molecular weight, peak area and retention time of the components present were ascertained

Statistical analysis

All measurements were carried out in three times. The results were shown as mean ± SD. Statistical comparisons were made by using the Student's test. $P < 0.05$ was set as statistical significance.

RESULTS AND DISCUSSION

The percentage yield and color of all five extracts are listed in following (Table 1) .Highest percentage was observed in ethyl acetate extract(7.8%w/w) and the lowest was noted in chloroform extract(0.85%w/w) .

Preliminary phytochemical analysis

Preliminary phytochemical analysis showed that most of the phytochemicals are present in ethyl acetate extracts. Detailed reports are listed in the following table (Table 2).The results suggest that ethyl acetate is the best solvent for the extraction of phyto constituents from *Ipomoea horsfalliae.*Tannins are present in petroleum ether extract at minor quantity. Chloroform extract contain flavonoids and phenols at minor extend. Ethyl acetate extracts showed high presence of steroids, flavonoids and phenols, also showed the presence of terpenoids, alkaloids, carbohydrate and alkaloids at lower extend.

Total phenolic content (TPC)

TPC activity is the process to figure out the amount of phenolic content in the samples. Phenolic compounds that contained in the plants have redox properties, and the properties allow them acting as antioxidants (A. B. Shoib et al.,2015 and M. A. Soobrattee et al., 2005) So far, plant phenolic form one of the main groups of compounds working as primary antioxidants or free radical scavengers. Plant polyphenols are effective as singlet oxygen scavengers, reducing agents and hydrogen atom donators. (Karaman S et al., 2010, Rice-Evans CA et al.,1996). Among the extracts analyzed, the ethyl acetate extract showed maximum phenolic content followed by ethanol, chloroform, water and petroleum ether extracts respectively. Petroleum ether extract showed the least phenolic content (Table 3). The ethyl acetate extracts were fractionated and found that the fraction 3 showed more phenolic content followed by fraction 4, fraction 1, and fraction 2 respectively. The phenolic compounds from natural sources possess antioxidant activity which can be neutralizing for the reactive oxygen species associated diseases (Tlili et al., 2013).

Many researchers reported that plant extracts have positive correlation between total phenolic content and antioxidant activity (Ahmed et al., 2019; Tlili et al., 2013). Many studies have concluded that the contents of total phenolics change depend on families and varieties of plants (Romani et al., 2003). Consumption of herb plant organs and/or their products have been related to reduced risk of chronic diseases, such as diabetics, obesity, and cardiovascular diseases and these health benefits have been associated with phenolics (Crozier et al., 2009; Del Rio et al., 2013; McDougall et al., 2005)

Total flavonoids content

Flavonoids are secondary metabolites with antioxidant activity, the potency of which depends on the number and position of free OH groups (Panche, A.N et al., 2016) . The total flavonoid content results were entirely synchronous with those of the total phenolic. It was successfully shown that samples with high level of phenolic content also contain flavonoids in great amount. The rich-flavonoid plants could be a good antioxidant source that would help

increase the overall antioxidant capacity of an organism and guard it against lipid peroxidation (F. Sharififar et al., 2009). The results showed that the total flavonoid content was higher in ethyl acetate extract followed by ethanolic, chloroform, water and petroleum extract respectively. Petroleum ether extract showed the least flavonoid content. Among the fractions, fraction 3(F3) have more phenolic content when compared with the other three fractions (Table 3).

Antioxidant studies

In this study, the antioxidant capacity of different extracts and selected fractions was tested by two different assays namely DPPH scavenging activity and ABTS assay. IC50 of ABTS and DPPH scavenging activities of each extracts were compared to IC_{50} of Ascorbic acid.DPPH is a stable free radical at room temperature and possesses a characteristic absorption at 517 nm (purple in color), which decreases significantly on exposure to radical-scavengers (by providing hydrogen atoms or by electron donation). A lower absorbance at 517 nm indicates a higher radical-scavenging activity of the extract. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical scavenging activity of specific compound or extracts (Amarowicz R et al.,2004) DPPH and ABTS assay results showed that ethyl acetate extract had the maximum antioxidant activity followed by the chloroform, ethanol, water and petroleum ether extracts respectively. Among the fractions, the order of anti-oxidant activity was Fraction F3 > Fraction F2 > Fraction F4 > Fraction F1 respectively in both DPPH and ABTS assays. The results are shown in Table 4.

GC-MS ANALYSIS

Knowledge of the chemical constituents of plants is important not only for the discovery of new therapeutic molecule, but also finding new sources of economic and potential phyto compounds for the synthesis of complex chemical substances and determining the actual significance of folkloric remedies (Milne et al., 1993). GCMS analysis was carried out for fraction F3 which was the most active fraction of ethyl acetate extract tested in this experiment. Mass spectrometry becomes a vital tool in the hands of the organic chemists and biochemists because of its potential to supply the definitive, qualitative and quantitative information on molecules based on their structural compositions. Gas chromatography is attached to a Mass Spectrometer (GC-MS) enables mixture of small molecules mainly organic compounds of low molecular weight (<600) which can be analyzed. The GC-MS analysis shows the presence of different compounds in Fraction F3 of ethyl acetate extract of the aerial parts of plant *Ipomoea horsfollia*. The compound present were identified through mass spectrometry attached with GC. Molecular weight, molecular formula, peak area and retention time of the compounds were ascertained. GC-MS analysis shows the presence of different phyto compounds in fraction F3 (Table-4) namely Gamma-Sitosterol (10.6%)Phytol(6.68%),2,4-bis(1-phenylethyl)-phenol(12.75%),Neophytadine(1.5%)etc are some important compounds identified

CONCLUSIONS

This work highlights the importance of aerial parts of *Ipomoea horsfalliae to use as* a rich source of natural antioxidants. The results reveal that ethyl acetate is the best solvent to extract antioxidant compounds from this plant. Antioxidant activity is more in ethyl acetate extract and fraction F3 in both DPPH and ABTS methods. This may be due to presence of high phenolic and flavonoid compounds .GC-MS analysis also support the presence of various antioxidant constituents in this plant. Further investigation into the isolation and identification of responsible antioxidant components and their mechanism of action is necessary to better understand their ability to control diseases that have a significant impact on quality of life.

Conflict of interest

The authors declare no conflicts of interest.

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Table 1.percentage yield of extracts.

S.No	Extracts	Color	Yield % (W/W)
1.	Petroleum ether	Greenish Yellow	4.80
2.	Chloroform	Green	0.85
3.	Ethyl acetate	Brownish Green	7.58
4.	Ethanol	Brown	5.24
5.	Water	Chocolate Brown	4.62

Table 2, Phytochemical analysis of *Ipomoea horsfalliae*

S.No.	Phytochemical constituents	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Water
1.	Alkaloids	-	-	+	++	-
2.	Corbohydrates	-	-	+	+	+
3.	Glycosides	-	-	+	+	++
4.	Terpenoids	-	-	++	-	-
5.	Proteins	-	-	-	-	++
6.	Amino acids	-	-	+	+	+++
7.	Steroids	-	-	+++	+	+
8.	Flavonoids	-	+	+++	+	-
9.	Phenols	-	+	+++	-	-
10.	Tannins	+	-	-	-	-
11.	Quinones	-	-	-	-	-
12	Anthraquinones	-	-	-	-	-
13	Saponins	-	-	-	-	++
+++ :highly present,++:moderately present, +:low,-:absence						

Table 3: Total phenolic and flavonoid content of extracts/fractions

Extracts/fractions	Total phenolic content (mg GAE/g extracts and fraction)	Total flavonoid content (mg QE/g sample)		
		2,4-DNPH method	$AlCl_3$ method	Total content
Petroleum ether	12.11±2.12	2.32±0.17	1.42±0.19	3.74±0.36
Chloroform	33.21±0.98	9.21±0.13	6.43±0.18	15.64±0.31
Ethyl acetate	82.09±1.11	21.22±0.12	37.13±0.81	58.3±0.93
Ethanol	52.38±1.28	14.12±0.79	9.21±0.78	23.33±1.57
Water	17.34±1.05	3.27±0.18	2.37±0.19	5.64±0.37
Fraction 1(F1)	29.13±2.11	4.12±0.12	4.82±0.17	8.96±0.29
Fraction 2(F2)	19.22±1.63	2.15±0.17	1.82±0.19	3.97±0.36
Fraction 3(F3)	73.11±2.13	19.32±1.19	33.21±0.27	52.53±1.46
Fraction 4(F4)	40.24±013	13.12±2.21	9.32±0.27	22.44±2.48

Values as mean ± SEM (n=3)

Table 4. Effect of extracts and fractions on DPPH and ABTS radical scavenging

Extracts/ Fractions	IC_{50} (µg/ml)	
	DPPH Assay	ABTS Assay
Petroleum ether	298.21±2.11	73.81±1.05
Chloroform	51.20±1.24	62.13±2.25
Ethyl acetate	22.12±2.31	19.25±2.85
Ethanol	112.53±1.23	79.33±013
Water	197.15±2.71	83.12±0.22
Fraction 1(F1)	29.31±2.01	33.13±1.02
Fraction 2(F2)	18.21±2.14	23.15±3.14
Fraction 3(F3)	13.42±1.27	12.12±1.38
Fraction 4(F4)	25.22±0.32	27.15±1.28
Ascorbic acid(standard)	8.18±0.32	10.21±0.92

Table 5. Phytocomponents identified in the Fraction F3 of *Ipomoea horsfalliae* by GC-MS

Sl no	Retention time	Compounds Name	Area %	Molecular weight	Molecular formula
1	4.162	(2rs,3sr,4rs.5rs)-2,3 : 4,5-diepoxyhexanamide	0.42	143	C6H9NO3
2	4.232	2,4-dione-pyrrolidine	9.36	99	C4H5NO2
3	5.382	1,2,3-trimethyl-benzene	2.44	120	C9H12
4	8.227	acetic acid, trifluoro-, octyl ester	1.1	226	C10H17F3O2
5	8.987	hexyl-cyclohexane,	0.87	168	C12H24
6	9.732	cyclohexasiloxane, dodecamethyl-	0.8	444	C12H36O6Si6
7	10.713	9-heneicosene, (e)-	0.42	294	C21H42
8	11.02	1-tetradecanol	2.38	214	C14H30O
9	11.125	dihexylsulfide	0.42	202	C12H26S
10	11.819	(1-methylethyl)-cyclohexane,	0.49	126	C9H18
11	11.942	tetradecamethyl- cycloheptasiloxane,	0.72	518	C14H42O7Si7
12	13.532	1-nonadecene	3.04	266	C19H38
13	13.914	2,4-bis(trimethylsiloxy)-, trimethylsilyl ester benzoic acid	0.56	370	C16H30O4Si3
14	15.073	phenol, 2-(1-phenylethyl)-	1.17	198	C14H14O
15	15.79	1-nonadecene	2.92	266	C19H38
16	15.862	hexane, 2,3,4-trimethyl-	0.37	128	C9H20
17	16.247	neophytadiene	1.55	278	C20H38
18	16.308	1-undecene, 9-methyl-	0.4	168	C12H24
19	16.501	16-heptadecenal	0.51	252	C17H32O
20	16.633	9-heptadecanone	0.9	254	C17H34O
21	16.695	7-octadecyne, 2-methyl-	0.69	264	C19H36
22	17.479	1,2-benzenedicarboxylic acid, dibutyl ester	1.03	278	C16H22O4
23	17.833	1-nonadecene	2.88	266	C19H38
24	17.896	1-undecene, 4-methyl-	0.53	168	C12H24
25	18.846	1-heptanol, 2-propyl-	0.71	158	C10H22O
26	18.947	phytol	6.68	296	C20H40O
27	19.7	1-docosanol	1.31	326	C22H46O

28	19.752	eicosyl isopropyl ether	2.1	340	C23H48O
29	19.815	heptadecyl acetate	0.68	298	C19H38O2
30	19.859	3,7,11,15-tetramethylhexadec-2-en-1-yl acetate	1.23	338	C22H42O2
31	20.62	eicosane	1.78	282	C20H42
32	21.41	1-hexadecanol	0.68	242	C16H34O
33	21.455	tetrapentacontane	2.86	758	C54H110
34	21.515	octadecyl 2-propyl ester sulfurous acid,	0.98	376	C21H44O3S
35	21.602	phenol, 2,4-bis(1-phenylethyl)-	3.87	302	C22H22O
36	21.732	2,4-bis(1-phenylethyl)- phenol	3.58	302	C22H22O
37	21.982	octadecamethyl-cyclononasiloxane,	0.8	666	C18H54O9Si9
38	22.196	2,4-bis(1-phenylethyl)- phenol,	5.31	302	C22H22O
39	22.257	tetrapentacontane	3.58	758	C54H110
40	22.499	2,4,6-tris-(1-phenylethyl)-phenol	3.8	406	C30H30O
41	22.823	diethyl(2-phenylethoxy)tetradecyloxy-silane,	4.07	420	C26H48O2Si
42	23.031	tetrapentacontane	4.56	758	C4H110
43	23.843	tetrapentacontane	3.31	758	C4H110
44	24.039	gamma-sitosterol	10.06	414	C29H50O
45	24.78	tetradecane	2.1	198	C14H30

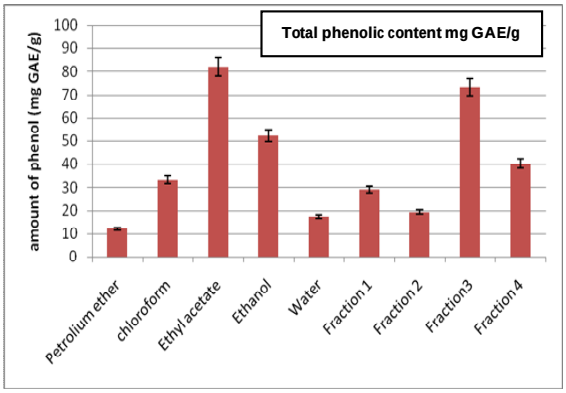

Figure 1.Total phenolic content of extracts and fractions.

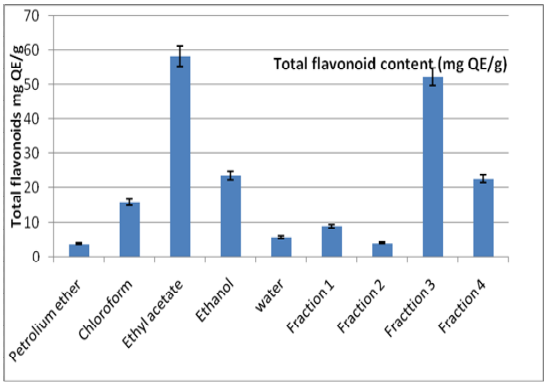

Figure 2: Total flavonoid content of extracts and fractions.

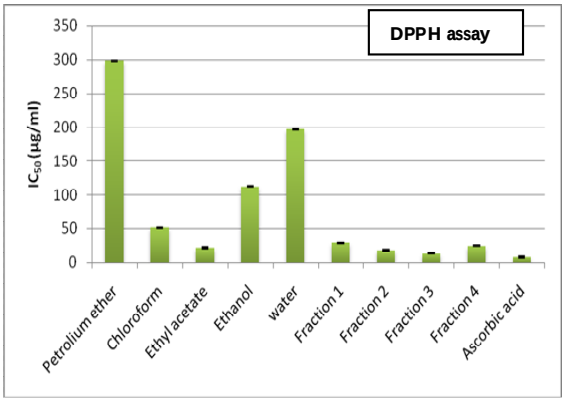

Figure 3:DPPH radical scavenging capacity of extracts and fractions

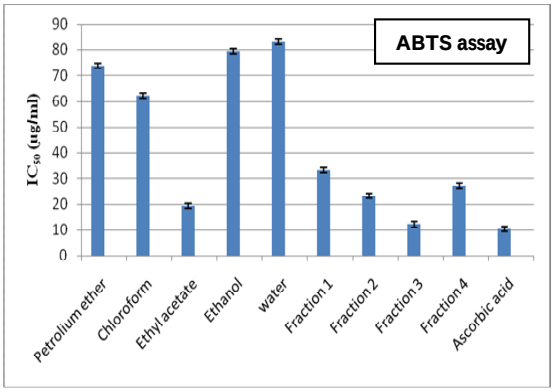

Figure 4:ABTS radical scavenging capacity of extracts and fractions.

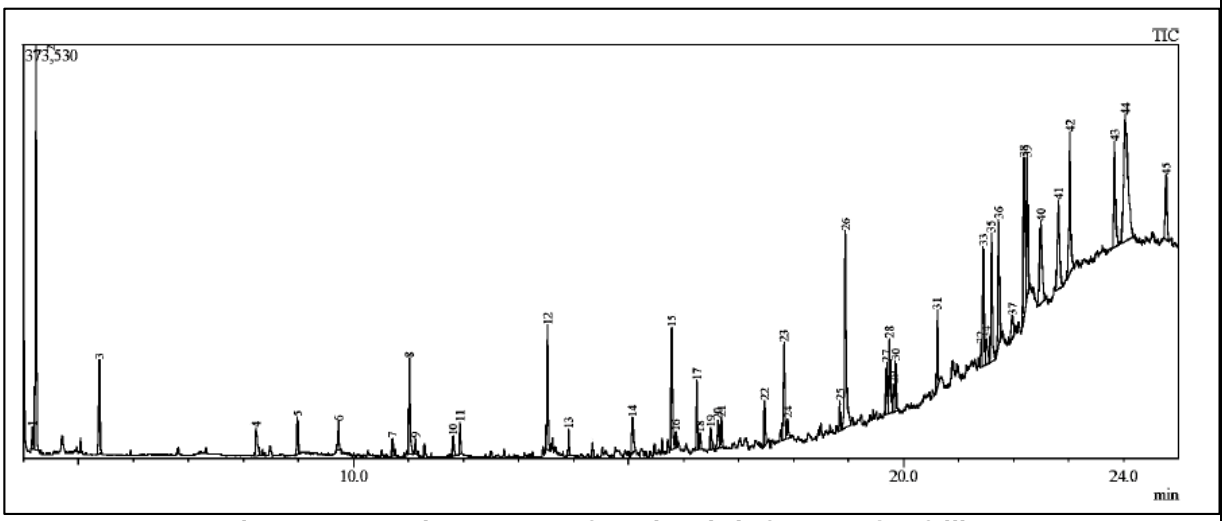

Figure 5: GC-MS chromatogram of Fraction 3(F3) of *Ipomoea horsfalliae*

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RESEARCH ARTICLE

The Influence of Adding Polycaprolactone and Polyethylene Grafted Maleic Anhydride on the Mechanical, Morphological and Biodegradation Properties of HDPE

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ABSTRACT

This study aims to prepare environment friendlypolymer blends in order to reduce the usage of the non-biodegradable polymers and contribute to the environment preservation. High density polyethylene (HDPE) and polycaprolactone (PCL) blends were prepared with different percentages, polyethylene grafted maleic anhydride (PE-g-MA) was added to the blend as a compatibilizer. The samples were prepared using the conventional injection molding technique. The mechanical properties were observed to have remarkable changes with the blend composition as the tensile strength and the elongation percentage at break increased significantly when compared to the neat HDPE. The morphology imaging by SEM showed that the addition of PE-g-MA improved the comptability and accordingly aided in obtaining a synergic blend with better properties. The soil burial test gave a significant biodegradation results and proved the blend's ability to biodegrade in soil which would encourage its usage as the main polymer for manufacturing single use products while preserving the environment.

Keywords: High density polyethylene, polycaprolactone, polyethylene grafted maleic anhydride, biodegradation, soil burial, mechanical properties.

INTRODUCTION

Nowadays, petroleum-based polymer products are still dominant because of their excellent mechanical, physical and thermal properties.These polymers are versatile and provide millions of tons of plastic products produced by the end of each year. These products are threatening the environmental preservation and our whole life. That's why; the society has been demanding the elimination of the non-biodegradable plastics usage. The polymer scientists have

suggested as using biopolymers and biodegradable polymers as an alternative to the petroleum-based polymers. Reducing the usage percentage of the petroleum-based polymers or the non-biodegradable polymers would aid the environmental preservation and decrease the ecological issues that we are facing currently (1-3).Thus, the purpose of this study is to prepare partially biodegradable blends that can biodegrade naturally over time and decrease the impact of the petroleum based plastics on the environment.

MATERIALS AND METHODS

The used HDPE grade (HDPE6070) has a melt flow rate of 7.6 g/10min at (190°C, 2.16 Kg) and a density of 0.960 g/cm^3. It was supplied by SidiKerir Petrochemicals Company (SIDPEC), Egypt in the form of powder. Polycaprolcatone with Mwt = 80,000 and melting temperature of 60°C was used, it was supplied by Sigma Aldrich, UK.High density polyethylene grafted maleic anhydride grade (Fusabond® E100) with a melt flow rate of 2 g/10min at (190°C, 2.16 Kg) and a density of 0.954 g/cm^3 was purchased from E.I. DuPont De Nemours and Company Inc.

Samples preparation

The different combinations of the blends used in this study are shown in Table 1. Materials were mixed and processed in a robot coupe Blixer 4 for 6 min with speed of 3000 rpm. The injection molding of the different samples using was carried out using Arburg allrounder 221k, Germanysingle screw-injection molding machine.

Methods

Mechanical Properties

The tensile properties of HDPE / PCL and PE-g-MA blends were measured using the universal testing machine with load cell 5 KN (Zwick /z005, Germany) as shown Figure 1. The dumbbell-shaped specimens, in accordance with ASTM D638 - 08, of all specimens were tested at room temperature. The samples were conditioned prior to testing at labconditions for 24 h. The tensile test was performed in uniaxial tension at a crosshead speed of 20 mm/min.

Scanning Electron Microscopy (SEM)

The test was used to reveal information on how a compatibilizer plays a role in improving composites properties. The test was carried out using JEOL, JSM-5300.

Biodegradation test

The samples were cut into circles Radius(r) = 2 mm and a thickness of 4mm and dried in a vacuum oven at 40 ° C till weight is constant then buried in soil mud which contains different types of Bacillus bacteria of the genus (*Bacillus Spp*), Other bacteria belong to the genus (*Corynebacterium Spp*) fungi such as (*Fusarium solani*), (*Fusarium oxysporum*), and (*Rhizoctonia solani*) with a pH of 7.41 as listed in the report performed at the faculty of Agriculture Alexandria University, Egypt. The temperature has never been much higher than room temperature (25 ± 2) ° C with a minimum airflow rate. Each sample was removed, washed with fresh water and dried again to constant weight. The degree of degradation was evaluated (referred as the percentage of weight loss). Samples kept wet in the soil by frequent water spraying. Weight loss percentage was calculated according to the following equation.

$$\% \text{ Weight Loss} = ((W_i - W_f))/W_i \quad \ldots\ldots\ldots\ldots\ldots (1)$$

The W_i and $W_f$are weight before soil burial and weight after soil burial, respectively.

RESULTS AND DISCUSSION

Mechanical properties

The tensile test was conducted on all of the blended samples and neat HDPE, yield strength and elongation percentage at break values were recorded (Table 2). The addition of PCL with different percentages of 1 wt%, 2 wt%,

4 wt% and PE-g-MA to the HDPE matrix seemed effective in improving the mechanical properties generally as shown in Figure 2. Regarding samples S11, S21 and S41, the tensile strength increased gradually from 26 MPa for neat HDPE to 28 MPa for S41. The elongation percentage at break also increased gradually from 1085% for neat HDPE to 1199% for S41. This improvement might be attributed to the comptability that was induced by the addition of PE-g-MA as a compatibilizer which enhanced the miscibility and interfacial adhesion between PCL and HDPE. The induced comptability can be resulted from thereaction between maleic anhydride with the hydroxyl (OH) end groups of PCL that may be taken place providing aninterface with higher performance. The neat PCL has high ductility which might also affect the elongation percentage at break positively and raised it higher than the neat HDPE (4-9).

