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RESEARCH ARTICLE

# HPLC Stability Indicating Assay Method for Marketed Herbal Antihypertensive Formulations Containing *Rauwolfia serpentina* Benth.

Sandhya. M. Bagde*, Smita.G.Mujbaile and P.S.Gangane.

Department of Pharmacognosy and Phytochemistry, Dadasaheb Balpande College of Pharmacy, Besa, Nagpur-440034,India.

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***Address for correspondence**
Sandhya. M. Bagde,
Assistant Professor ,
Department of Pharmacognosy and Phytochemistry,
Dadasaheb Balpande College of Pharmacy,
Besa, Nagpur-440034 (M.S.),India.
E.mail: sandhyahadke90@gmail.com.



## ABSTRACT

Stress degradation studies were carried out on Reserpine (Antihypertensive agent in *Rauwolfia serpentina* Benth. and marketed herbal Reserpine containing formulations following the conditions prescribed in the parent drug stability testing guidelines (Q1AR) issued by International Conference on Harmonization (ICH). The present study describes degradation of Reserpine under different ICH prescribed stress conditions (acid and base hydrolysis, oxidation, dry and wet heat degradation and photo-degradation) and establishment of stability indicating HPLC assay. The method employed HiQ sil C18 W size 4.5x250mm column as stationary phase. The solvent system consists of Acetonitrile: Ammonium chloride 1% w/v (1:1). The analysis of Reserpine was carried out at 268nm. This system was found to give well resolved peaks of Reserpine (RT 4.005 $\pm$ 0.02 min.). The drug undergoes degradation under acidic and basic conditions, oxidation, dry and wet heat treatment and photo degradation. All the peaks of degraded products were resolved from the standard Reserpine with significantly different Rt. As the method could effectively separate the drug form its degradation products, it can be employed as a stability indicating one.

**Key words:** Reserpine, *Rauwolfia serpentina*, HPLC, stability indicating, stress degradation.

# INTRODUCTION

The parent drug stability test guidelines (Q1A) issued by International Conference on Harmonization (ICH) requires that analytical test procedures for stability samples should be fully validated and the assays should be stability indicating [1]. Stress testing is a part of development strategy under ICH requirements and is carried out under more severe conditions than accelerated studies. Further, it is suggested that stress studies should be carried out to establish the inherent or intrinsic stability characteristics of the molecule by establishing the degradation pathways and help in validation of the analytical methods to be used in stability studies. The parent drug stability guidelines (Q1AR) requires that stress testing of drug substance should include the effect of elevated temperature, humidity, light and oxidizing agents, as well as the susceptibility across a range of pH values [2]. Accordingly, the purpose of the present study is to put ICH recommendations into practice by subjecting Reserpine to the variety of suggested stress test conditions to establish inherent stability of the drug and to develop the validated stability indicating HPLC assay. The endeavor was to quantify overall amount of degradation of Reserpine under different stress conditions. There is no report yet on these aspects for this drug. Reserpine (Figure 1) is chemically methyl-11, 17 $\alpha$ –dimethoxy-18 β-[(3, 4, 5-trimethoxybenzoyl) oxy]-3β, 20$\alpha$- yohimban-16 β- carbxylate[3].

Klyushnichenko (1995)[4] studied the separation of model mixture of six indol alkaloids by HPLC and HPTLC on the normal and reverse- phase Armsorb supports respectively. The retention time of Reserpine was about 42 minutes [4]. An exhaustive literature survey was done on available internet sites, chemical abstract and all other possible literature sources for the stability indicating assay method development on herbal drug substances and formulations Himani Agrawal (April 2004) [5] carried out Stress degradation studies on guggulsterone (the hypolipidemic agent in the gum-resin exudates of Commiphora mukul) following the conditions prescribed in the parent drug stability testing guideline (Q1AR) issued by International Conference on Harmonization (ICH). The study describes degradation of guggulsterone under different ICH prescribed stress conditions (acid and base hydrolysis, oxidation, dry and wet heat degradation and photo degradation) and establishment of a stability indicating HPTLC assay. M. J. Ansari (March 2005) [6] give Stability-indicating HPTLC determination of Curcumin in bulk drug and pharmaceutical formulations.

The International Conference on Harmonization (ICH) guidelines entitled 'stability testing of new drug substances and products' require the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. Susceptibility to oxidation is one of the required tests. Also the hydrolytic and the photolytic stability are required. An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products. The aim of the present work is to employ validated HPLC method for stability studies of Reserpine in presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its dosage forms.

# MATERIALS AND METHODS

## Chemicals and Materials

All solvents were of HPLC grade. All other materials were purchased of analytical grade E-Merck, Qualigens, and Rankem etc. Distilled water and Whatman filter paper Grade-I were used throughout the experimental work.A standard Reserpine marker compound was purchased from "Natural Remedies Pvt. ltd." Bangalore. The marketed formulations were purchased from the local market.

### HPLC Instrumentation

Jasco Gradient mode HPLC with HiQ sil C18 W (size 4.5mmx250mm, particle size 5µm) column was used for the study. Jasco UV 2075 is used as a detector and Jasco PU 2080 plus was the solvent delivery system. The system control and data processing were performed by BORWIN software (build Jasco).

### Mobile Phase

The mobile phase consists of Acetonitrile: Ammonium chloride 1%w/v (1: 1), pH- 5.6 and the flow rate was 1.5ml/min

### Calibration curves of Reserpine

Serial standard eight different concentration levels of Reserpine (5-40 µg/ml) were prepared. For HPLC analysis, a 20µL sample volume was injected 5 times. The chromatograms were monitored by UV at 268nm. The peak area of UV chromatograms were plotted versus the concentration and the calibration curve was constructed using a least square regression equation for the calculation of slope, intercept, and square of correlation coefficient.

### Analysis of Reserpine in prepared marketed formulations

The marketed formulation A (labeled claim: Sarpagandha 62.5mg,) and formulation B (labeled claim: Sarpagandha 48mg), the tablets were powdered and powder equivalent to 5 mg of Reserpine was weighed. Dissolved in 100ml distilled water. Filtered with whatman filter paper. Take the residue in a separating funnel and powder with five 25ml quantities of chloroform. Combine the chloform layer. Evaporate the chloroform layer and dilute the residue with 10 ml methanol (0.5mg/ml). This final stock solution of formulation A and B are coded as Solution B and C respectively. The standard stock solution of formulation A and B was further diluted with the mobile phase to get the final concentration of about 10 µg/ml of Reserpine.

### Validation of method

### Precision

Repeatability of sample application and measurement of peak area were carried out using five injections of same sample (10µg/ml of Reserpine). The intra and inter-day variation of the determination of Reserpine was carried out at two different concentration levels.

### Recovery studies

The analyzed samples were spiked with 50, 100 and 150% of the standard Reserpine and the mixtures were reanalyzed by proposed method. The experiment was conducted three times. This was done to check for the recovery of the drug at different levels in the formulation.

### Stability of Reserpine in standard and test solutions

A standard solution was initially prepared (std.no.1) and held under refrigerated condition ($4^0$ – $8^0$ c) for up to 30 days and tested against freshly prepared standard solution (std.no.2). A test solution of two different tablets brands were initially prepared (Test 1and 2) and held under refrigerated condition ($4^0$ – $8^0$ c) for up to 30 days and tested against freshly prepared test solution (Test 3 and 4).

### Forced Degradation of Standard Reserpine

A standard Reserpine solution (10µg/ml, 20µl injection volume) was injected before and after each sample analysis. Also blank experiments were carried out. A system suitability injection was developed during each working day.

### Preparation of Acid Degradation Products

In four different 5ml reaction vials a volume of 1ml of standard stock solutions A, B and C (0.5 mg/ml) were evaporated to dryness and a volume of 0.5 ml of 1M hydrochloric Acid was added to each vial. These vials are half inserted into a block heater at 9 0°C for 10 min. and cooled. A volume of 0.5ml of Sod. Hydroxide (1M and 5M resp.) was added for neutralization.

### Preparation of Base Degradation Products

In four different 5ml reaction vials a volume of 1ml of standard stock solutions A, B and C (0.5 mg/ml) were evaporated to dryness and a volume of 0.5 ml of 1M Sod. Hydroxide was added to each vial. These vials are half inserted into a block heater at 90°C for 10 min. and cooled. A volume of 0.5ml of hydrochloric Acid (1M and 5M resp.) was added for neutralization.

### Preparation of Hydrogen Peroxide Induced Degradation Products

In four different 5ml reaction vials a volume of 1ml of standard stock solutions A, B, and C (0.5 mg/ml) were evaporated to dryness and a volume of 1 ml of 6%Hydrogen peroxide was added. These vials are half inserted into a block heater at 90°C for 10 min. and cooled.

### Dry and Wet Heat Degradation Products

Two screw capped reaction vials were used. In the first vial put 1mg of Reserpine powder and in the second vial put 1mg of Reserpine with 10µl water as a source of moisture. Similarly take standard stock solutions A, B and C in separate vials and evaporate to dryness. Both stored at 9 0° for 2 hours in a hot air oven. The contents are dissolved in methanol and diluted to a given claimed concentration of 10 µg/ml and 20 µl was injected for HPLC analysis. The percentage amount of Reserpine that remained was calculated referring to standard Reserpine solution adjusted from the calibration curve.

### Photochemical Degradation Products

The photochemical stability of the drug was also studied by exposing the 1mg of Reserpine standard, triturated tablet powder and Sarpagandha powder to direct sunlight for three days (from 10.00 to 17.00 h on a wooden plank).The entire above degraded sample solutions were made up to a final concentration of 10 µg/mL with mobile phase which was used for degradation study.

## RESULTS AND DISCUSSION

### Development of the optimum mobile phase

Each mobile phase was filtered through 0.45 µ membrane filter. The mobile phase was allowed to equilibrate phase until steady baseline was obtained. The standard solutions containing Reserpine was run and different individual solvents as well as combinations of solvents were tried to get a good separation and stable peak. From the various mobile phases tried, mobile phase containing Acetonitrile: Ammonium chloride 1%w/v (1: 1), pH- 5.6 was selected as it shown sharp peak with symmetry and significant reproducible retention time for Reserpine. It is given in table 21 and chromatogram of Reserpine shown in Figure 1.

### Calibration curves

The linear regression data for the calibration curve as shown in table 1 showed a good linear relationship over the concentration range 5-40 μg/ml with respect to peak area.

### Validation of the method

### Precision

The repeatability of sample injection and measurement of peak area were expressed in terms of %RSD and results are depicted in Table 2, which revealed intra and intre-day variation of Reserpine.

### Recovery studies

The proposed method when used for extraction and subsequent estimation of Reserpine from marketed formulations after spiking with 50,100 and 150% of addingtinal drug afforded recovery of 97-99% as listed in table 3.

### Stability of Reserpine in standard and test solutions

Under refrigerated condition ($4^0 – 8^0$ c) standard and test solutions remains stable for up to 30 days (Table 4, 5).

### Analysis of Reserpine in prepared marketed formulations

The Reserpine content in marketed formulation A was found to be 98.05 with a %RSD of 0.2488 and in formulation B, 99.73% with a %RSD of 0.1807. It may therefore be inferred that the marketed formulations can be analysed using this new and simple method. The low %RSD value indicated the suitability of this method for routine analysis of Reserpine in pharmaceutical dosage forms.

### Stability indicating property

A stock solution containing 0.5mg/ml of standard Reserpine in methanol was freshly prepared. This solution was used for forced degradation

### Acid and base induced degradation products

The chromatogram of the acid degraded samples of Reserpine showed additional peaks at RT 3.392 and 8.292 min (Figure. 3A, Table 5) in standard which can be correlated with the degradation peaks having RT 3.050, 8.008 in the formulation A and formulation B respectively (figure 3B and 3C resp.). Apart from these, additional peaks are observed in formulation A and formulation B at RT 1.208, 1.792, 1.433, 0.292, 4.858. It indicates that Reserpine undergoes degradation under acidic condition.

The chromatogram of the base degraded samples of Reserpine showed additional peak at RT 0.683 min. (Figure. 4A, Table 5) in standard which can be correlated with the degradation peaks having RT 0.883, in formulation A. Apart from this additional peaks are observed in formulation A at RT 1.608, 2.33, 3.692, 3.95. The drug is completely degraded in formulation B (figure 4B, and 4C resp.). It indicates that Reserpine undergoes degradation under basic condition.

### Hydrogen Peroxide Induced Degradation Product

The chromatogram of the oxidative degraded samples of Reserpine showed additional peak at RT 3.383 (Figure. 5A, Table 5) in standard, additional peaks are observed in formulation A having RT 1.083. The drug is completely get oxidized in formulation B (figure 5B and 5C resp.). It indicates that Reserpine undergoes degradation in oxidized condition.

### Dry and wet Heat Degradation Product

The chromatogram of the dry heat degraded samples of Reserpine showed no additional peak (Figure. 6A, Table 5) in standard. But shows additional peak at RT 3.108 in formulation A (Figure. 6B). It indicates that Reserpine undergoes degradation under dry heat degradation condition.

The chromatogram of the wet heat degraded samples of Reserpine showed no additional peak (Figure. 7A, Table 5) in standard and in marketed formulations. It indicates that Reserpine does not undergo degradation under wet heat degradation condition.

### Photochemical Degradation Product

The chromatogram of the photochemical degraded samples of Reserpine showed no additional peak (Figure13, Table 21) in standard. But apart from this additional peaks are observed in formulation A and formulation B at RT 2.958 and 2.806. It indicates that Reserpine undergoes photochemical degradation.

About 5- 15% degradation was found in acid treatment, 40% - 85% in base treatment, about 25% - 80% degradation was found in oxide treatment. In other treatments (Dry heat, Wet heat, and photochemical) about 4% - 8 % degradation was found. Looking the above results we can say that most of the peaks we find into the marketed formulations do not match with the standard Reserpine degradation products. Thus it can be considered that most of the impurities present in the marketed preparations may have been formed due to degradation during processing and storage.A simple, selective, precise and stability indicating High-performance liquid chromatographic method of analysis of Reserpine in marketed herbal antihypertensive formulations was developed and validated. Statistical analysis proves that the method is repeatable and selective for the analysis of Reserpine in marketed herbal Reserpine containing formulations.

This study is a typical example of development of a Stability-indicating assay, established following the recommendations of ICH guidelines. It is one of the rare studies where forced decomposition studies were done under all different stressed conditions and the degradation products were well resolved. The HPLC technique employed is stability indicating since it resolves all the degradation products of Reserpine under all stressed conditions. This study may be extended to study the degradation kinetics of Reserpine and to predict degradation pathways. This method can be proposed for the analysis of Reserpine and its degradation products in stability samples in industry. Also the method is employed to estimate the content of Reserpine in other Reserpine containing plants. A new finding of this study is that the Reserpine is unstable in almost all forced degradation conditions.The method can be used to determine the purity of the drug available from various sources by detecting the related impurities. As the method separates the drug from its degradation products, it can be employed as stability indicating one.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. ICH, Q1A stability testing of new drug substances and products, in: International Conference on Harmonization, IFPMA, Geneva, October 1993.
2. ICH, Q1A Stability Testing of New Drug Substances and Products, in: Proceedings of the International Conference on Harmonization IFPMA, Geneva, 2000.
3. The United States Pharmacopoeia 26 NF 21, 2003; 1625.
4. Klyushichenko VE: Determination of indol alkaloids from R. serpentina and R. vomitoria by High-Performance Liquid Chromatography and High-Perfoemance Thin-Layer Chromatography. Journal of chromatography,A. 1995; 704:357-362.

5. Agrawal H, Kaul N, Paradkar A and Mahadik K: Stress Degradation Studies on Guggulsterone. Journal of Pharmaceutical and Biomedical analysis 2004; 36: 23-31.
6. Ansari M and Ahmad S: Stability-indicating HPTLC determination of Curcumin in bulk drug and pharmaceutical formulations. Journal of Pharmaceutical and biomedical Analysis 2005; 39:132-138.
7. Deore SL, Mohod MA, Baviskar BA and Khadabadi SS: HPTLC validated stability indicating assay method for marketed herbal antihypertensive formulations. Pharmaceutical methods Elsevier May 2013; 4(1):11-15.
8. Shrivastava A, Issarani R, and Nagori BP: Stability Indicating High-Performance Liquid Chromatography Method for the Estimation of Artemether in Capsule Dosage Forms. J Young Pharm. 2010 Jan-Mar; 2(1): 79–84.
9. Diego MD, Mennickent S, Godoy G, and Miranda GA: Validated stability-indicating LC method for simultaneous determination of enalapril and hydrochlorothiazide in pharmaceutical samples. Current Pharmaceutical Analysis 2011; 7(4):248–252.
10. Jing-Chun Wang, Qi Zhang and De-Fu Cai: Stability-Indicating Validated HPLC Method for Analysis of Berberine Hydrochloride and Trimethoprim in Pharmaceutical Dosage Form. Hindawi Publishing Corporation Journal of Chemistry 2013; Volume, Article ID 360812:1- 9.
11. Singh M, Younus-Kunju Thajudeen-Koya Kamal, Rabea Parveen, and Sayeed Ahmad: Development and Validation of a Stability-Indicating HPTLC Method for Analysis of Arjunolic Acid in a Herbal Formulation. Journal of Planar Chromatography 24 (2011); 2:172–175.

**Table 1: Linear Regression Data for the Calibration Curve.**

| **Parameters** | **Results** |
|---|---|
| Linearity range (μg/ml) | 5-40 |
| Correlation coefficient | 0.9992 |
| Slope ± S.D. | 51.93 ± 0.54 |
| Y intercept | 0 |

**Table 2: Intra and Interday Precision of HPLC Method.**

| **S.No.** | **Sample No.** | **% Drug Estimation** | | | |
|---|---|---|---|---|---|
| | | **Interday** | | **Intraday** | |
| | | **Formulation A** | **Formulation B** | **Formulation A** | **Formulation B** |
| 1 | I | 97.86 | 98.28 | 97.86 | 98.36 |
| 2 | II | 98.37 | 99.04 | 97.86 | 98.35 |
| 3 | III | 98.35 | 98.71 | 97.85 | 98.28 |
| | **Mean** | 98.19 | 98.676 | 97.8566 | 98.33 |
| | **±S.D.** | 0.2358 | 0.3111 | 0.0047 | 0.0355 |
| | **R.S.D.** | 0.2401 | 0.3153 | 0.0048 | 0.0361 |

**Table 3: Recovery Studies.**

| **Formulation A** | | | | | |
|---|---|---|---|---|---|
| **Sl. No.** | **Eq.Wt.of Tablet (g)** | **Amount of Pure drug added (mg)** | **Peak area of standard** | **Peak area of sample** | **% Recovery*** |
| 1 | 4.300 | 0.50 | 141985.68 | 163092.87 | 99.22 |
| 2 | 4..310 | 1.0 | 141985.68 | 176680.56 | 99.59 |
| 3 | 4.400 | 1.5 | 141985.68 | 209438.10 | 99.69 |
| | | | | **Mean** | 99.50 |
| | | | | **±S.D.** | 0.2475 |
| | | | | **R.S.D.** | 0.2488 |
| **Formulation B** | | | | | |
| 1 | 4.87 | 0.5 | 141985.68 | 174399.543 | 99.58 |
| 2 | 4.84 | 1.0 | 141985.68 | 188273.56 | 99.93 |
| 3 | 4.80 | 1.5 | 141985.68 | 143270.25 | 99.68 |
| | | | | **Mean** | 99.73 |
| | | | | **±S.D.** | 0.1802 |
| | | | | **R.S.D.** | 0.1807 |

* Each value is the mean of five observations

**Table 4: Results of Standard Solution Stability.**

| **Standard no.** | **1** | **2** |
|---|---|---|
| Concentration | 20 mcg/ml | 20 mcg/ml |
| Preparation date | 17/02/2009 | 20/03/2009 |
| Mean area | 294519.00 | 311889.40 |
| RSD | 0.4108 | 0.5155 |

**Table 5: Results of Sample Solution Stability.**

| **Test no.** | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| Concentration | 20 mcg/ml | 20mcg/ml | 20mcg/ml | 20 mcg/ml |
| Preparation date | 03/02/2009 | 03/02/2009 | 03/03/2009 | 03/03/2009 |
| Mean Assay | 98.19 | 98.676 | 97.8566 | 98.33 |
| RSD | 0.2401 | 0.3153 | 0.0048 | 0.0361 |

**Table 5: Forced Degradation of Standard Reserpine and Reserpine in Formulation A and Reserpine in Formulation B.**

| **Sl. No.** | **Sample exposure conditions** | **RT of degradation products** | **Figure No.** | **%Recovery** |
|---|---|---|---|---|
| 1 | Reserpine std., 1M HCl | 2(3.292, 8.392) | 3 A | 95.20% |
| 2 | Formulation A 1M HCl | 3 (1.208, 1.433, 1.792) | 3 B | 76.90% |
| 3 | Formulation B 1M HCl | 2 (0.292, 4.808) | 3 C | 85.20 |
| 4 | Reserpine std., 1M NaOH | 1 (0.683) | 4 A | 60.90% |
| 5 | Formulation A 1M NaOH | 4 (1.608,2.33,3.692,3.958) | 4 B | 22.20% |
| 6 | Formulation B 1M NaOH | 1 (3.392) | 4 C | 16.37% |
| 7 | Reserpine std., 20vol H2O2 | 1 (3.383) | 5 A | 76.35% |
| 8 | Formulation A, 20vol H2O2 | 1 (1.083) | 5 B | 21.88% |
| 9 | Formulation B, 20vol H2O2 | 4 (0.883,1.608,2.333,3.692) | 5 C | Not detectable |
| 10 | Reserpine std., day light | - | 8 A | Not detectable |
| 11 | Formulation A, day light | 2.958 | 8 B | 2.958 |
| 12 | Formulation B, day light | - | 8 C | Not detectable |

