



*A Special issue of:*

**“National Conference  
on  
Inclusive Development through Biotechnology”**

**Volume 10, SP – 11/Apr/2020**

**DOI: <http://dx.doi.org/10.22376/ijpbs/ijlpr.10.SP11/Apr/2020.1-96>**

*Publication partner*





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## **NATIONAL CONFERENCE ON INCLUSIVE DEVELOPMENT THROUGH BIOTECHNOLOGY**

**17<sup>th</sup> & 18<sup>th</sup> January, 2020**

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## About this Special Issue

Biotechnology is at the forefront of the battle, to improve the lives of millions throughout the world – by improving agricultural yields and food security, by developing alternative fuels. It has revolutionized mankind since its existence. It provides effective diagnostics, prevention and treatment measures including production of novel drugs and recombinant vaccines.

TO deliberate on emerging biotechnologies and novel approaches with the potential to achieve the goal of sustainability and striking a balance between developmental needs and environmental conservation, the Department of Life Sciences at Kristu Jayanti College organised the National Conference on Inclusive Development Through Biotechnology on 17<sup>th</sup> and 18<sup>th</sup> January 2020.

The event witnessed experts in the field sharing remarkable research and experiences that enlightened the academia.

It seems to be pertinent to publish the scientific articles presented in the conference that could be treated in a transversal way.

This Special Issue is a compilation of the research articles presented by authors on the varied applications of biotechnology for sustainable development. This issue will definitely be a great source of information for the recent researches in the relevant area.

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# HEAVY METAL CONTAMINATION IN PERIYAR RIVER: A REVIEW

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## ABSTRACT

Periyar river originates from the Sivagiri peaks of Sundaramala in Tamilnadu with a total length about 300kms in which 244kms in Kerala. Periyar river helps in power generation, domestic water supply, irrigation, tourism, industrial production, fisheries etc. River is undergoing eco degradation due to various anthropogenic stresses. Most prominent industries are situated on the banks of Periyar river. Effluents discharged from these chemical factories to Periyar river eventually reaches to Cochin estuary. Detection of toxic heavy metals which are bio accumulative and carcinogenic from various parts of river is alarming. Eloor, the industrial area situated at the banks of Periyar river recorded a higher rate of nervous system disorders, congenital malformations, circulatory and respiratory disorders, and a wide range of other diseases because of the action of toxic pollutants. Pollutants affect the river quality and even pollute the groundwater resources. Industries are ignoring this warning and none of the works is reported regarding the effluent treatment. There is no proper treatment system for these heavy metals which is discharging to the Periyar river by industries. There is an urgent need to develop an efficient, cost effective waste water treatment system to remove heavy metals and other toxic pollutants.

**KEYWORDS:** *Heavy metals, Bioaccumulation, Anthropogenic stresses, Toxic pollutants*

## 1.1 INTRODUCTION

Periyar river lies between latitudes 9°15'30"N and 10°21'00"N and longitudes 76°08'38"E and 77°24'32"E. Periyar basin spreads over an area of 5,398 square km most of which is situated in Central Kerala. The most industrialized zone of the Periyar river basin is Eloor-Edayar belt with large, medium and small scale industries. Several studies revealed that the river ecosystem has many dead zones and riverbed has high deposits of heavy metals. Greenpeace India identifies Periyar as one of India's most contaminated rivers and describes lower Periyar as a cess pool of toxins due to alarming levels of DDT, endosulfan, hexa and trivalent chromium, lead, cyanide, BHC. Pollutants turn the river into black and other colours which is being discharged through underwater pipes. Regular fish kills are common in Periyar river due to the discharge of this toxic pollutants to the river by these industries.<sup>1,2,3</sup>

## 1.2 INDUSTRIES AND HEAVY METALS

The most prominent industries on the banks of Periyar are Hindustan Insecticide Limited, Merchem Limited, Indian Rare Earths Limited, The Travancore Cochin Chemicals, Fertilisers and Chemicals Travancore, Binani Zinc Limited, etc. Hindustan Insecticide Limited produces DDT and other pesticides. Travancore Cochin Chemicals engaged in the manufacture of chlorine, caustic soda and allied chemicals. Fertilisers and Chemicals Travancore manufacture fertilizers like ammonium phosphate, ammonium sulphate, soda ash and so on. Merchem Limited produces rubber chemicals; Indian Rare Earths Limited mines beach sand for minerals, Binani Zinc contaminating the river with heavy metals like zinc, lead, cadmium, chromium, iron, and nickel, and this was the chief cause for the river turning red.<sup>1</sup> Heavy metals can result in reduced mental and central nervous system, damage to blood composition, lungs, kidney, liver and other vital organs. Heavy metals are associated with adverse health effects such as allergic reactions (e.g., beryllium, chromium), neurotoxicity (e.g., lead), nephron toxicity (e.g., mercuric chloride, cadmium chloride) and cancer (e.g., arsenic, hexavalent chromium).<sup>4</sup> Distribution of metals like mercury, copper, lead and nickel in the Periyar river was studied by Ouseph et al (1994).<sup>5</sup> The study by Anju et al (2011) revealed that heavy metal concentration in Periyar river near industries were high, particularly during premonsoon period.<sup>6</sup> Results of

heavy metal analysis in water and sediments revealed high concentration of heavy metals such as Cr, Cu, Mn, Pb and Zn in Kuzhikandam Creek, Eloor is a strong indication of industrial activities by HIL, FACT, TCC and Binani Zinc (Ciji, 2012).<sup>7</sup> Heavy metals present in the Kuzhikandam Creek sample from studies conducted by Ciji (2012) and Lekshmi et al (2017) is mentioned in Table 1 and Table 2.

**Table 1: Heavy metals present in the sample.<sup>7</sup>**

Heavy Metal (mg/l)	Kuzhikandam Creek
Chromium	1.205
Copper	2.59
Manganese	2.99
Lead	2.06
Zinc	16.26

**Table 2: Heavy metals present in the sample.<sup>4</sup>**

Heavy Metal (mg/l)	Kuzhikandam Creek
Cadmium	1.5
Iron	8.4
Copper	3.12
Manganese	4.01
Lead	3.18
Zinc	12.32

### 1.3 HEAVY METAL REMEDIATION

Bioremediation is a branch of biotechnology for removing contaminants, pollutants and toxins from water and soil using organisms such as bacteria, algae, fungi. The study by Binish et al (2015) found that *Clostridium bifermentans* isolated from sediments of Cochin estuary has exhibited multiple heavy metal resistance to three heavy metals lead, cadmium and copper. Minimal inhibitory concentration (MIC) of 12 ug/ml was noticed for lead and cadmium and 10ug/ml for copper. Resistant pattern of *Clostridium bifermentans* was lead=cadmium>copper.<sup>8</sup> Study conducted by Prashant et al (2016) indicated that increasing concentration of metal solution affect the bacterial growth at considerable level. Thirty bacterial strains were isolated from water and sediment samples of Periyar river and nutrient agar plate was used for isolation and multi-metal analysis. More than 85% of isolated strains were resistant to minimum level. 93.3% were resistant to copper solution and 90% resistant to chromium solution. Certain test cultures were sensitive to lowest concentration (10mM) and very few strains were resistant to high concentration (200mM).<sup>2</sup> Lekshmi et al (2017) in their study analysed heavy metals from two sites of Periyar river and carried out batch and equilibrium studies. Lead removal studies were done using *Citrus sinensis* and *Mangifera indica* and found out that orange peel has greater affinity for lead with 92.6% for site I (Cd>Pb>Fe>Zn>Mn>Cu) and 92% at site II (Cd>Zn>Pb>Mn>Cu>Fe) at concentration 2.5.<sup>4</sup>

### 1.4 CONCLUSION

Higher heavy metal concentration in the environment harm majority of native microflora and some of them evolve resistance mechanism towards heavy metals.<sup>9</sup> Total heavy metal content of sediments of Cochin estuary was higher when compared with average values reported from other Indian rivers.<sup>10</sup> Number of multi-resistant strains to several metals was higher in sea/river water samples containing lower level of faecal indicators.<sup>11</sup> Heavy metal accumulation in Periyar river has resulted in adaptation of bacterial distribution, diversity and their growth is proportionate to the extent of pollution. The river received various pollution sources throughout the river-line.<sup>2</sup> Most of the studies on heavy metal remediation involves isolation of heavy metal resistant bacteria at different concentrations but not experimented in reality to treat the industrial effluents. So studies should be conducted on bioreactors using consortium of microorganisms for efficient removal of heavy metals discharged from the industries.

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# A GREENER METHOD TO DETECT AND QUANTIFY SAPONIN CONTENT IN SOAPNUT OF THREE SPECIES- A COMPARATIVE STUDY

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## ABSTRACT

Saponin was extracted from soapnuts of three species found in India- *Sapindus mukorossi*, *Sapindus trifolatus* and *Sapindus emarginatus*. A modified Vanillin Sulphuric Acid Assay was employed to measure saponin content. Aqueous extract of dried soapnut pericarp was prepared by four methods- ultrasonication, ultrasonication using 50% ethanol-water, maceration and boiling. Saponin, being water soluble, does not require reflux or soxhlet extraction using organic solvents. The modified method used 1% aqueous vanillin solution and 65% Sulphuric acid. Full colour development occurred without heating. Results revealed that *S. trifoliates* has 34% more saponin than Quillaja standard. *S. emarginatus* and *S. mukorossi* contain 80% and 78% more sapogenin than *S. trifoliatus* respectively. There was no statistically significant difference in the yield of the four extraction methods. Boiling hastens filtration of extract. This method is quicker and needs fewer chemicals to assess saponin content in *Sapindus* spp.

**KEYWORDS:** Soapnut, Saponin, Vanillin, Quillaja, Ultrasonication, UV-Visible Spectrophotometer.

## 1. INTRODUCTION

Soapnut is a traditional cleaning agent that was widely used in India before the advent of laundry soaps and detergents. Three tree species in India yield soapnuts- *Sapindus mukorossi*, *Sapindus trifolatus* and *Sapindus emarginatus*. Soapnut has mild detergent action due to the presence of the hederagenin saponins.<sup>1</sup> It has found applications in pharmacology<sup>2</sup>, ayurveda<sup>3</sup>, biopesticides<sup>4</sup> and poultry feed supplement.<sup>5</sup> Besides natural shampoos,<sup>6</sup> soapnuts are being explored as eco-friendly and biodegradable replacement to synthetic detergents.<sup>7,8</sup> Soapnut oil also has potential to produce biodiesel.<sup>9,10,11</sup> The present work attempts to find the most convenient and cost effective method to detect and quantify saponin content in soapnuts.

## 2. MATERIAL AND METHODS

### 2.1 Soapnuts:

Soapnuts of *S. emarginatus* and *S. mukorossi* were purchased from markets in Hyderabad and Coimbatore respectively. Fresh *S. trifoliatus* fruit was collected from CUSAT and air dried under sunlight. All three fruit samples were verified by Dr. C. Murugan, Scientist and Taxonomist at Botanical Survey of India. Dried fruits were manually deseeded, crushed and stored in air-tight containers. Soapnut-based commercial laundry detergent was purchased online.

### 2.2 Chemicals:

AR grade Vanillin Merck, 98% Sulphuric Acid from Merck, Saponin *Quillaja* sp. with Sapogenin content 20-35 % from Sigma Aldrich.

### 2.3 Instruments:

Ultrasonic processor UP 100H from Heilscher, UV-Visible Spectrophotometer –Evolution 201 by Thermo Scientific and Digital Weighing Balance CPA225D from Sartorius.



**2.4 Extract preparation:**

Crude extract of soapnut was prepared by 3 methods: maceration, maceration followed by boiling and ultrasonication. All extracts were freshly prepared for assay.

**2.4.1 Maceration:**

5 gm of soapnut powder was soaked for 12 hours at room temperature in 40 ml double distilled water<sup>12</sup>. The mixture was gently ground manually and filtered through Whatman Filter Paper No 1. Care was taken to prevent froth formation.