Scanning electron microscope (SEM)

Scanning electron microscope (SEM) was used to investigate the cross section area morphology and the possible interfacial adhesion between the HDPE matrix, PCL and HDPE-g-MA as shown in Figure 3. The morphology of the blends is controlled by the processing conditions, composition, as well as the nature of the polymers (interfacial tension and viscosity ratio)[5, 10, 11].HDPE has homogenous, smooth, and uniform surface, unlike the blended samples which showed less homogeneity. By increasing the PCL content, the homogeneity decreased which hinted less comptability within the blend. The incorporation of PE-g-MA improved the homogeneity gradually, this might mean that the PE-g-MA played its role as a compatibilizer and enhanced the comptability between HDPE and PCL. It is very clear for samples S41, S42 and S44 that the gradual increase of the PE-g-MA percentage improved the morphology and the interfacial adhesion between the matrix components which would generally affect the other properties positively (1, 12, 13).

Soil burial test

The biodegradation of plastics proceeds actively under soil conditions according to their properties and the soil microorganism's optimal growth conditions[14, 15].It is noteworthy to mention that only biodegradation is measured without any consideration of UV degradation which also has a major importance in case of HDPE degradation.The weight loss percentage increased by raising the polycaprolactone content and with the factor of time. In four months, the weight loss increased gradually from 0.05 wt% for sample S11 that contains 1 wt% PCL to 0.1 wt% for sample S41 which contains 4 wt% PCL as shown in Table 3. The incorporation of PE-g-MA resulted in improving the performance of the biodegradation and increasing the ability of each PCL content to induce the biodegradability in the samples. For example, the weight loss percentage increased from 0.1 wt% to 0.13 wt% for samples S41 and S44, respectively, and that's when the PE-g-MA percentage was raised from 1 wt% to 4 wt%.

The presence of PCL and PE-g-MA induced the biodegradability within HDPE. PCL is biodegradable polyester affected by the action of microorganisms in the soil. The enhanced degradation of the blends compared to HDPE can be explained by the initiation of microbial degradation of PCL chains. Therefore, as the PCL concentration is increased, the biodegradation increased. Also, the PE-g-MA incorporation into the matrix improved the comptability between HDPE and PCL and enhanced the PCL biodegradable influence on the blend which raised the weight loss percentage. By increasing the PE-g-MA concentration, the moisture absorption and the swelling are increased too, leading to a faster biodegradation as well. This is shown in other studies that have similar results (15-17).

CONCLUSION

The incorporation of small percentages PCL into the HDPE matrix had a positive influence on its properties. The mechanical properties improved significantly after the addition of PE-g-MA as a compatibilizer which enhanced the comptability within the blend and influenced the interfacial adhesion. The biodegradation in soil gave remarkable results after four months with regard to the small PCL percentages used in this study. This study can represent a beginning to replace the totally non-biodegradable plastics with partially biodegradable products.

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Table 1. The design of the blended samples

SAMPLE	HDPE	PCL	PE-g-MA
S0	100%	0%	0%
S11	98%	1%	1%
S12	97%	1%	2%
S14	95%	1%	4%
S21	97%	2%	1%
S22	96%	2%	2%
S24	94%	2%	4%
S41	95%	4%	1%
S42	94%	4%	2%
S44	92%	4%	4%

Table 2.Tensile test results of neat HDPE and its blends

Sample	Tensile strength at yield (MPa)	Elongation at break (%)
S0	26	1085
S11	27	1136
S12	26	1145
S14	26	1115
S21	27	1186
S22	26	1049
S24	27	982
S41	28	1199
S42	26	1078
S44	26	1036

Table 3.Weight loss percentage of neat HDPE and its blends in burial soil test over four months

Sample	Weight loss%	Weight loss%	Weight loss%
	1 Month	2 Months	4 Months
S0	0	0	0
S11	0	0.02	0.05
S12	0	0.02	0.06
S14	0	0.03	0.07
S21	0	0.03	0.07
S22	0	0.03	0.08
S24	0	0.05	0.09
S41	0	0.07	0.10
S42	0	0.07	0.11
S44	0	0.09	0.13

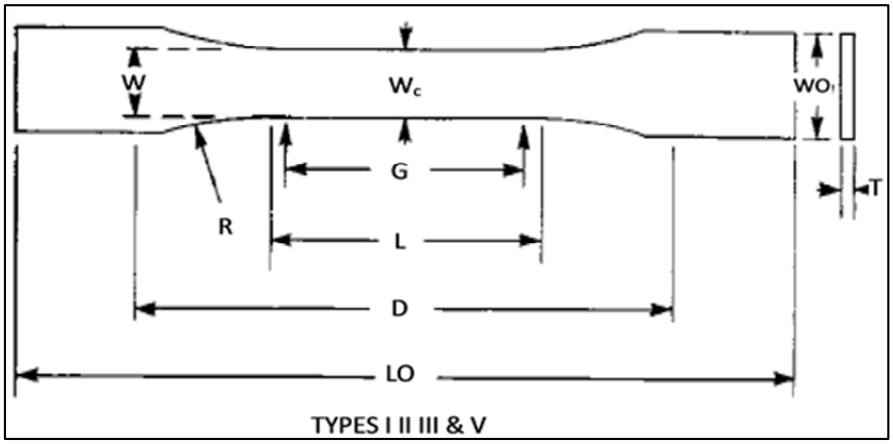

Figure 1.Sample dimension according to American Standard ASTM D638-08

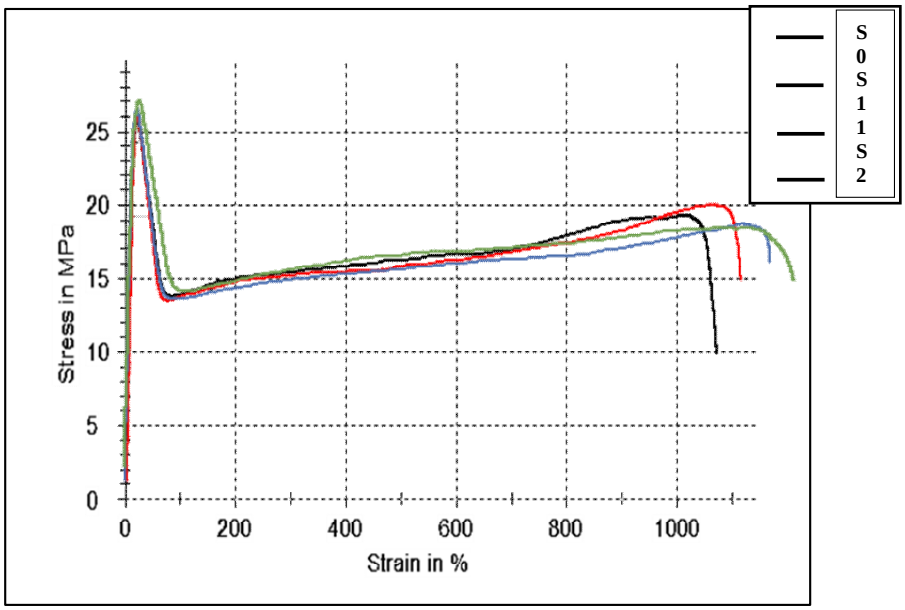

Figure 2.Stress-Strain curves of samples S0, S11, S21 and S41

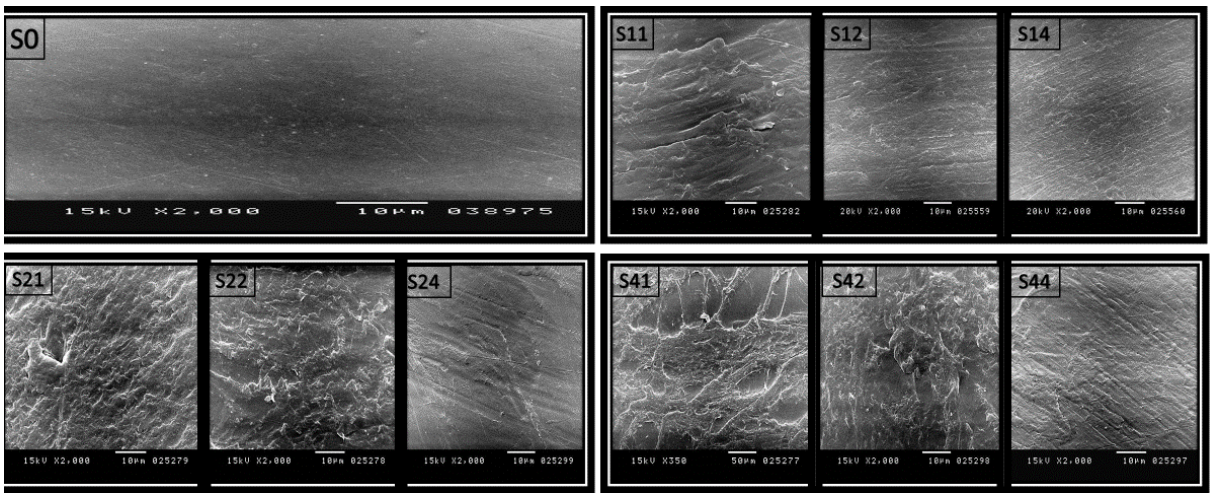

Figure 3.SEM micrographs of neat HDPE and its blends

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REVIEW ARTICLE

Directive Principles of State Policy in India

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ABSTRACT

Directive Principles of State Policy provide essential guide-lines both for the state as well as the citizens for establishing economic democracy in India. The Constitution makers in India did not force on the people any particular economic system but they only tried to suggest a system which could be most suited to Indian conditions. With the passing of Forty-Second Constitution Amendment Act, ii has been provided that India shall be a socialist democracy but socialism is not in the traditional sense but in the sense which suits Indian conditions. The Sapru Committee had recommended a division of fundamental rights into two classes — judiciable and nonjudiciable.The Constitution Act of India, 1935, itself provided for 'Instruments of Instructions' which were a fruitful idea. Ambedkar said. The above statement of Dr. Ambedkar makes it crystal clear that these principles are not binding upon the Government. The Government is to strive. If it does not fulfil these principles despite its striving, nobody can challenge it in the court or law. Liberal Intellectualistic,This category includes those things on which the liberal intellectuals have been insisting for the last many years, e.g., ft) The State shall endeavour to secure for the citizens a uniform civil code throughout the territory of India. Though Basu's fears are genuine, yet we must not efface from our mind a naked reality that the President's or Governor's powers to veto a Bill passed by the Legislature is only limited. In the words of Dr. Ambedkar"The Directive Principles cannot be pressed into service either by the President or the State Governor for the purposes of vetoing a law passed by the legislature". in a parliamentary form of government, the constitutional rulers cannot be assertive. If they are, they cannot be tolerated.

Keywords: Directive Principles, as against Fundamental Rights, are not justifiable in the courts of law, State activity, Justice, social, economic and political; Liberty; Equality; Fraternity.

INTRODUCTION

Directive Principles of State Policy provide essential guide-lines both for the state as well as the citizens for establishing economic democracy in India. The Constitution makers in India did not force on the people any particular economic system but they only tried to suggest a system which could be most suited to Indian conditions. With the passing of Forty-Second Constitution Amendment Act, ii has been provided that India shall be a socialist

democracy but socialism is not in the traditional sense but in the sense which suits Indian conditions. According to our Constitution Directive Principles, as against Fundamental Rights, are not justifiable in the courts of law. It is for the first time that a Part of Constitution (Part IV) has been devoted to these Principles. Earlier to this, the Government of India Act, 1935 also contained an Instrument of Instructions to the Governor-General and the Governors, but these instructions were for the executive government. Present Directives are however, for the Parliament and state legislatures. Whereas the Instrument of Instructions had no public opinion behind it, the directives have force of public opinion behind them. Constitution makers in India drew their inspiration from the Irish constitution for incorporating these Directives in the constitution. There are some other countries of the world which have also incorporated directive principles in their constitutions. Thus, Constitution of India is not the first constitution to contain Directive Principles of State Policy.

OBJECTIVES

The Constitution Act of India, 1935, itself provided for 'Instruments of Instructions' which were a fruitful idea. Ambedkar said. The above statement of Dr. Ambedkar makes it crystal clear that these principles are not binding upon the Government. Tshe Indian constitution becomes vested with the cope of sanctity essential to its durability, it will be difficult for any public figure to propose any legislative measures without making an appeal to Fundamental Rights or Direätive Principles.

SOME DIRECTIVE PRINCIPLES

Constitution makers wanted to give the people of India maximum rights but due to the country's social, economic, political and other conditions, it was not possible for them to give much to the people in the form of Fundamental Rights. Accordingly, whatever, they considered possible or feasible, they gave that the people in the form of Fundamental Rights and the rest they incorporated in the form of Directive Principles with the desire that with the passage of time, these might become justiciable rights. These Principles incorporate Gandhian philosophy and Socialistic ideas. These enjoin upon the governments to:

In more recent times, thinkers on political and social reforms who did not agree with the Marxian approach for the eradication of the ills and evils of modem society advocated such principles to be made the guiding force of State activity. The ideas of Jeremy Bentham, the political and social strand of the Liberal and Radical parties of Western Europe, the major principles of Fabian Socialism and, to some extent, those of Guild Socialism, are all taken to much of what is embodied in this Part of the Constitution. Ivor Jennings claims that the ghosts of Sidney and Beatrice Webb stalk through the pages of the Entire text and this part of the Constitution expresses Fabian Socialism without the word 'socialism', 'for only the nationalisaiton of the means of production, distribution and exchange is missing'.' But this would be to give an exaggerated importance to the Fabian influence, for one finds other documents and proclamations of more recent date that could have influenced the framers even more. Mention has already been made of the Irish Constitution. The Sapru Committee had recommended a division of fundamental rights into two classes —judiciable and nonjudiciable.2 The Constitution Act of India, 1935, itself provided for 'Instruments of Instructions' which were a fruitful idea. Ambedkar said.

It would however be wrong to suppose that the various principles embodied in this chap'ler are mere foreign borrowings or adaptations of principles of recent Western political or social philosophy. In fact, a number of these principles are entirely Indian, particularly those which formed an integral part of the very foundations of the national movement Provisions dealing with village panchayats, cottage industries, prohibition, protection against cow-slaughter, Scheduled Castes, Scheduled Tribes and other socially and Jucationally backward classes, are all formally and essentially Indian and some of these were the cherished ideals for the recognition of which Gandhi had striven throughout his life.

As the title itself indicates, the principles embodied in this chapter are directives to the various governments and government agencies (including even village panchayazs) to be followed as fundamental in the governance of the country. It shall be the duty of the State to apply these principles in making laws. Thus, they place an ideal before the legislators of India while they frame new legislation for the country's administration. They lay down a code of conduct for the administrators of India while they discharge their responsibilities as agents of the sovereign power of the nation. In short, the Directive Principles enshrine the fundamentals for the realization of which the State of India stands. They guide the path which will lead the people of India to achieve the noble ideals which the Preamble of the Constitution proclaims: Justice, social, economic and political; Liberty; Equality; Fraternity. It is this realization that impelled a member in the Constituent Assembly to demand the placing of this chapter immediately after the Preamble in order to give it 'greater sanctity' than others.3 There was also a suggestion to change the title of the chapter to 'Fundamental Principles of State.

The Advisory Committee on Fundamental Rights had made a definite recommendation to the Constituent Assembly for the inclusion of such a chapter. The committee reported: We have come to the conclusion that in addition to these fundamental rights, the Constitution should include certain directives of State Policy which, though not cognisable in any court of law, should be regarded as fundamentaL in the governance of the country.

There are nineteen articles of the Constitution that deal with the Directive Principles. These cover a wide range of State activity embracing economic, social, legal, educational, administrative, cultural and international problems. The most important of these are the following. Speaking about the purpose of this chapter, Ambedkar said: In enacting this Part of the Constitution, the Assembly is giving certain directions to the future legislature and the future executive to show in what manner they are to exercise the legislative and the executive power they will have. Surely it is to the intention to introduce in this part these principles mere pious declarations. It is the intention of this Assemble that in future both the legislature and the executive should not merely pay lip-service to these principles but that they should be made the basis of all legislative and executive action that they may be taking hereafter in the matter of the governance of the country.

1.To secure and protect a social order which stands for the welfare of the people. (Art. 38).

2. In particular, the State shall direct its policy towards securing:

(a) adequate means of livelihood to all citizens;

(b) a proper distribution of the material resources of the community for the common good;

(c) the prevention of concentration of wealth to the common detriment;

(d) equal pay for equal work for both men and women;

(e) the protection of the strength and health of the workers and avoiding circumstances which force citizens to enter avocations unsuited to their age or strength; and (1) the protection of childhood and youth against exploitation or moral and material abandonment. (Art. 39).

3. To provide free legal aid to ensure that opportunities for securing justice are not denied to any citizen by reason of economic or other disabilities (Art. 39A).

4. To organize village panchayats as units of self-government. (Art. 40).

5. To secure the right to work, education and public assistance in cases of undeserved want, such as unemployment, old age, sickness, etc. (Art. 41).

6. To secure just and humane conditions of work and maternity relief. (Art. 42).

7. To secure work, a living wage, a decent standard of life, leisure and social and cultural opportunities for people, and in particular to promote cottage industries.(Art. 43).

8. To secure the participation of workers in the management of undertakings engaged in any industry. (Art. 43A).

9. To secure a uniform civil code applicable to the entire country. (Art. 44).

10.To provide, within ten years from the commencement of the Constitution, free and compulsory education to all children up to the age of fourteen years (Art. 45).

The Constitution Eighty-sixth Amendment Act of 2002 has amended this provision as follows:

45. Provision for early childhood care and education to children below the age of six years : The State shall Endeavour to provide early childhood care and education for all children until they complete the age of six years.
Amendment of Article 5 1A
In Article 5 1A of the Constitution, after clause (J) the following clause shall be added, namely:
(k) Who is a parent or guardian to provide opportunities for education to his child or as the case may be, ward between the age of six and fourteen years.
11. To promote with special care the educational and economic interests of the weaker sections of the people, especially the Scheduled Castes and Tribes (Art. 46).
12. To secure the improvement of public health and the prohibition of intoxicating drinks and drugs (Art. 47).
13. To organize agriculture and animal husbndry on scientific lines and preserve and improve the breeds and prohibit the slaughter of cows, calves and other milch and draught cattle (Art.48).
14. To protect and improve the environment and to safeguard the forests and wild life of the country (Art. 48A).
15. To protect all monuments of historic interest and national importance (Art. 49).
16. To bring about the separation of the judiciary from the executive (Art. 50).
17. To endeavour to secure:
(a) the promotion of international peace and security;
(b) the maintenance of just and honourable relations between nations;
(c) foster respect for international law and treaty obligations in the dealings of organised people with one another; and (d) the settlement of international disputes by arbitration (Art. 51).
Taken together, these principles lay down the foundations on which a new democratic India will be built up. They represent the minimum of the ambitions and aspirations cherished by the people of India, set as a goal to be realized in a reasonable period of time. Indeed, when the State of India translates these principles into reality, she can justly claim of be a Welfare State.

Distinction between Fundamental Rights and Directive Principles

Before we enumerate Directive Principles of State Policy, it is essential to differentiate them from Fundamental Rights. (a) Fundamental Rights are Justiciabje, Directives Nonjusticiable. The Fundamental Rights, incorporated in Chapter 111 are justiciable while Directive Principles are not. If a Fundamental Right is encroached upon, the person concerned can approach the High Court or the Supreme Court to seek redress by getting the right enforced. However, if a Directive Principle as embodied in Chapter IV is violated, no court of justice can come to the rescue of the aggrieved party. For instance, if a person is illegally detained, a writ of 'habeas corpus' can be obtained by the detenu. If Goveent does not separate judiciary from the executive or introduce universal compulsory education, the courts cannot help the aggrieved. The Directive Principles are not mandatory principles. They confer no legal rights hence they make no provision for legal remedies either. Article 38 lays down that the "state shall strive". In the words of Dr. Ambedkar "the word 'strive' was purposely used because their intention was that however adverse the circumstances that stand in the way for a Government in giving effect to these principles and however unpropitious the time may be, they should always strive for the fulfillment of the principles. Otherwise, it would be open to the Government to say that the circumstances were not good and the finances were so bad that they could not implement them." The above statement of Dr. Ambedkar makes it crystal clear that these principles are not binding upon the Government. The Government is to strive. If it does not fulfil these principles despite its striving, nobody can challenge it in the court or law.

(b) Fundamental Rights Prohibitive Whereas Directives Positive Directions. Fundamental Rights lay down the negative obligations of the State. They are in the nature of the injunctions requiring the state not to do certain acts. In other words, they are prohibitive in character. For instance, no official of the State can arbitrarily debar the citizens from lawful enjoyment of such privileges. As freedom of speech and expression, movement, assembly and worship. If he does so, the aggrieved person is vested with the right to approach the court to get the wrong rectified. The Directive Principles, on the other hand, are positive obligations of the State towards its citizens. They declare it as the dnty of the State to promote certain social and economic objects. Gledhill remarks, "Fundamental Rights are

injunctions to prohibit the Government from doing certain things; the Directive Principles are affirmative instructions to the Government to do certain things."

(c) Directive Principles subsidiary to Fundamental Rights. Constitutionally, the Directive Principles are subsidiary to the Fundamental Rights. In the case of State of Madras v. Champakan Dorairajan, the Supreme Court of India observed, "The Directive Principles of State Policy which are expressly made unenforceable by a Court cannot override the provisions in Part III which.... are made enforceable by appropriate writs, orders or directions under Article 32."

The chapter on Fundamental Rights is sacrosanct and not liable to be abridged by any legislative or Executive acts or order except to the extent provided in the appropriate articles in Part III. The Directive Principles of State Policy have to conform to and run subsidiary to the chapter on Fundamental Rights....Howsoever so long as there is no infringement of any Fundamental Right to the extent conferred by the provisions in Part III, there can be no objection to the State acting in accordance with the Directive Principles In a number of such cases, Supreme Court expressed similar views. Decisions of Supreme Court establish the legal superiority of Fundamental Rights over Directive Principles, though the latter are considered fundamental in the governance of the land.

CLASSIFICATION OF THE DIRECTIVE PRINCIPLES

Since the Directive Principles have not been enumerated in the constitution in accordance with a logical plan, it is difficult to classify them. Dr. M.P. Sharma in his book 'The Republic of India' groups lose principles under three classes—Socialistic, Gandhian and Liberal Intellectualistic. We may however make an addition of another group 'General' which is not covered under three classes mentioned by Dr. M.P. Sharma. This 'General' group may include all those directives which are not covered up by his classification.

(a) Socialistic Principles. The bulk of the Directive Principles however aim at the establisfment of a welfare state based on socialistic pnnciples. Article 38 provides that the State shall strive to promote the welfare of the people by securing and protecting a social order in which justice, social, economic and political shall inform all the institutions of national life. Article 39 calls upon the State to direct its policy towards securing (z) that the citizens, men and women, equally have the right to an adequate means of livelihood, (ii) that the ownership and control of the material resources of the community are so distributed as to subserve the common good, (iW that the operation of the economic system does not result in the concentration of wealth and means of production to the conunon detriment, (iv) that there is equal pay for equal work for both men and women, (v) that the health and strength of workers, men and women and the tender age of children are not abused and that citizens are not forced to enter vocations unsuitable to their age, (vz) that childhood and youth are protected against exploitation and moral and material abandorinent.

Article 41 seeks to ensure the right to work, to education and to public assistance in cases of unemployment, old age, sickness and disablement and other cases of undeserved want. Article 42 states that the State shall make provision for securing just and human conditions of work and for maternity relief. Article 43 exhorts the State to secure all workers, agricultural, industrial or otherwise, work, a living wage, conditions of work ensuring a decent standard of life and full enjoyment of leisure and social and cultural opportunities. According to Article 46, the State is to promote with special care, the educational and economic interests of the weaker sections of the people and in particular of the Scheduled Castes and Scheduled Tribes and to protect them from social injustice and all forms of exploitations.

Article 47 imposes a duty on the State to raise the level of nutrition and the standard of living of its people and the improvement of the public health. These Directive Principles undoubtedly embody the main objectives of a socialistic pattern of society. In the words of Sir Ivor Jennings "the ghosts of Sidney and Beatrice Webb stalk through the text of Part N of the constitution."