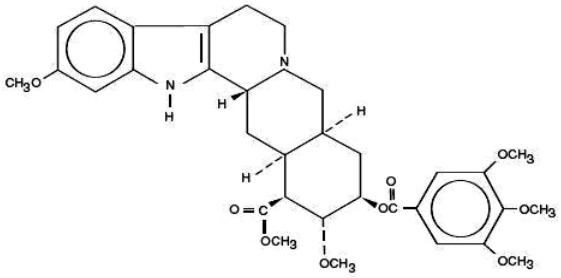


**Fig.1: Structure of Reserpine**

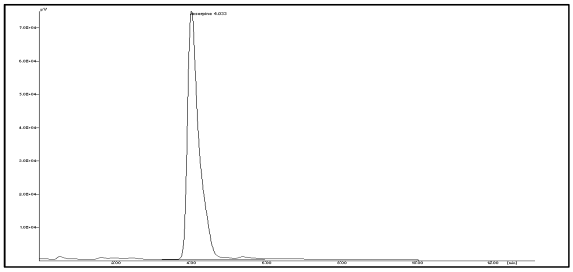

**Fig. 2: Chromatogram of Reserpine standard (10µg/ml)**

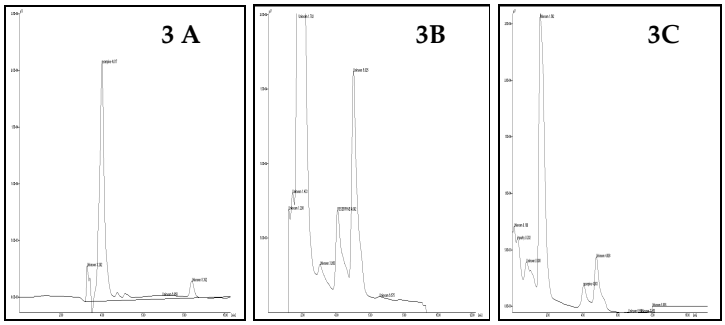


**Fig.3. Chromatogram acid degradation products of Reserpine in 3A) Standard 3B) Formulation A, 3C) Formulation B**

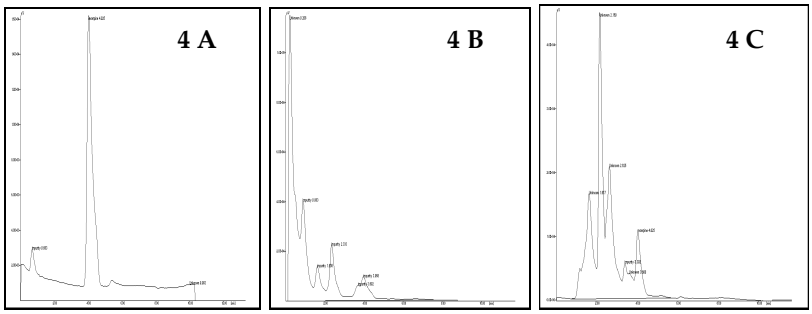


**Figure 4. Chromatogram of Alkali Degradation Products of Reserpine in 4A) Standard 4B) Formulation A, 4C) Formulation B**

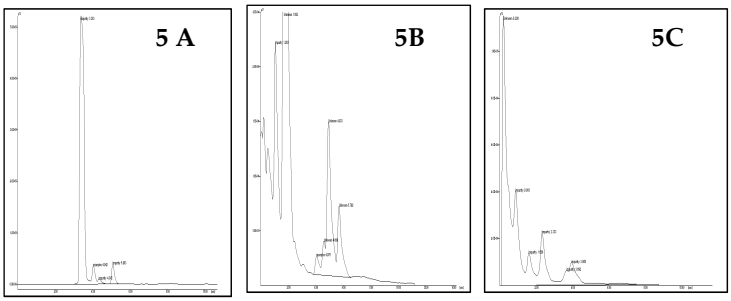


**Fig. 5. Chromatogram of Oxidative Degradation Products of Reserpine in 5A) Standard 5B) Formulation A, 5C) Formulation B**

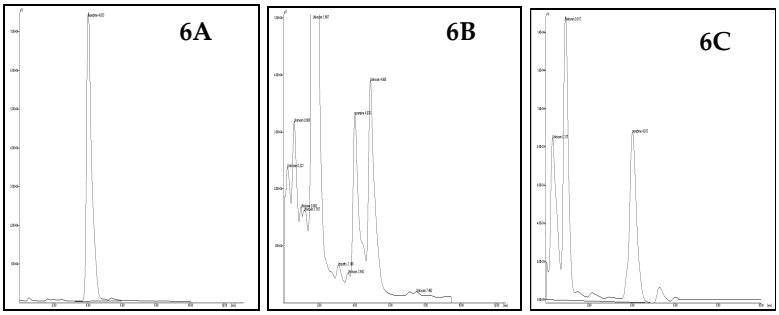


**Fig. 6. Chromatogram of Dry Heat Degradation Products of Reserpine in 6A) Standard 6B) Formulation A, 6C) Formulation B**

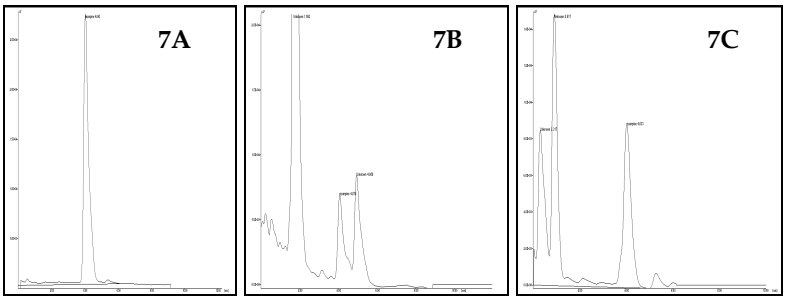


**Fig.7. Chromatogram of Wet Heat Degradation Products of Reserpine in 7A) Standard, 7B) Formulation A, 7C) Formulation B**

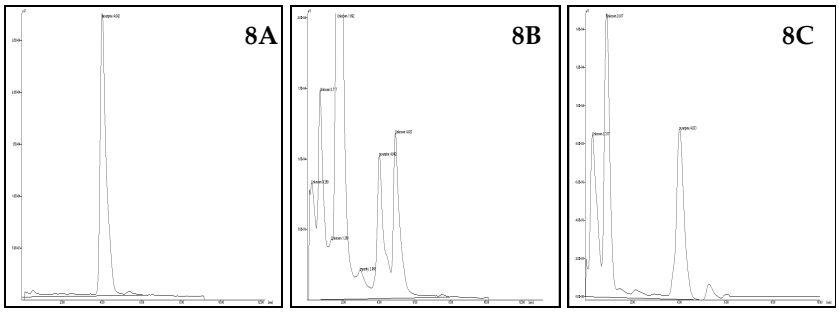


**Fig.8. Chromatogram and Its Day light Degradation Products of Reserpine in 8A) Standard, 8B) Formulation A, 8C) Formulation B**

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RESEARCH ARTICLE

# The Impact of Neighborhood on the Landform Classification Map.

Marzieh Mokarram* and Abdollah Seif

Department of Geographic Sciences and Planning, University of Isfahan, Iran.

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***Address for correspondence**
Marzieh Mokarram
Department of Geographic Sciences and Planning,
University of Isfahan,
Iran-81746-73441.
E.mail: m.mokarram.313@gmail.com.



**ABSTRACT**

The main objective of this study is to the impact of neighborhood on the landform classification in Dalani Mountain where located in Zagros mountain, Iran. In order to landform classification used Digital Elevation Models (DEMs) with 90 m resolution. In this study used semi-automated landform classification based on topographic position index (TPI). By using TPI, the study area was classified into landform category. In the study used circle, annulus, rectangle, and wedge neighborhood. The results show that there is variety of landform using difference neighborhood in the study area. But canyons/ deeply incised streams class and open slopes have maximum and minimum percentage respectively in all of the neighborhoods.

**Key words:** landform classification, Dalani Mountain, Digital Elevation Models (DEMs), Topographic Position Index (TPI), Neighborhood**.**

## INTRODUCTION

Geomorphometry provides a quantitative description of the shapes of landforms. According to Blaszczynski (1997), landforms are defined as specific geomorphic features on the earth`s surface, ranging from large-scale features such as plains and mountain ranges to minor features such as individual hills and valleys. Geomorphometric properties have been measured by calculating the geometry of the landscape manually (Horton, 1945; Miller, 1953; Coates, 1958). Recently, advances in computer technology, increased processing power, new spatial analytical methods and the increasing availability of digital elevation data have re-oriented geomorphometry (Pike, 1999). Landform units can be carried using various approaches, including automated mapping of landforms (Burrough et al., 2000; Meybeck et al., 2001; Schmidt and Hewitt, 2004; Saadat et al., 2008), classification of morphometric parameters, filter techniques,

cluster analysis and multivariate statistics (Dikau et al., 1995; Dikau, 1989; Adediran et al., 2004).The purpose in the study is the impact of neighborhood on the landform classification in Dalani Mountain where located in Zagros mountain, Iran.

**Case study**

The study area is Dalani Mountains, Iran, which is located at 34° 19′ 30″ to 34° 57′ 30″ N and 46° 49′ 12″ to 48° 18′ 34″ E, with area of 4,575.68 km$^2$ (Figure 1). The highest elevation in this area is 3302 m, which is located in the south of the basin, while the lowest elevation is 1284 m, which is located in the north of basin. The dataset for the area originates from a DEM with resolution of 90 m (SRTM), which was downloaded from http://srtm.csi.cgiar.org.

## MATERIALS AND METHODS

Weiss (2001) presented a very interesting to the concept of Topographic Position Index (TPI) and how it could be calculated (Weiss 2001).The TPI is the basis of the classification system and is simply the difference between a cell elevation value and the average elevation of the neighborhood around that cell. Positive values mean the cell is higher than its surroundings while negative values mean it is lower (Jenness 2006).TPI is naturally very scale-dependent. Scale is determined by the neighborhood used in the analysis. The TPI values reflect the difference between the elevation in a particular cell and the average elevation of the cells around that cell. The Neighborhood defines what cells are considered to be "around" that cell. In the study used from differentneighborhood that include: 1.Circle:A circular neighborhood defined by a radius length extending outward from the cell center. In the theory, this neighborhood should be composed of all grid cells whose cell centers lie within that distance of the focal cell center (Jenness 2006).2.Annulus:An annular neighborhood looks like a ring defined by an inner and outer radius length extending outward from the cell center.This neighborhood should be composed of all cells whose cell centers lie within this ring (Jenness, 2006).3.Wedge:A wedge-shaped neighborhood looks like a slice of pie cut out of a circular neighborhood and is defined by a starting angle, and ending angle, and a radius (Jenness, 2006). 4. Rectangle:A square or rectangular neighborhood defined by width and height, which will be centered around your focal cell center (Jenness, 2006).

## RESULTS

The results show that in the study area, there are 4 classes that consist of: canyons/deeply incised streams, plain small, open slope, and mountain top/high ridges. The impact of neighborhood on the landform classification for the study area show that Figure 6 to Figure 9 and Table 1 to Table 4.

## CONCLUSION

In this study, TPI in difference neighborhood such as circle, annulus, rectangle, and wedge was used to generate landform elements according to Weiss (2001). Digital elevation models used as inputs data in the study area. The results show that there is variety of landform using difference neighborhood in the study area. In all of the neighborhoods, canyons/ deeply incised streams class and open slopes have maximum and minimum percentage respectively.

## REFERENCES

1. Adediran, A.O., Parcharidis, I., Poscolieri, M., Pavlopoulos, K. (2004). Computer-assisted discrimination of morphological units on north-central Crete (Greece) by applying multivariate statistics to local relief gradients. Geomorphology 58, 357–370.

2. Blaszczynski, J.S. (1997). Landform characterization with geographic information systems. Photogrammetric Eng. Remote Sens., 63: 183-191.
3. Coates, D.R. (1958). Quantitative geomorphology of small drainage basins in Southern Indiana. 1st Edn., Columbia University, New York.
4. Dikau, R. (1989). The application of a digital relief model to landform analysis in geomorphology. In: Raper, J. (Ed.), Three-dimensional Applications in Geographical Information Systems. Taylor & Francis, London, pp. 51–77.
5. Dikau, R., Brabb, E.E., Mark, R.K., Pike, R.J. (1995). Morphometric landform analysis of New Mexico. ZeitschriftfürGeomorphologie, N.F., Suppl.-Bd., 101, 109-126.
6. Horton, R.E. (1945). Erosional development of streams and their drainage basins: Hydrophysical approach to quantitative morphology. Geol. Soc. Am. Bull., 56: 275-370.
7. Meybeck, M., Green P., Vorosmarty C.J. (2001). A New Typology for Mountains and Other Relief Classes: An Application to Global Continental Water Resources and Population Distribution, Mount. Research & Development 21, 34 - 45.
8. Miller, J.C. (1953). A quantitative geomorphic study of drainage basin characteristics in the cinch mountain area. Virginia and Tennessee, Technical Report 3.
9. Pike, R.J. (1999). A bibliography of geomorphometry, the Quantitative Representation of Topography-Supplement 3 (Open-File Report 99-140), US. Geological Survey.
10. Saadat, H., Bonnell, R., Sharifi, F., Mehuys, G., Namdar, M., Ale-Ebrahim, S. (2008). Landform classification from a digital elevation model and satellite imagery. Geomorphology 100, 453–464.
11. Schmidt, J., Hewitt, A. (2004). Fuzzy land element classification from DTMs based on geometry and terrain position. Geoderma 121, 243–256.
12. Weiss, A. (2001). Topographic Positions and Landforms Analysis (Conference Poster). ESRI International User Conference. San Diego, CA, pp. 9-13.
13. Jenness, J. (2006). *Topographic Position Index (tpi_jen.avx) extension for ArcView 3.x, v.* 1.3a. Jenness Enterprises, http://www.jennessent.com/arcview/tpi.htm, (last accessed on February 2010).

**Table 1. Wedge neighborhood for the study area.**

| Landform Classification | Area (km²) |
|---|---|
| Canyons, Deeply Incised Streams | 303.85 |
| Plains Small | 35.37 |
| Open Slopes | 9.50 |
| Mountain Tops, High Ridges | 220.10 |
| Sum | 568.84 |

**Table 2. Rectangle neighborhood for the study area.**

| Landform Classification | Area (km²) |
|---|---|
| Canyons, Deeply Incised Streams | 270.14 |
| Plains Small | 65.92 |
| Open Slopes | 26.74 |
| Mountain Tops, High Ridges | 206.03 |
| Sum | 568.84 |

**Table 3.Circle neighborhood for the study area**

| Landform Classification | Area (km²) |
|---|---|
| Canyons, Deeply Incised Streams | 270.14 |
| Plains Small | 65.92 |
| Open Slopes | 26.74 |
| Mountain Tops, High Ridges | 206.03 |
| Sum | 568.84 |

**Table 4.Annulus neighborhood for the study area**

| Landform Classification | Area (km²) |
|---|---|
| **Canyons, Deeply Incised Streams** | 276.70 |
| **Plains Small** | 60.76 |
| **Open Slopes** | 24.12 |
| **Mountain Tops, High Ridges** | 207.23 |
| **Sum** | 568.84 |

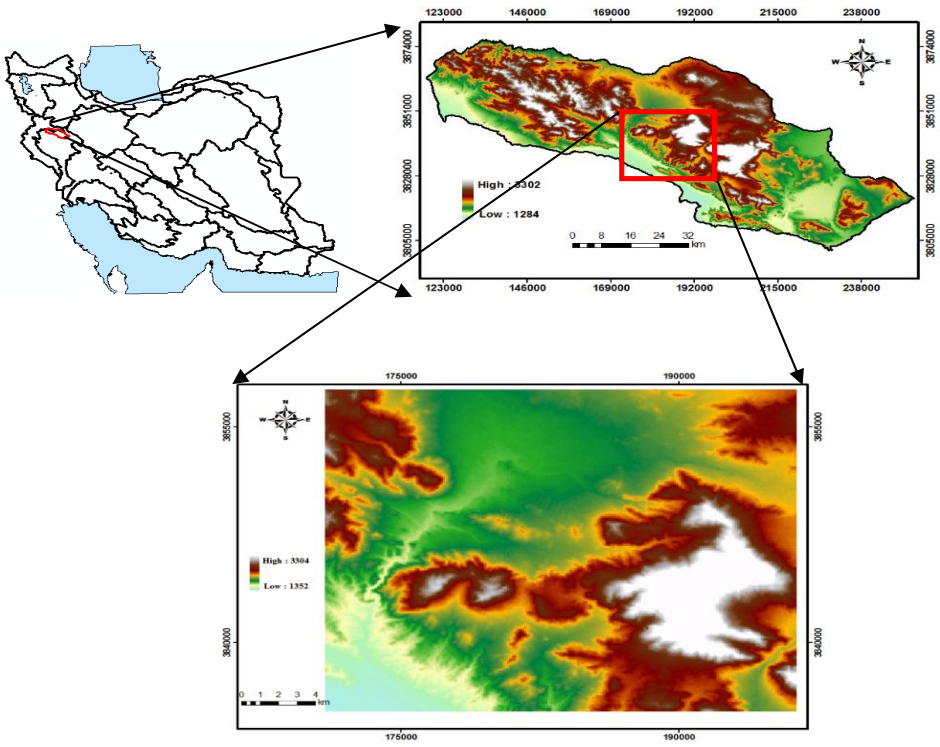


**Fig. 1: Location of the case study**

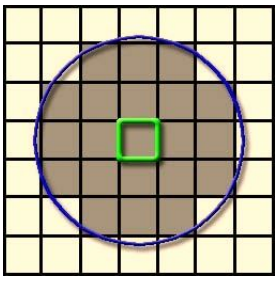

**Fig. 2: Circle neighborhood (Source:Jenness, 2006)**

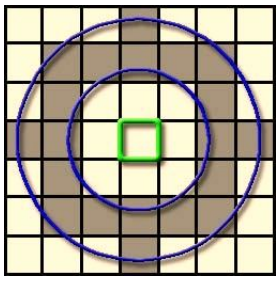

**Fig. 3: Annulus neighborhood (Source:Jenness, 2006)**

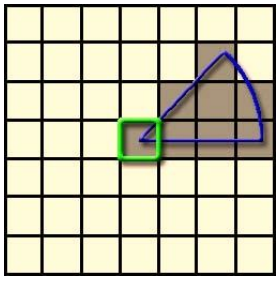

**Fig. 4: Wedge neighborhood (Source:Jenness, 2006)**

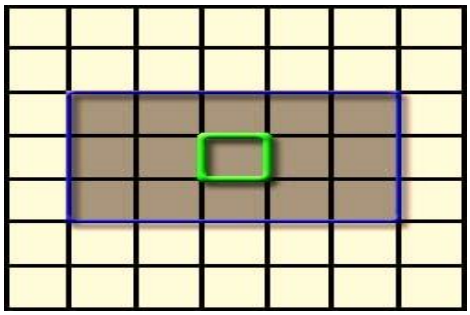

**Fig. 5: Rectangle neighborhood (Source:Jenness, 2006)**

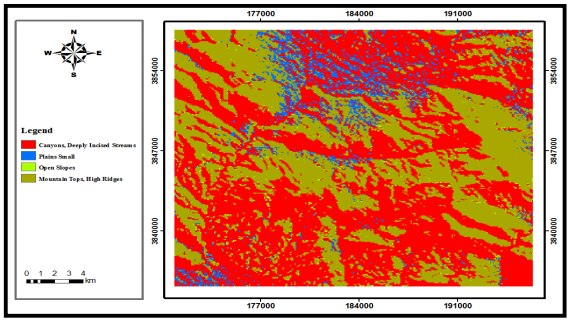


**Fig. 6: wedge neighborhood for the study area**

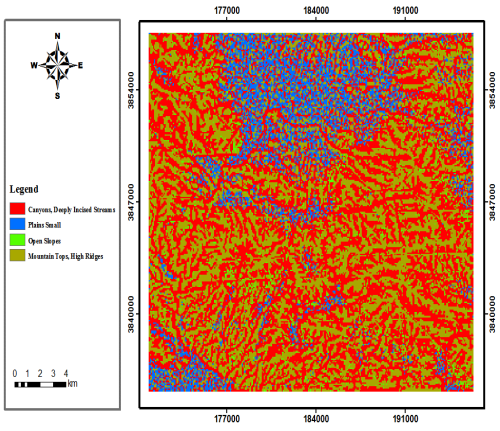


**Fig. 7: Rectangle neighborhood for the study area**

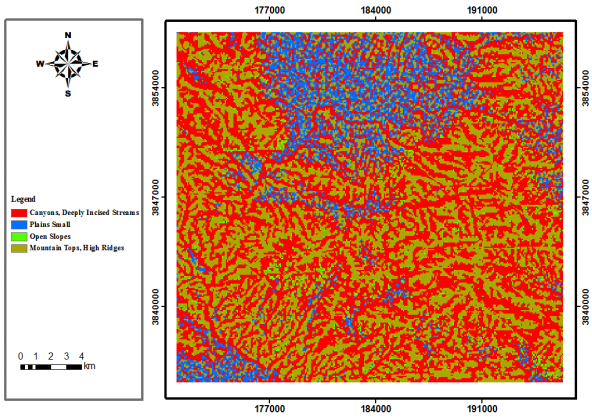


**Fig. 8: Circle neighborhood for the study area**

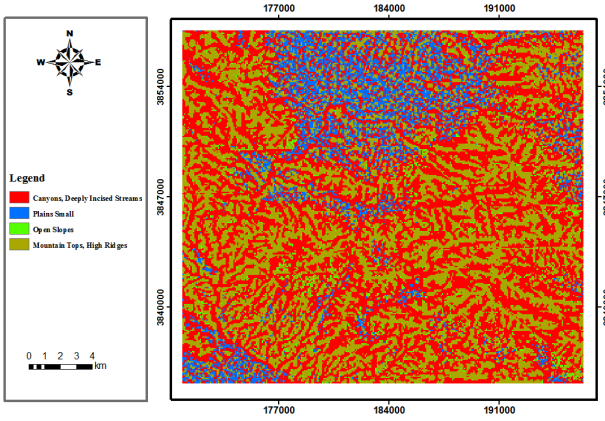


**Fig. 9: Annulus neighborhood for the study area**

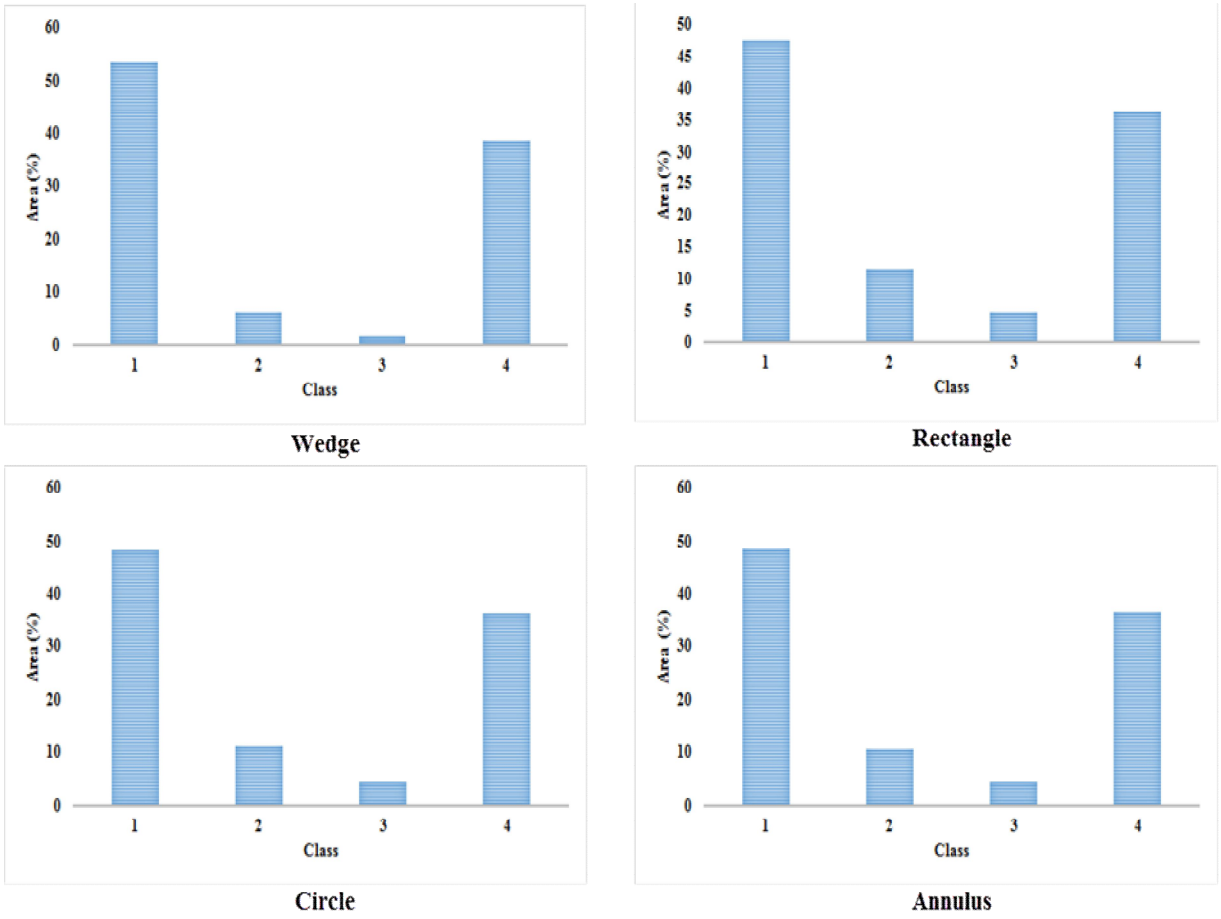


**Fig. 10: Percentage of each landform classes in the circle, annulus, rectangle, and wedge neighborhood**

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RESEARCH ARTICLE

# Composting of Korai Grass (*Cyperus corymbosus* Rottb.) Waste by Native Microbe and Earthworm Species in Ayyampalayam, Tiruchirappalli District,TamilNadu,India.