**2.4.2 Maceration followed by boiling:**

5gm deseeded fruit was soaked for 12 hours in distilled water and boiled for 2 minutes. The supernatant was filtered through Whatman filter paper no 1.

**2.4.3 Ultrasonication:**

5gm roughly crushed soapnut was placed in 40 ml double distilled water<sup>13</sup> and ultrasonicated for 5 min on 20% amplitude and 15 minutes on 40% amplitude. The supernatant was filtered.

**2.4.4 Ultrasonication with 50% ethanol mix:**

Based on Koseet *al* (2016)<sup>1</sup>, ultrasonication with 50% ethanol-water as solvent was prepared. The procedure was same as above.

**2.5 Vanillin Sulphuric Acid Assay:**

The Vanillin Sulphuric Acid Assay as reported by Hiai *et al* (1975)<sup>14</sup> requires 0.5 mL sample to mix with 0.5 ml 8% (w/v) vanillin in 50% water-ethanol mix in an ice bath. 2.5 mL of 72% H<sub>2</sub>SO<sub>4</sub> is added<sup>15</sup>. The mixture is heated to 60°C for 15 min for full colour development. The expected colour is pink to purple; the absorbance was recorded at 527 nm using UV-Vis spectrophotometer. Standard calibration curve is prepared with Oleanoleic Acid or *Quillaja* saponin. Since saponins are completely water soluble, this method was suitably modified as follows: 0.5 ml sample was added to 0.5 ml 1% aqueous vanillin solution. The mixture was chilled in an ice bath. 2.5 ml of 65% sulphuric acid was added drop by drop. The mixture was gently stirred for 3 minutes in the ice bath. Colouration began within 3 minutes. After 15min at room temperature, full colour development was recorded using UV-Visible Spectrophotometer in the range 450 to 600 nm. Readings were compared with standard curve of *Quillaja* saponin, expressed in *Quillaja* equivalent using the formula:

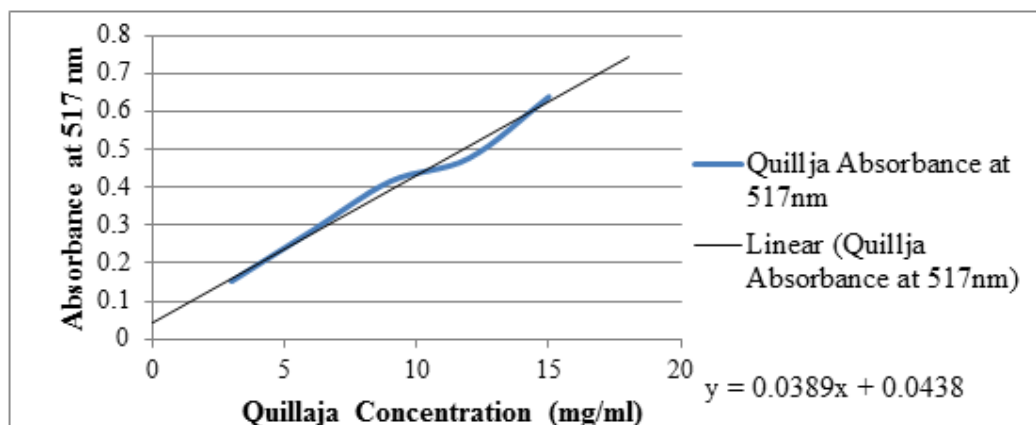
$$\frac{(\text{Reading- Blank}) \times \text{Extracted Volume} \times \text{Dilution factor}}{\text{Dry Sample Weight}}$$

The assay was conducted in triplicate for each soapnut sample. Using this method, the saponin content of a soapnut-based commercial laundry detergent was assessed.

**3. RESULTS**

Wavelength for maximum absorption  $\lambda$  max (nm) for different materials is as follows: *Quillaja*: 517nm, *Sapindus mukurossi*: 530.5nm, *S. emarginatus*: 532.5, *S. trifoliatius*: 531.5nm, Soapnut-based detergent: 532.5nm (at 15 min after reaction), 590nm and 450.5 (at 60 min after reaction).

A standard curve of absorbance for *Quillaja* saponin was prepared (Fig 1). Absorbance of the three soapnut samples, prepared through four different extraction methods (average of triplicate readings) was calculated using the Standard *Quillaja* Curve (Fig 1). This is presented in Table 1.

**Fig I: Standard Graph of Absorbance for Quillaja saponin by Vanillin Sulphuric Acid Assay.**

**Table 1**  
**Comparison of Sapogenin content in 'Quillaja Equivalent' for 3 species of Soap nuts by different extraction methods**

EXTRACTION METHOD	<i>S. trifoliatus</i>	<i>S. mukorossi</i>	<i>S. emarginatus</i>
Maceration	1.12	1.70	2.59
Maceration followed by boiling	1.65	2.69	2.75
Ultrasonication	1.33	2.98	2.17
Ultrasonication in 50% ethanol	1.25	2.17	2.14
<b>Avg. Quillaja equivalent</b>	<b>1.34</b>	<b>2.38</b>	<b>2.41</b>

A two-way ANOVA test revealed that there is no significant difference between extraction methods at 5% level of significance.

#### 4. DISCUSSION

This is the first study comparing the saponin content of the three species, to the best of the authors' knowledge.

##### 4.1 Vanillin-Sulphuric Acid Assay:

This assay quantifies the total saponins on the principle that saponins are digested by conc.  $H_2SO_4$  to release sapogenins, which are then oxidized by vanillin. The reaction turns the solution to pink- purple. Absorbance is measured in a UV-Vis Spectrophotometer at 450-600 nm. Le *et al.* (2018)<sup>15</sup> improved this method by adding a solvent evaporation step before spectrophotometry. They reported that solvents including methanol, acetone, butanol, acetonitrile, ethylacetate, dichloromethane, diethyl ether, chloroform and hexane interfere with the colourimetric analysis. Ethanol gave the least interference in the reagent blank, with a standard curve matching that of water as a solvent. In this modified assay we have eliminated solvent extraction with methanol, ethanol, hexane, or acetone and also the solvent evaporation step. Hence, soxhlet extraction and reflux were disregarded. Corresponding to this, vanillin solution was prepared in water. Vanillin is water soluble only up to 1.25% concentration.<sup>16</sup> It was observed that freshly prepared vanillin solution gives best colour development. Even a 24-hour old solution causes charring or discoloration during assay. 72% Sulphuric acid caused charring of vanillin and discolouration to dark brown. To prevent this, different concentrations of sulphuric acid were tried. Reaction did not occur below 60%  $H_2SO_4$ . Best results were produced with 65%  $H_2SO_4$ .

##### 4.2 Extraction methods:

While *S. mukorossi* fruit can be crushed and sieved, the other varieties are very hygroscopic. *S. trifolatus* has a sticky texture and high mucilage content. Boiling expedites filtration, but changes the aroma of the extract.

#### 4.3 Commercial soapnut detergent:

*S. emarginatus* was detected in the commercial soapnut detergent which claims to be free from chemical foaming agents. Two other peaks developed after 60 minutes. This could be due to essential oils present. Despite digestion with H<sub>2</sub>SO<sub>4</sub>, the solution continued to froth. It is possible that some non plant-based surfactant has been added.

## 5. CONCLUSION

Water is the most efficient solvent for saponin extraction and quantification from soapnut. This corroborates the work of Li et al (2013)<sup>17</sup> who used foam fractionation method for separation of total saponins from *S. mukorossi*. The Vanillin-Sulphuric acid assay with 1% aqueous vanillin solution and 65% sulphuric acid is an efficient method to quantify saponin content of soapnuts. *S. emarginatus* is marginally higher in saponin content than *S. mukorossi*, but nearly twice that of *S. trifolatus*. Suitable applications can be designed for the different soapnuts based on their saponin and mucilage content.

## 6. ACKNOWLEDGEMENT

We extend our sincere gratitude to the Director, School of Environmental Studies for providing facilities to carry out the research work. We thank Department of Applied Chemistry, CUSAT for their support with the UV-Visible Spectrophotometer and Botanical Survey of India for verification of plant material.

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## ANALYSIS ON PESTICIDAL PROPERTIES OF CHOSEN PLANT SAMPLES

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### ABSTRACT

In the present investigation the pesticidal properties present in essential oils of *Citrus medica* leaves and *Ixora coccinea* flowers were analyzed. The compounds present in the essential oil extracted from the plant sources were studied by preliminary screening and GC-MS method. Fumigation assay method was used to study the pesticidal effect of the essential oil against the adult insects of *Tribolium castaneum*. Further the molecular mechanism of pesticidal activity of the essential oils were screened by *in-silico* method by choosing the A cetylcholinesterase of the *Tribolium castaneum* as target. The compounds obtained from GC-MS which obeyed the Tice rule were used as ligand to study the molecular interaction using docking. The fumigation assay results showed the highest pest mortality of  $82 \pm 2.6\%$ ,  $64.33 \pm 4.0\%$  in 80% concentration of essential oil of *Citrus medica* leaf and *Ixora coccinea* flowers respectively on *Tribolium castaneum* in 48 Hrs. The molecular docking results showed the compounds Curan,16,17,19,20-tetradecahydro- of *Citrus medica* and 3H-Pyrazol-3-one,4-[[4-(dimethylamino)phenyl]imino]-2,4-dihydro-2,5-diphenyl of *Ixora coccinea* have significant molecular interaction with the target with the lowest energy value of -89.65 and -98.83 respectively. Hence the compounds which showed the pesticidal activity can be utilized to develop potent botanical pesticide against *Tribolium castaneum*.

**KEYWORDS:** Pesticidal activity, *Citrus medica* leaves, Tice rule, fumigation and *Ixora coccinea* flowers.

### INTRODUCTION

Biopesticides generally have several advantages when compared to conventional pesticides<sup>1,2</sup>. Biopesticides are naturally less toxic to humans and the environment, do not leave harmful residues, and are usually more specific to target pests. In the present study the *Citrus medica* and *Ixora coccinea* were used for analyzing its pesticidal activity against the pest *Tribolium castaneum*. *Tribolium castaneum* have been selected as model pest which affects economically important crops such as wheat. The red flour beetle (*Tribolium castaneum*) is a species of beetle in the family Tenebrionidae. It is a worldwide pest of stored products, particularly food grains, and also a model organism for ethological and food safety research. In the past decades, apart from the pyrethrum which has attained international and commercial approval due to its high effectiveness and broad spectrum pesticidal activity very few natural insecticides have been developed. Thus, the present study aimed to identify a bioactive compound from essential oil of *Citrus medica* leaves and *Ixora coccinea* flowers against *Tribolium castaneum* as an alternative for harmful chemical pesticides.

### MATERIALS AND METHODS

#### PRAPARATION OF ESSENTIAL OIL

The fresh leaves of *Citrus medica* were collected from the plant at Noombal, Thiruvallur district, Tamilnadu. The fresh flowers of *Ixora coccinea* were collected from the plant at Aranvoyaluppam, Thiruvallur district, Tamilnadu. The collected leaves and flowers were washed thoroughly under tap water and shade dried for two weeks. The shade dried plants were chopped into pieces and stored. From the chopped plant material, 30g of semidried chopped pieces of *Citrus medica* leaves and *Ixora coccinea* flowers was soaked in 200mL of Hexane separately. The contents were allowed to stand for 144 hours. Then the contents were filtered

using Whatman filter paper. Again the extract was soaked in 100mL of ethanol to extract the essential oil since essential oil is soluble in ethanol. The contents with ethanol were filtered. The mixture was then transferred to 500mL separating funnel and separated by a process called liquid-liquid separation process. The content of the separating funnel was allowed to equilibrium for 2 hours and the contents were separated into two layers depending on the density difference. The upper hexane layer and lower ethanol layer (along with natural essential oil) were separated and stored in dark bottles. The lower ethanol-oil layer was boiled for 5 minutes in a water bath at 78°C to remove the ethanol leaving the natural essential oil<sup>3,4</sup>.