(b) Gandhian Principles. Gandhian ideology finds quite a prominent place in some of these principles, as the ruling party was wedded to Gandhian philosophy since the time Gandhiii became undisputed leader of the Congress. Gandhian concept is discernible in the following principles:
(i) The State shall organise village panchayats and endow them with such powers as may enable them to function as units of self- government. (ii) The State shall promote with special care, the educational and economic interests ofilarijans, Scheduled Tribes and weaker sections of the community. Mahatma Gandhi's campaign to uplift clown-trodden backward communities is an open secret.
(V) That the State shall endeavour to promote cottage industries on an individual or co-operative basis in rural areas.
(iv) The State shall take steps for preserving and improving the breeds of milch and draught cattle, includiné cows and calves and for prohibiting their slaughter. (v,) The State shall endeavour to effect prohibition of the consumption, except for medicinal purposes, of intoxicating drugs and drinks which are injurious to health.
(c) Liberal Intellectualistic. This category includes those things on which the liberal intellectuals have been insisting for the last many years, e.g., ft) The State shall endeavour to secure for the citizens a uniform civil code throughout the territory of India. (ii) The State shall endeavour to provide within a period of ten years from the commencement of the constitution, for free and compulsory education for children below and up to 14 years of age. (iii) The State shall endeavour to organise agricultural and animal husbandry on modem and scientific lines. (iv,) The State shall take steps to separate the judiciary from the executive in the public service of the State. (v) The State shall endeavour to promote international peace and security; maintainjust and honourable relations between nations; foster respect for international law and treaty obligations; encourage settlement of international disputes by arbitration.
(d) Miscellaneous or General. Certain Articles of 'Directive
Principles' chapter can be kept in this category. Articles 36 and 37 are merely concerned with the definition and application of the Directive Principles. Article 36 provides that in this (part) unless the context otherwise provides, the State has the same meaning. Article 37 provides that these principles shall not be enforceable by any court of law and at the same time declares that they are nevertheless fundamental in the governance of the country and it shall be the duty of the State to apply these principles in making laws. Article 49, which makes it an obligation of the State to protect every monument or place or object of artistic or historic interest which Parliament of India has declared of national importance also refers to general matters. We cannot afford to include this article in any of the preceding group of principles.

THEIR VALUE

(a) Sanctions Behind the Principles. Despite such vehement criticism (as stated in the preceding paragraph), these principles are not as meaningless and useless as they are deemed to be. It is wrong to say that there is no force behind them. In this age of democracy 'vigilant public opinion' is the real force behind an institution which stands for the benefit of the individuals. The government in a parliamentary system of government is under a constant fire of criticism. The actions. of the government are subject to scrutiny by the masses and the distinguished leaders of the party. If the government pursues a policy in accordance with the principles of the constitution, people tolerate it, otherwise they oust it in the next elections. Since the Directive Principles have been embodied in the constitution, the governments are apt to implement them. There may not be the legal force behind them but the highest tribunal the public opinion stands behind them. No government can afford to ignore these Directives, if it is not keen to doom its future for all times to come.

(b) Their Constitutional Sanctity Unchallengeable. It is obvious from the above fact that though the Directive Principles are not enforceable, their constitutional sanctity is an undisputed fact. Their violation is as contrary to the constitution as violation of a constitutional principle legally enforceable. In the words of Gledhill, "If the Indian constitution becomes vested with the cope of sanctity essential to its durability, it will be difficult for any public figure to propose any legislative measures without making an appeal to Fundamental Rights or Direätive Principles. Measures will be attacked by the opposition as unconstitutional in so far as they conflict with the "Directive Principles." In Gopalan v. State of Madras; C.J. Kania opined that being a part of the constitution, the Directive

Principles do not "represent a temporary will of the majority, but the deliberate wisdom of the nation expressed through the Constituent Assembly... By declaring them to be fundamental in the governance of the country, the constitution has invested them with certain sanctity.

(c) An Insurance against Extremes. The framers of our constitution were judicious enough to realise that in a democratic system of government, the swing of public opinion may put different parties in power at different times. At one time; the party on the helm of affairs may be conservative iii outlook while at another time, the reins of government may fall in the hands of a party radical in leanings. These principles will induce the conservatives to introduce reforms necessitated by the exigencies of the time and also exercise restraint on the radicals if they would be keen to introduce reforms of sweeping nature. They would act as signposts to all succeeding governments. In the words of Dr. Ambedkar "They have left enough room for people of different ways of thinking with regard to reaching of the ideal of economic democracy." According to Raghavachariar "Whoever may capture the government power, they will have to respect this instrument of instructions. He may have to answer for them before the electorate when the next election.

(d) Importance of Moral Precepts. Even if the Directive Principles are to be considered as mere moral precepts or pious resolutions, still their significance cannot be mrnimised. According to Gledhill, "The lives of the countless millions have been shaped and directed by moral precepts impinging on their minds and it is not difficult to find instances of similar precepts directing the course of history and nations." In spite of the fact that constitutional landmarks like Magna Carta, the French Declaration of Rights and the Preamble to the American Constitution do not have any legal sanction behind them, yet their impact on the histories of countries concerned cannot be considered less vital. Just as the provisions of these landmarks cannot be declared ineffectual, the principles contained in Chapter IV also are apt to shape the destiny of our nation and guide the policies of our future government. Hence, they shall not be dismissed as ineffectual moral precepts.

(e) Fundamental Principles of Social and Economic Order. The principles embodied in the Directives are conceived as the fundamental principles of a new social and economic order which was the ultimate goal of the fathers of Indian constitution. It is definitely stated in the constitution that these principles though legally not enforceable are nevertheless fundamental in the governance of the country and it shall be the duty of the State to apply these principles in making laws. Article 38 prescribes, "The State shall strive to promote the wertare of the people by securing and protecting as effectively as it may, a social order, in which justice, social, economic and political shall inform all the institutions of national life." Article 39 goes further in enumerating principles of socialism. The framers of the constitution were conscious of the fact that political democracy alone is not enough, hence they were endeavouring to promote the conception of welfare state by laying down these fundamental principles of social and economic order which fearing the wrath of public, the legislators and the executives could not easily ignore. Dr. Ambedkar's statement in the Constituent Assembly makes this point perfectly clear. "Their intention was that however adverse circumstances that stand in the way for government in giving effect to these principles and howsoever impropitious the time might be, they should always strive for the fulfilment of these principles."

(f) Ambiguities of the Constitution Removable Through Directives. If we make an analytical appraisal of the decisions of the Supreme Court regarding certain ambiguities of the Fundamental Rights provisions, we find that Directive Principles have rendered a great assistance to the judges. Since the Directive Principles constitute a part of the constitution whenever a question regarding the Interpretation of a vague provision in the constitution crops up, a reference to the Directive Principles can be made to come Cut of the thicket of constitutional ambiguities. Article 19, for example, permits the imposition of reasonable restrictions on the Fundamental Rights. If a conflict on the interpretation of "reasonable restrictions" ensues, a reference to the Directive Principles can easily be made. A restriction promoting any objective embodied in the Directive Principles is usually considered reasonable by court of law; If a question as to whether a certain matter is in the interest of the public or not arises. reference to the Directive Principles is helpful. In Gopalan v. State of Madras Chief Justice Kania remarked It has no doubt been repeatedly

made clear that the courts cannot uphold a Directive Principle when it comes in conflict with a specific provision of the constitution, relating to Fundamental Rights. However in the interpretation of these provisions, the Directive Principles can serve a very useful purpose. Most of the Fundamental Rights are subject to reasonable restrictions which may be imposed for public purpose or in public interest. The courts can be and have been guided by the Directive Principles in determining whether the actual restrictions placed bylaw on the exercise of Fundamental Rights are reasonable or in public interest or whether they sub serve a public purpose." Prof. Alexandrowics is of the view that the courts "should give the greatest possible weight to the Directive Principles for the purpose of their interpretations of the provisions relating to Fundamental Rights..." In the State of Bombay v. KM. Balsara, the Supreme Court gave weight to Article 47—aiming at Prohibition, to support its decision that the restrictions imposed by the Bombay Proinbition Act was a reasonable restriction on the right to acquire any profession or carry on any trade. In Buoy Cotton Mills v. State of Ajnier, the Supreme Court upheld the constitutional validity of the Minimum Wages Act (1948 by taking into consideration Article 43. In the opinion of the Court, the fixation of wages for labourers did not violate freedom of trade under Article 19(5). Mr. M.C. Setaiwad, Attorney General of India, has beautifully summed up the utility of the Directives in this field "... These fundamental axioms of state policy though of no legal effect have served as a useful beacon light to courts...Restrictions imposed by law on the freedom of citizens may well be reasonable if they are imposed in furtherance of the Directive Principles.

(g) Nominal Executive Heads cannot exploit these Provisions. D.D. Basu, a well known commentator on the Indian constitution, apprehended that the President or the Governor might refuse to assent to a Bill passed by their respective legislatures on the plea that such a bill is inconsistent with the Directive Principles. It might therefore lead to deadlocks between the President and the Council of Ministers or the Governor and the Council of Ministers, since the latter are responsible for passing an important legislation. Though Basu's fears are genuine, yet we must not efface from our mind a naked reality that the President's or Governor's powers to veto a Bill passed by the Legislature is only limited. In the words of Dr. Ambedkar "The Directive Principles cannot be pressed into service either by the President or the State Governor for the purposes of vetoing a law passed by the legislature". in a parliamentary form of government, the constitutional rulers cannot be assertive. If they are, they cannot be tolerated.

THE VALUE OF THESE PRINCIPLES

A constitution framed in the middle of the twentieth century could hardly do without chapter on directive principles of the type the Indian Constitution has. The establishment of political democracy is a fundamental aim of the Constitution. But that in itself is not enough. The sustaining forces of that political democracy have to be carefully built up. The most effective force which will sustain a political democracy is the simultaneous existence of an economic democracy. Where there is no economic democracy, political democracy is bound to degenerate soon into a dictatorship. If the fundamental rights guarantee a political democracy in India, the Directive Principles ensure the eventual emergence of an economic democracy to sustain the former. Thus, the Directive Principles of State Policy become the greatest guarantee for a genuine democracy in India. In the light of these considerations, it would betray a lack of discernment to consider these directives as a mere political manifesto without any legal sanction,' or to characterize them as vague and indefinite serving no useful purpose2 or to dismiss them as a mere moral homily. The last five decades and more demonstrate that such criticism has neither substance nor relevance today. If K.T. Shah were alive now, he should certainly have revised the opinion that he expressed in the Constituent Assembly that these principles "are like a cheque on a bank payable when able, only when the resources of the bank permit."Another apparently weighty criticism of the Directive Principles is implied in the question whether it is worth-while to insert in a constitution of today a collection of political principles taken from the experience of the nineteenth century England or Western Europe, and to deem them to be suitable for India in the middle of the twentieth century.4 The question whether they would be suitable for the twenty-first century when the Constitution is hoped to be still in operation is difficult to answer. It is probable that they may become outmoded by then. Who can predict the precise nature of the potentialities of an atomic or a hydrogen age? It may revolutionize the whole economic system of the present day and convert India into a land of plenty where all human wants in the material

field are fully and instantaneously satisfied. In such a state of affairs, the Directive Principles will indeed look not only outmoded but even reactionary. But as far as the twentieth century was concerned, India had yet to reach in many spheres of economic activity a standard comparable to that which existed even in the nineteenth century in Western Europe. Thus, en sumiflg that the Directive Principles reflect the nineteenth century political ideas of the West, their value in twentieth century India was not lost. Besides, it is not quite correct to characterize these principles as borrowings from aborad. As has been pointed out elsewhere, there are many provisions in this chapter which prove the originality of the constitution and reflect the genius of the Indian people.

If and when the Directive Principles become outmoded, they can be suitably amended altogether abolished. The process of amending these provisions is simple. But by the time such amendments take place, India will have benefited immensely by the Directive principles, and an economic democracy will have sent its roots deep into the Indian soil and the present form in which these principles are embodied will have realized its goal. Moreover, these principles would have become part and parcel of the Indian heritage. Thus, one can see the immense educative value of these principles. They will instil in the minds and thoughts of the coming generations of Indian youth the fundamental values of a stable political order and a dynamic economic system. A constitution is primarily concerned with the present. The future will take care of itself if the present is built on solid foundations. It is quite unnecessary, therefore, to think of the distant future with reference certain provisions of a constitutional document.

The real importance of the Directive Principles is that they contain the positive obligations of the State towards its citizens. No one can say that these obligations are of an insignificant type or that even if they are fulfilled, the pattern of society in India will still remain more or less the same. In fact they are revolutionary in character and yet to be achieved in a constitutional manner. Herein lies the real value of embodying these principles as an integral part of the Constitution. Through the Directive Principles of State Policy, the Constitution of India will steer clear of the two extremes, a proletarian dictatorship which destroys the liberty of the individual and a capitalist oligarchy which hampers the economic security of the masses.

CONCLUSION

These Directives thus constitute the national objectives and the national conscience and whosoever is victorious at the polls will not be free to violate them. According to Mr. Alladi Krishnaswamy Ayyar, “No ministry responsible to the people can afford light heartedly to ignore the provisions in Part IV of the constitution.” They have served as a guide for the Union Parliament and state legislatures. They are cited by the courts to support decisions. The governmental bodies have been invariably guided by these provisions.”

If we make a critical estimate of the achievements of the present Government in implementing these policies, we feel encouraged. Panchayats are being established in the remotest villages of our country. They are being restored to their old pristine glory. Nationalization of certain industries, setting up of corporations. Heavier taxation of bigger incomes, recovery of deliberately concealed taxes and courageous steps to bring Dalmias in the clutches of law reflects that the State is doing its lest to avoid concentration of wealth. The establishment of the poor house at the capital to wipe out great slur on the fair name of Indian democracy, La, begging, is a substantial step towards provision of adequate means of livelihood. The state owned factories, industries and Government Corporations are expanding, hence more and more people are getting employment. The Employees State Insurance Act and the Workmen’s Compensation Act are very significant steps to provide assistance to the workers during old age, disablement or undeserved want. The cottage industries are being encouraged. The Minimum Wages Acts have been passed to ameliorate the lot of labourers of various categories. Efforts are being made to make available primary education to the children below the age of 14 years. The assistance to Scheduled Castes in the form of stipends and scholarships, remission of school and college fees, concession in the age and fees limits tör those applying for jobs through public Service Commission are effective steps to uplift the downtrodden class of Hindus. Agriculture is

being organised on scientific lines. The attempts to improve the breed of the cattle are being made. Slaughter of cows and calves in some of the States has been prohibited. The Ancient Monuments Acts are steps in the direction of the fulfilment of the Directives mentioned in Article 49. In some of the States as Andhra Pradesh, Gujarat, Haryana, Punjab,. Kerala, Madras, Mysore and Maharashtra judiciary has been separated from the executive. In the fulfilment of principles pertaining to international peace and security, our Prime Minister's efforts can hardly be minimised. "Panch Sheel" is a laudable step in this direction. Pt. Nehru was hailed as a cultural ambassador of spiritual East to the material West. He is raised to the pedestal of glory even by the pseudo-democrats of U.S.A. and Britain and orthodox and fanatic Muslims of the Arab world.

Still much is to be achieved. Political influences and economic and social disparities still persist. Standard of living of the people is yet to be raised. Unemployment is yet to be eradicated. Socialism is yet to be ushered in. But with all this, it can be safely concluded that government's efforts in the implementation of these objectives are really very commendable and fairly substantial.

Dirncultles on the way of Implementation: There are, however, several difficulties on the way of implementation of these Principles. One such difficulty is that the states have no adquate financial resources to implement the Directives. Implementation of every Directive means huge financial lay out which the country cannot afford. Then another difficulty is that the country is faced with so many problems that the Directives do not fall in high priority category. The people on the whole have not raised strong voice against the neglect of the Directives or at least paying less heed to these. But a serious difficulty on the way of implementation of Directive comes Directives when that comes to the implementation of a Directive which is directly or indirectly concerned with a religious community. Much hue and cry raised by the leaders of religious community when the Supreme Court suggested implementation of Directive about uniform civil code.

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RESEARCH ARTICLE

Simultaneous Estimation of Etoricoxib and Thiocolchicoside By RP-UPLC Method in Combined Dosage Forms

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ABSTRACT

A new simple, precise method was developed for Etoricoxib and Thiocolchicoside by using UPLC for Simultaneous Estimation. The compounds were separated by using column C- 18 BEH 2.1 x 50 mm (1.7 µm) at ambient temperature. Flow rate was 0.8 ml/min; wavelength of 255 nm, Mobile phase was acetonitrile: Buffer (50:50), Disodium hydrogen phosphate buffer was used with pH 3.5. The injection volume was 2 µl and Run time 4 min. The retention time of Thiocolchicoside and Etoricoxib was 1.4 and 2.6 mins respectively. The percentage purity was found to be 99.54 and 99.34 respectively. The validation parameters Specificity, Accuracy, linearity, LOD, LOQ and Robustness were studied. The linearity of Thiocolchicoside was 3.2 – 25.7 µg/ml and for Etoricoxib 36 – 288 µg/ml. They have good linearity with Correlation coefficient Value of 0.998, 0.992 respectively.

Keywords: UPLC, Thiocolchicoside, Etoricoxib, Simultaneous Estimation.

INTRODUCTION

Etoricoxib is 5 - chloro- 2 - (6-methyl pyridin-3-yl-3-(4-methylsulfonylphenyl) pyridine. It is used as a non- steriidal anti- inflammatory agent (1). It is selective inhibitor of COX -2 that deceases GI toxicity and is without effects on platelet function (2). Several methods have been reported for the analysis of etoricoxib in pharmaceutical dosage

form as well as in the biological fluids and tissues. i.e. spectrophotometry methods (3)(4) and chromotographic methods; HPLC (5)(,6) and LC/Mass spectrophotometry. (7)(8).(9).

Thiocolchicoside, chemically N- { 1,2- dimethoxy – 10 methyl sulfanyl - 9 - oxo-3- {3,4,5-trihydroxy-6 - (hydroxymethyl) oxan-2-yl) oxan -2 -yl) oxy-6,7 - dihydro -5 H- benzo (a)heptalen -7 - yl) acetamide,is a muscle relaxant, Which has been claimed to possess GABA mimetic and glycinergic action. It is used in the symptomatic treatment of painful muscle spasm (10). It has powerful convulasant activity and should not be used in seizure – prone individuals (11, 12). Thiocolchicoside is not official in any pharmacopoeia. On detailed literatiure survey, it was found that thiocolchicoside can be estimated by spectrophotometry (13, 14) HPLC (15, 16) and by HPTLC (17) methods individually or in combination with other drugs (18) (19-22). Since No RP-UPLC methods are reported for the simultaneous estimation of etoricoxib and thiocolchicoside in combination, therefore anttempt has been made to estimate both these drugs simultaneous by a simple RP-UPLC method. The proposed method was optimized and validated as per ICH guidelines. The structure of etoricoxib and thiocolchicoside are given in Figure.1

MATERIALS AND METHOD

All solvents were HPLC grade and Reagents were Analytical Grade. Acetonitrile from RANCHEM, Potassium di hydrogen phosphate from Merck, Ortho phosphoric acid from Merck. Etoricoxib and Thiocolchicoside were procured from Apex Pharma Chennai. The method was developed in Waters UPLC Acquity system,: C- 18 BEH column was used for the separation. The Mobile Phase consists of acetonitrile: Buffer (50:50), Disodium hydrogen phosphate buffer was used with pH 3.5. The injection volume was 2 µl with flow rate of 0.8 ml per minute and wavelength was 255 nm. The analysis was performed at constant column temperature at 35^{o} C. Mobile Phase was used as Diluent

Method Development

Selection of Wavelength

Isobestic point Etoricoxib and Thiocolchicoside was determined by dissolve it in Acetonitrile and scanned between 200 - 400nm and the Isobestic Point was found to be 255nm

Preparation of Standard Stock Solution

Etoricoxib 45mg was weighed accurately and dissolved in 50ml, sonicate about 10 minutes, then 10 ml of the above solution was Pipette out into 50 ml to get final concentration. Thiocolchicoside 20mg was weighed accurately and dissolved in 50ml; sonicate about 10 minutes, then 2 ml of the above solution Pipette out into 50 ml to get final concentration.

Preparation of Sample

Etoricoxib 10 tablets were weighed and powdered, the powdered drug was weighed equivalent to about 300 mg of Etoricoxib dissolved into50 ml Volumetric flask, dissolve it completely, add 5 ml of the above solution into 50 ml. Thiocolchicoside 10 tablets were weighed and powdered drug was weighed equivalent to about 300 mg of dissolved into 50 ml dissolved it completely, from this solution pipette out 5m and make upto 50ml to final concentration

VALIDATION

This method was validate according to the ICH Guidelines, the following parameters were studied System Suitability, Specificity, Linearity and Range, Accuracy, Robustness Limit of Detection and Limit of Quantitation

System Suitability

System suitability studies were performed by injecting 5 replicate of injections of Etoricoxib and Thiocolchicoside. It was performed to determine the resolution, theoretical plates, tailing factor, repeatability of retention time etc. All parameters were within the range.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. It was performed to identify the any impurity may present; it was done by using standard, sample, placebo dilutions of both of the drugs. It was observed there is no interference. (Fig 2)

Calibration Curve (Linearity)

Linearity of Etoricoxib was found to be 36µgm/ml to 288µgm/ml, with R^2 Value of 0.9998. Linearity of Thiocolchicoside was found to be 3µgm/ml to 24µgm/ml, with R^2 Value of 0.9992. (Table 2).

Accuracy

Accuracy is closeness of agreement between true value and Value to be found. For Accuracy studies three levels were selected 80%, 100% and 120% for Etoricoxib and Thiocolchicoside. Recovery was found with Average value of 99.30 % and 99.20 % respectively. (Table 1).

Robustness

Robustness study was done by changing the pH, wavelength, flow rate in both the drug. % RSD was within the limit as per ICH guidelines.

Limit of Detection

For Limit of detection the S/N Ratio should be ≥ 3 & ≤ 9. Limit of detection of Etoricoxib was 0.3 ppm and signal to Noise Ratio was 3.71. Limit of detection of Thiocolchicoside was 0.2 ppm and signal to Noise Ratio was 3.32. (Table 3)

Limit of Quantitation

For Limit of Quantitation the S/N Ratio should be ≥ 10 & ≤ 30, Limit of Quantitation of Etoricoxib was 0.8 ppm and signal to Noise Ratio was 12.63. Limit of Quantitation of Thiocolchicoside was 0.5 ppm and signal to Noise Ratio was 10.24. (Table 3)

CONCLUSION

It was concluded that the developed Method was simple, Accurate, Rapid and sensitive, it can be used in quality control laboratories for routine analysis. The method provides economically feasibility due to the lesser consumption of sample run time.