Malathi A.[1]*, C.D.Lethi[1] and Sujatha Ilangovan[2]

[1]PG and Research Department of Zoology, Holy Cross College (Autonomous), Tiruchirappalli- 620002. TamilNadu,India.

[2]Department of Biochemistry, Holy Cross College (Autonomous), Tiruchirappalli-620002. TamilNadu, India.

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***Address for correspondence**
Malathi A.
Research Scholar,
PG and Research Department of Zoology,
Holy Cross College (Autonomous),
Tiruchirappalli- 620002,TamilNadu,India.
Email: malathi.malar@yahoo.com



## ABSTRACT

Study was undertaken to decompose korai grass waste (*Cyperus corymbosus*), which contained high amount of cellulose, hemicellulose and lignin using native microbes and earthworms. Microbes were isolated from the korai field and their comparative efficiency to decompose the latter was studied using submerged culture method. The korai field predominantly harbored four species of microbes, namely *Bacillus firmus, B. circulans, Aspergillus niger* and *A.flavus.* Submerged culture of these microbes in korai containing media revealed that *B.firmus* exhibited maximum reduction in the weight, C content, C/N, cellulose, hemicelluloses and lignin of korai, indicating its effectiveness in decomposition of korai. Korai waste was pretreated with *B.firmus* for fifteen days and was subsequently composted with a local earthworm, *Lampito mauritii* in field condition. The pretreatment with the bacteria reduced the time duration for vermicomposting and the quantity and quality of the vermicompost was enriched compared to the control. The vermicast contained more of N,P and K.

**Keywords:** Microorganisms, *Cyperus corymbosus, B. firmus,* Vermicomposting, Physico-chemical characters.

## INTRODUCTION

The major crop in Ayyampalayam panchayat, Tiruchirappilli district, Tamil Nadu, India, which is located at 10.4593° N and 78.7027° E is korai grass (*Cyperus corymobosus* Rottb.). It is cultivated in 243 hectares out of 762 hectares of the cultivated land. A survey conducted by us has shown that the korai waste produced in this area is 14 t per hec/harvest, which is usually burned as a practice. Burning of such huge quantities of biomass from the two annual harvests would lead to environmental problems (Akimoto, 2003). Biodegradation through vermicomposting of Cyperus waste is not easy as it is hard with high amount of cellulose and lignin (Diab and Sandouka (2010). The rate of decomposition and the quality of compost can be enhanced by treating the wastes initially with certain efficient microflora (Singh and Sharma, 2002). *Pleurotus sajorcaju*, *Trichoderma viridae* and *Aspergillus niger* are known to degrade hemicelluloses and cellulose (Buswell and Chang, 1994; Banitez *et al*., 2000; Nedgwa and Thompson, 2001; Milala *et al*., 2009). This work was undertaken with the objective of isolating and using the native microbes to partially decompose the korai waste and to compost it fully using native earthworm species.

## MATERIALS AND METHODS

### Collection of water samples

Water samples were collected from the water channel in korai field in the study area. Samples were collected in sterile screw-cap bottles from eight sites. The bottles were wrapped in aluminium foil and transported to the laboratory. They were stored in refrigerator at 4°C for further analysis.

### Isolation of microorganisms

One ml from each sample was serially diluted ($10^{-1}$ to $10^{-7}$) in sterile conditions. From each dilution tube 1 ml of dilution fluid was transferred into the culture media. They were plated in triplicate on sterile nutrient agar plates and on sterile potato dextrose agar plates using pour plate method. The bacterial cultures were incubated at 37°C for 24 h. Incubations for fungi were carried out at room temperature for 7 to 10 days. The developed colonies of bacteria and fungi were picked randomly using a sterile inoculating loop and sub cultured by streaking on nutrient agar plates and potato dextrose agar plates respectively. The isolates were identified by Gram staining and morphological and biochemical tests using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

### Collection of korai waste

The korai waste was collected from field, washed thoroughly with water, dried in the oven at 70°C for 15 minutes. It was chopped into pieces of 2 to 3cm and stored in plastic containers.

### Submerged culture of isolated microbes in korai containing media

The korai degradation potential of the microbes was studied using the submerged culture method as reported by Sinegani *et al*., (2005). Nutrient broth (100ml) containing 10g korai was prepared in 250 ml conical flask. For each species 15 flasks were prepared. They were inoculated with bacteria from the pure cultures. They were incubated at the room temperature for 15 days. On every third day, the contents of three flasks were analyzed. In order to study the extent of degradation, the intact korai was removed, dried and weighed. From the difference between the initial and final intact korai weight, the percentage weight loss was calculated, which represented the degradation efficiency of the different species. These korai waste samples were analyzed for C, N, C/N ratio, cellulose, hemicelluloses and lignin. The degraded material from each flask was collected on a filter paper, dried to a biomass pellet and weighed.

For fungal culture, 1 cm diameter discs were cut from the fungal colonies and inoculated in 100 ml fungal culture medium in 250 ml conical flask and incubated at room temperature. Further processes were carried out as in the bacterial cultures.

### Stocking and mass culture of the selected microorganisms

The isolate of the microbe with the highest degradation potential was maintained and stored at 4 ± 1°C on slants of sub culture. It was sub cultured in 100 ml of the medium and incubated. After incubation, 10 ml of the inoculums was transferred to 1000 ml of the broth and kept in shaking incubator for mass multiplication.

### Pretreatment of korai waste with the microbe and vermin composting

The mass cultured microbes were applied in precomposting the korai waste in the field. Heap method (Sunitha *et al*., 1997) was used for this process. In each treatment the heap (6×2×2 feet size) of feed substrate was prepared using 650 kg of the korai waste with 60 – 70% moisture on a thin layer of sand on a polythene sheet. The compositions of the substrate used were 100% korai waste, 80% korai with 20% cow dung , 100% korai mixed with microbial culture and 80% korai and 20% cow dung mixed with microbes. The heaps were inoculated with 6.5 l culture of *B.firmus* and mixed well. All the four sets were prepared in triplicates. The time duration for the pretreatment was 15 days (Edwards and Bohlen, 1996). After the pretreatment, two thousand earthworms of the native species, *Lampito mauritii* were added to each heap. They were covered with gunny bags. The moisture content was maintained throughout. The time taken for the complete decomposition of the substrate in the different composition was noted. The compost was harvested, dried, weighed and analysed for its nutritive value. The C : N ratio was calculated from the measured values of C and N. The pH and organic matter were determined by the method of ISI Bulletin (1982) and total N, P, K, Ca and Mg as per the procedures described by Tandon (1993).

## RESULTS

### Microbes identified

The colony characteristics, biochemical tests and growth rate of the bacterial isolates from the culture of water samples from the fields of *C. corymbosus* are presented in Tables 1a, b and c respectively. Accordingly, the samples contained two species of bacteria, *Bacillus firmus* and *Bacilluus circulans.* The colony characteristic of the fungal isolates from the same sample is depicted in Table 2. The identified fungal species were *Aspergillus niger* and *Aspergillus flavus*.

### Korai decomposing ability of the native microbes

The submerged cultures carried out to find out the waste degradation potential of the selected microbes revealed that all the four microbes facilitated the process of degradation, but he bacterial species, *B.firmus* was observed to be more efficient than the others. This finding was evident from results of the weight loss of the korai waste (Table 3), the content of the C, N, C/N, cellulose, hemicellulose and lignin in the degrading waste (Table 4) and the fully degraded biomass pellets (Table 5) in submerged cultures of the isolated microbes. The percentage weight loss increased as a function of time in all the microbial cultures; but it was comparatively higher in bacterial cultures. The percentage weight loss of the korai was 48.70±0.75% and 51.53±1.60% respectively in the 15$^{th}$ day cultures of *B. circulans* and *B.firmus*. The amount of C reduced from 55.12±0.14 % to 52.06± 0.44 % during the 15 days of the culture of korai with *B.firmus*. On the other hand, the percentage N increased with in this culture. The N was 0.88±0.04 % and 1.22±0.07 % on the 0 and 15$^{th}$ day of the culture respectively. Consequently, the C/N in the *B.firmus* culture also exhibited a considerable reduction (59.68±1.07 and 40.56±0.72 respectively). A reduction in the C content was not observed with

the cultures of *B. circulans* and the fungi. Compared to the N in the decomposed waste by *B. firmus*, it was less in those produced from the cultures of *B.circulans* and the fungi species.

The cellulose, hemicelluloses and the lignin content gradually reduced in the degrading korai waste in the submerged cultures. On the 15th day of degradation, cellulose, hemicelluloses and lignin content was the least in the korai waste in the submerged culture of *B. firmus* (20.31±1.96%, 12.34±0.25% and 10.36±0.67% respectively). The cellulose, hemicelluloses and lignin contents of the degraded waste from the 15th day culture of *A. niger* were 24.11±1.25%, 17.36±0.31%, 18.34±0.24% respectively. The corresponding values for A.*flavus w*ere 23.14±1.66%, 15.33±0.37% and 18.22±0.54%. The highest percentage of cellulose 27.35±0.96%), hemicellulose (19.36±1.47%) and lignin (14.23±1.57%) was observed in the waste from the culture of *B.circulans*. The high potential of degradation of *B. firmus* was also apparent from the degraded pellet weight. The decomposed organic content was 5.15±0.16 g in the culture of *B. firmus* and it was 4.87±0.07 g, 3.90±0.15 g and 3.70±0.18 g respectively in *B.circulans, A. niger* and *A. flavus*. Thus, considering the results of the various parameters studied in the submerged culture, it was confirmed that *B. firmus* was the most efficient local microbial species to predecompose the korai waste.

**Duration for composting and compost out put**

The pretreatment of the korai waste with *B. firmus* accelerated the composting process. The time taken for the complete decomposition of the waste by *L. mauritii* in the two substrate media namely korai and korai with cow dung were 92.40±10.08 and 83.71±1.09 days respectively. When these media were pretreated with *B. firmus*, the corresponding number of days was 73.60±1.18 and 65.21±2.19 days.

**Nutritional characteristics of the vermicompost**

The nutritional characteristics of the vermicopost produced from the different composting combinations are presented in Table 6. Pretreatment with the microbe generated vermicompost with higher quantities of N, P, K and calcium. When the korai waste was pretreated with *B. firmus* and composted with *L. mauritii*, the N content in the vermicompost increased from 0.90±0.01 % to 2.21±0.00%. The corresponding values of N in the korai with cow dung combination were 1.25±0.00% and 2.50±0.00%. The amount of P in the compost was 0.71±0.01% and 0.86±0.02% respectively in the substrate media of korai and korai with cow dung. When these media were pretreated with *B. firmus*, the corresponding values were 0.91±0.01% and 0.94±0.00%. Similar trend was also observed with K and Ca. The decrease in the OC and C/N ratio after the composting process was pronounced with the pretreated substrates indicating a higher degree of the decomposition of the organic matter. In all the composting combinations, there was a decrease in the pH of the compost. However, this decrease was more pronounced in the substrate combination with cow dung.

## DISCUSSION

The water samples from the korai fields predominantly contained two bacterial species, *B.firmus* and *B.circulans* and two fungi, *A.niger* and *A.flavus*. *Bacillus* and *Aspergillus* are common genus found in biomass containing areas. Bacterial isolates from cabbage cultivated soil contained *B. stearothermophillus, B. circulans* and *B. macerans*. Gaddeyya *et al*., (2012) also report the occurrence of *A. flavus and A. niger* in agricultural fields.

The data from the submerged culture reveal that all the four microbes are decomposers of korai waste. The highest percentage of weight loss in korai waste observed with *B.firmus* is 51.53% in 15 days. Zaved *et al*., (2008) have recorded that solid kitchen waste is decomposed by *Bacillus sp. reducing* its weight. Hoitink (1980) has reported a weight loss of 25.76% in composted bark biomass using *Trichoderma* strain in 30 days. Decomposition with the microbes results in decreased C, C/N ratio, cellulose, hemicellulose and lignin content in the korai waste. The

percentage decrease in lignin from the third to fifteenth days is nearly 65%. The findings in this study are parallel to those obtained by Kumar and Shweta (2011), who demonstrated a decrease in cellulose, hemicellulose and lignin in wood waste treated with different microbes. According to them, *P. chrysosporium* is found to be an effective lignin degrader (66 ± 3.20 to 54 ± 2.20% decrease), though the best results (83 ± 7.0% decrease) in terms of ligno-cellulosic decomposition is recorded, when *T. reesi* is added to wood waste. The cellulose and lignin degrading capacity of microbes is attributed to their degrading enzymes (Benitez *et al*., 1999 and Kumar *et al*., 2010a). Lignin is known to be the most recalcitrant material present in the by-products of sugar industries and decomposes only at the later stage of decomposition (Manna *et al*., 2003). Cellulose, hemicelluloses and lignin decreased significantly during microbial pre-decomposition and subsequent vermicomposting (Kumar and Shweta, 2011).

Among the four microbes, *B.firmus* exhibits the greatest degree of korai waste degradation potential. Rao and Lakshmi (2007) suggest that *B. firmus* being active at mesophilic range of temperatures, has made the organism more applicable in developing environment friendly technologies, as the basic pre requisite for the development of any environment friendly technology is the temperature at which the organisms operate. Neutral and alkaline proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment friendly technologies.

The pretreatment of korai waste with *B.firmus* for fifteen days has considerably reduced the time duration for vermicomposting. Corroborative finding that microbial pre-decomposition of sugar cane waste from 40 to 20 days would enable its conversion into value added products in a short time period is reported by Kumar *et al*. (2010a). Kumar and Shweta (2011) have also recorded enhancement of wood waste decomposition by microbial inoculation prior to vermicomposting. The pretreatment with *B,firmus* improved the quality of the vermicompost with increased N, P and K content. Production of enriched vermicompost adding microbial inoculants and nutrients is now gaining popularity. (Singh and Sharma 2002; Anilkumar *et al*., 2007; Hashemimajd and Golchin, 2009; Kumar *et al*.,2010). Enriched quality of the vermicast on inoculation of a consortium of microorganisms such as *A. niger*, *P. sajor-caju*, *A. chroococcum* and *T.harzianum* during vermicomposting to crop residues and farm yard manure is documented by Singh and Sharma (2002). According to Kumar and Singh (2001), the inoculated microbial strains proliferated rapidly, fixed nitrogen, solubilised and added native phosphate.

## CONCLUSION

In conclusion pretreatment and subsequent vermin composting with locally available bacterial species, *B.firmus* and the earthworm, *L.mauritii* respectively have not only facilitated the process of decomposition of the korai waste with high cellulose and lignin content, but have also enhanced the quantity and quality of the compost. This approach is an ecofriendly procedure to manage the large amount of the korai waste generated in the village and convert it to value added product.

## REFERENCES

1. Akimoto, H., (2003) Global air quality and pollution. *Sci.* 302:1716-1719.
2. Diab. A. and Sandouka.M. (2010) Biodegradation of Polycyclic Aromatic Hydrocarbon (PAHs) in the Rhizosphere Soil of *Cyperus conglomeratus*, an Egyptian Wild Desert Plant. *Natur. Sci.* 8(12):144-153.
3. Anilkumar, A.S., Nair, K.H. and Sherief, A.K. (2007) "Utilization of enriched coir pith vermicompost in organic mediculture". *Plant Archives*.7:617-620.
4. Benitez, E., Nogales, R., Elvira, C., Masciandaro, G., and Ceccanti, B. (1999) Enzyme activities as indicators of the stabilization of sewage sludges composting with *Eisenia foetida*. *Bioresour*. *Technol*. 67:297-303.
5. Banitez, E., Nagales, R., Masciandraro, G., and Ceccanti, B. (2000) Isolation of isoelectric focusing of humic-urease complexes from earthworm (*Eisenia foetida*) processed sewage-sludges. *Biol*. *Fert*. *Soils*. 31:489-493.

6. Buswell, J.A. and Chang, Shu-ting. (1994) Biomass and extracellular hydrolytic enzymes production by six mushroom species grown on soybean waste. *Biotechnol. Lett*. 16:1317-1322. Edwards, C. A., and Bohlen, P.J. (1996) Biology and Ecology of Earthworms, Chapman and Hall, London, UK, 3rd edition.
7. Gaddeyya, G., Shiny, N. P., Bharathi, P. and Kumar P.K. (2012) Isolation and identification of soil mycoflora in different crop fields at Salur Mandal. *Adv. In. App. Sci. Res*. 3:2020-2026.
8. Hashemimajd, K. and Golchin, A. (2009) "The effect of iron-enriched vermicompost on growth and nutrition of tomato," *J. Agri. Sci. and Tech*.11: 613-621.
9. Hoitink, H.A.J. (1980) Composted Bark, A lightweight growth medium with fungicidal properties. *Plant Disease*. 64: 142-147.
10. Holt, J.G., Krieg, N.R., Sneathm, P.H.A. Staley, J.T. and Williams, S.T. (1994) Bergey's Manual of Determinative Bacteriology, 9th Edn. Baltimore, MD: Williams and Williams.
11. ISI Bulletin. Manak Bhadur, Bhadur Shah Jafar Marg, New Delhi 1982.
12. Kumar, R. and Shweta. (2011) "Enhancement of wood waste decomposition by microbial inoculation prior to vermicomposting," *Bioresour. Technol*. 102:1475-1480.
13. Kumar, R., Verma, D., Singh, B.L., Kumar, U. and Shweta. (2010a) "Composting of sugar-cane waste by-products through treatment with microorganisms and subsequent ermicomposting". *Bioresour. Technol*.101:6707-6711.
14. Kumar, V. and Singh, K.P. (2001) Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. *Bioresour*. *Technol*.76:173-175.
15. Manna, M.C., Singh, M., Kundu, S., Tripathi, A.K., and Takkar, P.N. (2003) Growth and reproduction of vermicomposting earthworm *Perionyx excavates* as influenced by food materials. *Biol. Fertil. Soils.* 24: 129-132.
16. Milala, M.A., Shehu, B.B., Zanna, H., and Omosioda, V.O. (2009) Degradation of agrowaste by cellulose from *Aspergillus candidus*. *Asian* J. *Biotechnol*. 1: 51-56.
17. Nedgwa, P.M. and Thompson, S.A. (2001) Effects of C to N ratio on vermicomposting of biosolids. *Bioresour*. *Technol*.75: 7-12.
18. Rao. K., and Lakshmi, M. (2007) Alkaline Protease from *Bacillus firmus* 7728. *Afri. J. of Biotech.* 6: 2493-2496.
19. Sinegani, S.A.A., Emtiazi, G., Hajrasuliha, S. and Shariatmadari, H. (2005) Biodegradation of some agricultural residues by fungi in agitated submerged cultures. *Afr. J. of Biotechnol*. 4:1058-1061.
20. Singh, A. and Sharma, S. (2002) "Composting of a crop residue through treatment with microorganisms and subsequent vermicomposting," *Bioresour Technol*.85:107-111
21. Sunitha, N.D., Giraddi, R.S. Kulkarni, K.A. and Lingappa, S. (1997) Evaluation methods of vermicomposting under open field conditions. *Kar. J. Agri. Sci*.10: 987-990.
22. Tandon, H.L.S. (Ed). (1993) Methods of analysis of soils, plants, waters and fertilizers. Fertilizers Development and Consultation Organisation, New Delhi, India.
23. Zaved, H.K., Rahman, M., Rahman, M., Rahman, S.M.Y., Artafat. and M. Rahman (2008) Isolation and characterization of effective bacteria for solid waste degradation for organic manure. KMITL Sci. Tech. J. 8: 44-55.