## PRELIMINARY TEST FOR ESSENTIAL OIL:

### *Evaluation of miscibility with alcohol*

The higher the miscibility with alcohol of low concentration the higher the percentage of oxygenated constituents. 1mL of heated ethanol extract (to remove ethanol) containing oil is completely miscible with 1mL of absolute ethanol.

### *Solubility with non-polar solvents*

The essential oil is soluble in non-polar solvents such as Benzene, Carbon disulfide and light petroleum. 1mL of heated ethanol extract (to remove ethanol) containing oil is completely miscible with 1mL of Benzene.

### *Solubility with water*

The oils are immiscible in water. The oil always floats to the top because it is less dense than water. Oil and water don't mix because water molecules are more attracted to each other than to oil molecules. 1mL of essential oil extract was added into 1mL water, the oil layer floats on the water layer.

### *GC-MS analysis*

GC-MS is used to separate the compounds present in the crude extract. This technique is used for the analysis of various organic volatile and semi-volatile compounds. An Agilent 6980 gas chromatogram was used and the helium carrier gas was set to 2mL/minute flow rate. The compounds were identified using NIST library V11.

### *Molecular property analysis*

The pesticidal properties of compounds identified from Gas chromatography-Mass spectrometry were analyzed based on the Tice rule using PUBCHEM database.

### *Pesticidal activity*

Adults of *Tribolium castaneum* were collected from affected *Triticum aestivum* (Commonwheat). The collected insects were kept in plastic cups closed with transparent lids for observation of their behavior for about three weeks. The insects were reared under laboratory conditions (26°C +2°C, 57-67% RH) separated and they were provided with fresh wheat grains placed in plastic cups closed with transparent plastic lid (plastic box) with holes for aeration. The feed was changed every week. The adults were reared to check whether the laboratory condition was suitable for its undisturbed lifecycle. The adult insects were again collected and reared in groups of ten to fifteen insects for acclimatization in laboratory conditions before two days of treatment and these groups were used for testing. Fumigation bioassay was carried out with *Tribolium castaneum* using essential oils extracted from *Citrus medica* leaves and *Ixora coccinea* flowers. Adult insects of uniform size were taken from the mass culture maintained in the laboratory. For each treatment 5 adult insects stored for 2 days were introduced in separate plastic petri plates containing feed of 1 gram fresh wheat grains. Both the essential oil extracts were applied on a whatman filter- paper slice and that was attached to the lower side of the petri plate's lid. Three replicates were maintained for each treatment. Feed without essential oil extracts was used as a negative control. There are three concentrations of both essential oils such as 20%, 40% and 80% in ethanol for assessing pesticidal activity. Feed with ethanol alone applied on the whatman filter- paper was also maintained as a control for checking whether ethanol has any effect on insect mortality. The entire experiment was conducted at 26°C +2°C, 57-67% RH. After 24 hours of feeding, the weight of the leftover feed and adult insects were recorded. And similarly after 48 hours, the weight of the leftover feed and adult insects were recorded for all concentrations of both



the essential oil extracts. All these treatments were repeated thrice for data validity for each concentration of essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers. Triplicates were maintained for 48 hours and for each and every concentration including the control and negative control without Ethanol treatment and the data were obtained from the maintained triplicates of each concentration of both essential oil extracts. Results of Percentage of larval mortality, Feeding deterrence index, and Consumption rate were estimated using the following formulae with the data collected from the maintained triplicates for each concentration of both essential oil extracts.

#### **Percentage larval mortality**

Calculated after a period of 24hours and 48hours using Abbott's formula<sup>5</sup>.

$$\% \text{Insect Mortality} = (T-C) / (100-C) * 100$$

Where, T –Percentage mortality in treated, C – Percentage mortality in control.

**Feeding deterrence index:** Calculated for each treatment using the formula of Ben Jannet *et.al.*<sup>6</sup>

$$\text{FDI} = (C-T) / (C+T) * 100$$

Where, C – Consumption in Control, T – Consumption in Treated.

**Consumption rate:** The following formula is used according to Waldbauer (1968) and Slansky and Scriber (1985)<sup>7,8</sup> to calculate CR (consumption rate).

$$\text{CR} = \text{Weight of food eaten} / \text{Duration of Experiment (Days)}.$$

#### **Bioinformatics approach for pesticidal studies**

The enzymes target (Acetylcholinesterase) of *Tribolium castaneum* was chosen from protein sequences databases such as NCBI. The structure of Acetylcholinesterase was not available in PDB database. So, the structure of Acetylcholinesterase of *Tribolium castaneum* was modeled using GENO3D software.

#### **Geno 3D online tool**

Geno3D (<http://geno3d-pbil.ibcp.fr>) is an automatic web server for protein molecular modelling. Five models were generated, the best structure model was chosen based on the least energy value as the compounds in nature exist in least energy state and also based on the Ramachandran plot generated for each of five modeled structures from GENO3D tool. Based on these two criteria best model was chosen and used for molecular docking with the selected phytochemical compounds of both the essential oil extracts.

#### **Molecular docking studies**

Molecular docking is a well-established computational technique which predicts the interaction energy between two molecules. This technique mainly incorporates algorithm like molecular dynamics .Monte Carlo stimulation, fragment based search method<sup>9</sup>. iGEMDOCK is a Graphical Environment for Recognizing Pharmacological Interactions and Virtual Screening. Pharmacological interactions are useful for identifying lead compounds and understanding ligand binding mechanisms for a therapeutic target. The PDB format files of selected compounds (ligands) and the modeled enzyme (Acetylcholinesterase) structure in PDB format were uploaded and docked.

## **RESULTS AND DISCUSSION**

#### **Preliminary test for essential oil**

As the essential oils have the capacity to mix with alcohol even at low concentration, we tested its miscibility with alcohol (70% ethanol). The essential oils of *Citrus medica* leaves and *Ixora coccinea* flowers were miscible in ethanol.



**Solubility with non-polar solvents:** As the essential oils are miscible in non-polar solvents such as Benzene, Carbon disulfide and light petroleum, we tested the miscibility of essential oils of *Citrus medica* leaves and *Ixora coccinea* flowers with Benzene. The extracts were completely miscible with Benzene.

**Solubility with water:** As oils are immiscible in water. The oil always floats to the top because it is less dense than water. Oil and water don't mix because water molecules are more attracted to each other than to oil molecules. We tested the miscibility of essential oil in water. The essential oils of *Citrus medica* leaves and *Ixora coccinea* flowers were immiscible with water and the oil layer floats on the water layer.

**GC-MS analysis:** Various compounds were identified from Gas chromatography-Mass spectrometry Analysis of both the essential oils from *Citrus medica* leaves and from *Ixora coccinea* flowers. The compounds identified from the essential oil were listed in Table 1 and 2.

**Table1**  
**Compounds in essential oil of *Citrus medica* leaves**

S.NO	COMPOUND	RT
1	$\alpha$ -Pinene	4.6
2	5-Caranol,trans,trans-(+)-	5.7
3	Terpineol,cis- $\alpha$ -	6.78
4	Caryophyllene	11.17
5	Flavone	15.85
6	Pentadecanoic acid,14-methyl-,methyl ester	16.9
7	Pentadecanoic acid,methyl ester	17.52
8	Phytol	18.65
9	Octadecanoic acid	19.28
10	Curan,16,17,19,20-tetradecahydro-	22.62
11	2,6-Bis(1,1-dimethylethyl)-4-phenylmethylenecyclohexa-2,5-dien-1-one	24.45
12	Coumarine,3-(2,4-dinitrophenyl)-	26.97
13	Terpineol	8.27
14	Bicyclo[3.1.1]hept-2-ene,2,6,6-trimethyl-,(n)-	3.72
15	$\gamma$ -Elemene	12.2
16	Phenol,2,6-bis(1,1-dimethylethyl)-4-[(4-hydroxy-3,5-dimethylphenyl)methyl]-	30.48

**Table2**  
**Compounds in essential oil of *Ixora coccinea* flowers**

S.NO	COMPOUND	RT
1	Spiro[1,3-dioxolane-2,2'(1'H)-naphthalene]-1'-methanol,3',4'-dihydro-	13.93
2	Piperazine,1-methyl-4-(1,2,3,4-tetrahydro-2-naphthyl)-	16.05
3	Hexadecanoic acid,ethyl ester	17.6
4	Pyrano[4,3-b]benzopyran-1,9-dione,5a-methoxy-9a-methyl-3-(1-propenyl)perhydro-	19.37
5	1,2-Benzenedicarboxylic acid,mono(2-ethylhexyl)ester	22.62
6	Quinazolin-4(3H)-one,3-(3-methoxyphenyl)-2-(2-phenylethenyl)-	24.43
7	3H-Pyrazol-3-one,4-[[4-(dimethylamino)phenyl]imino]-2,4-dihydro-2,5-diphenyl-	26.95
8	Isopropyl stearate	19.98

**Molecular property analysis:** All the compounds identified from the essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers through Gas chromatography –Mass spectrometry Analysis were assed for insecticide likeness properties. There were 5 and 6 compounds from the essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers strictly follows the Tice rule (Table 3 and 4) which possess the Molecular weight within 150 to 500 g/mol. Similarly, the number of rotatable bonds was below 12. The Log

P values of the compounds were within 0 to 5. The Number of hydrogen bond donors was less than or equal to 2. The Number of hydrogen bond acceptors was within 1 to 8.

**Table 3.**  
*Compounds extracted from Citrus medica leaves.*

S.No	Name of the compound	Molecular weight g/mol	Log P	No. of H bond donors	No. of H bond acceptors	No. of Rotatable bonds
1	5-Caranol,trans,trans-(+)-	154.253	2.6	1	1	0
2	Terpineol,cis- $\alpha$ -	154.253	1.8	1	1	1
3	Flavone	222.243	3.6	0	2	1
4	Curan,16,17,19,20-tetradecahydro-	278.399	2.7	1	2	0
5	Terpineol	154.253	1.8	1	1	1

**Table 4.**  
*Compounds extracted from Ixora coccinea flowers.*

S.No	Name of the compound	Molecular weight g/mol	Log P	No. of H bond donors	No. of H bond acceptors	No. of Rotatable bonds
1	Spiro[1,3-dioxolane-2,2'(1'H)-naphthalene]-1'-methanol,3',4'-dihydro-	220.268	1.1	1	3	1
2	Piperazine,1-methyl-4-(1,2,3,4-tetrahydro-2-naphthyl)-	230.355	2.9	0	2	1
3	Pyran[4,3-b]benzopyran-1,9-dione,5a-methoxy-9a-methyl-3-(1-propenyl)perhydro-	308.374	1.6	0	5	2
4	1,2-Benzenedicarboxylic acid,mono(2-ethylhexyl)ester	278.348	4	1	4	9
5	Quinazolin-4(3H)-one,3-(3-methoxyphenyl)-2-(2-phenylethenyl)-	354.409	4.5	0	3	4
6	3H-Pyrazol-3-one,4-[[4-(dimethylamino)phenyl]imino]-2,4-dihydro-2,5-diphenyl-	368.44	4.7	0	4	4

**Pesticidal activity:** The essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers have pesticidal activity against *Tribolium castaneum* and caused mortality. At 80% (higher) concentration of the essential oils, the percentage mortality of *Tribolium castaneum* was higher than the other concentrations such as 20% and 40% concentrations of essential oil extracts (Table 5). On comparison, the percentage of insect mortality was higher 40.66% in treatments involving 80% concentration essential oil extract of *Citrus medica* leaves against *Tribolium castaneum* in 24 hrs of treatment.

**Table 5**  
*Percentage of insect mortality on different concentrations of essential oil extract of Citrus medica leaves and Ixora coccinea flowers*

CONCENTRATION (V/V)	<i>Citrus medica</i> LEAVES		<i>Ixora coccinea</i> FLOWERS	
	24Hours	48Hours	24Hours	48Hours
20	0	42.66 $\pm$ 2.516	0	22.33 $\pm$ 2.516
40	24 $\pm$ 3.605	61 $\pm$ 1.732	0	41.66 $\pm$ 2.886
80	40.66 $\pm$ 2.081	82 $\pm$ 2.645	18.66 $\pm$ 1.527	64.33 $\pm$ 4.041

Control	0	0	0	0
Negative Control	0	0	0	0

\*Mean±Standard deviation

**Feed deterrence index:** The feed deterrence index was calculated on the basis of diet consumed on the respective treatment, it shows that both the essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers are feed deterrent to *Tribolium castaneum*. With the increase in concentration of essential oil extracts an increased deterrence index (DI) was observed (Table 6).