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Table 1. Assay of Etoricoxib and Thiocolchicoside

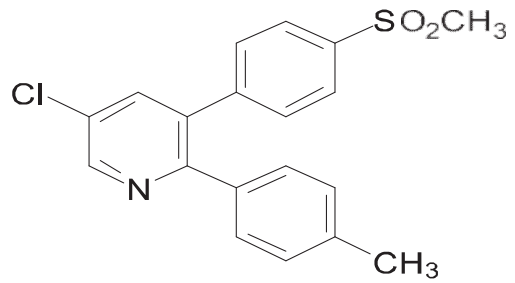
Commercial Formulation	Drug	Standard area	Sample Area	Label Claim (mg)	% Purity
	Etoricoxib	13964295	14088540	90 mg	99.54 %
	Thiocolchicoside	1101321	1126678	8 mg	99.34 %

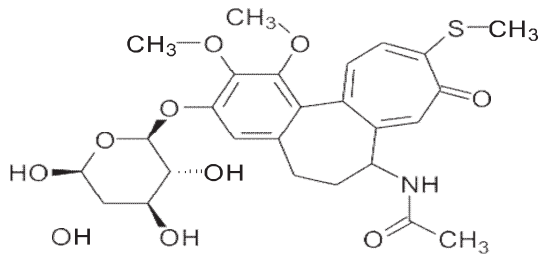
Table 2. Linearity and Range

S.No	Etoricoxib		Thiocolchicoside	
	Conc (mcg/mL)	Mean area	Conc (mcg/mL)	Mean area
1	36	1903617	3	146715
2	72	4800448	6	380021
3	108	8103589	9	612363
4	144	10991324	12	847008
5	180	14402644	15	1134365
6	216	17398920	18	1396486
7	252	20402986	21	1644830
8	288	23502280	24	1895112

Table. 3. LOD and LOQ

S. No.	Concentration (ppm)	S/N (signal to noise ratio)
LOD		
1	0.2 ppm (Thiocolchicoside)	3.32
2	0.3 ppm (Etoricoxib)	3.71
LOQ		
1	0.5 ppm (Thiocolchicoside)	10.24
2	0.8 ppm (Etoricoxib)	12.63





ETORICOXIB **THIOCOLCHICOSIDE**

Fig: 1. The structure of etoricoxib and thiocolchicoside

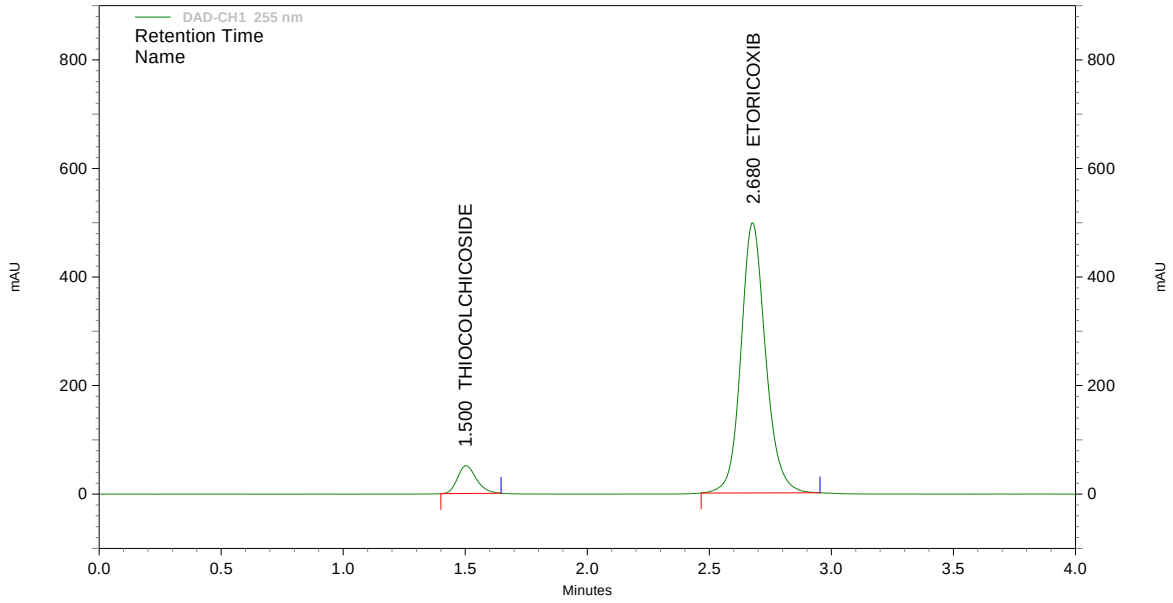


Fig: 2. Chromatogram of Etoricoxib and Thiocolchicoside

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RESEARCH ARTICLE

The Effect of Whole Body Irradiation on the Uterus of Mice

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ABSTRACT

Gamma (γ) radiation is known to have a number of side effects, one of which is premature ovarian failure. Radiation exposure is also reported to adversely affect the uterus. However, there are no detailed studies examining morphological alterations in the uterus following irradiation. In the present study, the exposure of adult female mice to a sub-lethal dose (6Gy) of whole body γ-radiation caused drastic changes in the uterine morphology. The endometrial epithelium appeared flat and endometrial glands were shrunken compared to epithelium with several folds and well developed glands in the endometrium of controls. Further, there was a significant reduction in the relative weight of the uterus and the endometrial thickness and a significant increase in the thickness of myometrium compared to those of controls. The results suggest that severe damage to the uterine endometrium may be cause of poor reproductive outcome reported earlier in human and animal studies. .

Key words: radiation, uterus, endometrium, mice

INTRODUCTION

Gamma (γ) radiation, which is widely used for radiotherapies, has a number of side effects, one of which is premature ovarian failure. Gamma radiation is reported to cause ovarian tumors [1,2], early ovarian failures [3,4] and reduction in follicular numbers [5,6]. Studies in animals also have shown radiation induced ovarian follicular loss and premature ovarian failure [7,8,9,10,11]. Apart from the follicular loss, radiation exposure also influences reproductive outcome. The studies in cancer survivors who have undergone radiotherapy reported preterm labour [12,13], early pregnancy loss and reduction in number of live births [14,15,16]. The direct exposure of abdominal region to radiation also caused permanent uterine damage in women, i.e. reduction in endometrial thickness and

uterine blood flow as assessed by ultra sound scanning [17,18]. It is also reported that an exposure to γ-radiation (0.1 Gy/ Day) continuously until parturition led to considerable reduction in litter size [19] and whole body irradiation caused significant reduction in litter size, physical anomalies in the foetuses and foetus resorption on 18th day of pregnancy [20] in mice. However, though there are studies portraying effects of radiation on reproductive outcome, there are no extensive studies on the effect of radiation on the uterus, though it has a crucial role in maintaining pregnancy. Since in humans, where ultra sound scan is used to assess irradiation induced damage in the uterus, an animal model will be most suited to investigate the radiation effect on uterine morphology. Further, for finding out methods to mitigate damaging effects of radiation on the uterus, preliminary studies on animal models reporting alterations in the uterus are necessary. Hence, the present study was undertaken to find out if exposure to a sub-lethal dose (6Gy) of γ – radiation results in any alterations in the uterus of adult female mice.

MATERIALS AND METHODS

Animals

Four months old Swiss albino mice, showing regular estrous cycles were procured from the central animal facility, University of Mysore, Mysuru. The mice had free access to food and water and were maintained under 12L/12D photoperiod. The experiment was approved by the Institutional animal ethics committee and the guidelines of the committee were followed for treatment of the animals.

Experimental design and Radiation exposure

The adult female mice were divided into two groups, control and irradiated, 5 mice in ach group. Each mouse in irradiated group was subjected to whole body exposure of 6 Gy γ-radiation using a gamma irradiator (source: ^{60}Co). The mice were autopsied on day 8 after the radiation exposure, day zero being day of the exposure. At autopsy, the weight of the body and the uterus was recorded and the uterus was weight was converted into relative weight as follows:

$$\text{Relative weight of uterus} = \frac{\text{Weight of the uterus x 100}}{\text{Weight of the animal}}$$

The uterus was fixed in Bouin's fluid for 24 hours, dehydrated through different grades of alcohol, embedded in paraffin and sections of 5μm thickness were cut from the mid-regions of the uterus. The sections were stained with haematoxylin and eosin and scanned for histological alterations if any. The endometrial thickness was measured using the ocular micrometer from 50 randomly selected areas per mouse and mean thickness of the endometrium and myometrium were computed considering the values of 5 mice per group.

Statistical analysis

The mean value of each parameter was computed using data of five mice/group and student T test was used to judge significant difference between the mean values of each parameter of control and irradiated mice.

RESULTS

Body weight

There was no significant difference between the body weight of the controls and irradiated mice.

The relative weight of the uterus

The relative weight of the uterus in the mice exposed to radiation was significantly lower than the controls.

Histology

The uterus of controls appeared normal with the presence of three distinct layers, viz. perimetrium, myometrium and endometrium. The endometrial layer in the controls was well developed with cellular stroma and was rich in well developed endometrial glands. The endometrial epithelium was clear and appeared wavy with a number of folds (Fig.1). The uterus of mice exposed to radiation, on the other hand showed thickened myometrium and reduced endometrium. The endometrial epithelium was conspicuous by being flat with the absence of foldings. The endometrial glands appeared shrunken and were few in number compared to the controls (Fig. 1). In radiation exposed mice the thickness of endometrium showed a significant reduction whereas that of myometrium was increased significantly when compared to these of controls (Fig. 2).

DISCUSSION

Adverse effects of radiation on various organs have been extensively studied despite the fact that it is widely used for therapeutic purposes. Among the different organs, ovaries were found to be more sensitive [21]. There are many factors influencing the effect of radiation on ovaries such as the age of the patient and the dose of radiation received. Likewise, the uterus is also vulnerable to the irradiation effects. Changes in uterine muscular vascularization and reduction in the uterine volume, both of which can impair fetus growth during the pregnancy, has been observed in women who have undergone radiotherapy in childhood [22]. Indeed, it is reported that abdominal and pelvic irradiation can cause preterm delivery [12], miscarriages and placental abnormalities[15] in humans. Studies have also shown that radiation can cause permanent uterine damage and affect uterine blood flow[23], which in turn can lead to miscarriages and resorption of the fetuses[24] in women. However the human studies use only ultra sound methods for assessing uterine changes. Similarly, whole body radiation exposure resulted in fetal abnormalities and resorption on day 15 of pregnancy in mice [20]. Though the studies report implications of radiation exposure on reproductive outcomes, detailed studies on uterine morphology are rare in irradiated animal models. The results of present study reveal that the exposure of adult mice to whole body γ-radiation at a dose of 6Gy causes remarkable changes in the uterus. It is shown that radiation exposure results in considerable reduction in the relative weight of uterus in mice, which is in agreement with the previous studies in humans, where the uterine volume was found to be reduced to 40% of the normal size due to radiation exposure in the childhood [18]. Further, the endometrium was most affected component of the uterus in the present study as there was distortion of epithelial layer, regression of endometrial glands and a significant reduction in the endometrial thickness. On the other hand, there was an increase in thickness of myometrium.

In addition, the endometrial epithelium in irradiated mice uterus appeared flattened with a very thin layer of epithelial cells, in contrast to a highly folded epithelium in the controls. Similar studies in rats showed that exposure to Cs-137 caused flattening of the endometrial surface of the uterus [25]. The endometrial glands also showed a significant reduction in mice exposed to radiation compared to the controls. The glands significantly contribute to nourishment of the fetus and maintenance of pregnancy and hence reduction in their number and epithelial foldings indicate impairment in supporting pregnancy and vulnerability to abortions. Earlier studies in humans have shown that radiation alters reproductive come, resulting in miscarriages [26], post-partum haemorrhage, preterm labour [12,15], small-for-gestational-age off spring [27], low pregnancy rates [13], higher incidents of live births and spontaneous abortions[16]. These observations are based on ultrasound scanning in women who have undergone radiation exposure at young age. In animals also, it is reported that radiation exposure causes fetal abnormalities and reduction in live fetuses [20]. Put together the results of these investigations and that of the present study, it can be

concluded that the reproductive outcomes may be influenced by the changes in uterine morphology. The findings of this study demonstrate that radiation exposure causes considerable damage to the uterus, which is crucial in reproductive outcomes as uterus plays a major role in maintaining pregnancy. Since γ-radiation is widely used for therapeutic purposes, the evaluation of its effects on uterus is equally important and in this regard, the present study provides information on the histological changes in animal model, which can form basis for further studies to mitigate radiation effects.

Authors' contribution

The first author executed the experiment, conducted all the assays to obtain data and tabulated the data. Second author has designed the study and critically reviewed the manuscript.

Conflict of interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors. The experiment was approved by the Institutional animal ethics committee and the guidelines of CPCSEA were followed for treatment of the animals.

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Table 1- Effect of Whole Body Irradiation on the Body Weight and Relative Weight of the Uterus in Adult Female Mice

Groups	Body weight (gm)	Relative weight of uterus (mg/gm)
Controls	34.40 ± 0.77	125.64 ± 17.68
Radiation exposed	32.98 ± 0.66	57.08 ± 4.66*
Student T test	0.25	5.9
P value	NS	P<0.05

* Significantly different (P<0.05) from controls as judged by Students T- test.

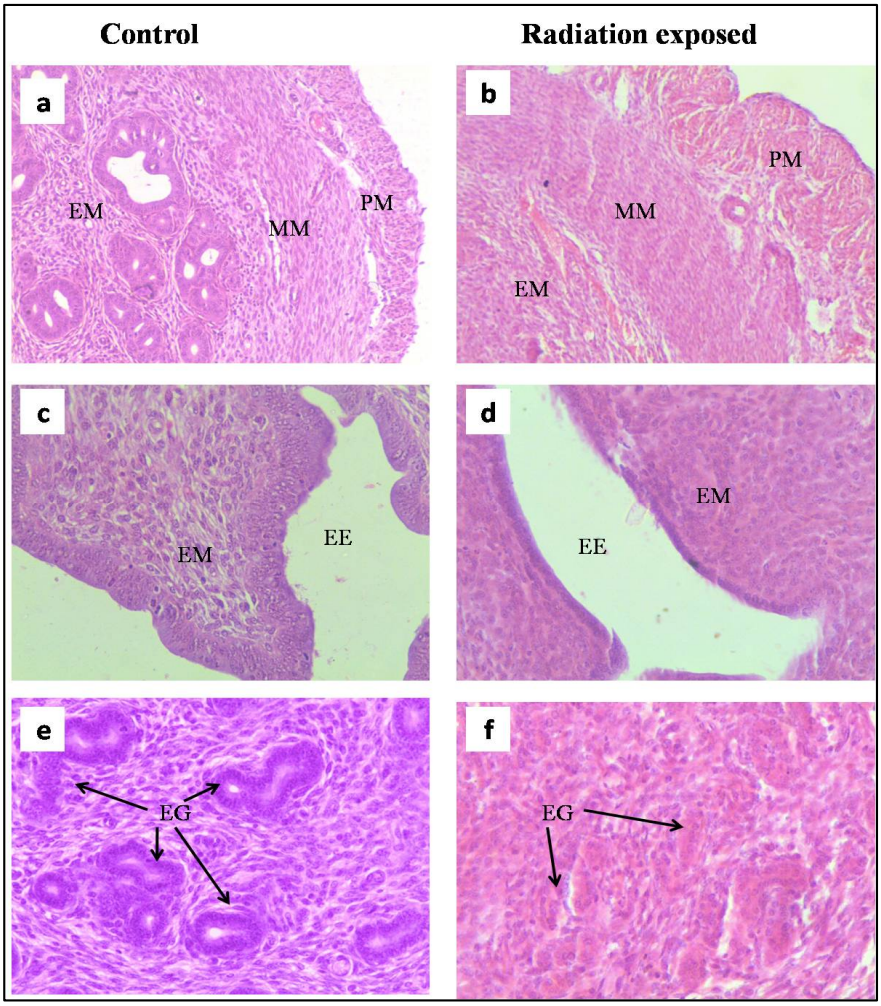

Fig. 1 (a- f) : Photomicrographs of the cross sections of the uterus showing the myometrium (a,b; 100X), endometrial epithelium (c, d; 200X) and endometrial glands (e, f; 200X) in control and irradiated mice. Note the presence of normal myometrium (a), endometrial folds (c) and endometrial glands (e) in control mouse compared to thick myometrium (b), flattened endometrial epithelium (d) and shrunken endometrial glands (f) in radiation exposed mouse (H&E). EE= endometrial epithelium, EG= endometrial gland, EM= endometrium, MM= myometrium, PM= perimetrium.

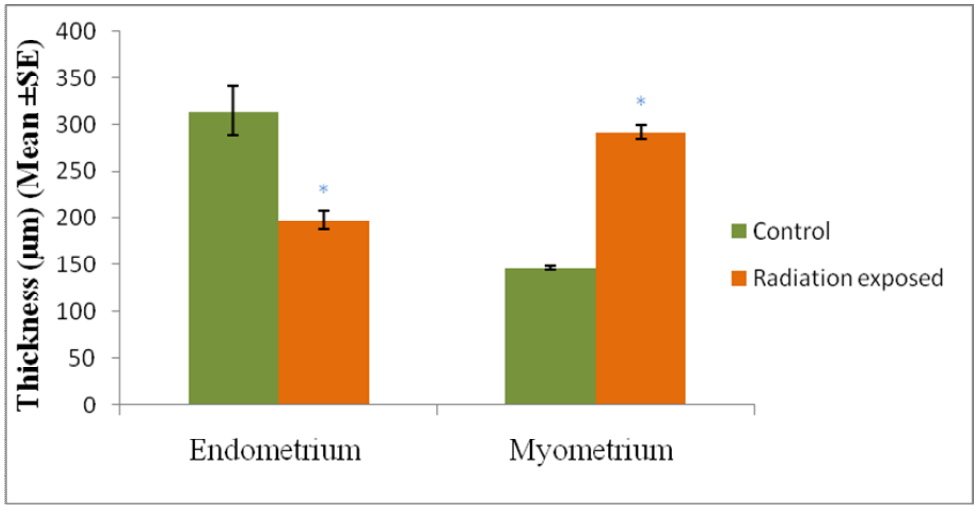

Fig. 2: Vertical bars showing thickness of endometrium and myometrium in mice following radiation exposure.
*** Significantly different (P<0.05) from controls, as judged by Students T- test.**

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RESEARCH ARTICLE

Nitric Oxide Protects Cucumber Plants against Drought Stress through Inducing Defense System of the Plants

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ABSTRACT

Nitric oxide (NO) is an important signaling molecule that plays a significant role in the protection of plants against various environmental stresses. The present work was carried out to evaluate the role of NO in the protection of plants against drought stress (DS). Results of the study show that exposure of plants to DS enhanced the synthesis of reactive oxygen species (ROS) namely hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\cdot-}$) which resulted in the damage to the cell membrane as shown by the increased level of electrolyte leakage. DS also induced osmotic stress and suppressed leaf relative water content (LRWC). However, application of NO donor sodium nitroprusside (SNP; 0,2 mM) to drought-stressed plants significantly enhanced the activities of antioxidant enzymes and synthesis of proline (Pro) and glycine betaine (GB) which countered oxidative and osmotic stress. Enhanced activities of antioxidant enzymes and Pro and GB resulted in reduced leakage of electrolyte and improved LRWC leading to improved growth of the plants. Therefore, it can be concluded that the application of NO induces tolerance against drought stress by enhancing the defense system of the plants.

Keywords Antioxidant enzymes, Drought, Osmotic stress, Oxidative stress, Proline

INTRODUCTION

Among several environmental stresses, drought stress is known to limit crop production and distribution worldwide. Drought stress induces excessive generation of reactive oxygen species (ROS) which induces oxidative stress and causes oxidation of macromolecules (1) that results in decreased crop yield. Being sessile, plants are always exposed to environmental stresses. However, plants possess a system for the defense to counter the detrimental effects of drought stress. This defense system is designed by a series of antioxidant enzymes such as ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), superoxide dismutase (SOD) and catalase (CAT). In addition to oxidative stress, drought stress also causes osmotic stress which reduces water uptake capacity of the plants and as a

result, the hydration level of the plants gets reduced. However, plants have been shown to neutralize osmotic stress by accumulating osmolytes such as proline (Pro) and glycinebetaine (GB). Regardless of the presence of various defense systems in plants, timely and precise activation of these defense systems is vital for the endurance of plants under stressful conditions. Therefore, the precise response of stress stimulus is vital which is performed by a network of signaling molecules. Nitric oxide (NO), a gaseous, small diffusible molecule is mainly synthesized by nitrate reductase (NR)-catalyzed reduction of NO_2^-(2) in plants. NO affects plants at almost every stage of their life cycle such as seed germination, adventitious root formation, flowering, stomatal closure and senescence (3,4). NO possesses antioxidant and cytoprotective properties (5-7). Moreover, NO plays a significant role in mediating responses to biotic and abiotic stresses in plants (8,9). Exogenous application of NO is capable to elevate the activities of antioxidative enzymes (6,7) and protects the plants against various abiotic stresses such as salinity, osmotic stress, drought, UV-B radiation, and heat (6,7, 10, 11).Keeping important role of NO in view, the present work was carried out to evaluate the effect of NO on the activation of the antioxidant system and osmolytes accumulation in plants under drought stress.

MATERIALS AND METHODS

Healthy and uniform seeds of cucumber (*Cucumis sativus* L.)were surface sterilized with 0.1% $HgCl_2$ for 10 min then vigorously rinsed with double distilled water (DDW). Surface sterilized seeds were sown in plastic pots (15 ×15 cm) containing a mixture of soil and vermiculite (1:1). After sowing the pots were well watered for 15 days and were kept under natural illuminated conditions. After 15 days, drought stress (DS) was induced by withholding water supply and plants were exposed to sodium nitroprusside [SNP: ($Na_2Fe(CN)_5NO$], used as NO donor. The treatments were given as: (i) DDW: control, (ii) Drought stress (DS), (iii) 0.2 mM SNP (NO), (iv) DS + 0.2 mM SNP (DS+NO). Plants that were irrigated daily and not exposed to SNP were considered as control. Each treatment was replicated three times. Eight days after the treatments, the response of the plants to DS and NO was evaluated.

Estimation of LRWC, electrolyte leakage, and hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\cdot-}$) content

The effect of DS and NO on LRWC was determined by the method of Yamasaki and Dillenburg (12). Whereas, the method of Lutts et al. (13) was adapted to measure electrolyte leakage (ELKG). Hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\cdot-}$)contents were determined by the method ofVelikova et al. (14), and Elstner and Heupel (15), respectively.

Assay of antioxidative enzymes

Before the determination of antioxidant enzyme activities, a crude enzyme extract was prepared. The activity of ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2) were assayed by the method of Nakano and Asada (16), and Foyer and Halliwell (17), respectively. Peroxidase (POX; EC 1.11.1.7) was assayed by the method of Upadhyaya et al. (18). Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (19).

Determination of Pro and GB content, and growth attributes

Proline (Pro) content was determined according to the ninhydrin method of Bates et al. (20). Glycine betaine (GB) content was estimated adopting the method of Grieve and Grattan (21). Effect of DS and NO on the growth attributes of cucumber plants was tested by measuring fresh weight (FW), and dry weight (DW). To analyze DW, plants were kept in an oven at 80 °C for 24 h. After 24 h dried plant material was weighed.

Statistical analysis

All the treatments were replicated three times. The data were analyzed statistically using SPSS-20 statistical software (SPSS Inc., Chicago, IL, USA). When F value was found to besignificant at 5% level of probability, Least SignificantDifference (LSD) was calculated. Values were expressed as means±Standard Error (SE).

RESULTS AND DISCUSSION

A perusal of the data shows that exposure of plants to DS reduced hydration of the plants as shown by the lower level of LRWC (Table 1). Plants under DS exhibited 38.4% reduction in LRWC as compared with the control. However, the application of NO to non-stressed, as well as drought, stressed plants elevated the level of LRWC by 25.9% and 16.0%%, respectively as compared with the drought-stressed plants (Table 1). Osmotic stress is one of the prime effects of DS which suppresses the water uptake capacity of the plants (7). However, plants counter osmotic stress by synthesizing osmolytes such as Pro and GB. The application of NO to drought-stressed plants enhanced the synthesis of these osmolytes (Table 2). Similar results were also reported by Khan et al. (6,7,22). Enhanced accumulation of Pro and GB improved the water uptake capacity of the plants and resulted in increased LRWC (Table 1).

The results show that DS induced oxidative stress as exhibited by the increased level of H_2O_2 and $O_2^{\cdot-}$ content (Table 1). Excessive generation of ROS (H_2O_2 and $O_2^{\cdot-}$) results when ROS generation surpasses the cellular antioxidant defense system that leads to oxidative stress. The ROS-induced oxidative stress made the membrane more permeable by peroxidation of membrane lipids (6,7,22) which is confirmed by higher values of electrolyte leakage (Table 1). The results show that antioxidant enzymes activities (APX, GR, POX, SOD) were enhanced under DS (Figure1A and B) but at the same time, H_2O_2 and O_2 content also increased which indicates that plants' antioxidant defense system was not able to counter DS-induced oxidative stress. However, application of NO to the stressed plants further elevated the activities of antioxidant enzymes (Figure 1 A and B) that suppressed the excessive generation of ROS (7, 23,24)which resulted in the maintenance of cell membrane as shown by decreased value of electrolyte leakage in the drought-stressed plants treated with NO (Table 1).

It is evident from Figure 1 that cucumber plants under DS exhibited a significant increase of 43.8%, 47.4%, 36.1% and 27.1% in the activities of APX, GR, POX, and SOD, respectively as compared with the control plants. It is well known that in response to oxidative stress the antioxidant defense system of the plants is activated (22). The results show that application of NO to the stressed plants further elevated the activities of APX, GR, POX, and SOD by 34.8%, 34.1%, 14.1%, and 8.06%, respectively than the drought-suffered plants that did not receive NO (Figure 1 A and B).Our results corroborate the findings of Praveen et al. (23), Siddiqui et al. (24), and Cao et al. (25).