**Table 1a:Morphological and growth characteristics of bacterial isolates from the pure culture of water sample collected from the fields of *C. corymbosus*.**

| Morphological and Growth Characteristics | D1 | D2 | D3 | D4 | D5 | D6 | D7 |
|---|---|---|---|---|---|---|---|
| Configuration | Circular | Circular | Circular | Circular | Circular | Circular | Circular |
| Margin | Irregular | Rhizoid | Irregular | Irregular | Irregular | Irregular | Rhizoid |
| Elevation | Flat | Slightly raised | Flat | Flat | Flat | Flat | Slightly raised |
| Opacity | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque |
| Colour | Cream | White | White | Cream | White | White | Cream |
| Gram's reaction | + | + | + | + | + | + | + |
| Cell shape | Rods | Rods | Rods | Rods | Rods | Rods | Rods |
| Size | 0.1 | 0.2mm | 0.1 | 0.1 | 0.2 | 0.3 | 0.1mm |
| Spores | + | + | + | + | + | + | + |
| Endospore | + | + | + | + | + | + | + |
| Motility | + | + | + | + | + | + | + |

Key: + Positive

**Table 1b: Results of the biochemical tests of the bacterial isolates from the pure culture of water sample collected from the fields of *C. corymbosus* and the identified genus.**

| **Biochemical Tests** | **D1** | **D2** | **D3** | **D4** | **D5** | **D6** | **D7** |
|---|---|---|---|---|---|---|---|
| Indole | _ | _ | _ | _ | _ | _ | _ |
| Voges proskaver | _ | _ | _ | _ | _ | _ | _ |
| Citrate utilization | _ | _ | _ | _ | _ | _ | _ |
| Mannitol | + | + | + | + | + | + | + |
| Catalase | + | + | + | + | + | + | + |
| Urease | _ | _ | _ | _ | _ | _ | _ |
| Key: + Positive | Bacillus | Bacillus | Bacillus | Bacillus | Bacillus | Bacillus | Bacillus |

**Table 1c Results of the microbial growth tests of the bacterial isolates from the pure culture of water sample collected from the fields of *C. corymbosus* and the identified species**

| **Sl.No.** | **D 1/D3/D4/D5/D6** | **D2/D7** |
|---|---|---|
| Growth at 40°C | - | + |
| Growth at 50° C | + | - |
| Growth at 60° C | - | - |
| Growth in 7% NaCl | + | + |
| Growth at pH<6 | + | + |
| Identification | *Bacillus circulans* | *Bacillus firmus* |

Key: + Positive

**Table 2 : Morphological and growth characteristics of bacterial isolates from the pure culture of water sample collected from the fields of *C. corymbosus*.**

| Parameters | C1 | C2 | C3 |
|---|---|---|---|
| Culture | | | |
| Colour | Fluffy white | White | Yellowish green |
| Mycella | Elevated | Elevated | Elevated |
| Slide | | | |
| Spore | Single celled | Single celled | Single celled |
| Conidiophore | + | + | + |
| Vesicle | + | + | + |
| Hyphae | + | + | + |
| Key: + Positive | *Aspergillus niger* | *Aspergillus niger* | *Aspergillus flavus* |

**Table 3: Weight loss (%) of the waste of korai, *C.corymbosus* in the submerged cultures of locally identified bacterial and fungal species on different days of degradation.**

| Experiment | 3rd day | 6th day | 9th day | 12th day | 15th day |
|---|---|---|---|---|---|
| *Bacillus circulans* | 4.66±0.70 | 21.43±1.11 | 29.26±1.13 | 34.80±3.93 | 48.70±0.75 |
| *Bacillus firmus* | 5.95±0.53 | 23.53±1.20 | 31.56±1.19 | 41.70±1.05 | 51.53±1.60 |
| *Aspergillus niger* | 18.86±1.17 | 21.90±1.85 | 27.26±1.41 | 32.03±0.65 | 39.06±1.55 |
| *Aspergillus flavus* | 17.20±0.91 | 22.56±1.05 | 26.13±0.61 | 31.13±1.10 | 37.00±1.83 |

All the values are mean and standard deviation of three replicates

**Table 4: C, N, C/N ratio, cellulose, hemicellulose and lignin content (%) in the waste of korai, *C.corymbosus* in the submerged cultures of locally identified bacterial and fungal species on different days of degradation.**

| Microorganisms | Parameters | Values | | | | |
|---|---|---|---|---|---|---|
| | | 3rd day | 6th day | 9th day | 12th day | 15th day |
| *B.circulans* | Cellulose (%) | 33.14±0.19 | 32.46±0.02 | 30.48±0.16 | 28.36±0.17 | 27.35±0.96 |
| | Hemicellulose(%) | 25.54±0.18 | 22.45±0.03 | 20.26±0.47 | 20.06±0.18 | 19.36±1.47 |
| | Lignin (%) | 35.16±1.26 | 28.34±1.34 | 19.03±0.36 | 15.49±0.14 | 14.23±1.57 |
| | Organiccarbon (%) | 59.12±0.37 | 56.25±0.41 | 54.12±0.78 | 54.28±0.35 | 52.16±1.25 |
| | Nitrogen (%) | 0.72±0.04 | 0.71±0.03 | 0.68±0.07 | 0.78±0.04 | 0.85±0.01 |
| | C : N ratio | 77.55±0.13 | 72.13±0.41 | 68.13±0.25 | 65.27±0.19 | 61.06±0.07 |
| *B.firmus* | Cellulose (%) | 35.14±0.19 | 32.46±0.07 | 30.48±0.15 | 25.36±0.16 | 20.31±1.96 |
| | Hemicellulose(%) | 23.54±0.18 | 22.45±0.03 | 19.26±0.47 | 14.06±0.18 | 12.34±0.25 |
| | Lignin (%) | 30.16±1.34 | 29.34±1.24 | 15.03±0.16 | 12.49±0.04 | 10.36±0.67 |
| | Organiccarbon (%) | 55.12±0.14 | 53.06±0.07 | 52.01±0.87 | 53.27±0.07 | 52.06±0.44 |
| | Nitrogen (%) | 0.88±0.04 | 0.91±0.03 | 0.96±0.21 | 1.12±0.06 | 1.22±0.07 |
| | C : N ratio | 59.68±1.07 | 56.42±0.08 | 50.28±0.17 | 42.17±1.35 | 40.56±0.72 |
| *A.niger* | Cellulose (%) | 33.04±1.19 | 29.41±0.72 | 25.48±0.35 | 22.26±0.56 | 24.11±1.25 |
| | Hemicellulose(%) | 28.53±0.16 | 21.45±0.02 | 19.26±0.42 | 16.06±0.28 | 17.36±0.31 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Lignin (%) | 31.16±1.32 | 28.34±1.23 | 22.03±0.13 | 18.49±0.02 | 18.34±0.24 |
| | Organiccarbon (%) | 61.37±0.09 | 60.41±20.12 | 60.18±0.07 | 57.25±0.19 | 55.21±0.09 |
| | Nitrogen (%) | 0.61±0.02 | 0.60±.0.05 | 0.63±0.01 | 0.67±0.04 | 0.68±0.07 |
| | C : N ratio | 97.35±0.41 | 91.32±0.48 | 91.36±1.78 | 83.15±0.16 | 74.07±0.19 |
| *A.flavus* | Cellulose (%) | 38.14±0.29 | 35.46±0.02 | 25.48±0.25 | 24.36±0.66 | 23.14±1.66 |
| | Hemicellulose(%) | 26.53±0.16 | 24.45±0.02 | 17.26±0.42 | 17.01±0.28 | 15.33±0.37 |
| | Lignin (%) | 35.16±1.32 | 27.34±1.23 | 20.03±0.13 | 20.49±0.02 | 18.22±0.54 |
| | Organiccarbon (%) | 65.13±0.51 | 65.07±0.27 | 64.36±0.35 | 58.15±0.61 | 58.02±0.15 |
| | Nitrogen (%) | 0.63±0.02 | 0.65±0.02 | 0.68±0.03 | 0.69±0.02 | 0.10±0.03 |
| | C : N ratio | 92.37±1.28 | 94.75±0.51 | 90.56±0.02 | 81.72±0.16 | 80.55±0.27 |

All the values are mean and standard deviation of three replicates.

**Table5 :Dry weight (g) of the biomass pellet from the submerged cultures of locally identified bacterial and fungal species in the medium containing the waste of korai (*C.corymbosus)* on different days of degradation.**

| **Experiment** | **3rd day** | **6th day** | **9th day** | **12th day** | **15th day** |
|---|---|---|---|---|---|
| *Bacillus circulans* | 0.46±0.07 | 2.14±0.11 | 2.99±0.21 | 3.21±0.33 | 4.87±0.07 |
| *Bacillus firmus* | 0.60±0.06 | 2.35±0.12 | 3.15±0.11 | 4.17±0.10 | 5.15±0.16 |
| *Aspergillus niger* | 1.88±0.11 | 2.19±0.18 | 2.72±0.14 | 3.20±0.60 | 3.90±0.15 |
| *Aspergillus flavus* | 1.74±0.13 | 2.25±0.10 | 2.61±0.05 | 3.11±0.11 | 3.70±0.18 |

All the values are mean and standard deviation of three replicates.

**Table 6: Nutritional characteristics of the vermicompost produced during the decomposition of korai waste by *L.mauritii* in two substrate media after pre-treatment with *B.firmus***

| **Parameter** | **Korai waste** | | | **Korai waste with cow dung** | | |
|---|---|---|---|---|---|---|
| | **Initial** | **Control** | **Pretreatment with *B.firmus*** | **Initial** | **Control** | **Pretreatment with *B.firmus*** |
| pH | 7.31±0.02 | 6.52±0.02 | 6.62±0.01 | 7.27±0.07 | 5.43±0.02 | 6.25±0.012 |
| OC % | 25.10±0.06 | 27.14±0.02 | 24.14±0.01 | 27.66±0.03 | 27.27±0.01 | 20.09±0.06 |
| OM % | 90.27±0.25 | 43.23±0.01 | 24.23±0.01 | 95.74±0.31 | 35.04±0.02 | 33.16±0.01 |
| N % | 0.30±0.02 | 0.90±0.01 | 2.21±0.00 | 0.79±0.06 | 1.25±0.00 | 2.50±0.00 |
| P % | 0.28±0.05 | 0.71±0.01 | 0.91±0.01 | 0.37±0.08 | 0.86±0.02 | 0.94±0.00 |
| K % | 0.22±0.01 | 0.82±0.01 | 0.86±0.01 | 0.30±0.02 | 0.71±0.01 | 0.90±0.00 |
| Ca% | 5.39±0.12 | 15.04±0.02 | 24.01±0.01 | 7.42±0.26 | 35.25±0.03 | 42.51±0.15 |
| C : N ratio | 78.55±0.08 | 29.80±0.04 | 10.90±0.02 | 32.51±0.13 | 21.39±0.01 | 8.03±0.02 |
| Mg% | 3.19±0.09 | 1.18±0.00 | 0.48±0.00 | 3.19±0.12 | 0.83±0.00 | 0.53±0.00 |

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RESEARCH ARTICLE

# Molecular Genetic Variation and Phylogeny of Genus *Rhynocoris* Based on Mitochondrial Cytochrome c Oxidase Subunit I Gene (Heteroptera: Reduviidae)

Arul Baskar[1]*, S. Dhanasekaran[2], K. G. Tirumurugaan[2], A. R. Vignesh[2] and B. Prakash[3]

[1]Department of Zoology, S. B. K. College, Aruppukottai - 626 101, Tamil Nadu, India.
[2]Department of Animal Biotechnology, Madras Veterinary College, Chennai - 600 007, Tamil Nadu, India.
[3]Department of Biotechnology, PGP College of Arts and Sciences, Namakkal - 637 206, Tamil Nadu, India.

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***Address for correspondence**
Dr.A.Baskar,
Assistant Professor,
Department of Zoology, S. B. K. College,
Aruppukottai- 626 101, Tamil Nadu, India.
E.mail: drarulbaskar@gmail.com



## ABSTRACT

The assassin bugs of the *Rhynocoris* species are well known for their role in bio-control potential of the insect pests, yet their taxonomic relationships have not been established at molecular level. In this study, we studied the genetic variations of mitochondrial gene cytochrome c oxidase subunit I (COI) of four *Rhynocoris* species. A phylogenetic analysis of 593 bp DNA sequence of the four *Rhynocoris* species was constructed and analyzed by neighbor-joining method. The results showed that the four *Rhynocoris* species formed two well-separated clades, which included *R. marginatus* as clade - I and *R. kumarii*, *R. longifrons* and *R. fuscipes* as clade-II in which *R. kumarii* and *R. longifrons* shared 100% identity among them and 96.1% with *R. fuscipes*. The *Rhynocoris* species represented in clade 1 shared 73.4% identity with *R. marginatus*, which is represented in clade 2. Vast variation in the nucleotide and amino acid sequence of *R. marginatus* was observed compare to other sequence, whereas no variation among *R. kumarii* and *R. longifrons* was observed.

**Keywords:** *Rhynocoris*, Phylogeny, mtDNA, COI, Taxonomic relationships.
**Abbreviations:** PCR: Polymerase Chain Reaction, COI: Cytochrome c oxidase subunit I, NJ Tree: Neibour Joining Tree.

## INTRODUCTION

The assassin bugs of the genus *Rhynocoris* are from diverse group of mostly predatory insect pests with currently close to 190 species described worldwide [23, 17]. Among the several Reduviids species, four *Rhynocoris* species were reported to possess the potential to act as bio-control predators against a number of insect pests of agricultural and horticultural plantations as well as the forest vegetation [2, 4, 5]. This genus was established by F.A. Kolenati in 1857, and revised by G.W. Kirkaldy in 1902. However, the genus *Rhynocoris* had very often manifested a significant variability, rather complicating the identification of species [22]. It could be retained as a taxonomic unit for purely practical positions, but was difficult to determine its phylogenetic and taxonomic relationship. In classification, 17 *Rhynocoris* species were recognized based on four morphological characters of Indian origin, out of which four species were considered in the present study: *R. marginatus*, *R. fuscipes*, *R. kumarii* and *R. longifrons*. In the recent years, several new species of *Rhynocoris* have been described in India, viz., *R. costalis*, *R. cruralis*, *R. fuscipes*, *R. kumarii*, *R. lapidicola*, *R. longifrons*, *R. marginatus*, *R. marginellus*, *R. monticola*, *R. nilgiriensis, R. nysiiphagus*, *R. pygmaeus*, *R. reuteri*, *R. shevroyensis*, *R. sequalus*, *R. tristicolor* and *R. varians* [17, 6]. The taxonomic relationship of the four *Rhynocoris* species was determined using morphological and cytological characters [4, 21]. However, the phylogeny and taxonomic relationships among *Rhynocoris* species at molecular level are still poorly understood and, hence remained unpublished.

Mitochondrial DNA (mtDNA) is being widely used for reliably elucidating the evolutionary relationships of insects both at species and intraspecific levels [1, 12, 29]. The insect mtDNA contains 13 protein coding genes, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes [26], in which the COI gene is one of the large protein-coding genes in the invertebrate mitochondrial genome. Due to its rapid evolutionary rate, the COI gene is suitable for the comparative studies of species within the same genus or family [18, 9]. In this study, we amplified and sequenced the partial COI gene from the mtDNA of four *Rhynocoris* species, namely *R. marginatus*, *R. fuscipes*, *R. kumarii and R. longifrons*, and then analyzed the taxonomic relationships within these *Rhynocoris* species at molecular level.

## MATERIALS AND METHODS

### Collection of *Rhynocoris* species

The adult insect samples representing four *Rhynocoris* species were collected from Ayyanar Kovil Tropical Rainforest bordering an agroecosystem (altitude 389 MSL, latitude 76. 39ºE and 10.45ºN) near Rajapalayam, Virudhunagar District, Tamil Nadu, Southern India, during 2008-2010. Minimum 10 insect samples were collected for each species and all the insect specimens were stored in absolute ethanol. Selected samples (n=5) were processed for DNA extraction following complete removal of ethanol. The list of species, their habitat, the specimen used and GenBank accession numbers are listed in Table 1. Total mtDNA was extracted from thoracic muscle or leg muscle of each individual of the four *Rhynocoris* species by phenol-chloroform method with minor modification as described by [10] by addition of 30µl of proteinase K (20mg/ml) and incubated for 16 hrs at 52°C.

### Polymerase Chain Reaction, sequencing and analysis

An approximate of 710 bp DNA fragment of the COI gene was amplified for each *Rhynocoris* species by two universal COI gene specific primers: LCO1490F (5′- GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198R (5′-TAAACTTCAGGGTGA CCAAAAAATCA-3′) as reported previously [11]. The PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. The PCR products were purified using the HiYield PCR/Gel extraction kit (RBC Biosciences, Taiwan) following the manufacturer's instructions. The purified amplicons were sequenced using the Big Dye Terminator Cycle sequencing ready reaction kit (Applied Biosystems Inc., USA) in the ABI prism 3100 Genetic analyzer. The sequencing of COI amplicons of each species (n=5) was performed with the

forward and reverse primer, and assembled to get the consensus sequence[20]**.**Sequenced COI gene of each *Rhynocoris* species was assembled using DNASTAR package (SeqMan) and analyzed. Assembled COI gene sequences of each species were used for construction of phylogenetic tree by Neighbor-joining (NJ) method PAUP 4.0b4a [28]. The Kimura two-parameter model was used for correcting possible multiple hits of nucleotide substitutions [14]. Percentage identity and divergence was rooted using *R. marginatus* as standard position (Table 2).

## RESULTS AND DISCUSSION

In this study, partial COI gene of four *Rhynocoris* species, i.e., *R. marginatus*, *R. fuscipes*, *R. kumarii and R. longifrons,* were sequenced and characterized for the first time and submitted in GenBank, the accession numbers were listed in Table 1. In our initial attempt for DNA extraction with phenol: chloroform method with 10µl of proteinase K (20ug/ml) yielded 12 ± 4.0 ng/ul of DNA and faint or no PCR amplification. Hence we modified the conditions for DNA extraction from *Rhynocoris* species. Increasing the proteinase K concentration from 0.2mg/ml to 0.6mg/ml and coupled with increase of incubation time to 16 hrs yielded 104.0 ± 7.0 ng/ul of DNA, this was applied for all the samples.

PCR amplification of *Rhynocoris* COI gene with specific primers produced single band of approximately 710 bp in all the species (Figure 1), upon sequencing we obtained partial nucleotide sequence of the amplified regions of the COI gene for four *Rhynocoris* species (Table 1). Since we obtained 593bp of sequence length for *R. Kumarii* and *R. longifrons*, remaining two species COI nucleotide sequences were trimmed accordingly. Multiple alignments of the COI gene sequences of *Rhynocoris* species demonstrate no nucleotide indels (insertion/deletion) within orthologs (Figure 3).The sequences can also be fully translated using mitochondrial code with an intervening stop codon (Figure 4). Base compositional information for the COI sequences was also estimated from the aligned sequences. For mainly *R. kumarii* and *R. longifrons*, approximately 593 bp sequences from the COI gene contained 171 variable sites (28.8%), which were not evenly distributed among the three codon positions with 101 (59.06%) substitutions were detected at the third codon position, 40 (23.39%) at the first codon position, and 30 (17.54%) at the second codon position (Figure 3). The distribution of variable sites reflected the occurrence of majority of substitutions at synonymous sites (codon third position and leucine codon first positions).

While analyzing the amino acid sequence of four species by multiple alignment, 33 amino acid variations in the *R. marginatus*, two amino acid variations in *R. fuscipes* and no variation among *R. kumarii* and *R. longifrons* was observed (Figure 4). The nucleotide composition of this COI gene was calculated from the four *Rhynocoris* species; as a result, it varied slightly depending on different *Rhynocoris* species in the same genus, but was approximately found to be adenine (30.4%), thymine (39.6%), guanines (14.5%) and cytocine (15.5%). Overall, the base composition shows extreme bias being AT 70% and GC 30% (Table 1). The AT bias is strongest at the second codon position (91.51%), much higher than the first (25.2%) and third (28.36%) codon positions.

Percentage identity and divergence of the partial nucleotide sequence of the COI gene from the four *Rhynocoris* species were summarized in Table 2, and were calculated using Mega version 3.1 [15]. In our molecular phylogeny *R. kumarii* and *R. longifrons* were found to be much closely related species supported by NJ tree, and the percentage identity between them is 100% (Table 2). According to the concept of DNA-based taxonomy [8] the percentage identity and divergence of partial nucleotide sequence of the mitochondrial COI gene of *R. kumarii* and *R. longifrons* showed similar values (Table 2). But, different percentages of identity and divergence values are observed from *R. marginatus* and *R. fuscipes* (Table 2).

Figure 2 showed a 50% majority rule NJ tree constructed from a set of four COI gene sequences. In NJ tree, the four *Rhynocoris* species can be clearly classified into two major evolutionary clades as follows. Clade I contains one *Rhynocoris* species, *R. marginatus* and, clade II contains three *Rhynocoris* species: *R. kumarii, R. longifrons* and *R. fuscipes*. In which *R. longifrons* was named as new species mainly according to its morphological characteristics. In

conclusion, the four species studied using COI gene provides preliminary idea about taxonomical relationship at molecular level. In the recent years, mitochondrial DNA (mtDNA) sequences are routinely used for wide range of taxonomic, population and evolutionary studies rather than 18s rDNA or cytochrome b genes. The mitochondrial gene cytochrome oxidase subunit I (COI) has been proposed as a bar-coding tool or to confirm the species delimitation for ecologic and evolutionary studies [24]. Hence we selected the COI gene for our study to establish the molecular taxonomical relation of these four species. The size and structure of COI gene has been well conserved in all the animal groups analyzed so far, a feature which makes it suitable genetic marker for evolutionary studies [16].

Although the insect genome has hundreds of mitochondrial pseudogenes [7], we obtained a unique amplification around 710bp in all the four species from all the specimens. Further the PCR amplified products subjected for sequencing yielded high quality sequence of 593bp in *R. kumarii* and *R. longifrons* species, whereas 657 bp in *R. marginatus* and 640bp in *R. fuscipes*. Since we obtained 593 bp sequence of COI gene in *R. kumarii* and *R. longifrons* species, we trimmed and carried out the phylogenetic analysis for other two species accordingly. Many previous reports utilized approximately 500 bp or less than 500 bp gene sequence for identifying the genetic variation and phylogeny of agriculturally important bugs; we carried out our phylogenetic analyses with 593 bp [13, 27].

As typical for a protein-coding gene, the variability in COI gene sequence of four species found to be minimum in the second position (17%), moderate in the first position (30.01%), and highest in the third position (59.5%) [25, 13]. Identification of variations in the mitochondrial GC content of taxonomic groups considered as another approach of diversity assessment, in this study we observed that the COI gene is rich in adenine and thymine with 30.4% and 39.6% respectively, and less of guanine and cytocine with 14.5% and 15.5% respectively. It was reported that a high AT bias in COI gene may be a common phenomenon in insects, and that an AT nucleotide bias, when present, trends to accumulate in hypervariable sites [25, 27]. Overall we observed the AT content was 70% and GC content was 30%.

In this study, similar nucleotide and amino acid sequence was observed between *R. kumarii* and *R. longifrons*. However, according to the morphological characteristics of *R. kumarii* is different from *R. longifrons* [4, 6]. Whereas *R. marginatus* has showed greater variability in nucleotide (153nt) as well as in amino acid sequence (37) level compare to other species, whereas *R. fuscipes* had 18 nucleotide and two amino acid change.The variations between of *R. marginatus* and *R. fuscipes* are identified based on their morphological feature: shape, rostrum and wing [4]. Therefore, they should be considered as different species [4]. Conversely this was not supported by any molecular studies. Although morphological characters are considered for the biodiversity assessments, it may lead to under or over estimation of biodiversity. To overcome such problems, a short, 593 bp sequence of COI used to identify the taxonomical relationship in this study. However, *R. marginatus*, *R. fuscipes*, *R. kumarii* and *R. longifrons* were classified under monophyletic group.