**Table 6**  
*Feed deterrence index of essential oil extract of Citrus medica leaves and Ixora coccinea flowers*

CONCENTRATION (V/V)	Citrus medica LEAVES		Ixora coccinea FLOWERS	
	24Hours	48Hours	24Hours	48Hours
20	40.33±1.527	48±2.00	32.77±0.692	39±1.732
40	50±1.154	59.66±1.527	33.11±1.017	48.66±1.527
80	98.66±1.527	97.66±2.081	98±2.00	59±1.00
Control	29.66±1.527	42.29±0.485	29.66±1.527	32.57±1.365
Negative control	9.33±1.154	24±1.00	18.66±1.527	13.82±0.717

\*Mean±Standard deviation

**Consumption rate:** As the feed deterrence index is increasing with increasing concentration of both the essential oils, the consumption rate of *Tribolium castaneum* is decreasing with increasing concentration of essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers (Table 7). The Consumption rate of *Tribolium castaneum* was lower in the treated samples rather than the untreated samples. The maximum lower Consumption rate 0% was found to be with 80% essential oil treatment for both the essential oil extracts.

**Table 7**  
*Consumption rate on different concentrations of essential oil extract of Citrus medica leaves and Ixora coccinea flowers*

CONCENTRATION(V/V)	Citrus medica LEAVES		Ixora coccinea FLOWERS	
	24Hours	48Hours	24Hours	48Hours
20	0.166±0.057	0.176±0.025	0.13±0.036	0.176±0.025
40	0.14±0.052	0.143±0.040	0.143±0.045	0.223±0.025
80	0±0	0±0	0±0	0.133±0.057
Control	0.283±0.076	0.25±0.05	0.21±0.036	0.30±0.05
Negative control	0.383±0.076	0.35±0.05	0.31±0.036	0.25±0.05

\*Mean±Standard deviation

## ENZYME (TARGET) STRUCTURE MODELING FOR MOLECULAR DOCKING

GENO3D online tool was used for modeling the structure of target enzyme Acetylcholinesterase of *Tribolium castaneum*. From three template chosen based on maximum Identity percentage values, five models were generated (Table 8). From the evaluation of five models generated, the model which has least energy value and whose majority of amino acids fall in the favoured region of generated Ramachandran plot was chosen as the best structure model. These two criteria are satisfied by the model 2. Hence, it was chosen as a best structure model and used for molecular docking with the selected compounds from both the essential oil extracts.

**Table 8**  
**Models Generated From Geno3d Online Tool**

S.NO	MODELS GENERATED	ENERGY(Kcal/mol)
1	Model 1	-22654.00
2	Model 2	-22997.00
3	Model 3	-9548.92
4	Model 4	-12052.40
5	Model 5	-22828.60

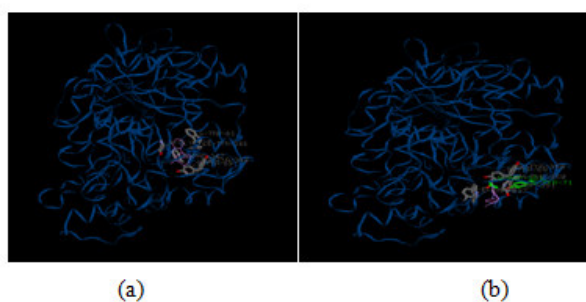
**Molecular docking studies:** The purpose of docking studies is to find the interaction of small molecules with their protein targets. The compounds that satisfy the Tice rule were docked with the enzyme target in *Tribolium castaneum* such as Acetylcholinesterase whose structure was modelled using Geno3d online tool. The software used for docking studies is iGEMDOCKv2.1. The post dock analysis showed, the compound Curan,16,17,19,20-tetradecahydro- of *Citrus medica* leaves and 3H-Pyrazol-3-one,4-[[4-(dimethylamino)phenyl]imino]-2,4-dihydro-2,5-diphenyl- of *Ixora coccinea* flowers bound with Acetylcholinesterase of *Tribolium castaneum* with least energy value -89.65 and -98.83 respectively compared to the energy of other docked structures with Acetylcholinesterase enzyme of *Tribolium castaneum* (Table 9,10 and Figure 1).

**Table 9.**  
**Docking results of the compounds of *Citrus medica* leaves essential oil**  
**With acetylcholine esterase**

S.No	Compound	Energy
1	Terpineol	-57.22
2	Flavone	-83.54
3	Terpineol,cis- $\alpha$ -	-58.6
4	5-Caranol,trans,trans-(+)-	-57.3
5	Curan,16,17,19,20-tetradecahydro-	-89.65

**Table 10.**  
**Docking results of the compounds of *Ixora coccinea* flowers essential oil with acetylcholinesterase**

S.No	Compound	Energy
1	Piperazine,1-methyl-4-(1,2,3,4-tetrahydro-2-naphthyl)-	-78.5
2	Spiro[1,3-dioxolane-2,2'(1'H)-naphthalene]-1'-methanol,3',4'-dihydro-	-77.8
3	1,2-Benzenedicarboxylic acid,mono(2-ethylhexyl)ester	-86.04
4	Pyrano[4,3-b]benzopyran-1,9-dione,5a-methoxy-9a-methyl-3-(1-propenyl)perhydro-	-78.7
5	3H-Pyrazol-3-one,4-[[4-(dimethylamino)phenyl]imino]-2,4-dihydro-2,5-diphenyl-	-98.83
6	Quinazolin-4(3H)-one,3-(3-methoxyphenyl)-2-(2-phenylethenyl)-	-90.7



**Figure 1**  
**best interaction complex of a) Curan,16,17,19,20-tetradecahydro- of *Citrus medica* leaves b) 3H-Pyrazol-3-one,4-[[4-(dimethylamino)phenyl]imino]-2,4-dihydro-2,5-diphenyl- of *Ixora coccinea* flowers with acetylcholinesterase**

## DISCUSSION

The pesticidal activity of the extracts was assessed by the Fumigation method of bioassay against adult insects of *Tribolium castaneum* and the results so obtained showed that the essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers acts as a pesticide against *Tribolium castaneum*. The insecticidal effect of plant extracts from plants supported well the current findings of the study<sup>10, 11, 12</sup>. Cineole which is a component of different species of Eucalyptus spp. and limonene is a constituent of Citrus spp. essential oils. These substances were bioassayed to determine possible fumigant, contact, and ingestion activity against *Rhyzopertha dominica* (F.) by Prates et al.<sup>13</sup> Essential oil of Thymus persicus (Ronniger ex Rech. f.) obtained from aerial parts of the plant and analyzed by GC and GC–MS. Carvacrol (44.69%) and thymol (11.05%) were the major constituents of the oil extracted. Fumigant toxicity of the essential oil was studied against *T. castaneum*, *S. oryzae* at  $27 \pm 1^\circ\text{C}$  and  $60 \pm 5\%$  RH in dark condition by Taghizadeh Saroukolai et al., 2009<sup>14</sup>. Similarly from the present study higher concentration (80%) of both the essential oil extracts was effective when compared with low concentrations of both the essential oils (20% and 40%). Molecular docking programs insights in to the possible interaction of various molecules in the discovery of novel pesticides were provided by many researchers<sup>15, 16, 17</sup>. In the present study also the molecular docking results also proved that the compounds isolated from both the essential oil extracts were interacting against with Acetylcholinesterase enzyme and so these essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers having compounds that possess pesticidal activity against *Tribolium castaneum*.

## CONCLUSION:

At higher concentrations, both the essential oil extract of *Citrus medica* leaves and *Ixora coccinea* flowers show potent pesticidal activity against *Tribolium castaneum*. The molecular docking studies strongly supports that the compound Curan, 16, 17, 19, 20-tetradecahydro- from essential oil of *Citrus medica* leaves and the compound 3H-Pyrazol-3-one, 4-[[4-(dimethylamino)phenyl]imino]-2, 4-dihydro-2, 5-diphenyl- from the essential oil of *Ixora coccinea* flowers have best interaction with Acetylcholinesterase with least energy values. Hence, the essential oil extracts of *Citrus medica* leaves and *Ixora coccinea* flowers may be considered to use as a novel potent pesticide against *Tribolium castaneum* without affecting the plants and the environment.

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# EVALUATION OF IMMUNOLOGICAL CHANGES IN FISH, *CATLA CATLA* ADMINISTERED WITH BACTERIAL PATHOGEN, *AEROMONAS HYDROPHILA*

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## ABSTRACT

In the present investigation various types of antigens were formulated using a bacterial pathogen *Aeromonas hydrophila*. The formulated antigens were characterized after administration in test animal of *Catla catla* for determination of immunomodulation. Immunomodulatory effect of antigens such as heat killed antigen, whole cell antigen, heat killed antigen with antiserum, whole cell antigen with antiserum and nucleotide antigens were evaluated using antigens administered animals with comparison of control animals. During test periods of three weeks test animals declined 20% of body weight and restless movement. B cell counts of all treated groups showed 50% reduction compared to control animals. Impact of immunomodulation in antigen administered animals reflects PFC formation. PFC assay shows 12.7 to 15.8 pfc cells per 10<sup>6</sup> spleen cells (23% reduction) compared to control (37.1 cells per 10<sup>6</sup> spleen cells). T cell counts of all treated groups showed 50% reduction compared to control animals. In DTH response showed high in DNA antigen and whole cell antigen administered animals. Lymphocytes migration assay showed whole cell antigen administered animals showed remarkable changes. From this study revealed that DNA antigen and serum combined antigens were effective to prevent bacterial pathogen in aquaculture system, it indirectly reflects immunity of human through food chain.

**KEYWORDS:** *A. hydrophila, Catla catla, B cell, T cell, Lymphocyte migration and immunological study.*

## INTRODUCTION

The humoral defense mechanism involves the production of antibodies an important mechanism to prevent bacterial disease through activation of classical complement system<sup>1</sup>. Toxins produced and secreted by bacteria are efficiently neutralized when antibodies bind to them. Prevention of bacterial adherence to fish epithelial cells, antibodies function as anti adhesins by binding to the adhesins on the bacterial surface. B cells play an important role in the humoral immunity. Together with the T cells, they make up the third line of defense differentiating into specialized antibody producing plasma cells and memory cells after activation<sup>2,3</sup>. Cellular immunity may also play an important role in combating mucosally infectious pathogen. These mucosally committed T cells may function either to prevent mucosal surface from injury by infectious pathogens or by exhibiting cellular cytotoxicity directed against intracellular pathogens<sup>4,5</sup>. Cytotoxic T cells recognize and destroy infected cells and activate phagocytes to destroy pathogens they have taken up<sup>6</sup>. The antigen specific aim of cell mediated immune response consisting, T-Lymphocytes as like as B-cells, which produce soluble antibody that could bind to specific antigen. Hypersensitivity and mixed lymphocyte migration are categorized in accordance with the effectors involved in these reactions. In the present study screening of stimulation of immune response in fish, *Catla catla* administered with bacterial antigen.

## MATERIALS AND METHODS

The infected fish samples *Carassius auratus* was collected in pre sterilized container from the fishing area in Srivilliputtur, (931°0.012"N, 7737°59.880"E) Tamilnadu, India. The collected fish sample was transported to the laboratory in an icebox for further analysis. From the infected fish, *C. auratus* pathogenic strains (*Staphylococcus aureus*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Escherichia coli* and *Vibrio* sp.)



were isolated and prepared the whole cell bacterial antigens, heat killed antigen, whole cell antigen with antiserum, heat killed antigen with antiserum and nucleotide antigen. LC<sub>50</sub> values of all bacterial pathogens in *Catla catla* were calculated. From the LC<sub>50</sub> value sublethal concentration of antigens were given to the test fish, *Catla catla* for further study.