The data in Table 2 exhibit that DS significantly induced the synthesis of Pro and GB. The DS enhanced Pro and GB by 16.8% and 24.7%, respectively as compared with the control.Plants counter DS-induced osmotic stress by osmotic adjustment carried out by the synthesis of osmolytes such as Pro and GB which possess the capability to enhance osmotic pressure and maintain hydration level of plants under stressful conditions (26-28). Although, plants show enhanced levels of Pro and GB under DS but at the same time reduced LRWC was also observed in drought-stressed plants. It shows that the enhanced synthesis of Pro and GB was not sufficient to counter osmotic stress. However, the application of NO to the stressed plants again elevated Pro and GB content by 9.9% and 19.3%, respectively than drought-stressed plants which did not receive NO (Table 2).Application of NO elevated Pro and GB content to a level required to counter osmotic stress that increased the osmotic pressure in the cells which causes more water uptake

and thus, observed a prominent increase in LRWC (Table 1).Xue et al. (29), Zhang et al. (30),and Khan et al. (7, 22) also observed the accelerating effect of NO on Pro and GB under various abiotic stresses.

The effect of DS on growth attributes of the plants was assessed by measuring their FW and DW. The results showed that DS adversely affected these growth parameters. Plants under DS showed 72.9% and 72.7% decrease in FW and DW, respectively as compared with the control plants (Table 2). The reduction in the growth of the plants under DS is not surprising because under DS the defense system of the plants was not able to counter DS-induced oxidative and osmotic stress. Enhanced oxidative and osmotic stress resulted in the damaged cellular membrane as shown by increased leakage of electrolytes and poor hydration levels of the plants (Table 1). All these together contributed to suppressed growth of the plants. It has been observed that DS induces defoliation and cessation of new leaf development (31). It is well known that water deficit coupled with the loss of cell turgor adversely affects cell expansion and cell growth resulting in reduced plant height (32)and thus, reduced FW and DW (Table 2). However, the application of NO to drought-stressed plants improved the FW and DW by 106.8% and 82.4%, respectively than the drought-stressed plants (Table 2). Application of NO protected the plants by enhancing the activities of antioxidant enzymes (Figure 1 A and B) and accumulation of Pro and GB (Table 2) that resulted in reduced leakage of electrolytes and increased LRWC (Table 1) coupled with improved FW and DW of the plants. NO is well known to improve the growth of the plants by reducing oxidative damage under various abiotic stresses (24,33).

CONCLUSIONS

The results of the study demonstrate that exposure of plants to DS induces excessive generation of ROS that created oxidative stress which results in the leakage of electrolytes. Under DS plants also face osmotic stress which suppresses the water uptake capacity of the plants.DS-induced generation of oxidative and osmotic stress adversely affected the growth of the plants. However, the application of NO (0.2 mM SNP; a NO donor), significantly countered the oxidative and osmotic stress by enhancing the antioxidant enzymes activities (APX, GR, POX, and SOD) and accumulation of osmolytes (Pro and GB). In conclusion, the application of NO induces drought-stressed tolerance in cucumber plants that resulted in improved growth of the plants.

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Table 1.Effect of nitric oxide and drought stress on leaf relative water content, electrolyte leakage, and H_2O_2 and O_2^- content

Treatments	Parameters			
	LRWC content (%)	Electrolyte leakage (%)	H_2O_2 content (µmol g^{-1} leaf FW)	O_2^- content (µmol g^{-1} leaf FW)
Control	71.68 ± 1.37	16.47 ± 0.871	27.84 ± 0.782	4.19 ± 0.238
DS	44.13 ± 0.90	28.32 ± 0.912	38.39 ± 0.715	5.08 ± 0.466
NO	55.51 ± 1.95	17.51± 0.446	27.24 ± 0.706	4.71 ± 0.517
DS + NO	51.18 ± 0.803	21.09 ± 0.395	29.57 ± 0.462	4.52 ± 0.349
LSD at 5%	6.59	2.46	1.82	1.15

Average of three determinations is presented with LSD at 5%±SE

Table 2. Effect of nitric oxide and drought stress on proline and glycine betaine content, and fresh weight and dry weight

Treatments	Parameters			
	Pro (mg g^{-1} leaf FW)	GB (mg g^{-1} leaf DW)	FW (g)	DW (g)
Control	18.47 ± 0.561	21.24 ± 0.652	5.39 ± 0.145	1.08 ± 0.002
DS	21.58 ± 0.448	26.49 ± 0.491	1.46 ± 0.007	0.295 ± 0.006
NO	16.39 ± 0.372	24.24 ± 1.169	5.62 ± 0.068	1.33 ± 0025
DS + NO	23.72 ± 0.493	31.59 ± 0.848	3.02 ± 0.359	0.538 ± 0.014
LSD at 5%	1.74	2.31	0.641	0.027

Average of three determinations is presented with LSD at 5%±SE

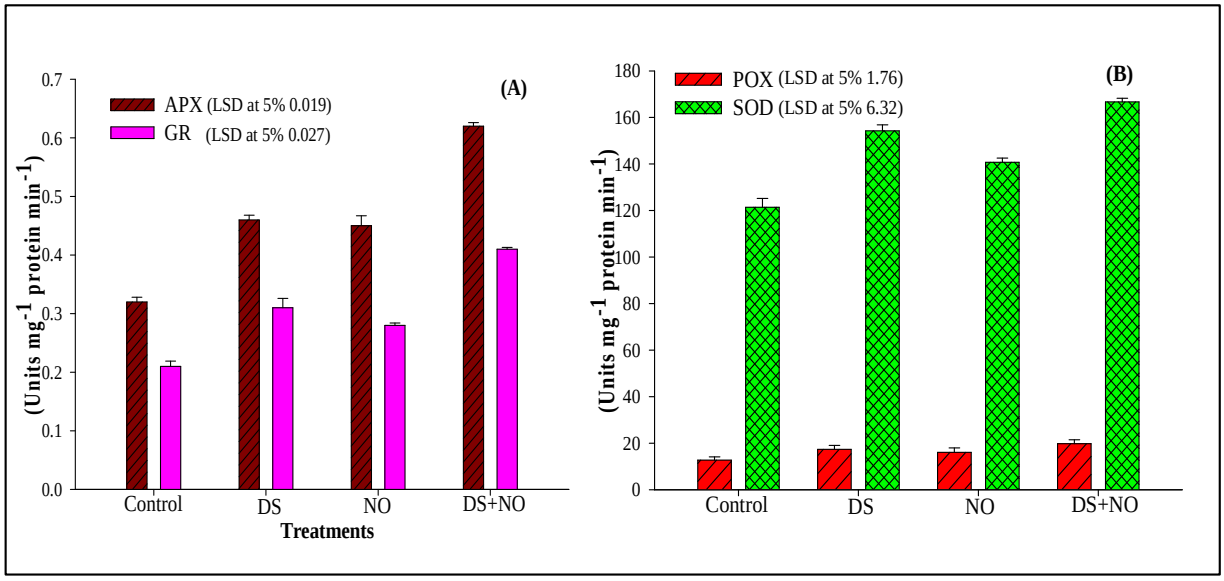

Figure 1.Effect of nitric oxide and drought stress on the activities of APX and GR (A), and POX and SOD (B). Average of three determinations is presented (LSD at 5%) with bars indicating SE

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RESEARCH ARTICLE

Retrospective Audit of Parotid Gland Tumor Cases Reported in Dr. Ruth K.M. Pfau Civil Hospital, Karachi

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ABSTRACT

To determine the pattern and histological type of parotid glandtumors presenting in a tertiary care hospital in Pakistan to determine the frequency of benign versus malignant disease. It was a retrospective study for which data was extracted from Department of Otorhinolaryngology, Dow University of Health Sciences, Dr. Ruth K.M.Pfau Civil Hospital, Karachi for the period between September 2010 to July 2019. The data of(n=82) patients with presenting complain of parotid gland tumors who came to the hospital for treatment was retrieved and analyzed. The results included those patients who underwent parotid gland surgery and excision of tumors,out of which 66(80.48%) had superficial parotidectomy and 16(19.51%) underwent total parotidectomy.Among those 40 (48.78%) and 42 (51.21%) were male and female respectively. More common were Benign tumors 67(81.7%) as compared to malignant tumors15(18.29%). Malignancy was more common in malesthan in females [12(14.6%) vs 3(3.65%)].

Keywords: Parotidectomy, Parotid tumors, Pleomorphic Adenoma, Mucoepidermoid carcinoma.

INTRODUCTION

Tumors of salivary gland belong to head and neck tumors, having a diverse histopathological presentation and accounting for 3% of all head and neck tumors. Of all the salivary glands, parotid gland is the most commonly affected gland, followed by sub mandibular gland, sublingual and minor salivary glands [1]. Among all the parotid tumors, benign tumors are the most common, with pleomorphic adenoma being the most common histopathological

subtype [2]. Malignancy is less common then benign, having mucoepidermoid carcinoma as the most common histopathological subtype [2, 3]. Surgical treatment for parotid gland pathologies dates to 16th century when it was performed only for calculi and ranula. With the passage of time, enucleation was done for tumor removal but due to high recurrence rate, concept of more extensive surgery was introduced and superficial and total parotidectomies were popularized. For benign parotid tumors, superficial parotidectomy was used and total was used for malignancies [3]. Patients having parotid gland pathology, present with a palpable mass at the site of gland. A detailed history and thorough examination, along with imaging and cytology via fine needle aspiration are performed. Fine needle aspiration cytology is routinely performed for parotid pathologies as it is helpful in finding out the nature of parotid pathology with 95% specificity and 75% sensitivity [4]. Ultrasound is of no major use in diagnosis of parotid pathologies [5]. To objective here is to determine the pattern and histological type of parotid gland tumors among patients who come for required necessary treatments in a tertiary care hospital in Pakistan. We also used the retrospective data to determine the frequency of malignant versus benign cases among the patients.

MATERIALS AND METHODS

A retrospective data analysis from September 2010 to July 2019 evaluated all those patients with presenting complain of parotid gland pathology, presented at the Department of Otorhinolaryngology, Dow University of Health Sciences, Dr.RuthK.M.Pfau Civil Hospital, Karachi. Total n=82 patients were included in this studyafter collecting their detailed history and examination reports, including imaged and cytology that were performed along with their surgeriesand post-operativehistopathological finding. Data recording of histopathological outcome, age,gender, and type of parotidectomy performed was also recorded.Data analysis was done using statistical package of Social sciences (SPSS V.21). Frequency and Percentages were computed for categorical variables and mean± standard deviation was presented for continuous data.

RESULTS

The mean age of the patients included was calculated to be 39.2 ±9.12. Parotid gland pathology was found to be the more common in female 42(51.21%) as compared to in males 40(48.78%). Histopathological analysis showed that benign tumors were more common 67(81.7%) than malignant tumors 15(18.29%). Moreover, Malignancy was more common in males than in females [12(14.6%) vs 3(3.65%)]. Mostly patients were presented to operation for Superficial parotidectomy 66(80.5%) than total parotidectomy 16(19.5%). The presentation of various parotid pathologies according to histopathology analysis is tabulated in (Table 1). Among 74 (90.2%) patients, there was no complication observed, however complication such as nerve was involved by tumor complete paralysis, all branches Neuroprexia, Marginal Mendibular Nerve Parasis were reported by 2(2.4%) patients respectively and Freys Syndrome and Temporal Branch Parasis, Buccal Branch Paresis by only 1(1.2%) patient in each type. Among all 67 benign cases, 59 (88%) opted for Superficial Parotidectomy and only 8(12%) went for total Parotidectomy, whereas 15 Malignant cases, 7(46.7%) underwent Superficial Parotidectomy and 8 (53%) total Parotidectomy.

DISCUSSION

Parotid gland tumors, which form a major portion of salivary gland tumor, have different histopathological subtypes belonging to benign and malignant subcategories. These parotid gland tumors, which present as a pre-auricular mass, are more common in males then females and malignancy is also more common in males [6, 7]. The salivary gland malignant tumors which are in the parotid gland are more prevalent as compared to other types located in submandibular and minor salivary glands. This is in accordance with the data reported from African countries, however variance in the distribution may be noted from the data reported from the western world [8]. There is no single characteristic feature which may point towards a specific diagnosis [1]. The most commonly presenting histological subtypes are pleomorphic adenoma, which is a slow growing, benign tumor of epithelial origin which

commonly occurs in superficial lobe [4]. The reported incidence of pleomorphic adenoma ranges between (40.8% to 70%) which accounts it to be the most frequent type and confirms with this study findings [10]. It is followed by mucoepidermoid carcinoma, and Adenoid cystic carcinoma which are types of malignant tumor [2-4, 9]. Tumors of parotid and submandibular nature are benign, and this agrees to the study findings in which histopathological analysis showed that 81.7% were benign tumors, and this is also reported by the published literature from other international countries. Regarding the sites of the malignant tumors they are found to be specific in minor salivary glands, and often the frequency of malignant tumors is low [11]. Benign tumors constituted of 75.3% of Pleomorphic Adenoma, this is also supported by the published literature, moreover its location in the submandibular gland also agrees with the results from other countries.

In this study the age of patients ranged from 23 to 65 years, with mean age of 39 years. We also found that pathology is more prevalent in females as compared to in males in the ratio (51% vs 49%), this is also like previous studies, but also differs considerably with the results which shows high prevalence among males [12]. As for the operative procedures which were performed following the clinical guidelines as per the extent of the disorder. Superficial parotidectomy was performed for most of the benign cases (88%), followed by 46.7% of malignant cases were handled with superficial parotidectomy. This was the only treatment of choice available and is also considered beneficial in protecting the facial nerve. Usually total parotidectomy is performed in benign tumors when deep lobe of the parotid gland is also affected. Moreover, if there is rupture of the capsule which is foremost to avoid, it may lead to recurrences [13].

CONCLUSION

Parotid gland tumor is a complex pathology to handle and primarily depend on the pathology, site of tumor as well as possible complications. Furthermore, there is a need to consider the age of patients, as well as predominance among females for which possible reasons of malignancy should be considered. The results of this study are very much like other researches in which data is reported from different parts of the globe. Therefore, we realize the need of standardization and improved reporting at the hospital levels considering the prevailing risk factors so that information from the patient can be used for better prognosis and implementation of best available surgical practices.

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CONFLICTS OF INTEREST

None.

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Table 1: Parotid pathologies as per histopathology analysis

Presentation of Tumor	n (%)
Benign Pleomorphic Adenoma	65 (75.3)
Malignant Mucoepidermoid Carcinoma	10 (12.2)
Adenoid cystic carcinoma	3 (3.6)
Tumor Warthin	1 (1.2)
Cystadenoma	1(1.2)
Ex-Pleomrphic Carcinoma	1(1.2)
Lymphoma	1(1.2)

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RESEARCH ARTICLE

Phenotyping of Lentil Genotypes for Drought Tolerance Using Polyethylene Glycol

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ABSTRACT

Lentil is generally grown as a rainfed crop in Bangladesh and often subjected to drought which is a major problem to plants grown in warm, arid and semiarid climates, because of low and erratic rainfall and a high rate of evapotranspiration. So it is an important strategy to overcome the drought associated problem by selecting and developing drought tolerant varieties through effective screening method. Seven lentil genotypes (Binamasur-6, BARI Masur-6, BARI Masur-7, LM-206-1, LM-206-2, LM-512-1 and LG-208) were subjected to four levels (0%, 10%, 15% and 20%) of polyethylene glycol 6000 (PEG-6000) to identify the drought tolerant genotypes. Seeds were exposed in drought to investigate the effect of drought on germination. Two week old seedlings were exposed to drought upto, maturity of the crop in hydroponic system. Germination percentage, plant height and nodes plant^{-1} after seven days of drought induction and after maturity shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, pods plant^{-1}, pod yield and seed yield plant^{-1} were recorded. The results revealed that all the aforementioned parameters were reduced with increasing drought stress in all genotypes. But at the highest level of osmotic stress (20% PEG-6000), the genotype LG-208 gave better results in all investigated traits than other genotypes. Therefore, it is clear that the line LG-208 is more drought

tolerant than other genotypes. These findings can also appreciate the future use of this genotype for obtaining higher yield in drought prone areas or can be used as breeding material.

Keywords: Lentil; Polyethylene glycol-6000; Drought stress; Germination; Plant growth; Yield.

INTRODUCTION

Abiotic stress is a major global hassle limiting crop productivity and worldwide agricultural productivity is subject to increasing environmental constraints in the form of abiotic stresses that negatively influence plants growth and development causing crop failure and reducing average yields greater than 50% (1). Drought stress is one of the most important and damaging abiotic stresses which lessen growth, development and production of plants (2, 3). Unpropitious soil moisture at sowing often negatively affects seed germination and results uneven seedling emergence, which eventually affects the establishment of a stand, with poor consequences at the yield (4, 5). Drought tolerance is a vital trait to increase production and profitability of a crop.The use of drought tolerant genotypes is one of the important means for good cultivation in drought condition.

Lentil is one of the most important food legume in many countries including Bangladesh. In Bangladesh, lentil covers an area of about 1,54,681 hectares and production is 1,68,837 metric tons (6). Lentil seeds are a rich source of protein, macronutrients like potassium, phosphorus, micronutrients like iron and zinc (7). It's straw is used as a valued animal feed in developing countries (8). Its capability in nitrogen and carbon sequestration improves soil nutrient status which in turn provides sustainability in production systems. Lentil is traditionally grown under rainfed environments and often encounters drought stress from limited and erratic rainfall in growing season (November-March). The north-western part of the country has started facing acute scarcity of water and increase in drought-proneness. According to Rahman et al. (9), due to the effect of drought the average crop production reduced 25-30% in the north-western part of Bangladesh. The temperature regime has begun changing the lengthy styles it was following and the rivers, river basins are drying up much quicker than before as well as water level depletion has started to increase. Due to excessive and indiscriminate use of surface and ground water in boro rice production our crop production is being threatened to the maximum level. In this context, government is discouraging boro rice production in High Barind Tract area and preferring crop with less water requirement. Lentil needs less water and cultivated in rainfed condition and drought tolerant lentil genotype can produce profitable and successful crop production in this area. However, lack of enough soil moisture in seedbed is a major barrier to establishment of this crop, because inadequate soil moisture can reduce emergence, slowdown seedling growth and reduce yield in rainfed crops (10).

Selection of plants with a superior drought tolerance in dry environment is very challenging (11). It is difficult to generate controlled and uniformly repeated simulation of drought in the field condition (12). Many researchers used Polyethylene glycol (PEG-6000) as abiotic stress inducer in numerous researches to screen germplasm which can tolerate drought stress (13). Kulkarni and Deshpande (14) showed that polyethylene glycol molecules are inert, non-ionic, and virtually impermeable to cellular membrane and can induce uniform water stress without causing direct physiological injury. Exposure to polyethylene glycol solutions has been efficaciously used to mimic drought stress with limited metabolic interferences as those associated to the utilization of low molecular weight osmolytes that can be taken up by the plant (15). PEG based screening for drought tolerance has been proven to be a felicitous technique to screen of germplasm successfully with good precision (14). Lentil genotype which can tolerate water stress can be achieved through means of exploring maximum genetic potential from available germplasm of lentil. In Bangladesh, lentil is least researched crop and there are also a few works available in lentil regarding drought screening with PEG globally. The objectives of this study was to evaluate the influence of drought stress on seven genotypes of lentil during germination and seedling to harvest under PEG induced drought stress and to select suitable lentil genotype(s) for drought tolerance.

MATERIALSAND METHODS

Experimental site and duration

The experiment was carried out at the laboratory and glasshouse of International Atomic Energy Agency, Department of Nuclear Sciences and Applications, Plant Breeding and Genetic Laboratory, Seibersdorf, Vienna, Austria during the period of July to October, 2014.

Experimental materials

A total of seven genotypes were used as genetic materials. Of them, three were most popular varieties in Bangladesh as Binamasur-6, BARI Masur-6, BARI Masur-7, three mutants, LM-206-1, LM-206-2 and LM-512-1 and one exotic line, LG-208 from International Centre for Agricultural Research in the Dry Areas (ICARDA).

Determination of germination percentage

Seeds were disinfected with 20% chlorox for three minutes and 3 times washed with sterilized distilled water. For each of seven genotypes, four replicates of 50-seeds were placed on two layers of filter paper in 9 cm Petri dishes with respective PEG treatments. Seeds of seven genotypes were incubated at 25±1ºC at different stress level of PEG-6000 i.e. 0% (control), 10%, 15%, and 20% with a relative humidity of 70%. The Petri dishes were covered to avoid the loss of moisture by evaporation under laboratory condition (25±2ºC) for 3 days. A seed was considered to be germinated when seed coat ruptured and plumule and radicle came out upto 2 mm in length. Germination percentage was calculated using the following formula (57).

$$\text{Germination } (\%) = \frac{\text{Number of seeds germinated}}{\text{Number of seeds tested}} \times 100$$

Glasshouse experiments

A total of 200 uniform full and good seeds of each of the materials were placed in nine centimeter Petri dishes with two layers of Whatman papers on 19 July 2014. After two days when small radicles came out, seeds were placed in a sandwich blotter for proper root elongation so that the seedlings can be placed in hydroponic system easily.

Hydroponic technique

The five days old seedlings (two days in Petri dishes and three days in sandwich blotter) were transplanted in hydroponic medium (KNO_3) (0.5mM), $Ca(NO_3)_2.4H_2O$ (0.5mM), $MgSO_4.7H_2O$ (0.2 mM), KH_2PO_4 (0.1mM), KCl (50 µM), H_3BO_3 (46 µM), Fe-EDTA (20 µM), $MnCl_2.4H_2O$ (2 µM), $ZnSO_4.7H_2O$ (1 µM), $CuSO_4.5H_2O$ (0.3 µM) and $NaMoO_4.2H_2O$ (0.5 µM) (58). pH of the nutrition solution was adjusted to 6.5 with 1M HCl or 1M KOH. The solution was aerated by bubbling air with an aquarium air pump and was replaced every five days. The experiment was conducted in a Completely Randomized Design (CRD) with three replications and each replication consisted of a sample of 10 seedlings. Drought stress was imposed in two week old-seedlings using poly ethylene glycol 10%, 15% and 20%. The untreated control plants were allowed to grow without drought induction. After 7 days plant height and nodes plant^{-1} were recorded. The experiment was sown on 19 July 2014 and matured different period depending on concentration of PEG, i.e., the highest concentration with drought induction (20%) was matured first on 02 October 2014 15% PEG induced drought was matured on 08 October 2014, 10% PEG induced drought as matured on 12 October, 2014 and control was 29 October 2014. After harvestshoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, pods plant^{-1}, pod yield and seed yield plant^{-1} were recorded. Shoot, root and pods were oven dried for 120 hours in 40^0 C.

Statistical analysis

The collected data on different parameters were statistically analyzed to obtain the level of significance using MSTAT-C package program. The mean differences among the treatments were adjudged following Duncan's Multiple Range Test (DMRT) (59).

RESULTS

There was a significant difference for all the traits and effect of genotypes and drought induction concentration was significant for all of the traits. Three concentrations of polyethylene glycol i.e., 10%, 15% and 20% for drought induction were markedly produced drought in hydroponic system for seven genotypes. As expected, traits as plant height after seven days of drought induction, number of nodes after seven days of drought induction, shoot length at harvest, root length at harvest, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, number of pod plant^{-1}, pod weight plant^{-1}, mean seed yield markedly reduced with an increase in PEG stress.

Germination percentage

The germination percentage in response to different treatments of PEG-6000 and control is presented in Table 2. All genotypes exhibited 100% germination in control. But it declined progressively with increasing stressed condition from 10% to 20% PEG than the control. In 10% , 15% and 20% PEG induced drought conditions, the highest values of this trait was found ingenotype LG-208 which were 95% , 65% and 56%, respectively.

Plant height after seven days of drought induction

The plant height was gradually reduced with increase of PEG concentration from 10% to 20% for drought induction (Table 3). Plant height was ranged from 17.79 cm to 12.66 cm for no treated genotypes which was 16.06 cm to 11.8 cm in 10%, 15.21 cm to 11.47 cm in 15%, 13.05 cm to 9.52 cm in 20% PEG treated seven genotypes. There was a significant variation in genotypes for plant height from 0%, 10%, 15% and 20% PEG induced drought stress. The genotype LG-208 gave the highest (15.21 cm, 13.05 cm) in 15% and 20% of PEG induced drought stress but in control, it gave the second highest height (16.43 cm).