Apart from molecular informations, morphological characters such as color, shape, nymphal habitat, alary polymorphism, egg architecture and pattern of oviposition could also be taken as additional supporting characteristics to understand the phylogeny and origin of reduviids [3, 19]. *R. longifrons* named as new species based on its morphological characteristics of egg, rostrum, wing and tibial pad, which are evidently different from other three *Rhynocoris* species [4, 6]. However, recent morphological, morphometrical, biological, behavioral and cytological studies of *R. marginatus*, *R. fuscipes*, *R. kumarii* and *R. longifrons* merely characterized and confirmed the local and extreme variations [6].

The present work concentrated the four important species i.e., *R. marginatus*, *R. fuscipes, R. kumarii* and *R. longifrons* for their biological control potential against insect pests. Although the analyses presented in this study were based on limited species and molecular markers, it examined the preliminary relationships of four narrowly distributed *Rhynocoris* species at molecular level for the first time. For further examination of more *Rhynocoris* species additional molecular tools would be required for robust assessment of taxonomic relationship of *Rhynocoris* species.

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## REFERENCES

1. Aikhionbare FO, Mayo ZB. Mitochondrial DNA sequences of greenbug (Homoptera: Aphididae) biotypes. Biomolecular Engineering 2000; 16: 199-205.
2. Ambrose AD, Ambrose DP. Biological tools in the biosystematics of three subfamilies of Reduviidae (Heteroptera: Reduviidae), Adeline publishers, Tirunelveli, India; 1996. p. 40-48.
3. Ambrose DP. Bioecology, ecophysiology and ethology of reduviids (Heteroptera) of the scrub jungles of Tamil Nadu, India, Ph.D., thesis. University of Madras, Madras, India; 1980. p. 42-74.
4. Ambrose DP. Assassin Bugs. Science Publishers, Enfield, New Hampshire, USA and Oxford and IBH Publishing Company Private Limited, New Delhi, India; 1999. p. 38-52.
5. Ambrose DP. Biocontrol potential of assassin bugs (Hemiptera: Reduviidae). Journal of Experimental Zoology of India 2003; 6: 1-44.
6. Ambrose DP. A checklist of Indian assassin bugs with taxonomic status, distribution and diagnostic morphological characteristics. Zoos' print journal 2006; 21: 2388-2906.
7. Bensasson D, Zhang D, Hewitt GM. Frequent assimilation of mitochondrial DNA by grasshopper nuclear genome. Molecular Biology and Evolution 2000; 17: 406-415.
8. Blaxter ML. The promise of DNA taxonomy. Philosophical Transactions of the Royal Society B: Biological Sciences 2004 ; 359: 669-679.
9. Boykin LM, Shatters RG, Rosell RC, McKenzie CL, Bagnall RA, De Barro P, Frohlich DR. Global relationships of *Bemisia tabaci* (Hemiptera: Aleyrodidae) revealed using Bayesian analysis of mitochondrial COI DNA sequences. Molecular Phylogenetics and Evolution 2007; 44: 1306-1319.
10. Chaudhry S, Sharma M. RAPD-PCR Based genomic DNA characterization of three population of *Anopheles* (Cellia) *stephensi* (Culicidae: Diptera). Journal of Cytology and Genetics 2006; 7: 121-130.
11. Folmer O, Black M, Hoeh W, Lutz R, Vrijienhoek, R. DNA primer for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrate. Molecular Biology and Biotechnology 1994; 3: 294-299.
12. Huang Y, Guillermo O, Marie S, Amy D, Anthony Z. Phylogenetic relationships of North American field crickets inferred from mitochondrial DNA data. Molecular Phylogenetics and Evolution 2000; 17: 48-57.
13. Ito K, Nishikawa H, Shimada T, Ogawa K, Minamiya Y, Tomoda M, Nakahira K, Kodama R, Fukuda T, Arakawa R. Analysis of genetic variation and phylogeny of the predatory bug, *Pilophorus typicus*, in Japan using mitochondrial gene sequences. Journal of Insect Science 2011; 11:18
14. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 1980; 16: 111-120.
15. Kumar S, Tamura K, Nei M. MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics 2004; 5: 150-163.
16. Lunt DH, Zhang DX, Szymura JM, Hewitt GM. The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Molecular Biology 1996; 5: 153–165.
17. Maldonado J. Systematic catalogue of the Reduviidae of the world (Insecta: Heteroptera). Caribbean Journal of Science Special Editions, University of Puerto Rico, Mayaguez; 1990. p. 694.
18. Martínez-Navarro EM, Galián J, Serrano J. Phylogeny and molecular evolution of the tribe *Harpalini* (Coleoptera, Carabidae) inferred from mitochondrial cytochrome-oxidase I. Molecular Phylogenetics and Evolution 2005; 35: 127-146.

19. Murugan C, Ravichandran G, Livingstone D. Phenotypic diversity of the rostrum of assassin bugs (Heteroptera: Reduviidae), Adeline Publishers, Tirunelveli, India; 1996. p. 317-322.
20. Pfeiler E, Bitler BG, Ramsey JM, Cardiel CP, Markow TA. Genetic variation, population structure, and phylogenetic relationships of *Triatoma rubida* and *T. recurva* (Hemiptera: Reduviidae: Triatominae) from the Sonoran Desert, insect vectors of the chagas disease parasite *Trypanosoma cruzi*. Molecular Phylogenetics and Evolution 2006; 41: 209-221.
21. Poggio MG, Bressa MJ, Papeschi AG. Karyotype evolution in Reduviidae (Insecta: Heteroptera) with special reference to Stenopodainae and Harpactorinae. Comparative Cytogenetics 2007; 1: 159-168.
22. Putshkov PV. *Rhynocoris persicus* (Heteroptera: Reduviidae): Three species or one? Zoological Herald 2002; 36: 27-34.
23. Putshkov VG, Putshkov PV. A catalogue of assassin bugs genera of the world (Heteroptera: Reduviidae). VINITI, Moskva; 1985. p. 138.
24. Schindel DE, Miller SE. DNA barcoding a useful tool for taxonomists. Nature 2005; 435: 17-17.
25. Simon C. Molecular systematics at the species boundary exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA. In: Hewitt, G.M., Johnston, A.W.B., Young, J.P.W. (Eds.), Molecular Techniques in Taxonomy. Springer, Berlin, Germany; 1991. p. 33–71.
26. Simon C, Fati F, Beckenbach A, Crespi B, Liu H, Flook P. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 1994; 87: 651-701.
27. Smith PT. Mitochondrial DNA variation among populations of the glassy-winged sharpshooter, *Homalodisca coagulata*. Journal of Insect Science 2005; 5: 41.
28. Swofford DL. PAUP: Phylogenetic Analysis Using Parsimony and other methods, version 4, Sinauer Associates, Sunderland, MA; 2000.
29. Weirauch C, Munro J. Molecular phylogeny of the assassin bugs (Hemiptera: Reduviidae), based on mitochondrial and nuclear ribosomal genes. Molecular Phylogenetics and Evolution 2009; 53: 287-299.

**Table 1: Summary of COI gene sequence obtained in this study from four *Rhynocoris* species.**

| Species | Locality | AT% | GC% | bp | Variations in DNA sequence | Variations in Amino acid sequence | GenBank No. |
|---|---|---|---|---|---|---|---|
| *R. marginatus* | India | 62.0 | 37.9 | 657 | 153bp (593) | 38 | GQ229415 |
| *R. fuscipes* | India | 69.8 | 30.2 | 640 | 18 bp (593) | 02 | GQ229414 |
| *R. kumarii* | India | 70.0 | 30.0 | 593 | 5 bp(593) | 0 | GQ229413 |
| *R. longifrons* | India | 70.0 | 30.0 | 593 | 5bp (593) | 0 | GQ229412 |

**Table 2: Percentage identity and divergence of partial nucleotide sequence of the mitochondrial COI gene from four *Rhynocoris* species.**

**Percentage identity**

**Divergence**

| | 1 | 2 | 3 | 4 | | Species |
|---|---|---|---|---|---|---|
| 1 | | 96.1 | 96.1 | 73.4 | 1 | *R. fuscipes* |
| 2 | 4.0 | | 100.0 | 73.4 | 2 | *R. kumarii* |
| 3 | 4.0 | 0.0 | | 73.4 | 3 | *R. longifrons* |
| 4 | 33.1 | 33.1 | 33.1 | | 4 | *R. marginatus* |
| | 1 | 2 | 3 | 4 | | |

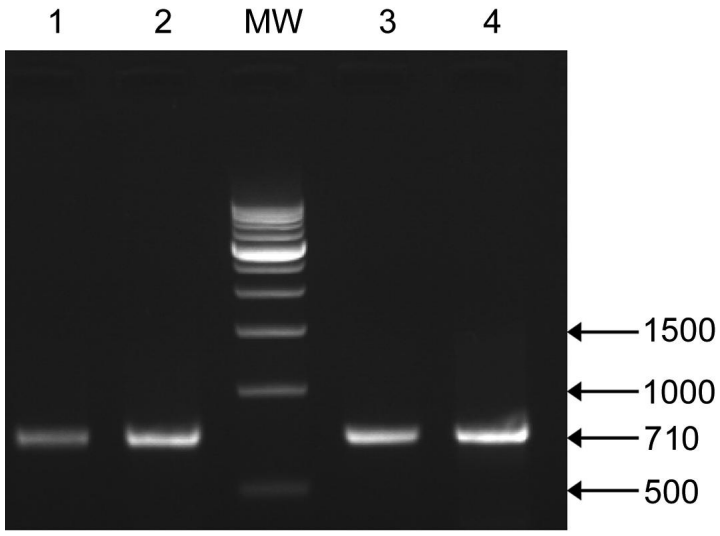


**Figure 1: Agarose gel electrophoresis (1.2%) of the 750 bp PCR amplicon of the COI partial gene from four different species of *Rhynocoris*: Lane 1, *R. marginatus*; Lane 2, *R. fuscipes*; Lane MW, 500 bp DNA ladder; Lane 3, *R. kumarii* and Lane 4, *R. longifrons*.**

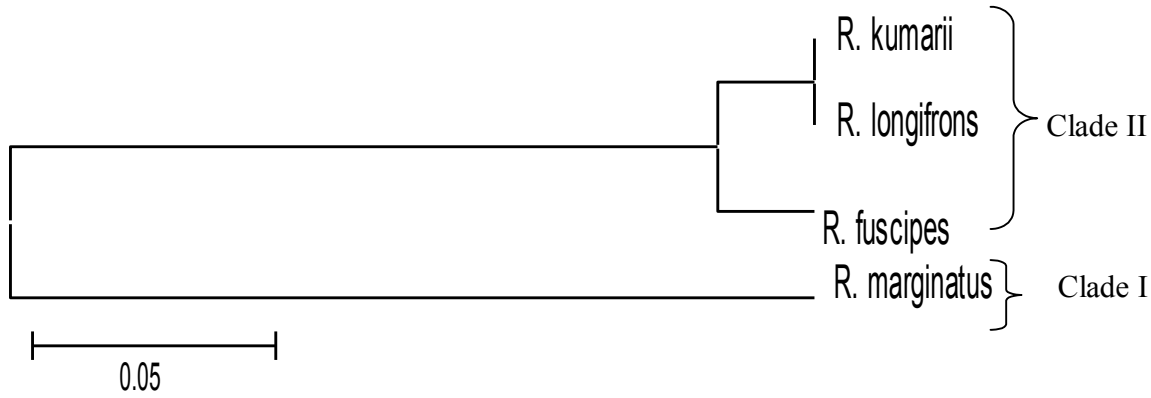


**Figure 2: Phylogenetic tree of four species of the genus *Rhynocoris* based on mitochondrial partial COI gene sequences. The neighbor-joining (NJ) tree (minimum evolution score = 0.35).**

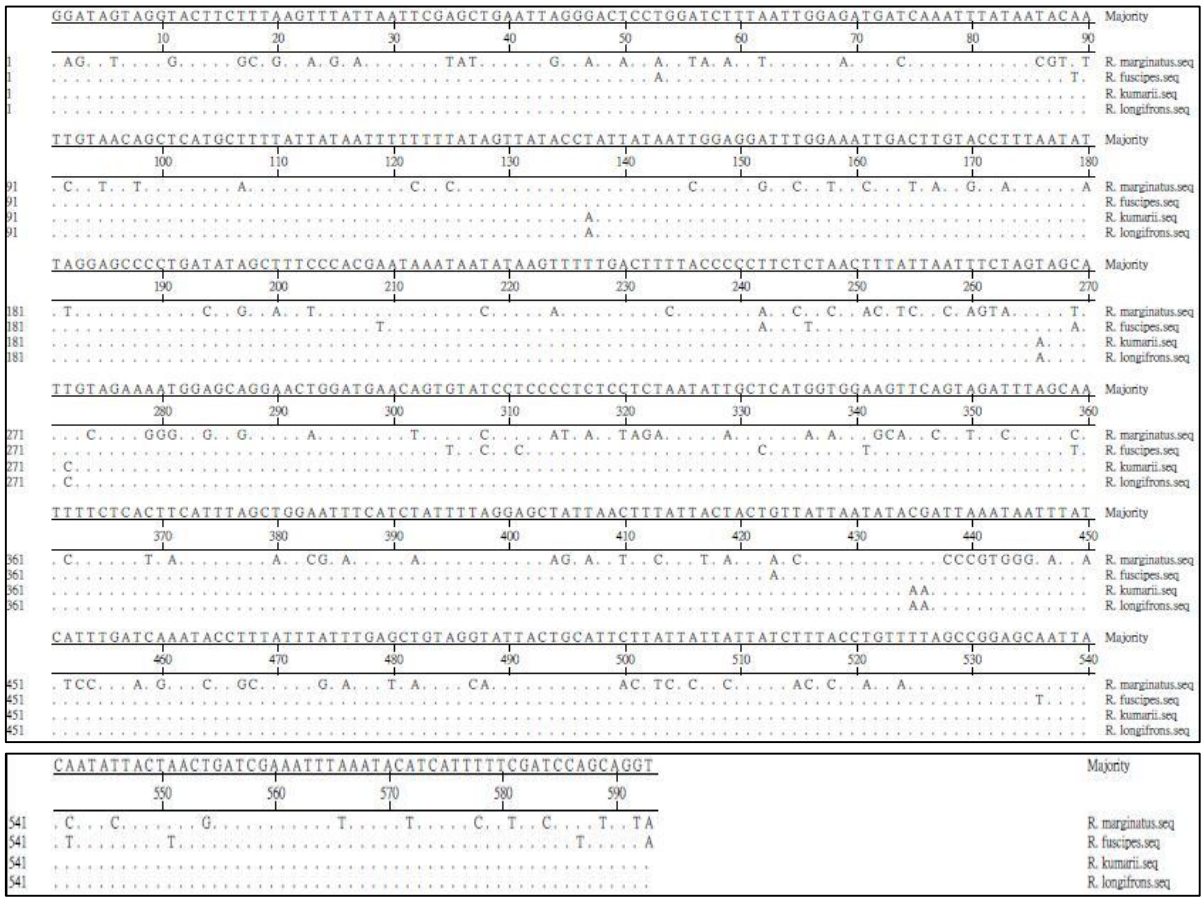


**Figure 3: Multiple alignment of four *Rhynocoris* COI gene nucleotide sequence.**

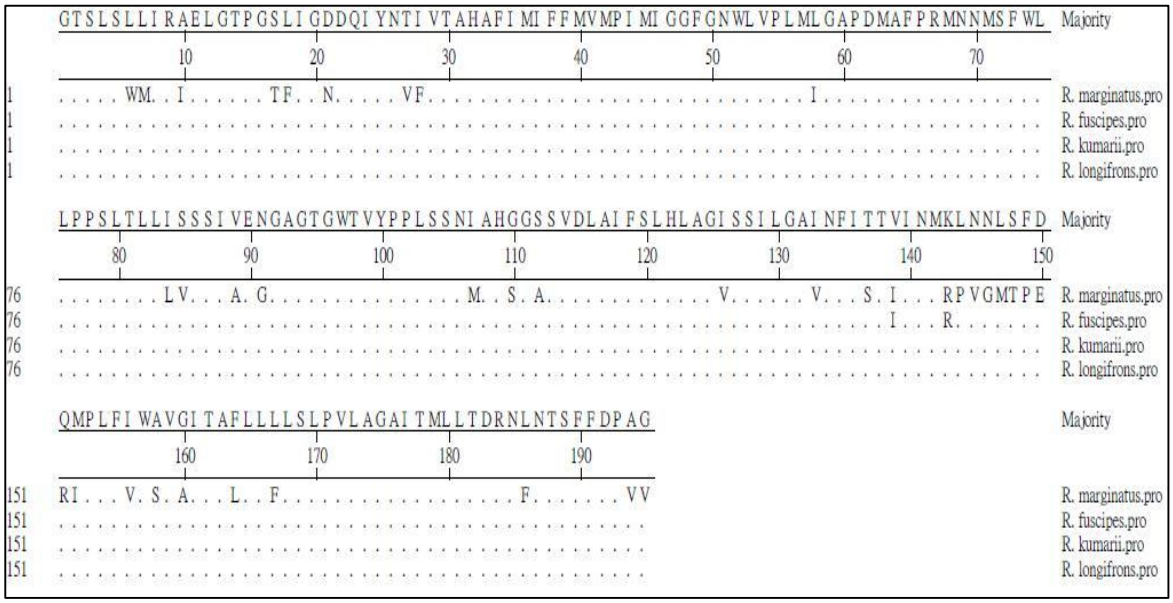


**Figure 4: Multiple alignment of four *Rhynocoris* COI gene amino acid sequence.**

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RESEARCH ARTICLE

# Extraction of Phytochemical Compounds from *Aegle marmelous* Linn. and Comparison of Natural and Chemotherapeutic Drugs for Diabetes Mellitus.

Jiyalhuk[1], K.Ramanathan[2*] and K.Geetha[1]

[1]Department of Biotechnology, Thanthai Hans Roever College, Perambalur, TamilNadu ,India.

[2]Department of Bioinformatics, Thanthai Hans Roever College, Perambalur, TamilNadu ,India.

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***Address for correspondence**
K.Ramanathan
Assistant Professor and Head,
Department of Bioinformatics,
Thanthai Hans Roever College of Arts and Science,
Perambalur, TamilNadu ,India
E.mail: ramanathanbio@gmail.com.



## ABSTRACT

The proposed study focused on the extraction of phytochemical compounds from *Aegle marmelous* and the comparison of natural and artificial drugs treated for diabetes mellitus. The phytochemical compounds were extracted and the effect of absorption was calculated based on Lipinski's criteria. The compounds that are satisfied the conditions of this criteria were selected for docking with the receptor. The Natural compound 6-octadecanoic acid was selected for docking with the target protein based on the parameters. The list of artificial compounds used to treat diabetes were retrieved and subjected to hydrophobic calculation. The artificial drug selected for docking and the score values were calculated. From these results, we found that 6-octadecanoic acid effectively docked with the receptor and produces high binding energy when compared with Troglitazone.

**Keywords:** Phytochemical Compounds, *Aegle marmelous*, Lipinski's Rule, ^-Octadecanoic acid, Troglitazone, Docking.

## INTRODUCTION

Medicinal Plants play an important role in the treatment of diabetes mellitus, especially in the developing countries due to their cost effectiveness. Diabetes mellitus, a metabolic disorder, is becoming a serious threat to mankind health. The prevalence of diabetes mellitus is expected to reach up to 4.4% in the world by 2030. Among all type of diabetes, type 2 diabetes is main complication[1].To perform phytochemical screening on all parts of *Aegle marmelos* so as to find out the identical phytocompounds and enhance its usage in drug preparation. Screening is performed for aqueous and methanol extract of various parts of the plant by High Performance Thin Layer Chromatography. Stem bark and root of aqueous extracts have similar phytochemical compounds. Marmelosin is found in all parts of the plant and its concentration is high in fruit. Stem bark could be substituted or added along with the root in any of the drug preparation where root is of important [2].

Traditional medicines derived from medicinal plants are used by about 60% of the world's population. Indian herbal drugs and plants used in the treatment of diabetes, especially in India. Diabetes is an important human ailment afflicting many from various walks of life in different countries. In India it is proving to be a major health problem, especially in the urban areas. Though there are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost [3].Diabetes mellitus is a metabolic disorder of endocrine system. This dreadful disease is found in all parts of the world and is becoming a serious threat to mankind health. There are lots of chemical agents available to control and to treat diabetic patients, but now currently several medicinal plants have been investigated for their beneficial use in diabetes. The effects of these plants may delay the development of diabetic complications and correct the metabolic abnormalities. Many chemical constituents are responsible for antidiabetic effects have been isolated from medicinal plants as nutraceuticals [4].

The antimicrobial activity of ethanolic leaf extracts of *Aegle marmelos* on selected microbial strains. The experiment shows that the phytochemicals present in the ethanolic leaf extracts of *A. marmelos* exhibit considerable antibacterial activity[5]. A Structured based virtual screening of differently substituted furocoumarins and analogues has been carried out against nuclear factor with the objective of selecting molecules able to inhibit the binding of this transcription factor to the DNA. The focus library was developed starting from chemical structures obtained from the literature, as well as retrieving compounds from available commercial databases [6]. The protein sequence was retrieved from Database and submitted to Hex tool for docking. Before docking the binding sites of the protein were calculated. The docking scores which produces high energy scores and activity. The molecular property evaluation was carried out by Insilco studies [7].

Diabetes mellitus is associated with disturbances of learning and memory and cognitive functioning. *Aegle marmelos* Corr. from Rutaceae family is widely used in Iranian folk medicine for the treatment of diabetes mellitus. It decreases blood glucose level by improving glucose tolerance and also has lipid-lowering and antioxidant properties. [8] In *Aegle marmelous,* all the parts of this tree including stem, bark, root, leaves, fruit and seeds at all stages of maturity has medicinal virtues and has been used in Ethno medicine to exploits its medicinal properties. The past studies showed that the pharmacognostical details [9].The *in vitro* antimicrobial activity of serial petroleum ether, chloroform and methanol extracts from leaves of *Aegle marmelos* were investigated against bacterial and fungal species [10].

## MATERIALS AND METHODS

*Aegle marmelous* were collected at various locations around Perambalur,TamilNadu,India. The sample were washed thoroughly under running tap water and dried under shade. They were then finely ground to a powder in an electric blender. The plant sample was subjected to GC-MS study for phytochemical analysis. About 2.0g of sample was soaked in 100ml methanol for 24 hours. The extract was filtered through what man no.1 and the filtrate was concentrated to dryness. The dried extract was diluted with GC methanol and was injected in to GC-MS.The

phytochemical compounds were identified from GC-MS study and these compounds were tested with Lipinski's Rule. The compounds which are satisfied the condition of Lipinski's criteria and these compounds were selected for docking studies.