**Immunization:** Six groups of experimental fishes were maintained for analysis of immune response. One ml of 10<sup>5</sup> cell of antigen was administered in 1 kg of mouse for analysis. Different types of antigen administered intramuscular in each group and marked for further analysis. One group maintain as a control animals.

**Serial bleeding:** The fishes were bleed serially using 1mL tuberculin syringe with 24 gauge needle from the common cardinal vein situated just below the gills<sup>7</sup> at regular interval of seven days for antibody response till 28<sup>th</sup> day intervals of 2 days for lysozyme and neutrophil assay till 10<sup>th</sup> day. For bleeding, the fish was held in the left hand with the right side of the fish facing the investigator. The operculum was lifted and kept open by the left hand thumb. A metal rod (3mm diameter and 12cm long) was used to lift the gill lamellae in order to expose the common cardinal vein. From the common cardinal vein nearly 0.2-0.3 mL of blood was collected from each fish using 1mL glass tuberculin syringe fitted with 24 gauge needle. The whole procedure from the handling of the fish to the end of the bleeding process took only 30-40 seconds causing minimal trauma to the fish. Quick and gentle handling of fish is required to avoid stress which is known to suppress immune system. Antigen administration and serial bleeding were always done between 14 hours and 16 hours to avoid possible influence of cardiac rhythmic variation on the immune response. The blood drawn was collected in eppendorf tubes.

**Antiserum collection:** Blood collected from immunized and normal fish was kept at room temperature for 15 minutes. The clot was freed from the wall of the micro centrifuge tube for efficient retraction and kept overnight at 4°C. The serum was separated by spinning down the clot at 3000 rpm for 15-20 minutes and collected in sterilized vials. The serum was stored in freezer at -20°C until use. In the present study humoral immune response was analyzed by antibody titration. B cells e rosette assay and plaque forming cell assay techniques are carried out by standard method of Sujatha *et al*<sup>9</sup>.. The test fish, *Catla catla* divided into 6 groups (each groups contain 6 animals) and treated with different types of antigen (One group served as control) prepared from *A. hydrophila*.

**DTH assay:** One of the parameters of immune assay DTH response was performed by standard techniques<sup>9</sup>. Test animals and control animals were injected 0.5 mL Dinitro chloro benzene (10 mg/ml) and saline respectively in subcutaneous region. After injections at regular intervals of 7 days measures thickness of paw. Fishes were sensitized by subcutaneous injection in the intranasal region with 0.5 mL of Freund's adjuvant containing 500 mg of antigen and boosted at 6<sup>th</sup> and 8<sup>th</sup> day by an intradermal injection to sterile phosphate buffer with a vernier caliper prior to challenge, i.e. 0<sup>th</sup>, 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup> and 12<sup>th</sup> hour post challenge, each with three readings. The increase in mean skin thickness (MST) of fishes was obtained after deducting the skin thickness of the same oil before challenge. Overall MST was obtained by taking the mean of individual fishes with a group.

**Lymphocyte migration inhibition test:** Blood is collected from antigen treated and control fishes using a heparin pretreated vials. On the same time two layers of agarose 1 and 1.5% (with antigen) were prepared in sterile plate. Then added layer of complement serum from guinea pig. Blood samples were taken in three by forth of capillary tube and marked. Capillary tube placed in the plate. Due antigen antibody interaction level of serum in capillary reduced and recorded.

## RESULTS AND DISCUSSION

Immune response in any living system were analysed by both humoral and cellular immunity and analysed by various parameters. Single assay not useful for immunological analysis. In this study B cell, PFC assay performed to evaluate humoral Immune response and T cell, DTH, lymphocyte migration assay were performed to evaluate cell mediated immune response in antigen administered animals. To optimize the serum dilution with saline, control serum was loaded over a range of dilution from 1:1250 to 1:320 and this appeared to be the highest antibody titre (Table 1 to 3 and

Plate 1). An estimation of antibody levels in the serum after an antigenic challenge will expose the functioning of humoral immune systems. In immunodeficient animal, antibody production was affected and hence humoral response against a disease causing antigen was less. But the immune complex treated animals enhance the production of antibody. In the present study, pathogen and heat killed pathogen treated fishes showed an antibody suppressive effect. The suppression of antibody reflects on the reduction of humoral immune response and this state subjects to easy infection. Immunomodulation of whole pathogen with antiserum treated fishes showed moderate change in antibody production. The immune complex of the samples tested were immune enhancer for antibody production. This was expected in animals were obviously exposed immune complex it will resist many intestinal pathogens. Earlier studies reported cross reaction of *Escherichia coli* with antibody of many other pathogens such as *Citrobacter* sp., *Brucella* sp.<sup>10</sup> and *Salmonella* sp.<sup>11</sup>. B Lymphocytes counts using rosette forming assay revealed significant decrement in pathogens exposed fishes than in control (Table 2). Of the two pathogens decrement in B lymphocyte it was much pronounced in *Escherichia coli* in the first and *Staphylococcus aureus* pathogens had more or less similar impact on B cell estimation. The present study, clearly confirms the decrement in B cell number in fish exposed to whole and heat killed pathogens. In this study conclude, impact of whole cell and heat killed pathogenic molecules on the synthesis, proliferation and activation of lymphocytes. Gebel *et al*<sup>12</sup>, reported that differentiation of B cell counts is affected by pathogens. Fishes exposed to pathogenic strains (1/10<sup>th</sup> sublethal concentration ) for 3 weeks showed reduction in PFC. Effect of pathogenic antigens in direct splenic plaque forming cells (1g M producing cells) showed a reduction in secondary plaque forming cell in the first 3 weeks and a time and dose dependent decrease in primary and secondary PFC response. The Peak antibody formation occurred in the first week of primary response and in the third week the secondary response in control as well as in treated animals was seen indicating that there was no delay in antibody formation. Moreover, the present study demonstrates that all pathogenic antigen exposure resulted in a suppression of primary IgM type plaque forming cell in response to antigens in fishes. Exposure to pathogenic antigen showed significant decrease in serum antibody titre to different antigen in time dependent patterns. The serum and response was more evident in pathogenic *Staphylococcus aureus* treated fishes administered intraperitoneally. However, other antigens reduce PFC about 3 fold in all the experimental weeks. The reduction in PFC is associated with decrease in serum antibody response to SRBC in pathogens exposed fishes. Genestier *et al*<sup>11</sup>, 1998 reported that immuno enhancive drugs enrich cell proliferations. B cell proliferations and modification depend on the exposure of antigens. But in the present study, nucleotide (DNA) produces moderate B cell proliferations. These studies support foreign substance to be genotoxic and moderate synthesis of DNA in cell [12]. B cell population such as, potentially auto reactive clones were selectively enumerated at B cell maturation and transforming growth factor of B cell repositories. Pathogens used here were found to be toxic and damage normal activity. This immunoglobulin gene rearrangement in B cell and growth factor(TGF  $\beta$ ) transforming growth factor -  $\beta$  might be affected by pathogens leading to reduction in B cell count, such induction by pathogens have been well documented by researchers. 'T' cell production of control and treated animals were estimated by rosette forming assay and recorded in Table. 4. The result showed significant changes in *Catla catla* fishes, when compared to control of five kinds of antigen treatment, the increment in 'T' lymphocyte number was much pronounced in *Catla catla* treated with heat killed antigen with antiserum. T cell is a vital component in cell mediated immune response, and it gets suppressed due to exposure of antigens (whole cell antigens). Immune response enhances the production of T cells due to pathogen tested (immune complexes and nucleotide antigens). It is found to be suppressive to T cell production so induction in cell mediate immunity has confirmed pathogenic potential of *A. hydrophila*. Dhasarathan *et al.*, (2006) and Muller *et al.*, (1997) had reported that the immunosuppressive drug inhibits cell proliferation and T-cell cytotoxicity. It also induces apoptosis in activated as well as testing cells<sup>13,14</sup>. T cell population which has reduced T cell counts, the inhibition of T cell activation, proliferation, immunity exclusion and co-operation with other cells had affected the overall immunity in fishes. So the immune complex of pathogens induces the T-cell counts compared to other treated and control fishes. The increment of T-cell activation and proliferation modulate the overall immunity in the fishes. The delayed type hypersensitivity reaction to tuberculin and DNCB antigen were tested in control and pathogen exposed fishes. The impact of pathogens on DTH response in fishes is recorded in Table 5. A comparative analysis of DTH response in fishes (in control and antigen exposed) revealed some interesting changes found due to pathogenesis in two antigens, whole and heat killed antigens show the high DTH responses compared to control. The size of skin edema also declined in whole cell and heat killed antigen exposed fishes. The reduced development of skin reaction in fishes after the exposure to two antigens suggests possible impairment in the immune capacity of the fishes. Seth *et al.*,

(2006) reported that the stress has been associated with a detrimental effect on immunity<sup>15</sup>. This is recognized that the immune cells produce peptide hormones which interact through shared ligand receptors and such peptides are capable of modulating various activities. Fishes exposed to immune complexes and DNA, DTH response was higher and size of the skin edema also increased when compared to control and other treated animals. The induced development of skin reactions suggest immune enhancement of fishes after exposure of immune complex and DNA antigens in the present study. T-cell counts also play a vital role in pathogenicity as T helper cells (T<sub>h</sub> cells) were one of the key factors that determine given antigens induces hypersensitivity reactions<sup>16</sup>. Further, T helper cells (T<sub>h</sub>-2) produce interleukin (IL-4) that in turn generate and maintain Ig E an important immunoglobulin produced during hypersensitive reactions. During DTH response, circulating T lymphocytes come in contact with antigen (mainly held by skin macrophages) and pre-sensitized cells present are stimulated to lymphokine production, and blast cell transformation. The lymphokines encourage the trapping of circulating mononuclear cells at the site of antigen and activation of non-sensitized "by-stander" cells into the reaction. A cascade effect is produced with necessary localization of mononuclear cells, which are clinically manifested as induction. These pathogen exposures have altered all these mechanism and induced DTH response in fishes. Lymphocytes are blood cells involved in immune response. The study of the migration of lymphocyte sheds light on the defense machinery inside the immune system. Pathogens inhibit defense machinery of the immune system. Migration of lymphocytes affects by cellular functioning, cell energetics and other cellular functions of immune cell which are involved in cellular immune responses and inhibition of lymphocyte migration. This could reduce the immunity and the animal may develop risk for defense mechanism. In the present study, significant changes were observed in heat killed antiserum of fishes than in normal fish. Among the different antigens exposed in fish, when compared with other antigens, the lymphocyte migration in fishes exposed to nucleotide antigen shows fastest migration. Lymphocyte migration was also significant in heat killed antiserum. Hence, the heat killed antiserum and nucleotide antiserum exposed fishes causes tolerance in immunity and provide defense against microbial infections and alterations. This study reflects the possible changes in animal system on exposure to vaccine molecules Table 6. and Plate 2. If the symptoms for hypersensitive reactions are expressed after days of antigenic challenge, it is called delayed type hypersensitivity some subpopulations of activated TH cells when encounter some types of antigen, they secrete cytokines that induce a localized inflammatory reaction. The presence of delayed type hypersensitivity reaction can be measured experimentally by injecting antigen intradermally into an animal and observing whether a characteristic skin lesion develops at the injection site. A positive skin test reaction indicates that the individual has a population of sensitized T<sub>h</sub>-1 cells specific for the test antigen. On injection of antigen, delayed type hypersensitivity response is diagnosed based on the development of a red, slightly swollen, firm lesion at the site of injection between 48 and 72 hrs later. The skin lesions result from intense infiltration of cells to the site of injection during a delayed type hypersensitivity reaction; 80% - 90% of these cells are macrophages. The presence or absence of delayed type hypersensitivity response in fish after exposing it to bacterial antigen highlights the functioning of the T<sub>h</sub>-1 cells. Hence, delayed type hypersensitivity analysis can be taken as one of the parameters to assess immunity changes that occur in fish after antigen treatment. An immune response mobilizes a battery of effectors molecules that act to remove antigen by various mechanisms. Generally, these effector molecules induce a localized inflammatory response that eliminates antigen without extensively damaging the host's tissue [16]. The inappropriate immune response is termed hypersensitivity or allergy. Hypersensitivity reactions may develop in the course of either humoral or cell mediated responses<sup>17</sup>. Cell mediated immunity plays a vital role in defence against the pathogens. The cell mediated immunity gets suppressed in *Catla catla* due to the exposure of whole cell bacterial antigen, whole cell bacterial antigen with antiserum, heat killed antigen, heat killed antigen with antiserum, nucleotide antigen. It is found to be suppressive to T cell production and induction in cell mediated immunity has confirmed the pathogenic potential of *A. hydrophila*. A comparative analysis of DTH responses exhibited by fish (in control and antigen administered) showed some interesting changes and these were found due to antigenicity of two types of antigens. The whole cell bacterial antigens and heat killed bacterial antigens show high DTH responses compared to control. Migration of lymphocytes affects cellular functioning, cell energetics and other cellular functions of immune cell which are involved in cellular immune responses and inhibition of lymphocyte migration could reduce the immunity and the *Catla catla* may develop risk for defense mechanism.