Nodes plant^{-1} after seven days of drought induction

The number of nodes plant^{-1} after seven days of drought induction was reduced with increasing the PEG concentration (Table 4). It was 9.793 to 8.17 in control, 9.32 to 7.42 in 10%, 8.95 to 6.73 in 15% and 8.63 to 6.61 in 20% PEG induced drought. In control, the genotype Binamasur-6 produced the highest number of nodes plant^{-1} (9.793) and in 10% PEG induced drought stress the genotype BARI Masur-6 exhibited the best performance (9.32) for this trait. But the genotype LG-208 produced the highest number of nodes plant^{-1} in, 15% (8.95), and 20% (8.63) PEG induced stress.

Shoot length at harvest

An increase in PEG concentration i.e., 10%, 15% and 20% for drought induction were markedly produced drought in hydroponic system for seven genotypes and decreased the shoot length of all genotypes at harvest compared to their relative controls (Table 5). At harvest the genotype Binamasur-6 gave the highest shoot fresh weight in control (34.00 cm). In 10% PEG induced drought stress, the genotype BARI Masur-7 gave the highest shoot length at harvest (24.68 cm). But in 15% and 20% PEG Induced drought stress, the genotype LG-208 gave the highest shoot length at harvest than that of the other six genotypes and the values were 23.17 and 15.77 cm, respectively.

Root length at harvest

Root lengths of all genotypes at harvest were decreased gradually with the increase of PEG concentration for drought induction (Table 6). It was 37.32 cm to 32.41 cm in control, 26.01 cm to 22.84 cm in 10%, 22.97 cm to 21.82 cm in 15% and 22.12 cm to 18.75 cm in 20% PEG induced drought stress. At harvest, the genotype BARI Masur-7 gave the highest root length (37.32 cm) in control and Binamasur-6 gave the highest root length (26.01 cm) in 10% PEG induced stress where the genotypes BARI Masur-6 and LG-208 gave the highest root length at harvest in 15% and 20% PEG induced drought stress which values were 22.97 and 22.12 cm, respectively.

Shoot fresh weight

Shoot fresh weight was gradually reduced with increase of the PEG concentration from 0% to 20% for drought induction. Significant variation was found in genotypes for shoot fresh weight from 0%, 10%, 15% and 20% PEG induced drought stress (Table 7). The genotype Binamasur-6 gave the highest shoot fresh weight in control (1.05 g), But in the cases of 10%, 15% and 20% PEG induced drought stress, the genotype LG-208 gave the highest shoot fresh weight and the values were 0.44, 0.404 and 0.103g, respectively.

Shoot dry weight

Shoot dry weights of different genotypes were changed by drought treatments (Table 8). The genotype Binamasur-6 gave the highest shoot dry weight in control (0.165 g) and 10% PEG induced stress (0.096 g). On the other hand, the genotype LG-208 gave the highest shoot dry weights in the cases of 15% and 20% PEG induced drought stress and the values were 0.0793 and 0.076 g, respectively.

Root fresh weight

Elevated PEG concentrations decreased root fresh weights of all seven genotypes (Table 9). In control and 10% PEG induced stress, the highest value of root fresh weight was found for the genotype Binamasur-6 and the values were 0.566 and 0.23 g, respectively. For this parameter, the genotype LG-208 gave the best performances in 15% and 20% PEG induced drought stress and the values were 0.1946 and 0.118g, respectively.

Root dry weight

Root dry weights of different genotypes were changed by different drought treatments (Table 10). An increase in PEG stress, the root dry weight of all genotypes at harvest were gradually reduced in comparison to their relative controls.Both the genotypesBARI Masur-7 and Binamasur-6 gave the highest root dry weightin control (0.029 g) and 10% PEGinduced stress (0.028 g). Alternatively, the genotype LG-208 gave the highest root dry weight within the instances of 15% and 20% PEG, induced drought stress and the values were 0.025, and 0.023 g, respectively.

Number of pod plant^{-1}

An increase in PEG stress markedly decreased the number of pod plant^{-1} of all genotypes at harvest compared to their relative controls (Table 11), Both in control and 10% PEG induced stress, the highest number of pod plant^{-1} was found for the genotype Binamasur-6 and the values were 6.88 and 2.53, respectively. But in 15% and 20%PEG induced drought stress conditions the genotype LG-208 produced higher number of pod plant^{-1} than that of other six genotypes and the values were 1.7 and 0.93, respectively.

Pod weight plant^{-1}

The pod weight plant^{-1} was reduced with the increase of PEG concentration from 0% to 20% for drought induction (Table 11). The genotype BARI Masur-7 gave the highest pod weight plant^{-1}, in control (1.5 g) and 10% PEG induced drought stress (0.85 g). In case of 15% PEG induced drought stress both the genotypes LG-208 and BARI Masur-6 gave the highest pod weight plant^{-1}, (0.57 g). The genotype LG-208 also gave the highest pod weight plant^{-1} , (0.17 g) in 20% PEG induced drought stress.

Mean seed yield

The mean seed yield performance to PEG-6000 induced drought stress of lentil is given in Table 13. This character of all seven genotypes of lentil was affected by drought stress and progressively decreased with increase of the PEG concentration from 0% to 20% for drought induction. Significant variation was found among the genotypes for mean seed yield from 0%, 10%, 15% and 20% PEG induced drought stress. It was 1.20 g to 0.41gin control, 0.6 g to 0.2933g in 10%, 0.36 g to 0.1 g in 15% and 0.19 g to 0.00g in 20% PEG induced drought. The genotype BARI Masur-7 gave the highest mean seed yield in control (1.2 g) and 10% PEG induced stress (0.6 g). On the other and, in 15% and 20% PEG induced drought stress, the genotype LG-208 gave the highest mean seed yield and the values were 0.36 and 0.19 g, respectively.

DISCUSSION

Scarcity of water due to drought is one of the most serious abiotic factors that limit seed germination, plants growth and yield (16). Water deficit affects several morphological features and physiological processes which are associated with growth and development of plants (17). Among Several methods to screen drought tolerant germplasm in plant species, PEG is considered as a superior chemical to induce dehydration condition (18). Molecules of PEG 6000 are small enough to influence the osmotic potential, however large enough to not be absorbed by plants (19). Because PEG does not enter the apoplast, water is withdrawn from the cell. Therefore, PEG solution mimic dry soil more closely than solutions of compounds with low molecular stress which infiltrate the cell wall with solutes (20).

The germplasm which shows better performance under water deficit condition can be considered as drought tolerant. Several reports showed that better growth under stress conditions as a trait to choose germplasm to enhance the yield (21). In this study, different traits examined such as germination percentage, plant height, root length, number of nodes plant^{-1}, shoot fresh and dry weight, root fresh and dry weight, number of pod plant^{-1}, pod weight plant^{-1} and mean seed yield gradually decreased with the increase of PEG concentration from 0% to 20% for drought induction. Seed germination is first crucial and the most sensitive step in the life cycle of plants (22) andit can be considered as a useful trait for the selection of water deficit tolerant germplasm (23). In the current study, water stress reduced germination percentage and this trait of all varieties reduced gradually with the enhancement of PEG concentration. The reduction in germination might be due to the less availability of free water to the seeds (24). The results were in agreement with the reports of Jamaati-e-Somarin and Zabihi-e-Mahmoodabad (25) and Muscolo et al. (26).The repressing impact of PEG on seed germination of various plants was recorded by several researchers, Ahmad et al. (27) on sunflower; Heikal and Shaddad (28) on cotton, pea and wheat;Partheeban et al.(29) on maize and Rana et al.(30) on wheat. According to Ayaz et al. (31), decrease in seed germination under stress conditions is due to some metabolic disorders. Water deficit stress may also cause degradation and inactivation of the fundamental hydrolytic and other group of enzymes required for germination (32).

Sarker et al. (33) opined that stem length and root length are key traits for drought tolerance in lentil. During stress situation stem acts as main reservoir of stored starch for the survival of plant. In this experiment, all the varieties showed common trend i.e. reduction in shoot length with the increment of PEG concentration. It is a commonly

observed phenomenon which depends on the tolerance capability of the plant. Decreasing in shoot length with increasing water stress was reported in lentil (34), wheat (35, 36), tomato (14) and soybean (37). Root length is one of the most important characters for water deficit stress because roots remain in contact with soil and absorb water from soil. For this reason, root length gives an important clue to the response of plants to drought stress. Roots are the primarily affected plant part under water stress conditions than any other plant parts (38). Generally, Root length decrease with the increase of drought level. Root system with better growth ability under stress conditions can be considered as tolerant germplasm (39). In this investigation a gradual reduction in root length with an increasing concentration of PEG was the common trend observed among all varieties. With the increase of PEG concentrations significant reduction in root length of different plants was reported byHamayun et al. (37) and Jajarmi et al. (40) and similar results like reduction in root length with increasing osmotic stress was identified in lentil (34), pea (41) and wheat (42, 43).

As pods are born on nodes, therefore, number of fruiting nodes plant^{-1} is an important trait which influences yield. In current investigation, a detrimental effect of water stress was found in number of nodes plant^{-1} at seven days after drought induction in all varieties. Number of nodes plant^{-1} of all genotypes were gradually decreased with the increment of PEG concentration. The negative effect of water stress on number of nodes plant^{-1} was also reported in lentil by Mishra (44). Higher plant fresh and dry weights under drought conditions are desirable characters. Water stress condition has a profound effect on shoot and root fresh and dry weight. In the present study a reduction in these traits were recorded in stressed conditions in all the genotypes. Decreasing in fresh and dry weights of roots and shoots due to water stress were reported in lentil (34, 45). Decrease in shoot dry weight due to drought stress were observed in marigold (*Tagetes erecta* L.) by Asrar and Elhindi (46), in faba bean (*Vicia faba* L.) by Xia (47). Liu et al.(48) reported that drought stress decreases shoot and root dryweight in Asian purple sage (*Salvia miltiorrhiza Bunge*).David and Park (49) observed decrease in dry weight of Phaseolus vulgaris under drought conditions which is similar to the results of this current investigation. Reduced biomass (fresh and dry weight) was also seen in water stressed soybean (37), common bean and green gram (50).

In this study both number of pod plant^{-1} and pod weight plant^{-1} were decreased with the enhancement of drought stress. Reduction in number of pod plant^{-1} due to water stress in lentil was also found by Mishra et al. (51). Under stress, the decrease on the number of pods, followed by fewer seeds pod^{-1} and smaller seed mass were reported in soybean by Dornbos et al. (52). Grain yield is a complex and quantitative character. It is well known that drought stress has negative effect on seed yield. In current investigation water stress also reduced the yield of all varieties and the reduction of yield was gradually increased with the elevation of PEG concentration. Mistra and Srivastava (53) found that that drought stress significantly decreased grain and biological yield in mint (*Mentha spicata* L.). Decreasing in yield due to water stress was reported in soybean (54, 55). Different lentil genotypes showed differential degree in reduction in germination percentage, plant height, root length, number of nodes plant^{-1}, shoot fresh and dry weight, root fresh and dry weight, number of pod plant^{-1}, pod weight plant^{-1} and mean seed yield in the present study. According to Kumar et al. (56), there can be important differences in the cultivars of the same plant species and in their tolerance to drought. In present investigation although for most of the traits the variety LG-208 didn't show best results in 0% and 10% PEG induced stress but with the increase of PEG concentration beyond 10%, it gave better results than other lentil varieties. This may be due to differential genetic sensitivity of the variety LG-208 to water deficit stress. Therefore, it can be inferred that the genotype LG-208 is more water deficit stress tolerant compared to other varieties.

CONCLUSIONS

Water stress because of drought is probably the most significant abiotic factor which adversely affects plant and also crop growth and development. Fertile lands are continuously rendered arid due to this stress. Development of new varieties is one of the ultimate techniques to overcome the problem related with the drought stress. New varieties can

be developed for higher drought tolerance by screening of germplasm. The present study was planned to identify the better lentil genotypes regarding drought stress that can be useful to scientific community and we found that PEG induced drought stress adversely affected germination percentage, growth and yield attributes of all genotypes at different levels. But in 15% and 20% PEG induced drought stress conditions, the genotype LG-208 showed better performance for most of the traits than the other genotypes. In conclusion, keeping in view the above stated research findings it can be concluded that the lentil genotype LG-208 is more drought tolerant at higher level of stress and can be used as high yielding lentil line/variety for successful lentil production in the drought prone areas of Bangladesh, especially in High Barind Tract of Chapainawabganj district. This line has also economic importance to other lentil growing areas.

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Table 1. List of genotypes used for the study with characteristics

Sl. No.	Genotypes	Parentage	Source of collection	Characteristics
1.	BARI Masur-7	ILL 7567 × Idlib 1	ICARDA	High yielding short duration lentil variety of Bangladesh developed by Bangladesh Agriculture Research Institute (BARI).
2.	LM-512-1	ILL 2601 × 1227516	Bangladesh	Mutant of a popular variety BARI Masur-5 developed by BARI.
3.	LG-208	ILL 7656	ICARDA	An introduced drought tolerant variety which was provided by ICARDA registered in 2016.
4.	LM-206-2	L-5 × 37047	Bangladesh	Mutant of a local variety of Bangladesh.
5.	LM-206-1	L-5 × 37047	Bangladesh	Mutant of a local variety of Bangladesh.
6.	BARI Masur-6	ILL 7677 × Idlib 1	ICARDA	Most popular and high yielding lentil variety of Bangladesh.
7.	Binamasur-6	ILL 5888 × ILL 5782	Nepal	High yielding variety developed by Bangladesh Institute of Nuclear Agriculture (BINA).

Table 2. Effect of various concentrations of Polyethylene Glycol on germination percentage of drought induction in seven genotypes of lentil.

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	100.00a	80.00bc	55.00b	47.00bc
LM-512-1	100.00a	80.00bc	49.00c	44.00c
LG-208	100.00a	95.00a	65.00a	56.00a
LM-206-2	100.00a	75.00c	51.00bc	37.00d
LM-206-1	100.00a	80.00bc	53.00bc	34.00d
BARI Masur-6	100.00a	80.00bc	61.00a	48.00bc
Binamasur-6	100.00a	81.00b	55.00b	51.00b
$LSD_{0.05}$	2.80	4.99	4.23	3.90
Level of significance	*	**	**	**
CV (%)	1.92	4.16	5.18	5.86

** = Significant at 1% level of probability, * = Significant at 5% level of probability

Table 3. Effect of various concentrations of Polyethylene Glycol on plant height (cm) after 7 days of drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	16.30b	15.23a	13.96ab	11.34bc
LM-512-1	12.66d	11.80c	11.47d	10.96c
LG-208	16.43b	15.42a	15.21a	13.05a
LM-206-2	13.75c	12.65bc	12.57cd	9.520d
LM-206-1	13.67cd	13.21b	12.71bcd	9.540d
BARI Masur-6	15.64b	15.31a	15.09a	11.87bc
Binamasur-6	17.79a	16.06a	13.62bc	12.01b
$LSD_{0.05}$	1.018	1.06	1.20	0.877
Level of significance	**	**	**	**
CV (%)	3.83	4.25	5.06	4.48

** = Significant at 1% level of probability

Table 4. Effect of various concentrations of Polyethylene Glycol on number of nodes/plant after 7 days of drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	9.480a	9.180a	8.810a	7.910ab
LM-512-1	8.180b	7.420c	6.730c	6.610c
LG-208	9.410a	9.260a	8.950a	8.630a
LM-206-2	8.170b	7.860b	7.670bc	6.500c
LM-206-1	8.180b	7.700bc	7.530c	7.070bc
BARI Masur-6	9.370a	9.320a	8.830a	8.390a
Binamasur-6	9.793a	9.230a	8.627ab	8.190a
$LSD_{0.05}$	0.492	0.367	0.986	0.910
Level of significance	**	**	**	**
CV (%)	3.14	2.46	6.90	6.82

** = Significant at 1% level of probability

Table 5. Effect of various concentrations of Polyethylene Glycol on shoot length (cm) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	33.10a	24.68a	17.92c	14.24a
LM-512-1	22.70b	17.66b	14.02d	10.02b
LG-208	32.00a	24.41a	23.17a	15.77a
LM-206-2	25.10b	17.46b	15.15d	9.660b
LM-206-1	24.50b	18.29b	15.28d	10.00b
BARI Masur-6	32.20a	24.04a	20.35b	14.78a
Binamasur-6	34.00a	24.34a	17.60c	14.71a
$LSD_{0.05}$	2.34	1.74	2.15	1.59
Level of significance	**	**	**	**
CV (%)	4.61	4.62	6.98	7.11

** = Significant at 1% level of probability

Table 6. Effect of various concentrations of Polyethylene Glycol on root length (cm) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	37.32a	25.08a	22.58a	20.94ab
LM-512-1	34.75abc	23.57b	22.84a	20.20bc
LG-208	33.31bc	22.84b	22.33ab	22.12a
LM-206-2	33.12bc	23.74b	21.82b	18.75c
LM-206-1	34.30bc	25.72a	21.75b	21.29ab
BARI Masur-6	32.41c	25.25a	22.97a	20.67 ab
Binamasur-6	35.97ab	26.01a	22.38ab	20.49abc
$LSD_{0.05}$	2.72	1.34	0.624	1.69
Level of significance	*	**	**	**
CV (%)	4.50	3.12	1.59	4.69

** = Significant at 1% level of probability, * = Significant at 5% level of probability

Table 7. Effect of various concentrations of Polyethylene Glycol on shoot fresh weight (g) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	1.020a	0.2700c	0.086bc	0.07b
LM-512-1	0.5800b	0.2000d	0.059e	0.054c
LG-208	1.020a	0.4400a	0.404a	0.103a
LM-206-2	0.5800b	0.2100d	0.062e	0.05cd
LM-206-1	0.6600b	0.2400cd	0.075d	0.046cd
BARI Masur-6	1.040a	0.3467b	0.092b	0.076b
Binamasur-6	1.050a	0.4200a	0.08cd	0.04b
$LSD_{0.05}$	0.123	0.055	0.0078	0.0095
Level of significance	**	**	**	**
CV (%)	8.16	9.17	4.04	8.52

** = Significant at 1% level of probability

Table 8. Effect of various concentrations of Polyethylene Glycol on shoot dry weight (g) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	0.148a	0.091a	0.0556bc	0.0546bcd
LM-512-1	0.087b	0.052c	0.0473cd	0.0353d
LG-208	0.151a	0.089a	0.0793a	0.076a
LM-206-2	0.066c	0.055bc	0.043 d	0.040d
LM-206-1	0.08bc	0.063bc	0.0513cd	0.0433cd
BARI Masur-6	0.152a	0.084ab	0.0693ab	0.0666ab
Binamasur-6	0.165a	0.096a	0.0715ab	0.0605abc
$LSD_{0.05}$	0.019	0.023	0.015	0.018
Level of significance	**	**	**	**
CV (%)	9.14	16.99	13.55	19.30

** = Significant at 1% level of probability

Table 9. Effect of various concentrations of Polyethylene Glycol on root fresh weight (g) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	0.222cd	0.1336bc	0.1243b	0.0853b
LM-512-1	0.1356e	0.117c	0.0486c	0.0263d
LG-208	0.2696c	0.221a	0.1946a	0.118a
LM-206-2	0.333b	0.0946c	0.0733c	0.034d
LM-206-1	0.1616e	0.0936c	0.06c	0.0466c
BARI Masur-6	0.1923de	0.1616b	0.141b	0.0836b
Binamasur-6	0.566a	0.23a	0.115b	0.088b
$LSD_{0.05}$	0.055	0.039	0.029	0.0095
Level of significance	**	**	**	**
CV (%)	11.22	14.76	15.40	8.17

** = Significant at 1% level of probability

Table 10. Effect of various concentrations of Polyethylene Glycol on root dry weight (g) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	0.029a	0.028a	0.02ab	0.017abc
LM-512-1	0.013b	0.013bc	0.012d	0.011cd
LG-208	0.028a	0.027a	0.025a	0.023a
LM-206-2	0.015b	0.012c	0.011d	0.008d
LM-206-1	0.014b	0.014bc	0.013cd	0.012bcd
BARI Masur-6	0.023ab	0.023ab	0.02ab	0.02ab
Binamasur-6	0.029a	0.028a	0.019bc	0.019ab
$LSD_{0.05}$	0.0095	0.0096	0.0055	0.0078
Level of significance	**	**	**	**
CV (%)	24.81	27.47	19.78	24.65

** = Significant at 1% level of probability

Table 11. Effect of various concentrations of Polyethylene Glycol on number of pod plant^{-1} at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	6.810a	1.990bc	1.440bc	0.21b
LM-512-1	4.770b	1.650d	1.230e	0.20b
LG-208	6.700a	1.800cd	1.700a	0.93a
LM-206-2	3.230c	1.620d	1.410cd	0.13c
LM-206-1	3.730c	2.080b	1.570ab	0.13c
BARI Masur-6	6.80a	2.140b	1.600a	0.20b
Binamasur-6	6.880a	2.530a	1.280de	0.23b
$LSD_{0.05}$	0.785	0.235	0.136	0.055
Level of significance	**	**	**	**
CV (%)	8.07	6.88	5.19	9.35

** = Significant at 1% level of probability

Table 12. Effect of various concentrations of Polyethylene Glycol on pod weight plant^{-1} (g) at harvest after drought induction in seven genotypes of lentil.

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	1.500a	0.8500a	0.4200b	0.03b
LM-512-1	0.6800d	0.5200d	0.2100d	0.01c
LG-208	1.280c	0.7000b	0.5700a	0.17a
LM-206-2	0.6000d	0.4600d	0.3600c	0.01c
LM-206-1	0.7000d	0.6000c	0.2400d	0.01c
BARI Masur-6	1.450ab	0.7900a	0.5700a	0.04b
Binamasur-6	1.350bc	0.8200a	0.4300b	0.01c
$LSD_{0.05}$	0.123	0.078	0.055	0.017
Level of significance	**	**	**	**
CV (%)	6.25	6.34	6.81	23.15

** = Significant at 1% level of probability

Table 13. Effect of various concentrations of Polyethylene Glycol on mean seed yield (g) at harvest after drought induction in seven genotypes of lentil

Genotypes	Different levels of Polyethylene Glycol concentration			
	0%	10%	15%	20%
BARI Masur-7	1.200a	0.6000a	0.2700b	0.01c
LM-512-1	0.5500b	0.3300d	0.1000d	0.00c
LG-208	1.103a	0.4900b	0.3600a	0.19a
LM-206-2	0.4100c	0.2933d	0.2100c	0.01c
LM-206-1	0.5200b	0.3933c	0.1300d	0.00c
BARI Masur-6	1.140a	0.5600a	0.3300a	0.04b
Binamasur-6	1.130a	0.5900a	0.2600bc	0.00c
$LSD_{0.05}$	0.095	0.055	0.055	0.021
Level of significance	**	**	**	**
CV (%)	6.03	7.86	12.24	33.47

** = Significant at 1% level of probability

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RESEARCH ARTICLE

First Report of Class 1 and Class 2 Integrons in Quinolones Resistant *Klebsiella pneumoniae* Isolates from Najaf, Iraq

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ABSTRACT

Quinolone-resistant *Klebsiella pneumonia* is considered a seriousglobal threat. However, little is known regarding their multidrug resistance (MDR) and the rule of integron classes in this phenotype. The present study was conducted to investigated the antimicrobial susceptibility and prevalence of class 1and 2 integrons inquinolone resistance clinical isolates of *K. pneumoniae* from Najaf, Iraq patients. Methods of 109 *K. pneumonia* were isolated, 74 were shown resistant toquinolone antibiotic were chosen for antibiotic resistance test and presence of class I and II integron by PCR. Results of the 74 quinolone resistant *K. pneumoniae* isolates, 47(63.5%) isolates were MDR, while 23 (31%) were considered as XDR, and PDR isolates were identified in 4 (5.4%) isolates that resistant to all agents in all antimicrobial categories tested. Among the 74 quinolone resistance *K. pneumoniae* isolates the class 1 integron present in 41(55.4%0), while present in only 10 (28.5%) of quinolone susceptible*K. pneumoniae* isolates. Conclusion the present study demonstrated strong association between the presence of class 1 integrons and resistance to the antibiotics used, suggested that integron may be assisting forward the spread of quinolone-resistant in Najaf.

Keywords: *K. pneumoniae,* quinolone-resistant,*intI1, intI2*.