There are four parameters which were calculated based on Lipinski's Rule. The H- Bond Donor, H-Bond Acceptor, Molecular weight and Log P retrieved were calculated. Based on the effects of absorption, the phytochemical compound was selected for docking with the target Receptor. Then the list of artificial compounds used to treat diabetes mellitus was retrieved and the Log P values were calculated. Based on the Log P value, the artificial compound was selected for docking with the receptor. The docking was performed by Hex tool and the score values for Natural and Artificial docking were compared.

## RESULTS

**Table 1: Analysis of Phytochemical compounds from *Aegle marmelous* L.**

| S.No. | Peak Name | Retention time | Peak area | %Peak area |
|---|---|---|---|---|
| 1. | Name: 1,1-Ethanediol, diacetate<br>Formula: $C_6H_{10}O_4$<br>MW: 146 | 3.70 | 1620705 | 0.1472 |
| 2. | Name: 1,6-Octadiene, 2,7-dimethyl-<br>Formula: $C_{10}H_{18}$<br>MW: 138 | 7.40 | 2298529 | 0.2088 |
| 3. | Name: Phenol<br>Formula: $C_6H_6O$<br>MW: 94 | 8.02 | 31791866 | 2.8881 |
| 4. | Name: cis-Linaloloxide<br>Formula: $C_{10}H_{18}O_2$<br>MW: 170 | 9.48 | 1598475 | 0.1452 |
| 5. | Name: Phenol, 2-methoxy-<br>Formula: $C_7H_8O_2$<br>MW: 124 | 9.69 | 4455256 | 0.4047 |
| 6. | Name: 2-Methyl-1-phenyl-1-pentanol<br>Formula: $C_{12}H_{18}O$<br>MW: 178 | 11.58 | 3701401 | 0.3362 |
| 7. | Name: 1,4:3,6-Dianhydro-à-d-glucopyranose<br>Formula: $C_6H_8O_4$<br>MW: 144 | 12.39 | 2465737 | 0.2240 |
| 8. | Name: Benzofuran, 2,3-dihydro-<br>Formula: $C_8H_8O$<br>MW: 120 | 12.69 | 40784084 | 3.7050 |
| 9. | Name: Benzenemethanol, 2-(2-aminopropoxy)-3-methyl-<br>Formula: $C_{11}H_{17}NO_2$<br>MW: 195 | 13.70 | 1903738 | 0.1729 |

| | | | | |
|---|---|---|---|---|
| 10. | Name: 2-Methoxy-4-vinylphenol<br>Formula: $C_9H_{10}O_2$<br>MW: 150 | 13.92 | 6283780 | 0.5708 |
| 11. | Name: Phenol, 2,6-dimethoxy-<br>Formula: $C_8H_{10}O_3$<br>MW: 154 | 14.51 | 4961020 | 0.4507 |
| 12. | Name: Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1à,2á,4á)]-<br>Formula: $C_{15}H_{24}$<br>MW: 204 .á-Elemene, (-)- | 15.01 | 10472431 | 0.9514 |
| 13. | Name: p-Vinylbenzohydrazide<br>Formula: $C_9H_{10}N_2O$<br>MW: 162 | 15.10 | 238105 | 0.0216 |
| 14. | Name: trans-Cinnamic acid<br>Formula: $C_9H_8O_2$<br>MW: 148 | 16.44 | 1667677 | 0.1515 |
| 15. | Name: 7-Octen-2-ol, 2-methyl-6-methylene-<br>Formula: $C_{10}H_{18}O$<br>MW: 154 Myrcenol | 16.63 | 767850 | 0.0698 |
| 16. | Name: Phenol, 4-(3-methyl-2-butenyl)-<br>Formula: $C_{11}H_{14}O$<br>MW: 162 | 16.74 | 2215205 | 0.2012 |
| 17. | Name: Benzene, 1,3,5-tris(1-methylethyl)-<br>Formula: $C_{15}H_{24}$<br>MW: 204 | 16.91 | 238988 | 0.0217 |
| 18. | Name: Benzoic acid, 2-hydroxy-, hydrazide<br>Formula: $C_7H_8N_2O_2$<br>MW: 152 | 17.03 | 929127 | 0.0844 |
| 19. | Name: Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-<br>Formula: $C_{12}H_{16}O_3$<br>MW: 208 Elemicin | 17.66 | 4533603 | 0.4118 |
| 20. | Name: 1,6-Anhydro-á-D-glucopyranose (levoglucosan)<br>Formula: $C_6H_{10}O_5$<br>MW: 162 | 17.91 | 14206335 | 1.2906 |
| 21. | Name: Ethanone, 1-(3,4-dimethoxyphenyl)- | 18.28 | 952878 | 0.0866 |

| | | | | |
|---|---|---|---|---|
| | Formula: $C_{10}H_{12}O_3$,<br>MW: 180 | | | |
| 22. | Name: Crotonic acid, o-formylphenyl ester<br>Formula: $C_{11}H_{10}O_3$<br>MW: 190 | 20.30 | 1327616 | 0.1206 |
| 23. | Eudesm-7(11)-en-4-ol<br>Formula: $C_{15}H_{26}O$<br>MW: 222 | 20.75 | 16498074 | 1.4987 |
| 24. | Name: 2-Propenamide, 3-phenyl-<br>Formula: $C_9H_9NO$<br>MW: 147 | 21.56 | 4074099 | 0.3701 |
| 25. | Name: Pyrimidin-4(3H)-one, 6-hydroxy-2-phenyl-<br>Formula: $C_{10}H_8N_2O_2$<br>MW: 188 | 23.68 | 4387478 | 0.3986 |
| 26. | Name: N-Furfuryl-p-toluidine<br>Formula: $C_{12}H_{13}NO$<br>MW: 187 | 24.95 | 6547112 | 0.5948 |
| 27. | Name: 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester<br>Formula: $C_{16}H_{22}O_4$<br>MW: 278 | 25.34 | 2774583 | 0.2521 |
| 28. | Name: Pentadecanoic acid<br>Formula: $C_{15}H_{30}O_2$<br>MW: 242 | 25.49 | 1285533 | 0.1168 |
| 29. | Name: 2-Decanone<br>Formula: $C_{10}H_{20}O$<br>MW: 156 | 25.95 | 1647749 | 0.1497 |
| 30. | Name: Hexadecanoic acid, methyl ester<br>Formula: $C_{17}H_{34}O_2$<br>MW: 270 | 26.33 | 6992899 | 0.6353 |
| 31. | Name: Isoquinolin-6-ol, 7-methoxy-1-methyl-<br>Formula: $C_{11}H_{11}NO_2$<br>MW: 189 | 27.40 | 68116339 | 6.1879 |
| 32. | Name: 2H-1-Benzopyran-2-one, 6,7-dimethoxy-<br>Formula: $C_{11}H_{10}O_4$<br>MW: 206<br>Coumarin, 6,7-dimethoxy | 27.92 | 59674732 | 5.4210 |

| | | | | |
|---|---|---|---|---|
| 33. | Name: 2H-1-Benzopyran-2-one, 3,4,7-trimethoxy-<br>Formula: $C_{12}H_{12}O_5$<br>MW: 236 Coumarin, 3,4,7-trimethoxy- | 28.60 | 218342 | 0.0198 |
| 34. | Name: Isoquinoline-4-carboxamide, 2,3,5,6,7,8-hexahydro-3-oxo-<br>Formula: $C_{10}H_{12}N_2O_2$<br>MW: 192 | 28.72 | 8082150 | 0.7342 |
| 35. | Name: 9,12-Octadecadienoic acid, methyl ester<br>Formula: $C_{19}H_{34}O_2$<br>MW: 294 | 29.10 | 1098323 | 0.0998 |
| 36. | Name: 9-Octadecenoic acid (Z)-, methyl ester<br>Formula: $C_{19}H_{36}O_2$<br>MW: 296 | 29.16 | 6002745 | 0.5453 |
| 37. | Name: 4-Aminocinnamic acid<br>Formula: $C_9H_9NO_2$<br>MW: 163 | 29.23 | 353280 | 0.0321 |
| 38. | Name: 7H-Furo[3,2-g][1]benzopyran-7-one, 9-hydroxy-<br>Formula: $C_{11}H_6O_4$<br>MW: 202 Xanthotoxol | 29.37 | 4510572 | 0.4098 |
| 39. | Name: Octadecanoic acid, methyl ester<br>Formula: $C_{19}H_{38}O_2$<br>MW: 298 | 29.51 | 3299264 | 0.2997 |
| 40. | Name: 6-Octadecenoic acid, (Z)-<br>Formula: $C_{18}H_{34}O_2$<br>MW: 282 | 30.09 | 190042752 | 17.2641 |
| 41. | Name: Octadecanoic acid<br>Formula: $C_{18}H_{36}O_2$<br>MW: 284 | 30.33 | 22283146 | 2.0243 |
| 42. | Name: 2,2-Dimethyl-3,4-dihydro-2H,5H-pyrano[3,2-c]chromen-5-one #<br>Formula: $C_{14}H_{14}O_3$<br>MW: 230 | 30.80 | 5751300 | 0.5225 |
| 43. | Name: 2-Hydroxy-3,4-tetramethylene-6-methoxy quinoline<br>Formula: $C_{14}H_{15}NO_2$<br>MW: 229 | 31.44 | 21935918 | 1.9927 |
| 44. | Name: 2H-1-Benzopyran-2-one, 6-[(3,3-dimethyloxiranyl)methyl]-7-methoxy- | 32.63 | 7926114 | 0.7200 |

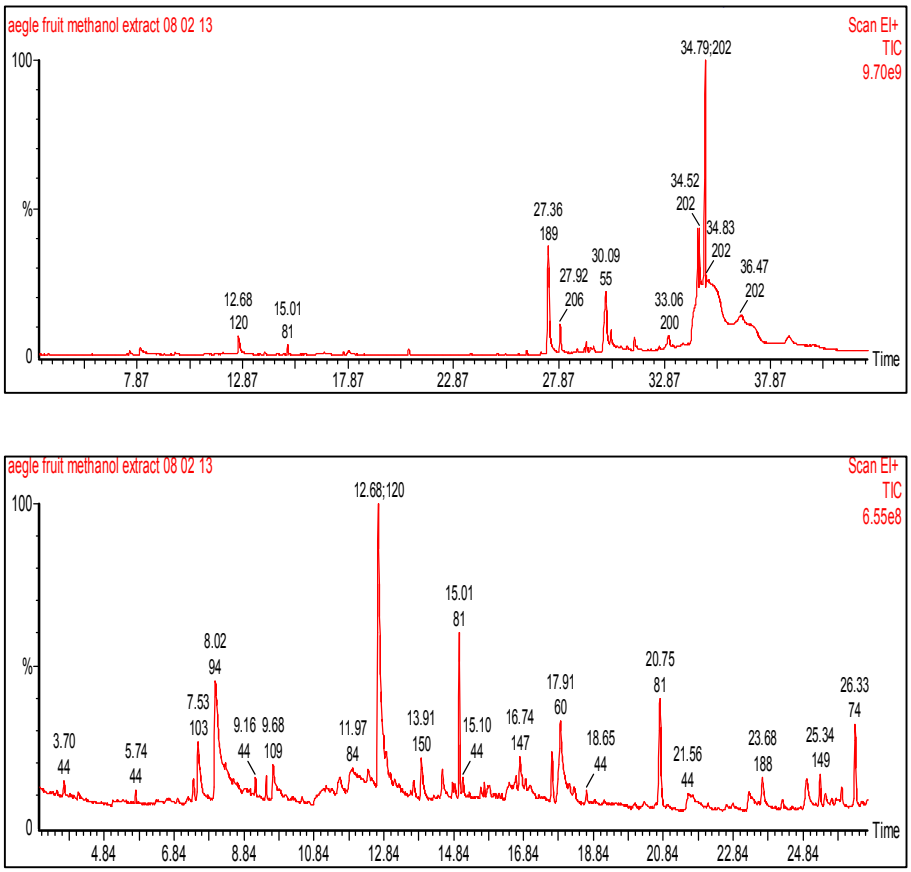
| | | | | |
|---|---|---|---|---|
| | Formula: $C_{15}H_{16}O_4$<br>MW: 260 | | | |
| 45. | Name: 2,3-Dihydro-4-hydroxymethylfuro(3,2-c)quinoline<br>Formula: $C_{12}H_{11}NO_2$<br>MW: 201 | 33.05 | 34399008 | 3.1249 |
| 46. | Name: Prangenin<br>Formula: $C_{16}H_{14}O_5$<br>MW: 286 | 34.52 | 52680052 | 4.7856 |
| 47. | Name: Prangenin<br>Formula: $C_{16}H_{14}O_5$<br>MW: 286 | 34.79 | 335085632 | 30.4403 |
| 48. | Name: 2H-1-Benzopyran-2-one, 7-[(3,7-dimethyl-2,6-octadienyl)oxy]-, (E)-<br>Formula: $C_{19}H_{22}O_3$<br>MW: 298 Aurapten | 36.47 | 95715256 | 8.6951 |

**Table 2: Effect of absorption for the Ligands.**

| Name of the Ligand | Effect of absorption |
|---|---|
| Metformin | + |
| Voglibose | - |
| Troglitazone | ++ |
| Acetohexamide | + |
| Miglitol | + |
| Glimepride | - |
| Sitagliptin | - |

**Table 3: Comparison of docking scores for the ligands.**

| Target Receptor | Ligand | Docking Scores |
|---|---|---|
| IRAK | 6-octadecanoic acid | 82.17 |
| IRAK | Troglitazone | 63.21 |




**Graph 1: Chromatogram for phytochemical compounds from *Aegle marmelous*L.**

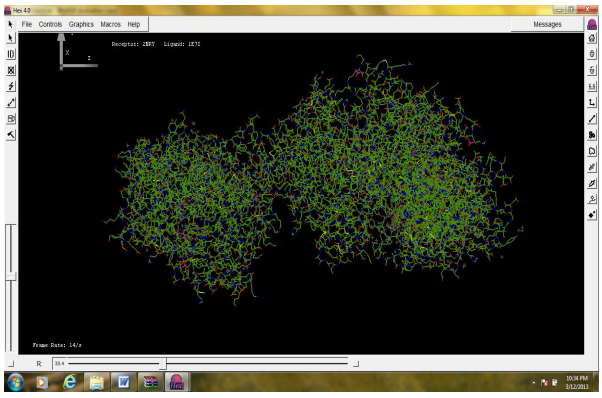


**Fig 1: Structure for the IRAK Receptor.**

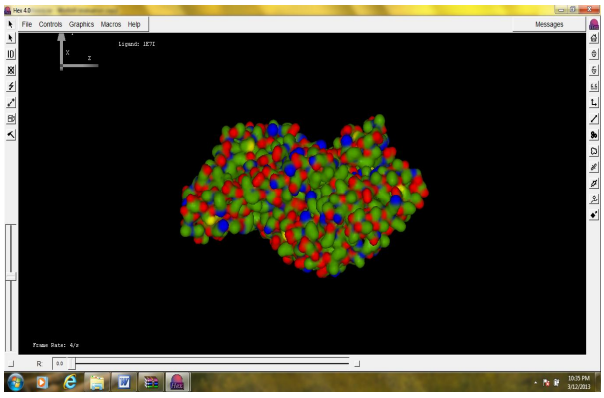


**Fig 2: Structure for the Ligand 6-Octadecanoic acid.**

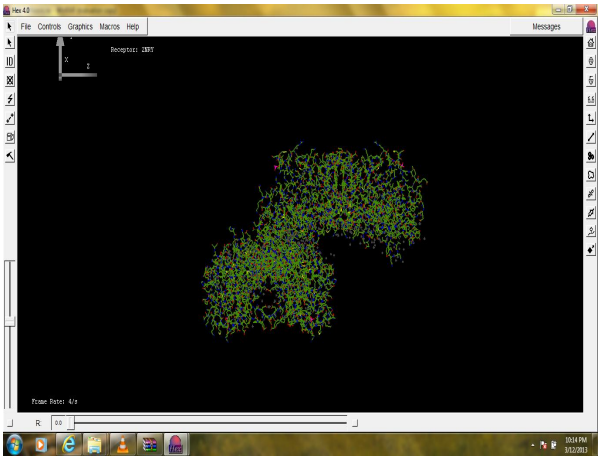


**Fig 3: Before docking the Receptor and the Ligand 6-Octadecanoic acid.**

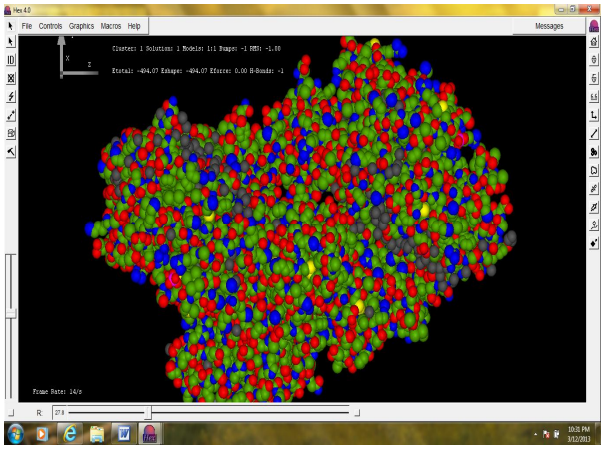


**Fig 4: After docking the Receptor and the Ligand 6-Octadecanoic acid**

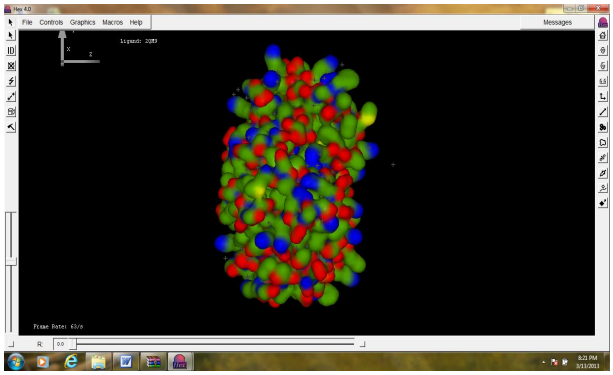


**Fig 5: Structure for the artificial compound Troglitazone.**

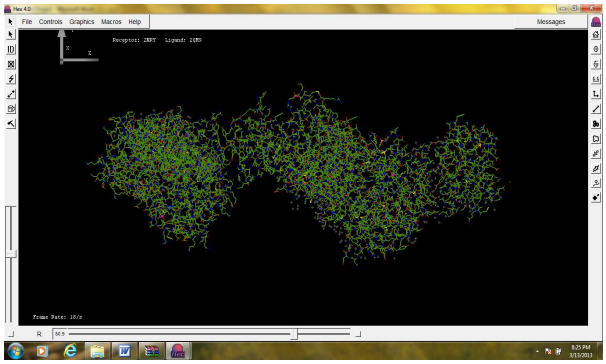


**Fig 6: Before docking the Receptor and the Ligand Troglitazone.**

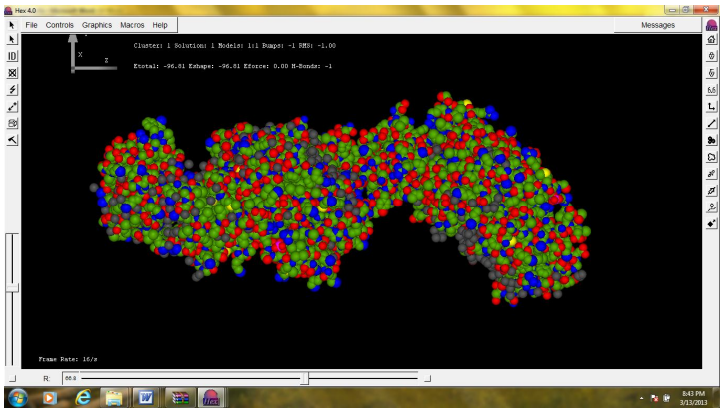


**Fig 7: After docking the Receptor and the Ligand Troglitazone**

## DISCUSSION

The Plant sample *Aegle marmelous* was collected and subjected to GCMS study for phytochemical analysis. There are 48 phytochemical compounds were present in the sample and these compounds were consolidated (Table 1). Graph 1 which shows that the chromatogram for the phytochemical compounds. Based on the peak area, the phytochemical compounds were selected for docking with the target protein. Among these phytochemicals, 6-octadecanoic acid shows the highest peak area. The Target Protein Structure was retrieved from Protein Data Bank and displayed in Fig 1 and the structure of the Ligand 6-octadecanoic acid displayed in Fig 2.The Receptor and the ligand were ready to interact with each other (Fig 3). The Target protein IRAK docked with the ligand 6-octadecanoic acid showed in Fig 4. The docking parameters for the natural compound were showed in Table 4. The Docking score was calculated from Hex tool. The list of artificial compounds which are treated for diabetes mellitus was retrieved from drug bank and the effect of absorption was calculated. The compound Troglitazone has the good absorption effect (Table 2).The structure of the artificial compound Troglitazone was retrieved from Drug Bank and showed in Fig 5. The Ligand Troglitazone was ready to dock with the IRAK receptor in Fig 6. Fig 7 shows that the docked structures of Receptor and the Ligand. The comparison of docking scores for both the compounds was showed in Table 3.

## CONCLUSION

The Plant sample *Aegle marmelous* was collected and the phytochemical compounds were identified by GC MS Study. The target protein structure was retrieved from Protein Data Bank and subjected to docking by Hex tool. Then the lists of artificial drugs were collected from drug bank and the artificial compound Troglitazone shows the good absorption effect and docked with the target protein.The score values were calculated and compared. The phytochemical compound 6-octadecanoic acid shows the docking score (82.17) and the artificial compound Troglitazone shows that 63.21. From these results, we concluded that the phytochemical compound 6-octadecanoic acid has effectively docked with the target protein and it act as a best anti diabetic agent. The compound 6-octadecanoic acid plays a significant role in *Aegle marmelous* and it could address the problem of diabetes mellitus.