**Table 1.**  
***Character analysis of Catla catla exposed to antigens***

S. No	Character	Heat killed bacterial antigen	Whole cell bacterial antigen	Heat killed bacterial antigen with antiserum	Whole cell bacterial antigen with antiserum	Nucleotide antigen
1	Weight of the animal (gm)	20	20	25	28	20
2	Movement of the animal	Resting	Abnormal	Restless	Abnormal	Abnormal
3	Adulation days	24	27	32	24	9

**Table 2.**  
***Enumeration of B cells using rosette forming assay in fish Catla catla exposed to different sublethal concentrations of A. hydrophila antigens***

S.No	<i>A. hydrophila</i> Test antigens	Number of B cells Rosette formed in 100 lymphocyte observed		
		I week	II week	III week
1.	Control fishes	18	18	18
2.	Heat killed bacterial antigen	8	6	9
3.	Whole cell bacterial antigen	8	7	9
4.	Heat killed bacterial antigen with antiserum	9	8	11
5.	Whole cell bacterial antigen with antiserum	6	6	8
6.	Nucleotide antigen	8	6	9

**Table 3.**  
***Plaque Forming Assay of fish Catla catla at different Time intervals***

S.No	<i>A. hydrophila</i> Test antigens	Distribution of PFC/10 <sup>6</sup> Spleen cells		
		I week	II week	III week
1.	Control fishes	36.3	36.4	37.1
2.	Heat killed bacterial antigen	10.2	11.4	12.8
3.	Whole cell bacterial antigen	11.2	12.1	15.8
4.	Heat killed bacterial antigen with antiserum	10.7	11.6	12.9
5.	Whole cell bacterial antigen with antiserum	11.1	12.3	12.7
6.	Nucleotide antigen	10.3	11.7	12.8

**Table 4.**  
***T cell counts in primary and secondary immune response against pathogens at different time intervals***

S. No.	Bacterial Strains	% of T cell production at different weeks							
		Primary Immune Response				Secondary Immune Response			
		Initial day	Week			Initial day	Week		
			I	II	III		I	II	III
	Normal	64	62.2	61.7	63.8	64	62	62	64
1.	Heat killed antigen	64	40.1	45.4	32.1	64	46	52	58
2.	Whole cell antigen	64	51	54	56	64	51	52	58
3.	Heat killed antigen with antiserum	64	40	44	31	64	42	46	48

4.	Whole cell bacterial antigen with antiserum	64	36.8	37.8	36.3	64	40	48	52
5.	Nucleotide antigen	64	35.3	37.2	36.1	64	48	50	54

**Table 5.**

*Delayed type hypersensitivity in primary and secondary immune response against pathogens at different time intervals (HK)*

S. No.	Bacterial Strains	Delayed type hypersensitivity at different times							
		Primary Immune Response				Secondary Immune Response			
		Initial day	Week			Initial day	Week		
			I	II	III		I	II	III
1.	Heat killed antigen	-	-	-	-	-	-	-	+
2.	Whole cell antigen	-	-	-	-	-	-	-	-
3.	Heat killed antigen with antiserum	-	-	-	-	-	-	-	-
4.	Whole cell antigen with antiserum	++	++	++	++	+	+	+	-
5.	Nucleotide antigen	+		+	+	+	+	+	+

+ Erythema alone ++ Erythema with oedema - No significant change over control

**Table.6.**

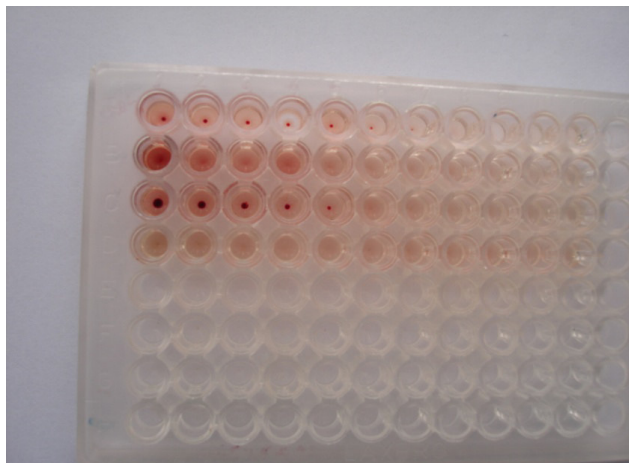
*Lymphocyte migration assay in primary and secondary immune response against pathogens at different time intervals (WC)*

S. No.	Bacterial Strains	Lymphocyte migration assay at different weeks (The values are measured in cm)							
		Primary Immune Response				Secondary Immune Response			
		Initial day	Week			Initial day	Week		
			I	II	III		I	II	III
1.	Heat killed antigen	1.2	1.1	0.9	0.7	1.2	0.9	0.7	0.6
2.	Whole cell antigen	1.2	1.1	0.9	0.8	1.2	1.0	0.9	0.8
3.	Heat killed antigen with antiserum	1.2	1.0	0.7	0.7	1.2	0.7	0.6	0.5
4.	Whole cell bacterial antigen with antiserum	1.2	1.1	1.1	0.9	1.2	1.0	1.0	0.9
5.	Nucleotide antigen	1.2	1.1	0.9	0.8	1.2	0.9	0.9	0.8



**Plate 1.**

***Evaluation of antibody titre of Catla catla exposed to antigens***

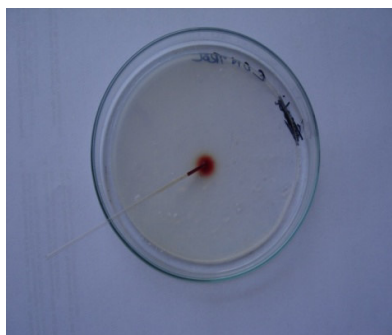


**Plate 2.**

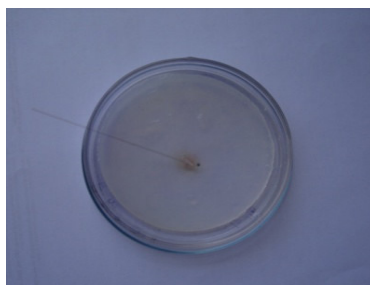
***Lymphocyte migration assay of whole cell antiserum, heat killed antiserum and Nucleotide treated antiserum***



***Lymphocyte migration in whole cell antiserum***



***Lymphocyte migration in heat killed antiserum***



***Lymphocyte migration in Nucleotide treated antiserum***

## Conclusion

In the present investigation evaluated immune modulation efficiency of various antigen prepared from a bacterial pathogen *Aeromonas hydrophila* using fish model *Catla catla*. From the study various parameters whole cell antigen with serum is highly effective to control pathogen. It helps to prepare a novel vaccine in disease management. From this study revealed that DNA antigen and serum combined antigens were effective to prevent bacterial pathogen in aquaculture system, it indirectly reflects immunity of human through food chain

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## ANTI-PROLIFERATIVE ACTIVITY OF CALOTROPIS GIGANTEA (FLOWER EXTRACT) ON COLORECTAL CANCER CELL LINE

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### ABSTRACT

The flower extract of *Calotropis gigantea* is rich in bioactive compounds and have potential to decrease various risk factors in the human body. The flavonoids in the flower extract of *Calotropis gigantea* have shown to possess potent anti-proliferative activity against colorectal cancer cell lines. Colorectal cancer is the cancer found in colon or the rectum, the cancerous growth starts in the inner lining of the colon or the rectum and these growths are called polyps. The polyps would change or develop into cancer with time. In this study, the therapeutic potential of *Calotropis gigantea* flower extract on colorectal cancer cell lines was analyzed. The anti-cancer properties of *Calotropis gigantea* flower extract have been attributed to its ability to induce apoptosis in cancer cells and inhibit cancer cell proliferation. Comparative analysis of the normal cell lines L929 (fibroblast cell lines) and HCT-116 (colorectal cancer cell lines) treated with *Calotropis gigantea* flower extract revealed its anti-cancerous property due to morphological changes of the HCT-116 cells, typical to apoptotic cells, leading to cell death, whereas there was no toxic effect on the L929 cells. The results confirm the anti-proliferative property of *Calotropis gigantea* flower extract against human colorectal cancer cell lines (HCT-116) which can induce death in cancer cells while protecting the non-cancerous cells.

**KEY WORDS:** *Calotropis gigantea*, Colorectal Cancer, HCT-116 cells, L929 cells, anti-cancer.

### INTRODUCTION

Cancer is a serious health issue in different parts of the world. Cancer is defined as abnormal cell growth, which tends to invade other parts of the body. Cancer is of 2 types- Benign and Malignant. Benign tumors do not spread whereas malignant tumors tend to spread, divide, and lead to severe conditions. The risk factors are mostly associated with tobacco, obesity, excessive alcohol, certain infections, poor diet and lack of physical activity.<sup>1</sup> The treatment includes radiation therapies, chemotherapy, surgery and targeted therapy. The chance of survival depends upon the type of cancer and the pre-treatment given.<sup>2</sup> In children, the most common cancers are acute lymphoblastic leukemia and brain tumors. The most common types of cancers in the male are Lung Cancer, Prostate Cancer, Colorectal Cancer, and Stomach Cancer. Whereas in females, the most occurred cancers are – Breast Cancer, Colorectal Cancer and Cervical Cancer.<sup>3</sup> Colorectal Cancer is the cancer found in colon or the rectum, the cancerous growth starts in the inner lining of the colon or the rectum and these growths are called polyps. The polyps would change or develop into cancer with time. Severe side effects of radiation and chemotherapy have given rise to interest in natural compounds that show evidence of low toxicity towards normal cells while effectively killing cancer cells.<sup>4</sup> Flavonoids are a large group of polyphenolic compounds that occur ubiquitously in plants as secondary metabolites.<sup>5</sup> *Calotropis gigantea* whose common name is crown flower is a plant, which has various medicinal properties. It plays a very important role in curing asthma and shortness of breath by using the root and leaves.<sup>6</sup> The bark is used for liver and spleen disease. The latex also contains important uses in conditions such as arthritis, cancer, antivenom. Mainly the leaves extracts of *Calotropis gigantea* have shown the highest zone of inhibitions on organisms such as Salmonella typhi, Shigella sonnei.<sup>7</sup> Escherichia coli and have proved to be very good antioxidant potential and antibacterial activity.<sup>8</sup> *Calotropis gigantea* has shown enormous protective properties and provides wide treatment opportunities hence it was analyzed for anti-proliferative activity on colorectal cancer. The latex, leaves and other parts contain a rich source of biologically active compounds.<sup>9</sup> The extracts from the leaves contain alcoholic and hexane groups and the

extract from the latex contains the triterpenoids and lupeol which shows medicinal properties like anti-angiogenic, anti-oxidative, anti-inflammatory and gives an inhibition to tumor growth.<sup>10</sup> The plant extract has been reported to have a strong cytotoxic effect on lymphoma cancer. The crude extracts from the different parts of *Calotropis gigantea* have shown cytotoxic activity,<sup>11</sup> and have proved to have potential to inhibit the growth of various cancer cell lines,<sup>12</sup> the flower extracts have shown effective cytotoxic activity, and hence it can be a powerful drug for cancer treatment.<sup>13</sup> In this study, the flower extracts of *Calotropis gigantea*, was analyzed for their anti-proliferative property against human colon cancer cell line (HCT-116) mostly used in drug screenings and therapeutic research.<sup>14</sup> The L929 is a fibroblast cell line is derived from L strain of normal subcutaneous areolar and adipose tissue which was also used for comparative analysis.