INTRODUCTION

Klebsiella pneumoniae is an important opportunistic pathogen associated with nosocomial infections and one of the leading causes of many diseases, and become public health concern especially when characterized as multidrug resistance. due to its responsibility of treatments failure (1). Transfer of antibiotic resistance genes between different

pecies of bacteria is associated with mobile DNA elements such as transposons and plasmids. Recently, a significant part of resistance genes occur in mobile genetic elements of Gram-negative bacilli, have been detected in DNA elements called integrons(2). Integrons encode the antibiotic resistance genes through site-specific recombination and are capable of capturing, integratingand mobilizing gene cassettes (3). Various species of Gram negative bacteria that conducted from hospital environments can carry integrons.(4). The definition of integron based on their respective integrase (*intI*) genes, which located in the 5´ conserved segment (5´CS)(3). This mobile genetic element also contains a 3´ conserved segment that carries the ethidium bromide and quaternary ammonium resistance gene (qacEΔ1) and sulfonamide resistance gene (sul1), which confer resistance to ethidium bromide and quaternary ammonium compounds and to sulfonamide, respectively transfer(4). The nucleotides sequence of integron gene revealed five class this mobile genetic element. Integron class 1 and 2 are frequently detected in clinical isolates of *Enterobacteriaceae*, including *K. pneumonia* (4).This study was conducted to investigate the antimicrobial susceptibility and prevalence of class 1and 2 integrons inquinolone resistance of *K. pneumonia* isolates from Najaf, Iraq patients.

MATERIALS AND METHODS

Bacterial Isolates

A total of 1590 clinical specimens,including urine, burn wound seminal fluid, wound abscesses and sputum were collectedfrom two teaching hospitals in Najaf (Al-Sader Medical City, Al-Hakeem General Hospital, and Al-Zahra Maternity and Children) from December 2012 till June 2013. Among of these 109, non-duplicated *K. pneumoniae* were collected. The isolates were collected from urine (n=59) followed byburn wound (n=46), seminal fluid (n=2), wound abscesses (n=1) and sputum (n=1).

Detection of Quinolones Resistant Phenotype

All *K. pneumoniae* isolates were classified as quinolones resistant according to susceptibility or resistant tonalidixic acid and ciprofloxacin antibiotics (Cypress, Belgium), and confirmed by MIC Strip (Liofilchem, Italy). According to the CLSI breakpoint criteria, the MIC standerd for ciprofloxacin resistance was ≥ 4 μg/mL, and > 32 μg/mL for nalidixic acid, according to the CLSI breakpoint criteria.

Antibiotic Susceptibility Phenotype

Antibiotics disks (Cypress, Belgium) were used to test the susceptibilityof quinolones resistant *K. pneumoniae* isolates,using the Kirby-Bauer method according to CLSI guidelines(5):Amoxicillin (25 μg), piperacillin (25 μg), amoxicillin-clavulanic acid (30 μg), ampicillin-sulbactam (20 μg), piperacillin-tazobactam (10 μg), ticarcillin-clavulanic acid (85 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefepime (30 μg), cefoxitin (30 μg), aztreonam (30 μg), imipenem (10 μg), meropenem (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), gatifloxacin (5 μg), levofloxacin (5 μg), lomefloxacin (10 μg), moxifloxacin (5 μg), norfloxacin (10 μg), ofloxacin (5 μg), amikacin (30 μg), tobramycin (10 μg), gentamicin (10 μg), kanamycin (30 μg), netilmicin (30 μg), chloramphenicol (30 μg), sulfamethoxazole (50 μg), trimethoprim (5 μg). The ATCC standard strain *E. coli* (ATCC 25922) was used as a positive control.

Detection of class 1 and 2 integrons by PCR

The *K. pneumoniae* DNA was extracted as described previously by Cheng and Jiang (6), after which the DNA samples were used as a source of templates for the polymerase chain reaction (PCR) amplification. The intI1 and intI2 genes were amplified by PCR using primers obtained from bioneer (Daejeon, South Korea) listed in table 1. The PCR amplifications were performed in a 20μl reaction mixture including 10μl KAPA Taq Ready Mix mixture (Kapa

Biosystems, Massachusetts, US), 25 pmol of each primer of the single gene, 5 µl of genomic DNA, and nuclease-free water to complete the volume. The PCR amplification was performed in Tprofessional thermal cycler (Biometra, Germany) in the following sequence: 5 min at 94ºC, followed by 35 and 30 cycles for integron class 1 and class 2 respectively of 30 s each at 94ºC (30 s min at 55ºC for integron class 1 and 1 min at 62ºC for integron class 2), 1 min at 72ºC, and a final extension step at 72ºC for 10 min. Using gel electrophoresis (Biometra, Germany), the PCR products were separated in 1.5% agarose gels with ethidium bromide stainedand visualized with gel documentation system (Biometra, Germany).

Statistical Analysis

Chi-square (χ^2) and Fisher's exact test were used to determine the relation between the presence of integrons and antibiotic resistance in SPSS software (SPSS 16, USA). A P value of < 0.05 was consideredas statistically significant.

RESULTS

Of the 109 *K. pneumoniae* isolates tested, 74 (67.8%) were shown resistant toquinolone was chosen forfurther studies. Among the clinical specimens, a total of 40 (54%) and 30 (40.5%) of quinolones resistant isolates were obtained from urine and burn wound respectively, while the residual isolates were recovered from seminal fluid 2(2.7%), wound abscesses and sputum1 (1.3% each) (table 2). The 74 *K. pneumoniae* isolate had shown resistance to nalidixic acid and/or ciprofloxacin antibiotics were involved in this study. Among the 31 detected antibiotics, the highest drug resistance rate were ampicillin and amoxicillin (100%) and similarly for amoxicillin-clavulanicacid, while, the lowermost drug resistance rate were 36.4% for imipenem and gatifloxacin, 37.8%Meropenem, Amikacinand 39.1% for Chloramphenicol (table 3). According to Magiorakos *et al*.(8),of the 74 QRKP isolates, 47 (63.5%) isolates were MDR, while 23 (31%) were considered as XDR, that susceptible to two or fewer antimicrobial categories and PDR isolates were identified in 4 (5.4%) isolates that resistant to all agents Known (Table 4). The present study indicatethat integrons are widespread in *K. pneumoniae* isolates. Among the 109 isolates, 51 (46.7%) were observed to have integrons, whereas, no class 2 integrons were detected. Among the 74 QRKP isolates the class 1 integron present in 41(55.4%0), while present in only 10(28.5%) of quinolone susceptible *K. pneumoniae* isolates, and were found strongly associated with QRKP isolates (p=0.008). class 1 integron was presented in all XDR and PDR and less existent in MDR isolates (Table 5).

DISCUSSION

The emerging quinolone-resistant clinical isolates and multidrug-resistant *K. pneumoniae* strains have been increased among clinical isolates in worldwide and become a serious therapeutic challenge. Integron has been recognized as one of the major sources of genes that responsible for antimicrobial resistance and is suspected to be a source of resistance genes to *Enterobacteriaceae*. Our study analyzed 109 *K. pneumonaie* clinical isolates obtained from hospitals in Najaf, Iraq, comprehensively for possession of integrase genes in quinolone and multidrug resistance isolate, and the association between them. The results of this study indicated that 74 (67.8%) of *K. pneumoniae* isolates had displayed quinolone resistance, these results were higher than studies from other countries (9,10). Among these 74 quinolone resistance isolates, high resistance to penicillins and generations of cephalosporins were observed, being in the range of (97-100%) and (55.4-89.1%) respectively, which is quite high. The present results also revealed the resistant pattern to fluoroquinolone a range of (36-87%), in addition to other antibiotic drugs, which is relatively high. Similar results were revealed previously in United States (11). The present study demonstrated an unexpectedly high rate of MDR isolates in present antibiotic resistance profile that represents 63.5% in addition 31% where XDR, and extraordinary 5.4% where PDR. Maybe the increased use of antibiotics during recent years in an uncontrolled way could be the cause of this. Continued used of certain antibiotics also supports the selection of certain resistance elements and promotes the perseverance of MDR bacteria (12). Similar result was established in previously in Iran

(2). Till now, there are no published studies estimated the presence of integrons (class 1 and 2) in QRKP isolates from the Najaf, Iraq. Therefore, the present study first reported the class 1 integronwere associated with 41(55.4%0) of QRKP. A study from Iran (13) reported integron present in 66.6% of *K. pneumoniae* isolates. Similar finding previously reported revealed this association of integron and QRKP isolates. This association may be due to increasing the rate of mutation of the bacterialcell, and/or the presence of resistance genes on integrons that responsiblefor decreasedthe permeability of membrane or improvedefflux pump (14). Coexistence of quinolone resistance with the presence of integron is animportant public health problem and requests for incessant surveillance, monitoring, and adjustmentof the antibiotic use policies. In conclusion, quinolone resistance becomes the one of leading concern in global public health. Findings of this study clearly obviouslyshow that resistance to this antibiotics is associated with the existence of class 1 integrons suggests that integron may be assisting forward the spread of quinolone-resistant in Najaf. A serious threat to human health may associate with quinolone resistance bacteria among worldwide. Additional studies of integrons are required to understand the mechanisms of possessing of MDR genes in quinolone resistance clinical isolates.

Conflict of interest

None to declare. All authors have no conflict of interest

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Table 1: Primers Used to Detect Integron Type I and Type II in this Study

Target	Primer name	Primer sequence (5´-3´)	References
intI1	Int1F	CAGTGGACATAAGCCTGTTC	7
	Int1R	CCCGAGGCATAGACTGTA	
intI2	intI2F	CACGGATATGCGACAAAAAGGT	7
	intI2R	GTAGCAAACGAGTGACGAAATG	

Table 2: Frequency of quinolone resistant *K. pneumoniae* isolates in clinical specimen

Clinical specimen	Total No (%) of *K. pneumoniae* isolates	No (%) of isolates had quinolone resistant
Urine (n= 1340)	59 (4.4)	40 (67.8)
Burn wound (n= 225)	46 (20.4)	30 (65.2)
Sputum (n= 7)	1 (14.3)	1 (100)
Wound abscess (n= 7)	1 (14.3)	1 (100)
Seminal fluid (n= 11)	2 (18.2)	2 (100)
Total (n= 1590)	109 (6.9)	74 (67.8)

Table 3: Antibiotic susceptibility pattern expressed by quinolone resistance *K. Pneumonia* isolates (n= 74)

Antibiotic	No. (%) of isolates showed	
	Resistance	Susceptible
Ampicillin	74 (100)	0(0)
Amoxicillin	74 (100)	0(0)
Piperacillin	72(97.3)	2(2.7)
Amoxicillin-clavulanic acid	74 (100)	0(0)
Ampicillin-sulbactam	70 (94.6)	4 (5.4)
Piperacillin-tazobactam	57(77)	17 (22.9)
Ticarcillin-clavulanic acid	65(87.8)	9 (12.2)
Cefotaxime	66(89.1)	8 (10.8)
Ceftazidime	64(86.4)	10 (13.5)
Ceftriaxone	64(86.4)	10 (13.5)

Cefepime	55(74.3)	19 (25.7)
Cefoxitin	41(55.4)	33 (44.6)
Aztreonam	63(85.1)	11 (14.8)
Imipenem	27(36.4)	47 (63.5)
Meropenem	28(37.8)	46 (62.2)
Nalidixic acid	66(89.1)	9 (12.2)
Ciprofloxacin	65(87.8)	10 (13.5)
Gatifloxacin	27(36.4)	47 (63.5)
Levofloxacin	33(44.5)	41 (55.4)
Lomefloxacin	64(86.4)	10 (13.5)
Moxifloxacin	60(81)	15 (20.2)
Norfloxacin	39(52.7)	35 (47.3)
Ofloxacin	39(52.70	35 (47.3)
Amikacin	28(37.8)	46 (62.1)
Gentamicin	43(58.1)	31 (41.9)
Kanamycin	44(59.4)	30 (40.5)
Netilmicin	30(40.5)	44 (59.4)
Tobromycin	57(77)	17 (22.9)
Chloramphenicol	29(39.1)	45 (60.8)
Sulfamethoxazole	64(86.4)	10 (13.5)
Trimethoprim	66 (89.2)	8 (10.8)

Table 4: Multiple antibiotic resistance phenotypes of 74 quinolone resistant *K. pneumoniae* isolates

Resistance Pattern No.(%)	No. of antibiotic categories(n=11)	No. of resistance isolates (%)	Isolate code No.
MDR 47(63.5)	3	5(6.7)	Kp 10,21, 31,35,65
	4	1(1.3)	Kp 33
	5	2(2.7)	Kp 6, 32
	6	4(5.4)	Kp9, 50, 97, 98
	7	12(16.2)	Kp4, 14, 20, 36, 47, 68, 91, 94, 105, 117, 118, 123
	8	14(18.9)	Kp3, 7, 46, 62, 48, 72, 76, 74, 73, 83, 85, 108, 115, 110
	9	9(12.1)	Kp22, 49, 67, 77, 102, 111, 114, 120, 130
XDR 23(31)	10	12(16.2)	Kp1, 15, 45, 55, 56, 57, 58, 60, 66, 93, 95, 128
	11	11(14.8)	Kp12, 18, 23, 37, 38, 69, 84, 87, 100, 103, 113
PDR 4(5.4)	11	4(5.4)	Kp63, 64, 104, 92

Table 5: Frequency of class 1 integron gene in 74 quinolone resistance *K. pneumoniae* isolates

Type of Resistance	Quinolone resistant			P Value
	intl +	intl -	Total	
MDR	14	33	47	0.012
XDR	23	0	23	0.00
PDR	4	0	4	0.00
	41	33	74	

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RESEARCH ARTICLE

Cytotoxic and Apoptotic Inducing Activity of Ethanol Extract of *Naravelia zeylanica* in Human Breast Cancer Cells

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ABSTRACT

Breast cancer is one of the leading cause deaths among females throughout the world. Current treatment methods have limited therapeutic success in breast cancer and also it becomes resistant to therapy. Therefore, better therapeutic methods are immediately required for breast cancer.The study intended to determinecytotoxicity of ethanol extract of *Naravelia zeylanica* against MCF-7 breast cancer cell line.The cytotoxicity of extract evaluated by dose dependent manner at concentrations ranging from 100-1000 µg/mL and viability of treated cells checked by MTT assay.The mode of cell death was analyzed by using different techniques viz., acridine orange & ethidium bromide (AO/EB) staining technique, single cell gel electrophoresis (comet) assay and Hoechst staining technique and JC1 staining technique.The leaves of *Naravelia zeylanica* were collected from Kolli hills. Extraction process was done by using Soxhlet apparatus. Human breast cancer cells MCF-7 was obtained from National Center for Cell Science (NCCS), Pune, India.Ethanol extract of *N. zeylanica* showed potent cytotoxicity and induced apoptosis in MCF-7 cell line. The results showed potent cytotoxicity against MCF-7 breast cancer cells at the dose of 150µg/ml has been taken as IC50 value for our further study. And also, the ethanolic extract of *N. zeylanica* showed significant signs of apoptosis such as cell shrinkage, membrane blebbing and nuclei DNA fragmentation.From this study we conclude that ethanolic extract of *N. zeylanica* having significant anticancer activity against MCF-7 cell lines and it could be good therapeutic nature for further investigation to develop anticancer agents.

Keywords: Comet assay, MCF-7, MTT assay and *Naraveliya zeylanica*.

INTRODUCTION

Breast cancer is one of commonest cancer among womenpopulation throughout the world. It is a lifetime risk estimated to be one in eight in developed countries. Burden of breast cancer will be increased to almost double of the present cases by 2030 [1].It is caused due to modifiable life style and different environmental threat factors, including usage of tobacco and alcohol, diet, deficiency of regular consumption of fruit and vegetables, lack of physical activity, obesity, chronic infections and exposure to ionizing and non-ionizing radiation [2].Conservative drugs are commonly used for treatment of cancer patients. Nevertheless, it causes many side effects. Thus, the research on medicinal plants and their derivatives for cancer treatment has been deepened [3].Medicinal plants and its derived compounds play key role for treating many diseases due to of lesser side effects as compared to synthetic drugs [4].Medicinal plants have rich sources of natural compounds exhibiting anti-proliferation properties [5].The plant *N. zeylanica* has been used for treatment of various diseases. It showsmany pharmacological activities like antioxidant, antimicrobial and antihelmintic [6].Due to their immense value of herb, the present study was carried out to evaluate anticancer activity of ethanol extract of *N. zeylanica* against MCF-7breast cancer cell line.

MATERIALS AND METHODS

Plant collection and extraction

The leaves of *N. zeylanica* were collected from kolli hills, Tamil Nadu, India and It was authenticated (BU 002) by The Rapinat Herbarium, St Josephs College, Trichy.Collected plant leaves were shade-dried and coarsely powdered.Then,powder was successively extracted with ethanol (80°C) using Soxhlet apparatus. The solvent was removed by vacuum distillation in a rotatory evaporator at 60°C. The extract was filtered through Whatman No. 1 filter paper and concentrated on a water bath to a syrupy mass. The dried substance was dissolved in ethanol and stored in cold room for future use.

Preliminary Phytochemical Tests

Ethanol extract of *N. zeylanica* was subjected to qualitative phytochemical test to identify the phytoconstituents using standard procedure [7].

Cell culture

Human breast cancer cells MCF-7 was obtained from National Center for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM medium supplemented with 10 % FBS and antibiotics Penicillin at 100 U/mL and streptomycin at 100µg/mLin a humidified atmosphere of 5% of CO2at 37°C.

Cell viability assay

The MTT (3- [4,5-dimethylthiazol-2-yl] -2,5 diphenyl tetrazolium bromide) assay[8]is based on the conversion of MTT into formazan crystals by mitochondrial enzyme of living cells. This assay was used to measure the in vitro cytotoxic effects of ethanol extract of *N. zeylanica* on breast cancer cell line MCF-7. The extract were taken as concentration range of 100-1000µg/ml and dissolved in 100 % dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and prepared to final dilution was added to the wells containing 5 X 103 MCF-7 cells per well. DMSO solution was used as the solvent control. After 24 h, 20 µl of MTT solution (5mg/mL in PBS) was added to each well and the plate was wrapped with aluminum foil and incubated for 3 h at 37 °C. The purple formazan product was dissolved by addition of 100 µL of DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference). Data were

collected for three replicates, each in triplicate wherein the three average values were used to calculate the means and the standard deviations. The percentage inhibition was calculated from this data using the following formula:

$$\text{Percentage inhibition} = \frac{[\text{Mean OD of untreated cells}]-[\text{Mean OD of treated cells}]}{\text{Mean OD of untreated cells (control)}} \times 100$$

From the values thus obtained, the IC_{50}values for the ethanol extract of *N. zeylanica* were calculated by plotting percentage inhibition against different concentrations.

Acridine orange (AO) and ethidium bromide (EB) fluorescent assay for cell death

AO and EB staining was performed as described by Spector et al. (1998)[9].MCF-7 breast cancer cells were cultured separately in 6-well plates and treated with IC50 concentration of ethanol extract of *N. zeylanica* for 24 h, when DMSO (0.02 %) was used as solvent control. The treated and untreated cells (25 µL of suspension containing 5000 cells) were incubated with acridine orange and ethidium bromide solution (1 partof 100 µg /mL each of acridine orange and ethidium bromide in PBS) and examined in a fluorescent microscope (Carl Zeiss, Jena, Germany) using a UV filter (450–490 nm). Three hundred cells per sample were counted, in triplicate, for each time point and scored as viable or dead, and if dead whether by apoptosis or necrosis as judged from the nuclear morphology and cytoplasmic organization. The percentages of apoptotic and necrotic cells were then calculated. Morphological features of interest were photographed.

Hoechst 33528 staining

MCF-7 breast cancer cells were cultured in separate 6-well plates and treated with 24 h IC_{50} concentrations of ethanol extract of *N. zeylanica*. After incubation for the respective periods, the treated and control cells were harvested and stained with Hoechst 33258 stain (1 mg/mL, aqueous) for 5 min at room temperature [10].A drop of cell suspension was placed on a glass slide, and a cover slip was laid over to reduce light diffraction. At random 300 cells, in triplicate, were observed at 400x in the fluorescent microscope fitted with a 377–355 nm filter, and the percentage of cells that reflected pathological changes was calculated [11]. Data were collected for three replicates each and used to calculate the percentage of abnormal nuclei.

Assessment of mitochondrial membrane potential (JC1 staining)

JC-1 dye is widely used in apoptosis studies to monitor mitochondrial membrane health. Mitochondrial membrane potential ($\Delta\psi m$) was assessed using the fluorescent probe JC-1, which produces orange-red fluorescence when accumulated in the mitochondria of healthy cells but fluoresces green when leached out into the cytosol due to loss of membrane [12].The MCF-7breast cancer cells were grown in glass cover slips (22x9x22 mm) placed in the wells of 6-well plates and treated with the ethanol extract of *N. zeylanica* at the 24 h IC_{50} concentration, and 0.02 % DMSO was used as solvent control. The cells were stained with JC-1 dye after 12h exposure which was observed in the fluorescent microscope and the pathological changes in the cells were observed and recorded.

Single cell gel electrophoresis (Comet assay)

Single cell gel electrophoresis (SCGE) or comet assay is a rapid and sensitive method which measures the DNA strand breaks in individual cells [13]. The cells were treated with the ethanol extract of *N. zeylanica* for 24 h.The harvested cells were suspended in low melting point agarose in PBS and pipetted out to microscope slides pre-coated with a layer of normal melting point agarose. The slides were chilled on ice for 10 min and then immersed in lysis

solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 0.2 mM NaOH [pH 10.01], and Triton X-100)and was incubated overnight at 48ºC in order to lysis the cells and permit DNA unfolding. Thereafter, the slides were exposed to alkaline buffer (300 mM NaOH, 1 mM Na2-EDTA, [pH>13]) for 20 min to allow DNA unwinding. The slides were washed with buffer (0.4 M Tris, pH 7.5) to neutralize the excess alkali and to remove the detergents before staining with ethidium bromide (20 mL in 50 mg/mL). Photomicrographs were obtained using the fluorescent microscope. 150 cells from each treatment group were digitized and analyzed using CASP software. The images were used to determine the DNA content of individual nuclei and to evaluate the degree of DNA damage representing the fraction of DNA in the tail. Data were collected for three replicates each and used to calculate the percentage of damaged cells.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of ethanol extract of *N. zeylanica*

Phytochemical screening confirm the presence of alkaloids, cardiac glycosides, flavonoids, phenols, saponins, tannins and steroids in ethanol extract of *N.zeylanica*.

Effect of ethanol extract of *N. zeylanica* treatment on the viability of breast cancer cells

Cytotoxic activity of ethanol extract of *N. zeylanica* on human breast cancer cell was determined by adopting MTT assay. The extract exhibited high toxic to the MCF-7 cells even in the low dose, however it increases when the dose increased (Table 1). The IC50 concentration of the extract was obtained at $150\mu g/ml$. It indicates that the extract has potent nature of cytotoxity against MCF-7 cells (Figure 1). It might be due to the presence of secondary metabolites like alkaloids, cardiac glycosides, flavonoids, phenols, saponins [14]. Earlier [15], Seong*et al*., (2018) reported that alkaloids and flavonoids possessed toxic effect. Result of the present phytochemical analysis also confirmed the presence of these phytochemicals.

Morphological changes in ethanol extract of *N. zeylanica*treated MCF-7 cells

Ethanol extract of *N. zeylanica* induce apoptosis in MCF-7 cell was identified by adopting acridine orange (AO) and ethidium bromide (EB) staining, the fluorescence patterns depend on viability and membrane integrity of thecells. Apoptosis is programmed cell death which removes unwanted cells in living system [16]. Uniformly green fluorescing nuclei with a highly organized cellular structure indicated live cells. Green fluorescing nuclei, with perinuclear chromatin condensation as revealed in brightgreen patches or fragments indicated cells in earlyphase of apoptosis. Orange to red fluorescing nuclei with highly condensed or fragmented chromatin indicated cells in a late stage of apoptosis. Orange to red fluorescing nuclei with no indication of chromatin fragmentation but the entire cells as well asnuclei were swollen to large size indicated necroticcells (Figure 2). Inducing apoptosis is one of main characteristic feature of anticancer agents [17]. Data on MCF-7 cells indicating apoptotic and necrotic changes produced by ethanol extract of *N. zeylanica* for 24h. It was collected from manual counting, and it is revealed that the extract is highly efficient in induce apoptosis (39%) compared than necrosis (10%). We adopted Hoechst stainingfor detect apoptotic nature ethanol extract of *N. zeylanica* against MCF-7 cells. Generally, apoptosis is characterized by chromatin condensation, cell shrinkage, DNA fragmentation and plasma membrane blebbing [18]. The observations suggested that the treatment of extract bring chromatin fragmentation, binucleation, cytoplasmic vacuolation, membrane blebbing and late apoptosisindication and although some of the cells indicated features of necrotic death (Figure3). Data collected from manual counting of cells with 49% of abnormal nuclear features. It indicates that the ethanol extract of *N. zeylanica* has potent inducing power of apoptosis in MCF-7 cells.