## REFERENCES

1. DK Patel, R Kumar, D Laloo, S Hemalatha (2012), Natural medicines from plant source used for therapy of diabetes mellitus: An overview of its pharmacological aspects, Asian Pacific Journal of Tropical Disease. 5 (3): 239-250.
2. Nirupama GS, Padmasri G, Ramesh RV, Vasanthi M (2012).Comparative analysis of phytochemical constituents present in various parts of *Aegle marmelos*. Asian Pacific Journal of Tropical Disease. S774-S777.
3. Pritesh Patel, Pinal Harde, Jagath Pillai, Nilesh Darji and Bhagirath Patel (2012). Antidiabetic Herbal Drugs A Review, *Pharmacophore* 3 (1): 18-29.
4. Uttara Singh, Sadhana Singh, Anita Kochhar (2012), Therapeutic potential of antidiabetic nutraceuticals. *Phytopharmacology* 2(1): 144-169
5. Subramanian Ramya1, Ramaraj Jayakumararaj, Gopinath Krishnasamy, Nagoorgani Periathambi, Aruna Devaraj (2012). Antibacterial Activity of Ethanolic Leaf Extracts of *Aegle Marmelos* (L) Corr. Int. *Res J Pharm. App Sci*. 2(6): 74-79 ISSN: 2277-4149.
6. Laura Piccagli, Monica Borgatti, Elena Nicolis, Nicoletta Bianchi, Irene Mancini, Ilaria Lampronti, Daniela Vevaldi, Francesco, Cabrini, Roberto Gambari ( 2010). Virtual screening against nuclear factor jB (NF-jB) of a focus library: Identification of bioactive furocoumarin derivatives inhibiting NF-Jb dependent biological functions involved in cystic fibrosis. *Bioorganic & Medicinal Chemistry* 18 (2): 8341–8349.
7. Manjunatha B.K. and Amith. R. Rupani (2011). Alloimperatorin From Aegle Mermelos -A Promising lead molecule for hepatoprotection- *In Vivo* And *In Silico* Approach. *International Journal of Current Research*. 3(12): 005-010.

8. Amir Farshchia B, Golbarg Ghiasia B, Samira Ghiasic (2010). *Aegle marmelos* Extract Can Enhance Memory in Rats. *Iranian Journal of Pharmaceutical Sciences Winter.* 6(1): 25-32
9. Gupta Deepika, John P, Kumar Pankaj, Pradhan Chandan, Mohammad Shuaib, Gupta Manoj Kumar (2011). Establishment of Monograph of *Aegle marmelos* Corr. Fruits. Pharm Ana & Qual Assur 11(3)
10. Saroj Kothari, Vaibhav Mishra, Savita Bharat and Shrinivas D, Tonpay (2011). Antimicrobial activity and phytochemical screening of serial extracts from leaves of *Aegle marmelos. Acta Poloniae Pharmaceutical Drug Research*, 68(5): 687 – 692.

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**RESEARCH ARTICLE**

# Optimization and Purification of Alkaline Phosphatase Producing *Bacillus sp.*

Gnanasankar.C[1]., R.Ajazhaja mohideen[2]*, K. R. Narayanan[1] and A. G. Murugesan[3]

[1]Department of Zoology, Sri Paramakalyani College, Alwarkurichi-627 412, TamilNadu, India.

[2]Department of Microbiology, Sadakathullah Appa College, Rahmath nagar, Tirunelveli,Tamil Nadu.

[3]SPK Centre of Excellence in Environmental Science, Manonmaniam Sundaranar University, Alwarkurichi-627 412, TamilNadu, India.

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***Address for correspondence**
R.Ajazhaja mohideen,
Department of Microbiology,
Sadakathullah Appa College,
Rahmath nagar, Tirunelveli-627 011,
Tamil Nadu ,India.
E.mail: microajaz@gmail.com.



## ABSTRACT

Isolation of alkaline phosphatase producing microbes from pond water of Tirunelveli district, TamilNadu.The individual bacterial colonies were screened for phosphatase enzyme production on phosphate agar medium by well assay method. Production, optimization and partial purification of alkaline phosphatase enzyme by *Bacillus sp.* was the aim of this study. *Bacillus sp.* was allowed to grow in broth culture for purpose of inducing alkaline phosphatase enzyme. The factors like pH, temperature, salinity and substrate concentration which were expected to affect the production of phosphatase by the selected strain was optimized by selecting one parameter at a time. Optimal conditions for Phosphatase production by *Bacillus sp* were; an optimum substrate concentrations 0.7 %; optimum incubation period, 24 hrs.; optimum incubation temperature was37 $^{o}$C; the optimum pH was 9.0; Mass scale cultivation processes was done in NaCl-3%, pH 9, glucose-1%, beef extract-0.5%, $CaCl_2$-0.01%, Casein-0.8% and incubated at 35$^{0}$C for 24h in shaking speed as 150rpm.

**Keywords:** Alkaline phosphatase, Bacillus sp., Phosphate solublizing bacteria.

## INTRODUCTION

Phosphorus (P) is the major essential macronutrient for biological growth and development. The insoluble precipitated inorganic phosphates in soil and water is solubilized by the action of organic acids produced by bacteria and made available to the crops plants [1].The organic phosphorus compounds are also decomposed and mineralized by enzymatic complexes of heterotrophic bacteria [2]. As the name suggests, alkaline phosphatase are most effective in an alkaline environment. In bacteria, alkaline phosphatase is usually located in the periplasmic space to generate free phosphate groups for uptake and use. This hypothesis is in accordance with the fact that alkaline phosphatase is usually secreted by bacteria during the phosphate starvation only [3].The alkaline phosphatase researchers commonly used Bacterial alkaline phosphatase (BAP). Alkaline phosphatase (AP) is a hydrolase enzyme responsible for removing phosphate groups in the 5- and 3- positions from many types of molecules, including nucleotides, proteins, and alkaloids. Microorganisms produce a large variety of enzymes; some are involved to hydrolyze the insoluble phosphates [4]. Many bacteria acquire phosphate by releasing alkaline phosphatases, enzymes that generate free phosphate normally under conditions of phosphate starvation.

These bacteria are general saprophytes, which mean that they gain nourishment directly from dead or decaying organic matter. They secrete extracellular enzymes and adsorb the soluble break-down products of the interaction of these enzymes with insoluble polymers, such as polysaccharides and starch. Alkaline phosphatase is a metalloenzyme with two $Zn^{2+}$ ions and one $Mg^{2+}$ ion at each activation site [5]. Enzyme activity is stimulated by large additions of calcium and magnesium and/or smaller additions of sodium, potassium, or iron [6]. Alkaline phosphatase activity is highest at pH values between 8 and 10 with the optimum at pH of 8.7 to 9 [7]. Compared to other proteins, phosphatases require a high investment of nitrogen (8-32% N) indicating that phosphatase production is dependent on nitrogen availability [8] creating a nutrient paradox in oligotrophic systems.

The purpose of the present study was to examine, under laboratory conditions, the ALP activities of the bacteria in water samples from pond ecosystem in Tirunelveli district, TamilNadu, India. Optimization and purification of extracellular forms of alkaline phosphatase from *Bacillus sp.* to determined optimal enzymatic properties.

## MATERIALS AND METHODS

*Bacillus sp* was used as a producer of alkaline phosphatase. *Bacillus sp.* was cultured in low phosphate medium [9] (Fig. 1). Volume of 10 ml of this medium in 100-ml conical flasks was inoculated directly from a plate colony. The mixture was incubated at 37°C with shaking by a rotary shaker (150 rpm/min) and after that was centrifuged for 20 min at 10000 rpm and 0-4°C to remove the cells.

### Optimization of culture conditions for alkaline phosphatase production

The factors like pH, temperature, salinity and substrate concentration which were expected to affect the production of ALP by the selected strain was optimized by selecting one parameter at a time [10].

### Effect of pH on growth

Different pH ranging from 4-10 were maintained in the medium and incubated at 35°C. Growth and enzyme activity were assessed for every 6h until decline phase.

### Effect of Temperature on growth

Different temperatures ranging from 25°C, 30°C, 35°C and 40°C were tested for growth and enzyme activity and assessed for every 6h until decline phase.

### Effect of NaCl concentration on growth

Different salinity ranging from 0 – 3.5% were maintained in the medium and incubated at 35°C. Growth and enzyme activity were assessed for every 6h until decline phase.

### Effect of various carbon sources on growth

Different carbon sources such as sucrose, glucose, maltose and starch were added in the medium separately in the concentration of 1% in the medium and incubated at 35°C. Growth and enzyme activity were assessed for every 6h until decline phase.

### Effect of nitrogen sources on growth

Different nitrogen sources such as beef extract, yeast extract, ammonium nitrate and ammonium sulphate were added in the medium separately at the concentration of 0.5% in the medium and incubated at 35°C. Growth and enzyme activity were assessed for every 6h until decline phase.

### Effect of calcium chloride and metal ions on growth

Different metal ions such as $CaCl_2$, $MgSo_4$, $(Na_4)_2HPO_4$, $K_2HPO_4$ and $KH_2PO_4$ were added in the medium separately in the concentration of 0.01% and incubated at 35°C. Growth and enzyme activity were assessed for every 6h until decline phase.

### Effect of substrate concentrations on alkaline phosphatase production

Different concentrations of phosphate (0.2-1%) were maintained in the medium and incubated at 35°C. Growth and enzyme activity were assessed for every 6 h until decline phase.

### Purification of protease enzyme by using DEAE Sephadex A-50 column

The column was selected at a height and internal diameter of 30cm and 1.2cm respectively and was gradually filled with the suspension of DEAE Sephadex A-50. DEAE Sephadex A-50 slurry was prepared by mixing 0.5g of dry powder DEAE Sephadex A-50 with binding buffer (20mM Tris buffer pH 8.2) at a ratio of 75% settled medium to 25% buffer. Swelling factor depends on the buffer used. Complete swelling took 1-2 days at room temperature or 2h at 100ºC in boiling water with vigorous stirring.

## RESULTS AND DISCUSSION

### Effect of pH on alkaline phosphatase (ALP) production

Effect of pH on the growth of *Bacillus sp*. showed that higher growth and maximum enzyme activity (215.4 U/ml/min) was observed at pH 9.The lowest growth and minimum enzyme activity were found at pH 4 (140 U/ml/min) Fig 2. The organisms at neutral pH produced 83.56% of the enzyme activity. As the phosphatase active at higher pH it was confirmed as an alkaline phosphatase. Kumar et al. [11] isolated two strains namely Bacillus species strain $S_4$ and Pseudomonas species strains $S_{22}$ and reported that alkaline phosphatase production was maximum at pH 7.5 and pH 9 for respectively. Similarly previous workers [12] reported maximum alkaline phosphatase production at 9-13.

### Effect of temperature on ALP production

Effect of temperature on phosphatase production was observed in the range of 25°C to 40°C. The temperature 35°C was found to be optimum so that a maximum of 240.6U/ml/min enzyme activity was achieved (Fig. 3). Minimum activity (148.2U/ml/min) of enzyme was observed at 25ºC.The enzymatic activity of phosphatase from *Bacillus sp.* increased with temperature from 45°C with maximal activity at 45°C [13]. Kalaiarasi et al. [14] optimized the alkaline phosphatase production from Pseudomonas fluorescens optimum conditions for phosphatase production was 37ºC.

### Effect of sodium chloride on ALP production

In the present investigation the effect of ALP production from Bacillus sp. in the medium with various NaCl concentration (0-3.5%), influenced the growth and enzyme activity. Maximum enzyme activity of 345 U/ml/min was observed at 3% of NaCl (Fig. 4). Minimum enzyme activity (48.4U/ml/min) at 1.5% of NaCl. Tolerance of enzyme upto 5M of NaCl over 24h without losing original activity was reported early workers [15]. Similarly Porro et al. [16] was reported production medium with 2% NaCl required for maximum phosphatase production from Pseudomonas spp.

### Effect of carbon and nitrogen sources on phosphatase production

In the present study *Bacillus sp.* strain was grown in medium with different carbon sources such as maltose, glucose, sucrose and starch. In glucose maximum enzyme activity (419.4U/ml/min) was found and in sucrose minimum enzyme activity (339.6U/ml/min) was noticed (Fig. 5). Gassesse et. al. [17] reported that phosphatase production in Bacillus, Pseudofirmus AL-89 increased in the presence of glucose, whereas in Nestenkonia spp. AL-20 glucose suppressed the phospatase production.

In the present investigation phosphatase from *Bacillus sp.* was grown on medium with different nitrogen sources. Beef extract showed maximum activity with 192.6U/ml/min and minimum activity was noted in ammonium nitrate (72U/ml/min) (i.e.) 63% of activity reduced from the optimum level (Fig. 6). The inorganic nitrogen source such as ammonium sulfate showed 78% of enzyme activity produced. Saha and Bhattacharyya, [18] reported in *Bacillus sp.* that the parent strain grew best in presence of ammonium nitrate.

### Effect of calcium chloride and metal Ions on protease production

The calcium chloride showed maximum phosphatase activity of 194.4U/ml/min (Fig. 7). $MgSO_4$ showed 77.16% of activity (150U/ml/min). $K_2HPO_4$ and $KH_2PO_4$ showed minimum enzyme activity with 38% and 48% respectively. Metal ions and trace elements are often required by bacteria for their growth and physiological activities. Rahman et al. [19] reported phosphatase production by P. aeruginosa was increased with metal ions such as $Mg^{2+}$, $K^+$ and $Ca^{2+}$.

### Purification of ALP enzyme

In the present study, purification of enzymes was carried out by precipitation of protein from the cell free dialysate with ammonium sulfate at the saturation level of 60%. This resulted in 3.14 folds of purification with yields of 91.4% and specific activity of 507.77U/mg. Similar results were already reported as partial purification of the two enzymes was carried out by precipitation of protein from the cell free dialysate with ammonium sulfate at the saturation level of 60%. This resulted in 3.1 folds of purification in case of beta-glucosidase and 2.87 folds in case of phosphatase with yields of 89.46% and 82.73% of the original activities respectivesly. Precipitated enzymes were then dialyzed and purified by gel filtration through Sephadex G-50 resulting in purification of 5.96 folds with yields 46.2% and specific

activity of 962.5U\ mg. In this step, phosphatase were purified 10.88 and 11.39 folds with yields of about 81.51% and 74.4%, and specific activities of 59.22 and 21.75 U mg [20].

## CONCLUSION

The Alkaline Phosphatases are the most important group of enzymes in the aquatic environment. The investigations on the Alkaline Phosphatase production by bacteria from pond ecosystem shows in ALP production Bacillus is a dominant genus in sediment soil samples of the pond Most of the facultative alkalophiles produce ALP when the pH is above 8.5.and the temperature 35$^{o}$C was found to be optimum so that a maximum of 240.6U/ml/min enzyme activity.

## REFERENCES

1. Fleischer, S., Bengtson, M. and Johansson, G. 1988. Mechanism of aerobic Fe(III)-P solubilisation at the sediment water interface. Verhandlungen des Internationalen Verein Limnologie, 23 :1825-1829.
2. Aaronson, S. and N.J. Patni 1976. The role of surface and extracellular phosphatases in phosphorus requirement of Ochromonas. Limnol. Oceanogr., 2 1 : 838-845.
3. Garen, A. and Levinthal, C. 1960. Fine Structure genetic and chemical study of enzyme alkaline phosphatase of E. coli. I: Purification and characterization of alkaline phosphatase. Journal of Biochimica et Biophysica Acta, 38 :470-473.
4. Andrade, V. S.; Sarubbo, L. A.; Fukushima, k.; Miyaji, M.; Nishimura, K. and de Campos-Takaki, G. M., (2002). Production of extracellualr proteases by Mucor circinelloides using D-glucose as carbon sources/substrate. Brazilian Journal of Microbiogy 33: 106-110.
5. Kim JW, Peterson T, Bee G, Hulett FM. Bacillus licheniformis MC14 alkaline phosphatase I gene with an extended COOH-terminus. FEMS Microbiol Lett 1998;159:47-58.
6. Aleksieva, P. and Peeva, L., (2000). Investigation of acid protinase biosynthesis by the fungus Humicola Lutea 120-5 in an airlift bioreactor. Enzyme Microb. Technol., 26: 402-405.
7. Jansson, M. 1988. Phosphate uptake and utilisation by bacteria and algae. Hydrobiologia, 56:175-180.
8. Tarafdar, J.C. and Marschner, H. (1994): Phosphatase activity in the rhizosphere and hyphosphere of VA mycorrhizal wheat supplied with inorganic and organic phosphorus. Soil Biol. Biochem., 26: 387 – 395.
9. Kostadinova, S., M. Marhova. (2008) Anniversary Ecological Counference (proceedings), 1th November 2008, 285-292.
10. Ikezawa, H. M. Matsushita, M. Tomita, R. Taguchi. (1986) Arch. Biochem. Biophys., 249, 588-595.
11. Kumar, A., Sachdev, A., Balasubramanyam, S.D., Saxena, A.K. and Lata, A. (2002). Optimization of conditions for production of neutral and alkaline protease from species of Bacillus and Pseudomonas. Ind. J. Microbiol., 42: 233-236.
12. Borriss, R. (1987). Biology of enzymes. In:Biotechnology (Rehm H and Reed G. Ed.) Weinbeim, Verlag chemistry., pp-35–62.
13. Engel, L.S., Hill, J., Caballero, A.R., Green, L.C. and Callaghan, R.J. (1998). Protease IV. A unique extra cellular protease and virulence factor from Pseudomonas aeruginosa. J. Biol. Chem., 273: 16792-16796.
14. Kalaiarsi,. K. and Sunitha, P.U. (2009). Optimization of alkaline protease production from Pseudomonas fluorescens isolated from meat waste contaminated soil. Afr. J. Biotechnol., 8(2): 7035-7041.
15. Jana, M., Chattopadhayay, D.J. and Pati, B.R. (1997). Thermo stable, high salt tolerant amylase from Bacillus megaterium VUMB–109. Acta Microbiol. Immunol. Hug., 45(2): 281–289.
16. Porro, S.C., Mellado, E., Bertoido, C., Antranikian, G. and Ventosa, A. (2003). Screening characterization of the protease produced by moderately halophilic bacterium Pseudomonas spp strain P76. Extremophiles., 7: 221–228.
17. Gessesse, A., Kabul, R.H., Gashe, B.A. and Mattiasson, B. (2003). Novel alkaline protease from alkalophilic bacteria grown on chicken feather. Enz. Microbial Technol., 32: 519-524.

18. Saha, N. and Bhattacharyya, B.C . (1990). Strain improvement: Mutagenesis and random screening procedure for Rhizopus oryzae IITKG – 1. Proceeding of International Symposium on Industrial Biotechnology. Department of Microbiology. Osmania. Univ. Hyderabad.
19. Rahman, A.R.N.Z., Geok, L.P., Basri, M. and Salleh, A.B. (2006). An organic solvent stable alkaline protease from Pseudomonas aeruginosa strain K enzyme purification and characterization. Enz. Microbial Technol., 39: 1484-1491.
20. Fawzi, E.M. (2003). Purification and characterization of the pectin lyase and protease produced by Penicillium velutinum grown under solid state fermentation. Annals Microbiol., 59 (4): 755-761.

**Fig.1: Phosphatase activity of *Bacillus sp. on* phosphate agar plate.**

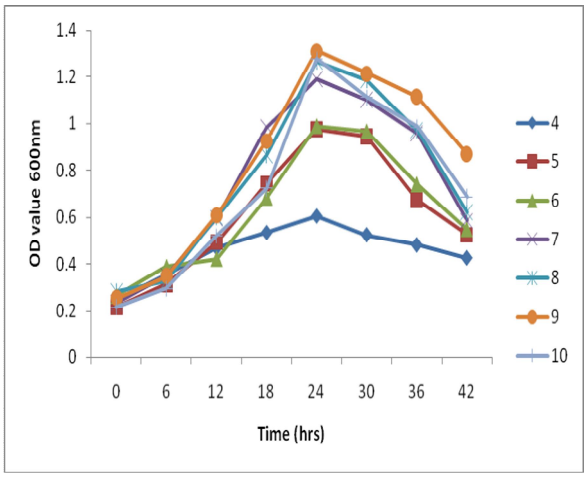


**Fig.2 :Effect of pH on Growth.**

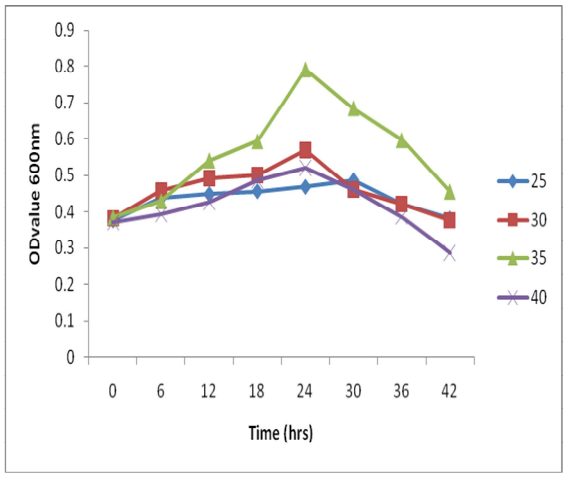


**Fig. 3: Effect of Temperature on Growth.**

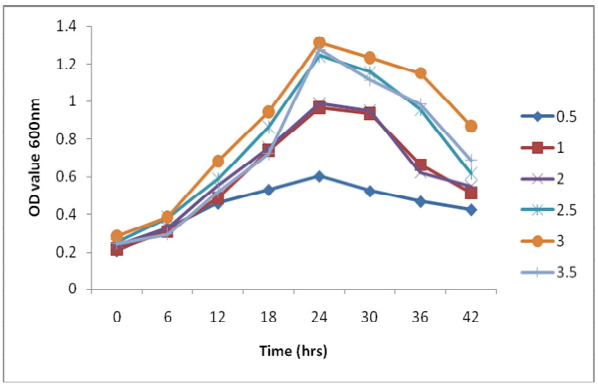


**Fig.4:Effect of NaCl on Growth.**

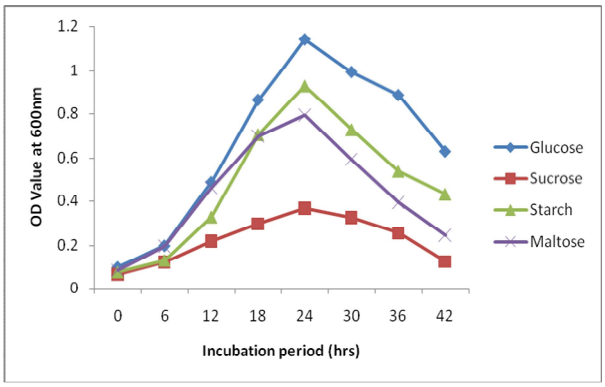


**Fig.5:Effect of Carbon sources on Growth.**

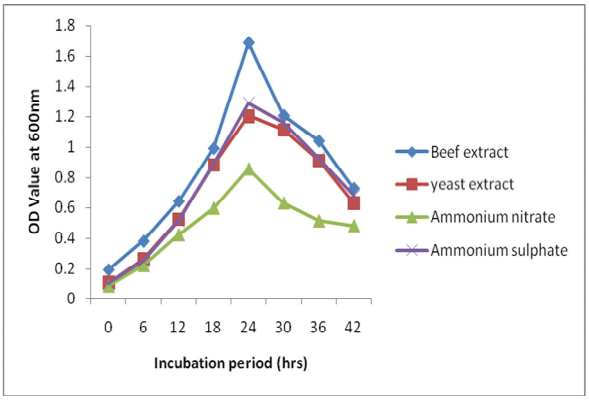


**Fig.6:Effect of Nitrogen sources on Growth.**

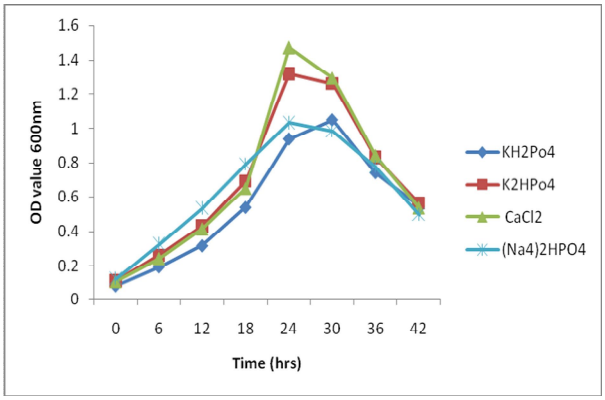


**Fig.7:Effect of substrate concentration on enzyme activity.**

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RESEARCH ARTICLE

# Pharmacological Studies on *Euphorbia fusiformis* Buch – Ham. ex. D. Don (Euphorbiaceae).

Kavitha K.S* and K.Raju

Post graduate and Research Department of Botany, Kandaswami Kandar's College, P. Velur, Namakkal – 638 182, Tamil Nadu, India.