## MATERIALS AND METHODS

### Sample Collection

The *Calotropis gigantea* samples were collected from K. Naryanapura, Kothanur, Bangalore, India. The samples were washed and *Calotropis gigantea* flowers were separated, pulverized and dried in hot air oven at 50°C for 24 hours. The extract was obtained using soxhlet apparatus with further evaporation and concentration.

### Phytochemical Analysis

The preliminary qualitative phytochemical screening test is done for alkaloids, flavanoids, tannins, saponins, phenols, carbohydrates, terpenoids, quinones by using standard test procedures. The extracts were subjected to different qualitative test for the detection of different phytochemicals. The standard method was employed for the detection of phytochemicals.<sup>15</sup>

### FRAP Anti-oxidant assay

Ferric reducing antioxidant power assay (FRAP) is used to analyze and measure the total antioxidant activity. The principle is based on the anti-oxidant property which is directly proportional to the reaction mixture; higher the absorbance higher is the antioxidant property.<sup>8</sup> The sample containing the antioxidant compound form a complex with ferric chloride and 246 tripyridyl-5-triazine solution (TPTZ) which is measured at 570 nm, calorimetrically.

### FTIR

Fourier- transformed infrared spectroscopy is a specialized technique done to obtain the infrared spectrum of the absorption of a solid, liquid or gas, this technique converts the raw data into spectrum. FTIR is a tool used to identify the various chemical bonds, functional groups present in the compound of the plant extract. The crude extract obtained after extraction was used as the sample. The sample (flower extract) is put into the IR-light source and the absorbance of the sample is used to measure the bonds of carbon atoms.<sup>16</sup>

### MTT assay

MTT assay is a type of colorimetric assay which is used for finding cell metabolic activity, the number of viable cells depends on the cellular oxidoreductase enzyme, this enzyme causes reduction of the tetrazolium dye MTT 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to the insoluble formazan which appears to be purple colour. The reduction of tetrazolium dye is highly dependent on the NAD(P)H oxidoreductase enzyme which shows the reduction is dependent on the cellular metabolic activity.<sup>17</sup>

- (i) Negative control (medium with cells but without the experimental drug/compound)
- (ii) Positive control (medium with cells and 15µM of Doxorubicin)

## RESULTS AND DISCUSSION

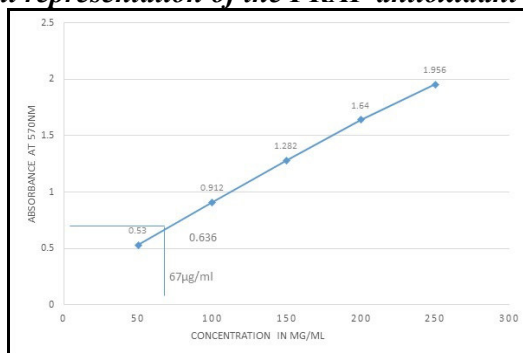
Cancer has paved its way in all the countries, with maximum mortality rate, it is one of the top- ranked diseases which have maximum limitations with the high cost and various side effects and the cure seems to have a high price to pay with a low rate of success. These combined limitations create a very urgent need to reach for novel molecules. Various studies conclude that phytochemicals have the capacity to kill cancer cell lines but the results remain underestimated. *Calotropis gigantea* was identified as a toxic plant by previous research whereas the purified extracts from different parts of *Calotropis gigantea* have shown enormous protective properties. The flower extract of *Calotropis gigantea* taken for this study has shown to have very

high cytotoxic potential against some cancer cell lines along with its use in traditional medicine in terms of various kinds of infections, bites, and diseases. Hence, the cytotoxic and antioxidant effect on colorectal cancer cell lines (HCT116) was analyzed. The phytochemical analysis of the flower extract of *Calotropis gigantea* using methanol as a solvent proved the presence of various bioactive compounds such as alkaloids, terpenoids, saponins, reducing sugars, carbohydrates, tannins, glycosides are responsible for metabolic processes resulting in better health. The phytochemical analysis of the flower extract of *Calotropis gigantea* shows positive result stating that it is rich in bioactive compounds and have potential in decreasing various risk factors in the human body. FRAP anti-oxidant assay revealed that the flower extract of *Calotropis gigantea* is composed of anti-oxidant compounds as shown in table 1; which have numerous advantages for human health, which is good for the heart, helps to lower the risks of infections and decreases the risk of cancer. Figure 1 shows the graphical representation of different absorbance values of the standard (ascorbic acid) and the test (flower extract) sample. The inhibition percentage of the test sample was found to be 67% stating that the test sample is rich in antioxidant compounds which play a very important role in reducing oxidative stress caused by the high concentration of the free radicals in cells and tissues. FTIR is a tool used in identifying the various chemical bonds, functional groups present in the compound of the plant extract. Table 2 shows the peak assignments which gives the different stretch and bending present between different chemical elements also shows the compounds present such as amines, alkyl, aromatic compounds etc., each plays a vital role for the benefit of the human body. Figure 2 shows the graphical representation of the peak value, consisting of high and inverted peaks. The MTT assay performed with normal fibroblast cell line (L929) and colon cancer cell line (HCT116) revealed that the flower extract of *Calotropis gigantea* at a concentration ranging from (25 - 400 $\mu$ g/ml) did not show any toxicity towards L929 (Figure 4) with IC50 value denoting non- toxic and around 74% of the cells were viable with very less morphological changes. Whereas the flower extract showed very high toxicity leading to cell death of HCT116 with IC50 value at 221.61 $\mu$ g/ml and only 35% were viable cells (Figure 3). It was observed that the flower extract at concentration 25-100 $\mu$ g/ml had less morphological changes of the cells similar to the negative control while 200 and 400 $\mu$ g/ml concentration revealed higher toxicity (Figure 5), morphological changes and cell death similar to the positive control. The anti-proliferative activity of flower extract of *Calotropis gigantea* showed promising results leading to the death of 75% cancer cells.

**Table 1**  
**Observation table for FRAP antioxidant assay**

S.No.	Standard (ascorbic acid) ml	Concentration $\mu$ g/ml	Water (ml)	FRAP(ml)	Incubation in the water bath for 1 hr at 37°C	OD at 570 nm
Blank	-	-	1	3.8		0.00
S1	0.2	50	0.8	3.8		0.531
S2	0.4	100	0.6	3.8		0.912
S3	0.6	150	0.4	3.8		1.282
S4	0.8	200	0.2	3.8		1.640
S5	1	250	-	3.8		1.956
Test	0.5	-	0.8	3.8		0.636

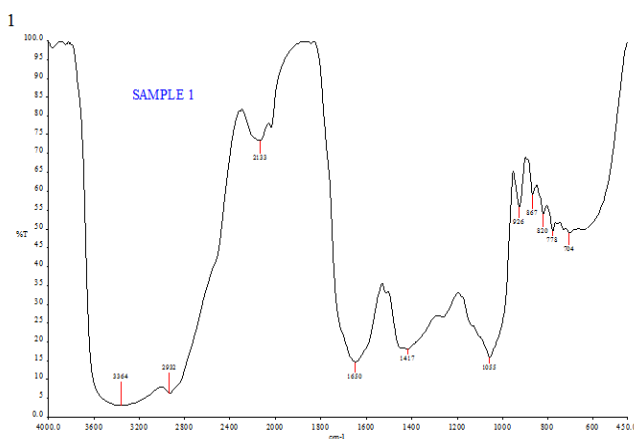
**Fig.1**  
**Graphical representation of the FRAP antioxidant values**



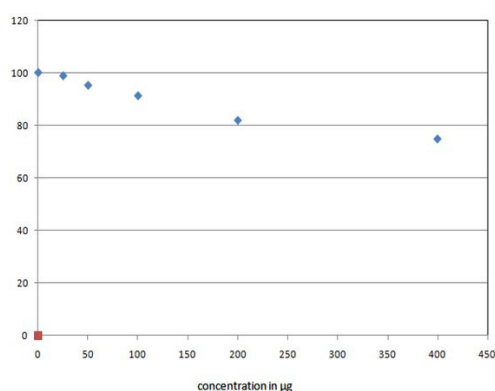
**Table 2**  
**Peak assignments and their related functional groups of methanolic extract of *Calotropis gigantea* flowers.**

Sl. No	Peak value (cm <sup>-1</sup> )	Peak assignments	Compounds
1.	3364	N-H stretch	Amine
2.	2932	C-H stretch	Alkyl
3.	2133	C C stretch	Alkynyl
4.	1650	C=C stretch	Alkenyl
5.	1417	C-H stretch	Alkyl
6.	1055	C-H stretch	Vinyl
7.	926	C-H stretch	Vinyl
8.	867	C-H bending	Aromatic
9.	820	C-H bending	Aromatic
10.	778	C-H bending	Aromatic
11.	704	C-H bending	Aromatic

**Fig 2**  
**FT-IR spectrum of methanolic extract of *Calotropis gigantea* flowers.**

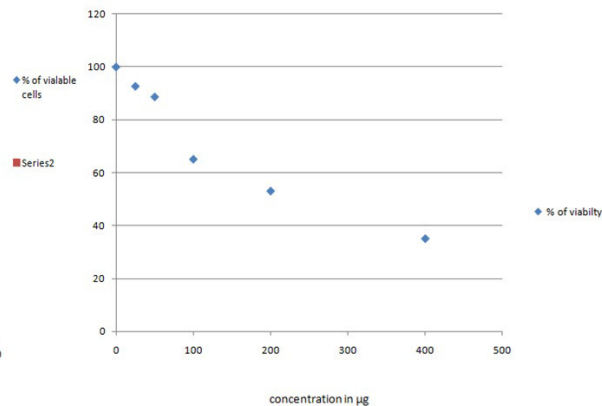


**Fig 3.**  
**MTT analysis for % of viability in L929 and HCT116**



IC50 value: Non toxic

Sample (flower extract) v/s L929 ( fibroblast cell-line)