Treatment of ethanol extract of *N. zeylanica* brings about loss of mitochondrial membrane potential

An assay to detect changes in mitochondrial function employs the fluorescent cation JC-1, which emits red fluorescence when sequestered into the mitochondria of healthy viable cells. Mitochondrial membrane stress is one of most significant signaling of apoptosis [19]. Apoptotic cells which are induced by ethanol extract of *N. zeylanica*no longer able to sequester the JC-1 cation due to loss of mitochondrial membrane potential. Figure 4 shows the results of JC-1-staining of MCF-7 cells treated with ethanol extract of *N.zeylanica* at its 24 h IC_{50} concentration. The result led to a high proportion of apoptotic cells in MCF-7 breast cancer cell line by ethanol extract of *Naravelia zeylanica*.

Treatment of ethanol extract of *N. zeylanica* induces DNA damage

DNA is an important molecular target for cancer cell killing [20]. Single cell gel electrophoresis assay (comet assay) is adapted to measure DNA damage in MCF-7 cells treated with ethanol extract of *N. zeylanica*. This assay is considered to be rapid, simple, visual and sensitive. DNA of damaged cells gives the appearance of a comet, with head and tail regions (Figure 5). The CASP image analysis software is used analysis the result. The results revealed that the extract induced 17% of slightly damaged cells, 29% of damaged cell and 39% highly damaged DNA in breast cancer cells (MCF-7).

CONCLUSION

This study unambiguously showed that the ethanol extract of *N. zeylanica* has stronger antiproliferative effect on A549 lung cancer cell line. The mechanism underlying morphological changes produced, nuclei altered nucleus structure of cells, DNA damage induced and alteration in mitochondrial transmembrane potential. Our finding suggested ethanol extract of N. zeylanica has DNA as well as mitochondrial membrane changing effect on MCF-7 breast cancer cell line and is therefore, worthy of further investigation needed in this plant.

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Table 1: Cell viability assay (MTT) on MCF-7 cells.

Concentration	% of Viability	% of toxicity
Control	100	0
100 µg	62	38
200 µg	36	64
300 µg	17	83
400 µg	14	86
500 µg	9	91
600 µg	9	91
700 µg	8	92
800 µg	7	93
900 µg	4	96
1000 µg	4	96

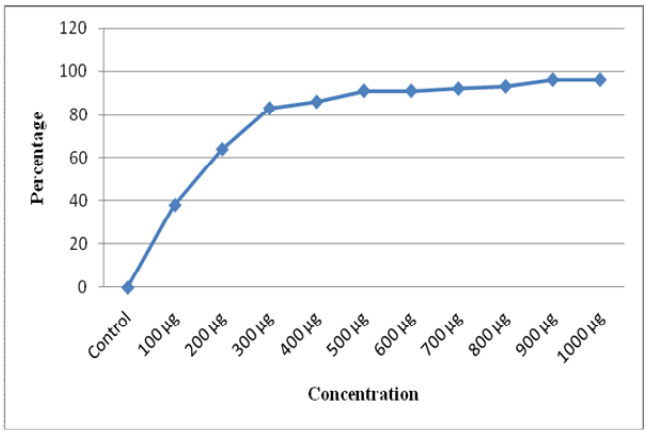
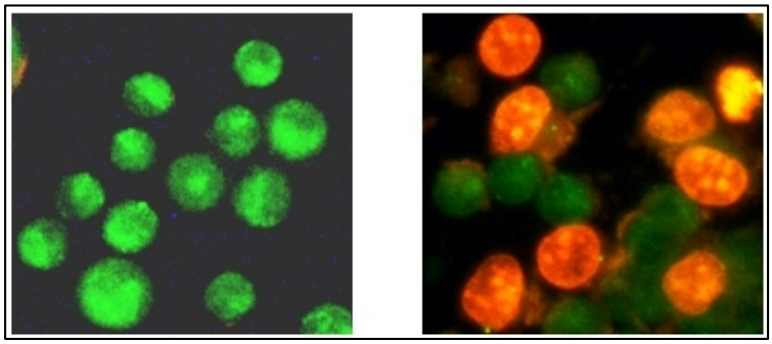
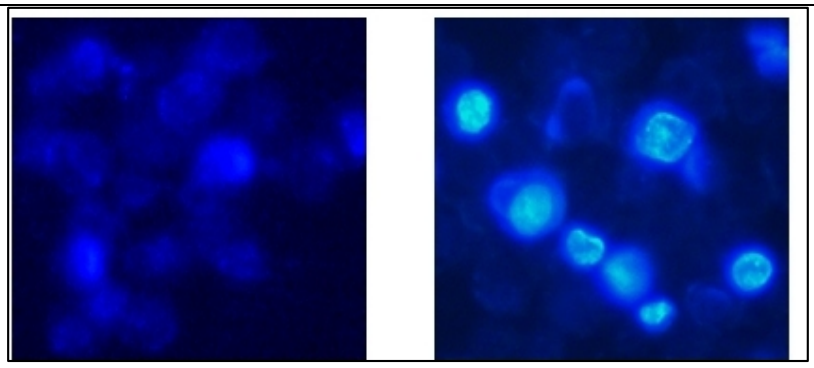


Figure 1. Inhibitory concentration of ethanol extract of *N. zeylanica* to MCF-7 breast cancer cell lines



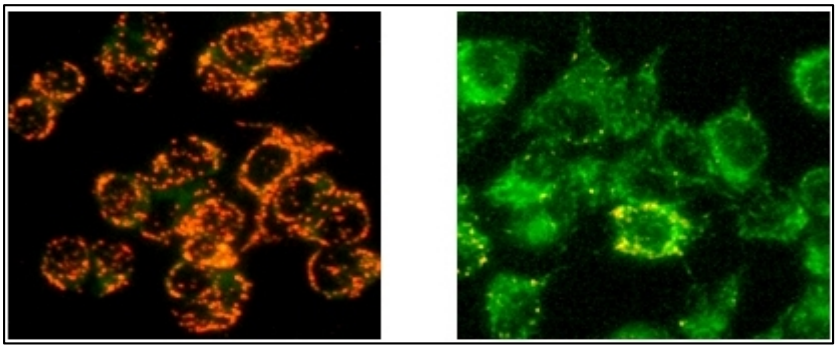
Control Cells Extract treated cells

Figure 2. Morphological changes observed in control and ethanol extract of *N. zeylanica* treated MCF-7 cells stained with AE/BO stain



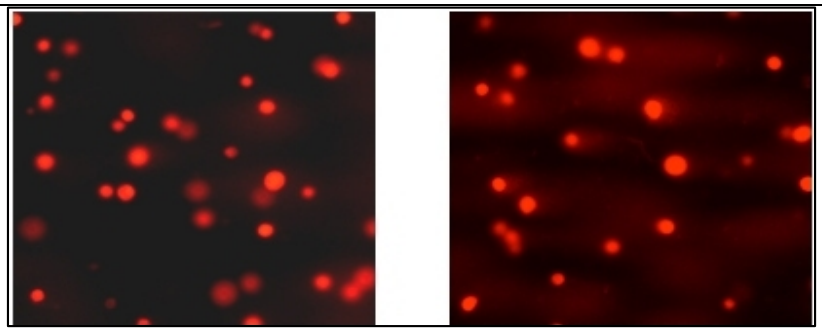
Control cells Extract treated cells

Figure 3. Morphological features of nuclei observed for control and ethanol extract of *N. zeylanica* treated MCF-7 breast cancer cells stained with Hoechst 33258



Control cells **Extract treated cells**

Figure 4. photomicrographs of MCF-7 breast cancer cells, JC1 dye accumulated in the Mitochondria of healthy cells as aggregates (red- orange fluorescing); in cells treated with ethanol extract of *N. zeylanica* due to collapse of mitochondrial membrane potential, the JC-1 dye retainedin to the cytoplasm in its monomeric form, which fluoresced green



Control cells **Extract treated cells**

Figure 5: DNA damage in ethanol extract of *N. zeylanica* treated lung cancer cells is revealed in the comet assay

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RESEARCH ARTICLE

Physicochemical Parameters of Costal Water in South East Coast of India, Tamil Nadu, India

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ABSTRACT

The present study was carried out to determine water quality parameters, pH, electrical conductivity, salinity, total dissolved solid, dissolved oxygen and biological oxygen demand, in east coastal region of India i.e. Chennai, Cuddalore, Nagapatinam, Rameshwaram and Tuticorin. The study revealed that the salinity, electrical conductivity and biological oxygen demand were very low in Nagapatinamduring pre-monsoon season, and it was observed to be high inTuticorin during monsoon season. The change in the physico-chemical character in the costal water could be due to agriculture, domestic sewage and industrial effluent discharge in to the sea nearby.

Key words: pH, salinity, dissolved oxygen, total dissolved solid, electrical conductivity, biological oxygen demand, Coastal water, Tamil Nadu coast.

INTRODUCTION

The hydrological study is a prerequisite in any aquatic system for the assessment of its potentialities and to understand the realities between its different trophic levels and food webs. Further, the environmental conditions such as topography, water movement, salinity, oxygen, temperature and nutrients characterizing the particular water mass also determine the composition of its biota. Thus the nature and distribution of flora and fauna in an aquatic system are mainly controlled by the fluctuations in the physical and chemical parameters of the water body (Damotharan *et al*., 2010). The marine environment, as a complex system is mainly influenced by various physical, chemical and biological processes. The open ocean is more stable compared to the near shore waters, where the interaction with the terrestrial zone is more effective in bringing about variations in different physicochemical parameters. Hence a thorough knowledge of hydrography is indispensable to estimate the quality of the

environment and its influence on biological fertility (Poonam and Rahul, 2012). Rapid industrialization along the coastal areas have brought a considerable decline in the water quality. They are subjected to a variety of socioeconomic drivers, producing increased pressures and impacts, which can lead to environmental stress or affect public health (Cave *et al*., 2003; Belzunce*et al*., 2004; Sundaramanickam*et al*., 2009). Such problems have been assigned mostly to an excess of nutrients, associated with industrial and municipal wastewater (Bell, 1991). The subsequent increase in nutrient loads produces an ecological impact over biological communities (Karlson*et al.,* 2002), associated mostly with eutrophication process (Wang, 1999). Hence, it is imperative to know the inter relationships between the organisms and environmental parameters in order to evaluate the stability and function of the ecosystem. In recent years, due to increase in domestic sewage, industrial inputs and various anthropogenic activities, water quality has been a serious concern. These activities may result in decline of species abundance, diversity and change the biology of the species. This zone is very sensitive and fragile. Continuous monitoring and assessment of this zone is must. Therefore, the present study has been aimed to study seasonal variations of hydrological parameters of surface waters at five stations along Tamil Naducoast for a period of one year from January to December 2014.

MATERIALS AND METHODS

Total of 5 stations/ coastal cities belong to the Tamil Nadu coast were selected for this study. Around 20 costal water samples were collected from these sites during four different seasons in 2014, Sampling dates: Post-monsoon (January to March), summer (April to June), Pre-Monsoon (July to September), monsoon (October to December).All the sea water and sea surface samples were collected from the shoreline of the each coastal stations. Sea water samples were collected from 0 –20cm below the sea surface water in sterile 2500 mL bottles (Vignesh*et al.,* 2012). All samples were kept in iceboxes and processed within 12 h of collection. The sampling station were: Chennai (S1), Cuddalore (S2), Nagappattinam (S3),Rameshwaram (S4) and Tuticorin (S5) (Fig; 1). The pH was determined using ELICO-LI 127 pH meter. The pH of water sample was directly determined with the electrode while pH of the water sample was determined by preparing suspension in distilled water. The contents were stirred well and allowed to settle and supernatant was used to check the pH.

Electrical Conductivity (EC) to determine soluble salts in the samples,it is preferred to keep the samples at the field condition and measured to get true picture. For this purpose saturated sample water extract was prepared. Sample extract is first prepared by taking 20g sediment into clean 100ml beaker, add 50ml of conductivity water then suspension is stirred for 30 minutes and the suspension was filtered by using ordinary filter paper. EC of the water samples and samples suspension was measured by using ELICO EC-TDS meter (CM 183, Make-India) where electrode was directly dipped into the respective solutions for the direct display of result on a digital scale. It was reported in micro Siemens (μS). The clear supernatant used for pH was also used for EC measurement. Salinity was determined by Mohr Knudsen argentometric titration method, using standard solution of silver nitrate (Merck) as an indicator to form silver halides, presence of excess silver ions lead to the formation of red silver chromate (the endpoint of titration).

Dissolved oxygen (DO) content of the water samples was measured by using Winkler's method (modified azide method). The sample was collected in 300 ml bottle and DO was fixed on site by using 1 ml each of Manganoussulphate and Alkaline-iodide-azide. The precipitate formed was dissolved in laboratory by using sulphuric acid and titrated with sodium thiosulphate using starch as an indicator. The end point of titration was blue to straw pale colour. The dilution method was followed to determine the BOD after three days at 27^{o}C. For the same, dilution water was prepared with the addition of nutrients namely phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride. The diluted sample was transferred to BOD bottles of 300 ml capacity. After determining initial DO, final DO was estimated from the bottles kept for incubation period for three days.

RESULT AND DISCUSSION

The range of pH, EC, TDS, salinity, DO and BOD levels in costal water samples were pH: 7.21 – 8.40, EC:41568 – 57156 µS/cm, TDS: 26188 – 36008 mg/l, Salinity: 25 – 33 ppt, DO: 4.4 – 6.7 mg/l and BOD: 6.7 – 8.3 mg/l, respectively. The pH of water is an important environmental factor, the fluctuation of pH is linked with chemical changes, species composition and life processes. It is generally considered as an index for suitability of the environment (Rani *et al.*, 2012). It was observed that the pH range of coastal water was minimum 7.21 at Nagapattinam during Summer season, and maximum 8.45 at Tuitucorin during Summer season (Fig 1). The highest value may be due to the Industrial Effluent because it contained many chemicals, salts and dissolved solids (Mishra and Saksena, 1993). Higher EC indicates the presence of high amount of dissolved inorganic substances in ionized form (Murhekar, 2011). ECwas found to be minimum 34640 µS/cm at Rameshwaram during Premonsoon season, and maximum 57156 µS/cm at Tuitucorin during Postmonsoon season. The TDS was found to be minimum 21823 mg/l at Nagapatinam during premonsoon season, and maximum 36008 mg/l at Tuticorin during postmonsoon season.

The salinity acts as a limiting factor in the distribution of living organisms, and its variation caused by dilution and evaporation and influence the fauna of the intertidal zone (Gibson, 1982; Balasubramanian and Kannan, 2005). In the present study, salinity was found to be minimum 20 ppt at Nagapatinam during premonsoon season, and maximum 52 ppt at Nagapatinam during post-monsoon season. The value of dissolved oxygen is crucial in determining the water quality criteria of an aquatic ecosystem. The DO is regulator of metabolic activities of organisms and thus governs metabolisms of the biological community as a whole and also acts as an indicator of trophic status of the water body (Saksena, 1994). DO was found to be minimum 4.9 mg/l at Nagapatinam during summer season, and maximum 8 mg/l at Nagapatinam during monsoon season.DO is the most important indicator of the health of a water body and its capacity to support a balanced aquatic ecosystem of plants and animals. Waste water containing organic pollutants depletes the DO and may lead to impact benthic communities by producing acute changes in their distribution, abundance, and diversity of species (Raffaelli, 2000).

BOD is the amount of oxygen required by the living organisms engaged in the utilization and ultimate destruction or stabilization of organic water. It is a very important indicator of pollution status of a water body. In the conducted experiments, BOD was high in Domestic and Industrial Effluent due to high organic load and excessive growth of total microorganisms (Kandhasamy, and Santhaguru, 1994). BOD was found to be minimum 6.7 mg/l at Cuddalour and Nagapatinam during postmonsoon season, and maximum 9.8 mg/l at Tuticorin during monsoon season.

CONCLUSION

In the present study disrupts normal functioning of the ecosystem, causing a variety of problems such as a lack of oxygen in the seawater, needed for fish and crustaceans to survive. Salinity is extremely important from the standpoint of monitoring seawater quality. The purpose of this study has been to determine the effect of drainage system and type of land use on the load of mineral nutrient compounds derived from the environment and present in coastal ecosystems. As coastal water originating from rain and snow is the main medium shaping soil processes and transport of minerals, particular attention has been paid to variations in atmospheric precipitation during the analyzed time period. Increased human activities over the recent past are imposing a greater stress on these ecosystems, resulting on changing their water quality and loss of biodiversity. In the present study tidal and diurnal variations in a large spectrum of physicochemical fraction was irrigates for this coastal environment.

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Table 1: Physicochemical parameters of all seasons and stations

Season/Station	pH	EC (µs/cm)	TDS (mg/l)	Salinity (ppt)	DO (mg/l)	BOD (mg/l)
POMS1	8.18	48498	30552	28	6.7	7.8
POMS2	7.8	51331	29291	30	6	6.7
POMS3	7.7	48135	26188	52	5.8	8.3
POMS4	8.2	45698	33285	29	6.1	7.5
POMS5	8.4	57156	36008	33	5.6	7.9
SS1	7.51	38620	32358	30	5.5	8.2
SS2	8.3	51382	28361	32	6.1	7.3
SS3	7.92	43691	24320	29	4.9	6.7
SS4	7.8	48368	31355	30	6.7	7.9
SS5	8.42	54384	34264	31	5.1	7.4
PMS1	7.35	48533	26691	26	5.8	6.8
PMS2	7.72	50965	28705	28	6.5	7.7
PMS3	7.21	41376	21823	20	7.4	7.1
PMS4	7.6	34640	32734	25	6.8	7.8
PMS5	8.14	51960	31308	30	5.9	8.3
MS1	8.31	47127	33414	29	6.3	7.6
MS2	7.95	45380	31411	30	7	8.1
MS3	8.1	60620	35865	31	8	8.3
MS4	7.88	43300	27290	25	5.5	7.8
MS5	8.45	55641	38190	33	7.6	9.8

Table 2: physicochemical characters of all seasons

S.No	Parameters	Postmonsoon	Summer	Premonsoon	Monsoon
1	pH	8.056±0.294415	7.99±0.371618	7.604±0.360735	8.138±0.239937
2	EC (µS/cm)	50163.6±4389.975	47289±6252.061	45494.8±7344.109	50413.6±7386.448
3	TDS	31064.8±3760.22	30131.6±3887.159	28252.2±4283.732	33234±4188.405
4	Salinity	34.4±10.01499	30.4±1.140175	25.8±3.768289	29.6±2.966479
5	DO	6.04±0.415933	5.66±0.74027	6.48±0.66106	6.88±1.003494
6	BOD	7.64±0.598331	7.5±0.578792	7.54±0.594138	8.32±0.870057

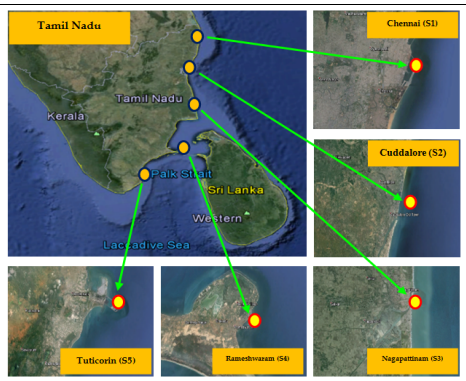

Fig 1: Study area

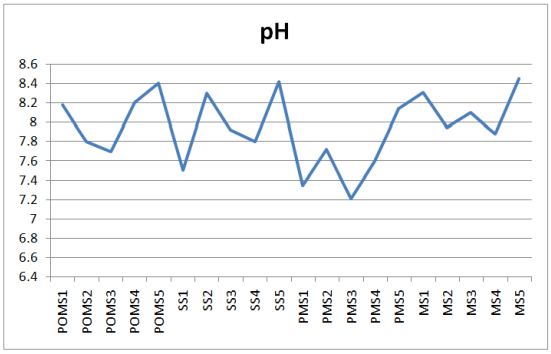

Fig 2 : pH physicochemical Parameters and stations

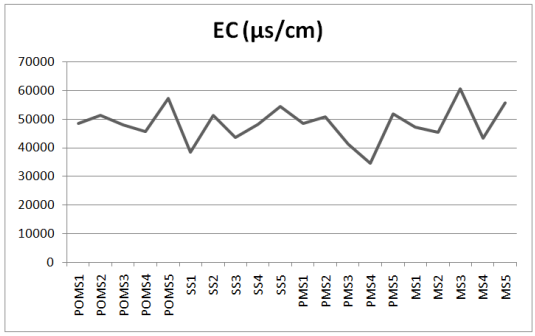

Fig 3 : EC(µS/cm) Physicochemical Parameter and different stations

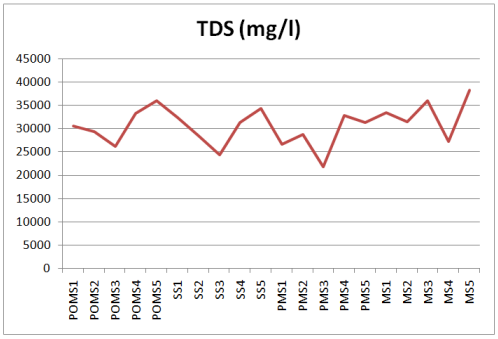

Fig 4 : TDS (mg/l) Physicochemical Parameter and different stations

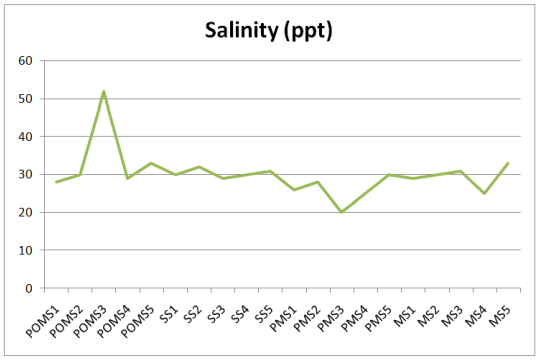

Fig 5: Salinity (ppt) Physicochemical Parameter and different stations.

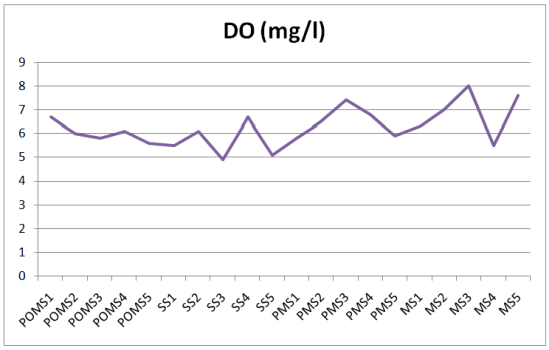

Fig 6: DO (mg/l) Physicochemical Parameter and different stations.

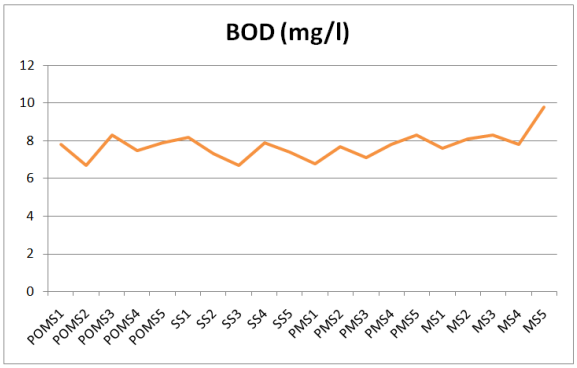

Fig 7 : BOD (mg/l) Physicochemical parameter and different stations