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***Address for correspondence**
Kavitha K.S,
Post graduate and Research Department of Botany,
Kandaswami Kandar's College, P. Velur,
Namakkal – 638 182,
Tamil Nadu, India.
Email: kavithamarimuthu@yahoo.co.in



**ABSTRACT**

This paper evaluated pharmacological investigation of *Euphorbia fusiformis* (*Palperukki kilangu in Tamil),* Buch – Ham. ex D.Don (Euphorbiaceae) at Kalrayan hills of Eastern Ghats in Tamil Nadu, it is situated nearest town of Salem.The information obtained may lead to directions of discovery of new drugs. On ***in vitro*** studies *Euphorbia fusiformis* the air dried, powdered leaf and rhizome was successively extracted in soxhlet extractor with chloroform, acetone, ethanol and water. Determination of total phenolics, tannins and total flavonoid content are examined. Free radical scavenging activity was evaluated using DPPH free radical. The extracts of *Euphorbia fusiformis* have antioxidant activities and acute toxicity was observed.

**Keywords:** Pharmacological, *Euphorbia fusiformis*, Euphorbiaceae , Antioxidant, Acute toxicity.

## INTRODUCTION

Plants are an important source of biologically active substance; therefore they have been used for medicinal purposes. Since ancient times, the use of medicinal plants is usually based on traditional knowledge from which their therapeutic proprieties are often ratified in pharmacological studies [1].Some of medicinal plants are believed to enhance the natural resistance of the body to infections [2]. The versatility of biological actions can be attributed to the huge amount and wide variety of secondary metabolites in plant organisms, belonging to several chemical classes

as alkaloids, flavonoids, phenols and tannins etc. The medicinal proprieties of plants have been investigated in the recent scientific developments throughout the world, due to the potent antioxidant activities [3]. An antioxidants have been reported to prevent oxidative damage caused by free radicals, it can interfere with the oxidation process by reacting with free radicals and also by acting as oxygen scavengers [4,5].Free radical scavenging activity was determined using the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH)[6].The scavenging effect on the DPPH radical increases sharply with the increasing concentration of the samples and standards to a certain extent[7].The toxicity study which is essential for an adaption of the traditional medicine was conducted to identify the tolerance limits of plant extracts[8].The ethanol extract of *Euphorbia fusiformis* rhizome showed remarkable hepato protective effect against rifampicin induced hepatic damage in Wistar albino rats[9].So that, the present study was conducted on *Euphorbia fusiformis*, to analysis the total phenolics, tannins, flavonoids content and antioxidant capacities of the plant extracts and evaluate their acute toxicity in mice.

## MATERIALS AND METHODS

The genus *Euphorbia* contains 195 species found in India with diverse medicinal active constituents and properties. The present work was carried out on *Euphorbia fusiformis*.Buch – Ham. ex D. Don Euphorbiaceae family, Common Tamil name : *Pal perukki kilangu.*

### Plant description

A succulent herb, rootstock cylindrical fusiform, buried in the ground, 12 to 85 cm long and 3 to 5.5cm in diameter, with 5 to 8 roots. Leaves glabrous, appearing after flowers, fleshy, numerous, arching mostly irregular in shape, broadly to narrowly lanceolate to oblanceolate, 12 to 25cm long and 1.5 to 5cm broad, obtuse with a reddish prominent midrib on the lower surface. Cymes yellow green 3.5 to 4.5cm long. Fruits capsule 7.5 to 8mm in diameter. Seeds ovate, smooth, very dark grey in colour.

### Habitat

In open or semi-shady places amongst grasses, present only on hill slopes[10].

### *In vitro* studies

### Solvent extraction

The air dried powdered leaf and rhizome of *Euphorbia fusiformis* was successively extracted in soxhlet extractor with chloroform, acetone, ethanol and water. Each time, before extracting with the next solvent, the powdered material was dried in hot air oven at 40°C. All the extracts were evaporated to remove even the final traces of the respective solvents. The percentage yield was also calculated. All the solvent extracts were used for the following in vitro studies.

### Determination of total phenolics

The total phenolic content was determined according to the method described by Siddhuraju and Becker (2003)[11].Ten microlitre aliquots of the extracts (10mg/2ml) were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test

tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as gallic acid equivalents.

Using the same extracts the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP)[12]. One hundred milligrams of PVPP was weighed into a 100×12 mm test tube and to this 1 ml distilled water and then 1 ml of the sample extracts were added. The content was vortexed and kept in the test tube at 4°C for 4h. Then the sample was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured as mentioned above and expressed as the content of non-tannin phenolics (tannic acid equivalents) on a dry matter basis. From the above results, the tannin content of the sample was calculated as follows:

Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%)

### Determination of total flavonoid content

The flavonoid content was determined by the use of a slightly modified colorimetry method described previously by Zhishen et al. (1999)[13]. A 0.5ml aliquot of appropriately (10mg/2ml) diluted sample solution was mixed with 2ml of distilled water and subsequently with 0.15ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5ml, and then the mixture was thoroughly mixed and allowed to stand for another 15min. Absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

### Free radical scavenging activity on DPPH

The antioxidant activity of the different solvent extract was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois (1958)[14]. The sample extracts at various concentrations (60 - 300 µg) was taken and the volume was adjusted to 100 µl with methanol. 5 ml of 0.1 mm methanolic solution of DPPH was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Gallic acid and BHA were used as positive control. Percentage radical scavenging activity of the sample was calculated as follows:

% DPPH radical scavenging activity = (control OD-sample OD / control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

### *In vivo* studies

### Solvent extraction

The air dried powdered leaf and rhizome material of *Euphorbia fusiformis* was extracted in soxhlet extractor with ethanol. The ethanolic extract was evaporated to remove even the final traces of ethanol. The percentage yield was calculated and the dry extract obtained was used for the following ***in vivo*** studies. The extract was dissolved in water prior to use.

### Experimental animals

Animals used in the present study were procured from the small animals breeding station, Mannuthy, Kerala, India. They were housed in polypropylene cages (38 x 23 x 10cm) with not more than six animals per cage and maintained under standard environmental conditions (14h dark /10h light cycles; temp 25±2°C; 35-60% humidity, air ventilation) and were fed with standard pellet diet (M/s. Hindustan Lever Ltd., Mumbai, India) and fresh water ad libitum. The animals were acclimatized to the environment for two weeks prior to experiment use. Animals were fasted over night before the experimental schedule, but have free access for water ad libitum. The experiment was carried out according to the guidelines prescribed by Animal Welfare Board and with the prior approval of animal ethic committee.

### Acute toxicity

Acute oral toxicity studies were performed according to OECD (Organization for Economic Co-operation and Development) guidelines (Ecobichon, 1997)[15].Swiss albino male mice (n = 6/each dose) selected by random sampling technique were employed in this study. The animals were fasted for 12 h with free access to water only. Ethanol extract of *Euphorbia fusiformis* leaf and rhizome (dissolved in water) were administered orally at a dose of 5 mg/kg initially to mice and mortality was observed for 3 days. If mortality was observed in 4/6–6/6 animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one mouse out of six animals then the same dose was repeated with higher doses such as 1000, 2500, 5000 and 10000 mg/kg. The general behaviors such as mortality and clinical signs, which includes changes in skin fur, eyes and mucous membranes, were observed for the first 1 h and after 24 h of test drug administration. The gross behaviors like body positions, locomotion, rearing, tremors and gait was observed.The effect of plant extract on passivity, grip strength, pain response, stereotypy, vocalization, righting reflex, body weight and intake were also observed.

## RESULTS AND DISCUSSION

**Table 1.Yield percentage, total phenolics, tannins and flavonoids of different solvent extracts of leaf and rhizome of *Euphorbia fusiformis*.**

| Sample | Solvent | Percentage yield (%) | Total Phenolics (mg TAE/g extract) | Total Tannin (mg TAE/g extract) | Flavonoids (mg RE/g extract) |
|---|---|---|---|---|---|
| Leaf | Chloroform | 1.0 | $39.42 \pm 1.22^{c}$ | $7.02 \pm 1.56^{a}$ | $5.23 \pm 0.21^{d}$ |
| | Acetone | 4.8 | $65.41 \pm 0.25^{e}$ | $23.56 \pm 0.41^{c}$ | $11.09 \pm 1.31^{f}$ |
| | Ethanol | 8.8 | $99.94 \pm 0.83^{f}$ | $69.32 \pm 0.69^{d}$ | $29.90 \pm 0.10^{g}$ |
| | Water | 4.5 | $42.32 \pm 2.30^{d}$ | $17.22 \pm 5.20^{b}$ | $10.23 \pm 1.60^{e}$ |
| Rhizome | Chloroform | 4.0 | $15.52 \pm 0.19^{a}$ | $5.55 \pm 0.69^{a}$ | $0.62 \pm 0.02^{a}$ |
| | Acetone | 1.5 | $41.71 \pm 0.51^{d}$ | $7.65 \pm 0.58^{a}$ | $3.30 \pm 0.20^{c}$ |
| | Ethanol | 2.5 | $31.95 \pm 0.88^{b}$ | $7.65 \pm 0.88^{a}$ | $2.02 \pm 0.02^{b}$ |
| | Water | 23.0 | $15.53 \pm 0.19^{a}$ | $6.99 \pm 0.33^{a}$ | $0.23 \pm 0.01^{a}$ |

Values are means of three independent analyses ± standard deviation (n = 3). Mean value followed by different superscript letters indicate significant statistical difference ($P<0.05$).
TAE – Tannic acid equivalent.RE – Rutin equivalent.

Percentage yield of the air dried, powdered leaf and rhizome of *Euphorbia fusiformis* extracts obtained using different solvents such as chloroform, acetone, ethanol and water(Table1). The total phenolic content was determined according to the method described by Siddhuraju and Becker (2003). The analysis was performed in triplicate and the results were expressed as gallic acid equivalents. Using the same extracts tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP). From this result, the tannins content of the sample was calculated. The flavonoid content was determined by the use of a slightly modified colorimetry method described previously by Zhishen *et al*. (1999). The analysis was performed in triplicate and the results were expressed as rutin equivalent. This study offers a base of using *Euphorbia fusiformis* as herbal alternative. Phenol, falvonoids and tannins are associated with various degrees of anti-inflammatory, analgesic[16] and antioxidant activities[17,18]. Phenol, falvonoids and tannins are good antioxidant substances which prevent or control oxidative stress related disorders[19,20].

**Table 2.DPPH radical scavenging activity of different solvent extracts of *E. fusiformis* leaf.**

| Solvent | Concentration (µg) | Percentage activity (%) |
|---|---|---|
| **Chloroform** | 60 | 9.85 ± 1.23 |
| | 120 | 20.22 ± 0.24 |
| | 180 | 31.51 ± 1.52 |
| | 240 | 40.87 ± 0.03 |
| | 300 | 56.30 ± 1.12 |
| **Acetone** | 60 | 15.40 ± 0.20 |
| | 120 | 30.63 ± 0.19 |
| | 180 | 43.50 ± 0.39 |
| | 240 | 56.00 ± 0.70 |
| | 300 | 67.76 ± 0.15 |
| **Ethanol** | 60 | 17.32 ± 4.1 |
| | 120 | 24.92 ± 1.01 |
| | 180 | 40.19 ± 0.04 |
| | 240 | 52.41 ± 1.32 |
| | 300 | 80.63 ± 0.10 |
| **Water** | 60 | 7.22 ± 1.02 |
| | 120 | 16.23 ± 1.47 |
| | 180 | 20.2 ± 0.25 |
| | 240 | 37.5 ± 0.52 |
| | 300 | 40.8 ± 0.03 |

Values are means of three independent analyses ± standard deviation (n = 3).

**Table 3.DPPH radical scavenging activity of different solvent extracts of *E. fusiformis* rhizome.**

| Solvent | Concentration (µg) | Percentage activity (%) |
|---|---|---|
| **Chloroform** | 60 | 2.22 ± 0.41 |
| | 120 | 6.45 ± 0.43 |
| | 180 | 10.08 ± 0.41 |
| | 240 | 14.00 ± 0.28 |
| | 300 | 18.78 ± 0.26 |
| **Acetone** | 60 | 5.06 ± 0.28 |
| | 120 | 10.96 ± 0.13 |
| | 180 | 16.61 ± 0.31 |
| | 240 | 21.28 ± 0.18 |
| | 300 | 26.84 ± 0.22 |
| **Ethanol** | 60 | 6.73 ± 0.29 |
| | 120 | 9.20 ± 0.24 |
| | 180 | 15.10 ± 0.45 |
| | 240 | 19.87 ± 0.26 |
| | 300 | 26.15 ± 0.42 |
| **Water** | 60 | 1.93 ± 0.18 |
| | 120 | 4.52 ± 0.18 |
| | 180 | 6.73 ± 0.09 |
| | 240 | 8.66 ± 0.10 |
| | 300 | 11.10 ± 0.22 |

Values are means of three independent analyses ± standard deviation (n = 3).

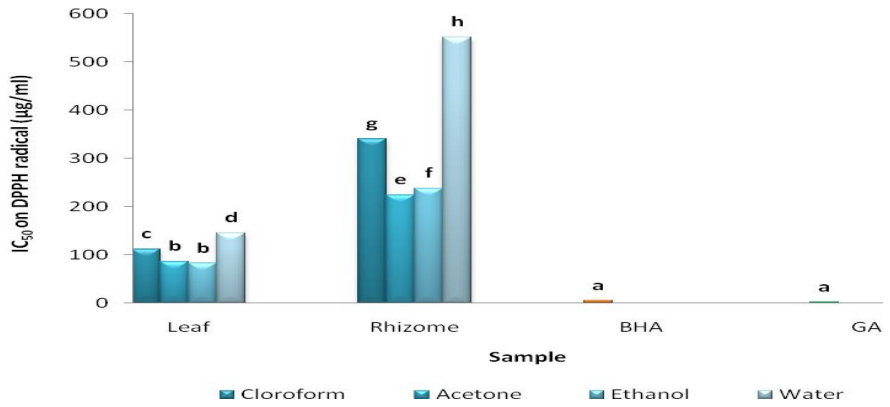


**Fig1.$IC_{50}$ of different solvent extracts of *Euphorbia fusiformis* leaf and rhizome on DPPH radical.**

Mean values followed by different superscript letters indicate significant statistical difference (P<0.05). BHA – Butylated hydroxyanisole; GA – Gallic acid.The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity [21]. From the above results, the amount of phenols, tannins and flavonoid content was observed in *Euphorbia fusiformis.* The presence of phenolic compounds may be due to the presence of tannins and flavonoids [22] which are known to possess antioxidant activities [23,24] From this studies leaf and rhizome extracts is taken by using the solvent chloroform, acetone, ethanol and water. The analysis was performed in triplicate. In this study, prove that *Euphorbia fusiformis* have the DPPH scavenging ability and when we increase the solvents extracts level the percentage of scavenging ability also increases (Table 2 &3)(Fig.1).

**Acute toxicity**

Acute oral toxicity studies were performed according to OECD (Organization for Economic Co-operation and Development) guidelines (Ecobichon, 1997).Swiss albino male mice (n = 6/each dose) selected by random sampling technique were employed in this study. Ethanol extract of *Euphorbia fusiformis* leaf and rhizome (dissolved in water) were administered orally at a dose of 5 mg/kg initially and then the same dose was repeated with higher doses such as 1000, 2500, 5000 and 10000 mg/kg. The general behaviors such as mortality and clinical signs, which includes changes in skin fur, eyes and mucous membranes, were observed for the first 1 h and after 24 h of test drug administration. From this observation the ethanol extract of *Euphorbia fusiformis* leaf and rhizome had no mortality and observable acute toxic effect on the experimental animals and therefore can be considered as non-toxic and safe for rat.

## CONCLUSION

From the above observation, the total phenolic content, tannins and flavonoid content was determined. DPPH radical scavenging ability and to evaluate their acute toxicity in mice. In the DPPH radical scavenging activity is observed in leaf and rhizome extracts of *Euphorbia fusiformis*. These results clearly indicate that *Euphorbia fusiformis* is effective against free radical mediated diseases. For the acute oral toxicity studies the plant extracts did not produce any toxic effect to the animal or no mortality up to 10,000 mg/kg body weight. So that we finally concluded the plant extracts is quite safe. For that, further study for detailed investigation is needed.

## REFERENCES

1. Kirley M. Canuto, Edilberto R. Silveira, Antonio Marcos E. Bezerra, Luzia Kalyne A.M. Leal and Glauce Socorro B.Viana; Phytochemistry, Pharmacology and Agronomy of medicinal plants. *Amburana cearensis*, an interdisciplinary study. Empresa Brasieira de Pesquisa Agropercuaria, Universidade Federal do Ceara, Brazil. 354 – 374.
2. Atal, C.K., ML.Sharma, A.Kaul and A.Khajuria; Immunomodulating agents of plant origin. 1:Preliminary screening 1986, J.Ethnopharmacol., 41:185-192.
3. Vinay R. Patel, Prakash R. Pate and Sushil S.Kajel; Antioxidant activity of some selected medicinal plants in Western Region of India 2010, Advan. Biol. Res., 4(1):23-26.
4. Shahidi, F. and P.D. Wanasundara; Phenolic antioxidants 1992, Cri. Rev. Food Sci. Nutr., 32:67-103.
5. Buyukokuroglu M.E.,I.Gulsin,M.Oktay and O.I.Kufrevioglu;*In-vitro* antioxidant properties of dantrolene sodium 2001, Pharmacol. Res., 44:491-494.
6. Koleva, I.I., T.A. Van Beeek, J.P.H. Linssen, A. de Groot and L.N. Evstatieva; Screening of plant extracts for antioxidant activity: a comparative study on three testing methods 2002, Phytochemical analysis, 13:8-17.
7. Motalleb, G., P. Hanachi, S.H. Kua, O. Fauziah and R. Asmah;Evaluation of phenolic content and total antioxidant activity in *Berberis vulgaris* fruit extract 2005, J. Biol. Sci.,5:648-653.

8. H.Wagnert;New approaches in Phytopharmacological research,1999. Pure Appl.Chem., Vol.71,No.9,pp.1649-1654.
9. Anusuya N, Raju K, Manian S; Hepato-protective and toxicological assessment of an ethnomedicinal plant *Euphorbia fusiformis*, Buch – Ham. ex D. Don 2010, J. Ethnopharmacol, 127 (2): 463-467.
10. Soumen Aditya; A revision of geophytic *Euphorbia* speicies from India, April 2010, Vol. 6. N.1.
11. Siddhuraju, P. and Becker; K.,Antioxidant properties of various solvent extracts of toal phenolic constituents from three different agroclimatic origins of Drumstick tree (*Moringa oleifera* Lam.)leaves 2003, Journal of Agricultural and Food Chemistry, 51:2144- 2155.
12. Siddhuraju, P. and Manian, S.; The antioxidant activity and free radical scavenging capacity of dietary phenolic extracts from horse gram (*Macrotyloma uniflorum* (Lam.) verdc.) seeds 2007, Food Chemistry , 105:950 – 958.
13. Zhishen, J., Mengcheng, T. and Jianming, W.; The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals 1999, Food Chemistry, 64: 555 – 559.
14. Blois, M.S.; Antioxidant determinations by the use of a stable free radical 1958,Nature, 26:1199 – 1200.
15. Ecobichon, D.J.; The basis of toxicology testing 1997, CRC Press, New York, pp.43-86.
16. Wang JR, Zhou H, Jiang ZH, Wong YF, Liu L; *Invivo* anti-inflammatory and analgesic activities of a purified saponin fraction derived from the root of *Ilex pubescens* 2008, Biological and Pharamaceutical Bulletin 31:643- 650.
17. Molina MF, Sanchez-Reus I, Iglesias I, Benedi J., Quercetin, a flavonoid antioxidant, prevents and protects against ethanol induced oxidative stress in mouse liver 2003,; Biological and Pharamaceutical Bulletin 26: 1398-1402.
18. Gholivand MB, Nasrabadi MR, Batooli H, Ebrahimabadi AH; Chemical composition and antioxidant activities of essential oil and methanol extracts of *Psammogeton Canescens* 2010, Food and Chemical Toxicology 48:24-28.
19. Vinson J A, Jang J, Dabbagh Y A, Serry M M, Cai S.; Plant phenols exhibit lipoprotein – bound antioxidant activity using an invitro model for heart disease. 1995a. J. Agric Food chem.43: pp2798-2799.
20. Vinson J A, Jang J, Dabbagh Y A, Serry M M,Jang J;Plant flavonoids, especially tea flavonoids, are powerful antioxidents using an *in vitro* oxidation model for heart disease. 1995, J. Agric Food chem.43: pp2798-2799.
21. Hein, K.E., A.R Tagliaferro and D.J Bobilya;Flavonoid antioxidant; Chemistry, metabolism and structure – activity relationships 2002, J.Nutr. Biochem., 13:572-584.
22. Nacoulama, O.G.;Plantes medicinales of pratiques medicinales Traditionnelles au BURKINAFASO:Cas du plateau central T1 and T2 1996, Doct. Theses D' Etat es Science NAI. Universite de Ouagadougon.
23. Aderogba ,M.A., E.K. Okoh and T.O. Idowu;Evaluation of the antioxidant activity of the secondary metabolites from *Pilostigma reticulatum* (DC.) 2005, Hochst. J. Biol. Sci., 5:239-242.
24. Badami, S., S. Moorkoth, S.R. Rai, E. Kannan and S. Bhojraj;Antioxidant activity of *Caesalpinia sappan* heartwood. 2003, Biol. Pharm.Bull.,26:1534-1537.

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6. Chapter in a book: Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995. p. 465-78.

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those applicable to full papers. Review articles need not be divided into sections such as materials and Methods and Results and Discussion, but should definitely have an Abstract and Introduction, if necessary.

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