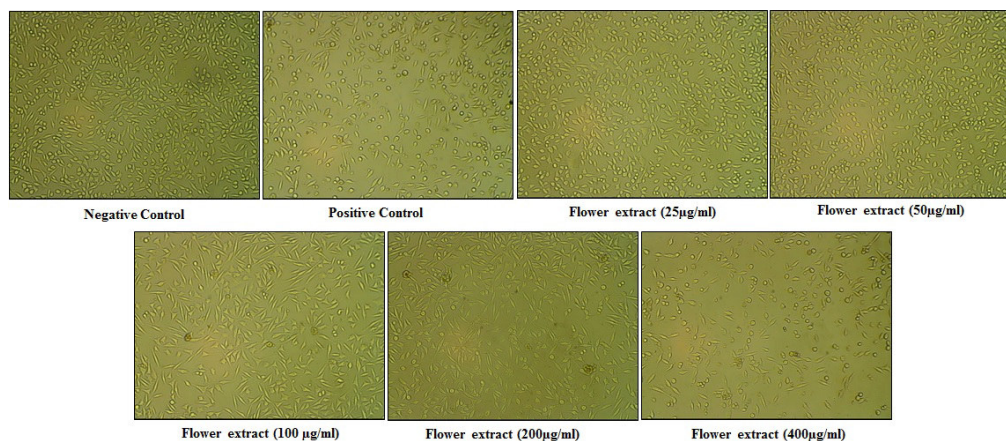


IC 50 Value = 221.61 µg/ml

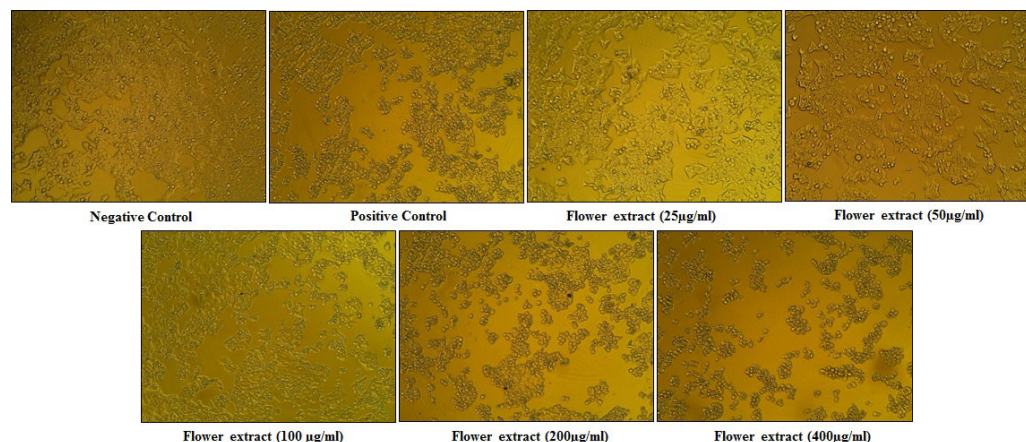
Sample (flower extract) v/s HCT116 (colorectal cancer cell-line)



**Fig 4**  
**Effect of flower extract normal fibroblast cell-line (L929)**



**Fig 5**  
**Effect of flower extract on colorectal cancer cell line (HCT116)**



## CONCLUSION:

The present study suggests that the mechanism by which the flower extract of *Calotropis gigantea* exerts cytotoxic action on the cancer cells is by apoptosis and cell cycle disruption. Cytotoxic activity on the HCT116 cancer cell line with higher cell death confirms the anti-proliferative property of flower extract of *Calotropis gigantea*. Further studies have to be performed to analyze the mechanism of action. The results reveal that the flower extracts have possible therapeutic potential against colorectal cancer cell lines by inducing apoptosis leading to the death of cancer cells whereas it protects the non-cancerous cells.

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## EFFECT OF ORGANIC MANURES AND BIOFERTILIZERS ON VEGETATIVE GROWTH OF *PHASEOLUS VULGARIS* L. (FRENCH BEAN)

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### ABSTRACT:

The attempt was conducted in pots at Kristu Jayanti College during December to March 2018-19 with triplicates having seven treatments comprised of organic manures with biofertilizers (*Azotobacter*, *Pseudomonas* and *Rhizobium*). The observations were recorded on growth and yield attributes. Among the seven treatments given, plant height was observed high in combinations of R:A:P (8 :8 :4 – 59cm) followed by sole applications than control, number of leaves were noticed high in mixed inoculation R:A:P (8 :8 :4 – 34 cm), number of leaves were observed high in sole inoculation of *Pseudomonas* - 33 Nos., chlorophyll content was recorded high in sole inoculation of *Pseudomonas* and in mixed inoculation 8:4:8 as 0.90 µg/ml followed by all mixed inoculants. Similarly fresh and dry weight also observed high in mixed inoculation compared with sole than control

**KEYWORDS:** *Biofertilizer, organic fertilizer, Azotobacter, Rhizobium, Pseudomonas*

### INTRODUCTION:

Biofertilizers are economical, eco-friendly, more efficient and are expected to reduce the inputs of synthetic fertilizers and pesticides. Biofertilizers enriches the soil environment with all nutrients, plant growth regulating substance and biodegradation of organic matter in the soil (Sinha *et al.*, 2014). Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable farming. (Khosro and Yousef, 2012). Organic farming is one of such strategies that not only ensures food safety but also adds to biodiversity of soil (Raja, 2013). Microbial products are most favored for crop production provides both macro and micronutrients which perform a crucial role in plant growth and development. For sustainable agricultural practices microbial biofertilizers are advantageous. In addition, vermicompost is a scientifically proven miracle plant growth enhancer. Vermicompost add significant value to the end users like farmers for replacement of chemical fertilizers and procuring better prices for the organic produce using such composting material locally available at much lower cost (Yvonne *et al.*, 2019). Vermicompost also improves soil structure, soil aggregation and improve water retention capacity (Anil Kumar *et al.*, 2018). Vermicompost is good quality manure that contains several essential nutrients needed by the crops such as nitrogen, phosphorus, nutrition. It also has a positive influence on vegetative growth, stimulating shoot growth and root development (Edwards *et al.*, 2004). The other positive influence of vermicompost application include alterations in morphology of crop plants such as increased leaf area and root branching (Lazcano *et al.*, 2009) and stimulated flowering, increase in the number and biomass of flowers (Arancon *et al.*, 2008; Atiyeh *et al.*, 2002) and overall increase in fruit yield (Arancon *et al.*, 2004a, 2004b; Atiyeh *et al.*, 2000; Singh *et al.*, 2008). In this context, an experimental study was made to check the efficiency of microbial biofertilizers on growth and yield parameters of French bean with sole and combined applications of microbial biofertilizers with vermicompost. Application of organic manure play an important role on yield and its attributes as well as nutrient uptake and directly increase the soil physical condition. In many situations combination of organic and inorganic fertilizers have produced higher yields than alone (Blackshaw, 2005). There is ample scope to increase the yield of bush bean by applying proper dose of biofertilizer and management of weeding regimes (Uddin *et al.*, 2018). French bean



(*Phaseolus vulgaris* L.) is known as common bean belongs to Legminaceae family extensively grown because of its short life span and nutritive values. Hence, the present investigation was carried out to study the response of organic fertilizers on French bean

## MATERIALS AND METHODS

### **Method of isolation**

1g of garden soil was weighed and made upto 10ml for serial dilution. From that, 1ml was taken and serially diluted with each test tube containing 9ml of sterilized distilled water to isolate the colony of *Azotobacter* sp. and *Pseudomonas* sp. Similarly, to isolate *Rhizobium* strain, root nodules were excised, thoroughly washed with distilled water and squeezed by using sterile glass rod in a beaker containing 5ml of distilled water.

### **Isolation of bacterial colony**

The dilutions of  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  were prepared and 0.1ml was transferred from these dilutions to petri plates individually containing Ashby's medium, Yeast Mannitol Agar Medium and *Beef extract broth* and the petri plates were incubated at 28°C for 48hrs. The individual colonies from each media was observed for the specific colony characters were transferred to slants containing respective medium, incubated at 28°C for 48hrs and were maintained at 4°C in refrigerator for further use.

### **Carrier materials**

Carrier cocopeat was packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 °C. The neutralized and sterilized carrier material was spread in a clean, dry sterile plastic tray. Bacterial culture drawn from various source were added to the sterilized carrier and mixed well by manually. The inoculants are packed in low density grade polythene bags and labeled for further treatments.

### **Soil testing and treatments**

Soil nutrients testing were done in laboratory using soil testing kits. Certified bean seeds were purchased and treated with below mentioned combinations of organic and prepared biofertilizers and sown in pots

### **TREATMENT DETAILS OF THE EXPERIMENT**

T<sub>1</sub> – Control (C)

T<sub>2</sub>- *Rhizobium* sp. (R)

T<sub>3</sub>- *Azotobacter* sp. (A)

T<sub>4</sub>- *Pseudomonas* sp. (P)

T<sub>5</sub>- Combination of *Rhizobium* sp., *Azotobacter* sp. & *Pseudomonas* sp. (8:8:4)

T<sub>6</sub>- Combination of *Rhizobium* sp., *Azotobacter* sp. & *Pseudomonas* sp. (4:8:8)

T<sub>7</sub>- Combination of *Rhizobium* sp., *Azotobacter* sp. & *Pseudomonas* sp. (8:4:8)

The saplings in the pots of different treatments were monitored and irrigated regularly. Weeds were handpicked whenever necessary. Parameters were recorded periodically from each treatments and the necessary results were tabulated.

## RESULT

### **SOIL NUTRIENTS**

Soil sample was strongly acidic indicated the pH of 5, available Nitrogen was recorded high (> 300), available phosphorous was noticed high (>15) and available potassium was observed high (> 150)

### **PLANT HEIGHT (CM)**

Plant height was observed in combined treatments in the ratio 8:8:4 (59cm), followed by sole application of *Azotobacter* (58cm), *Pseudomonas* (56cm), mixed application 4:8:8 (54cm), 8:4:8 (50cm) compared with control (43cm)

### NUMBER OF LEAVES

The number of leaves from each treated pots were counted at 15<sup>th</sup>, 30<sup>th</sup> and 60<sup>th</sup> DAS.

### NUMBER OF FLOWERS AND PODS

The plants were observed for number of flowers and fruits in each treatment at 15<sup>th</sup>, 30<sup>th</sup> and 60<sup>th</sup> DAS. It was observed the number of flowers were more in mixed inoculant 8:8:4 (30 Nos.) followed by 23 Nos. in sole inoculants (R & A), 8:4:8 (20 Nos.) compared with control 17 Nos. Similarly number of pods were observed high in sole inoculant of *Pseudomonas sp.* treated pots (22 Nos.) followed by *Azotobacter sp.* and combinations in the ratio 8:8:4 (19 Nos.) then with other sole applications and combinations ranging from 16-17 numbers compared with control observed only 13 numbers.

### ESTIMATION OF CHLOROPHYLL (ARNON'S 1949)

Total chlorophyll content was recorded high in sole inoculation of *Pseudomonas* and in mixed inoculation 8:4:8 as 0.90 µg/ml followed by all mixed inoculants (0.85 and 0.83), *Rhizobium*, *Azotobacter* than control

### DETERMINATION OF FRESH AND DRY WEIGHT

Fresh weight of pod was observed high in combined inoculation of ratio 8:4:8 (90.98%) followed by 4:8:8 (7.67%), *Pseudomonas* (4.78%), *Azotobacter* (3.67), *Rhizobium* (2.78%), combined inoculation (2.73%) compared with control (2.64%). Similarly dry weight was high in sole inoculation of *Rhizobium* (93%) followed by *Pseudomonas* (89%), 4:8:8 (88%), *Azotobacter* and mixed inoculation 8:8:4 and 8:4:8 as (86%) than control (84%).

**Table 1**  
**Effect of bio & organic fertilizers on vegetative growth of French bean**

Treatments	Parameters														
	Plant height (cm)			No. of leaves			No. of flowers			No. of fruits			Total chl. µg/ml	Fresh weight %	Dry weight %
	30 <sup>th</sup>	60 <sup>th</sup>	90 <sup>th</sup>	30 <sup>th</sup>	60 <sup>th</sup>	90 <sup>th</sup>	30 <sup>th</sup>	60 <sup>th</sup>	90 <sup>th</sup>	30 <sup>th</sup>	60 <sup>th</sup>	90 <sup>th</sup>	90 <sup>th</sup> day	90 <sup>th</sup> day	90 <sup>th</sup> day
Control	19	28	43	11	21	26	0	11	17	0	6	13	0.73	2.64	84
<i>Rhizobium</i> (R)	21	31	48	14	24	29	0	14	23	0	9	17	0.75	2.78	93
<i>Azotobacter</i> (A)	23	36	58	16	24	28	0	13	23	0	10	19	0.74	3.67	86
<i>Pseudomonas</i> (P)	28	45	56	15	26	27	0	19	29	0	13	22	0.90	4.78	89
R:A:P 8:8:4	27	40	59	16	22	34	0	18	30	0	11	19	0.85	2.73	86
R:A:P 4:8:8	29	39	54	11	24	32	0	17	23	0	10	17	0.83	7.67	88
R:A:P 8:4:8	23	33	46	14	23	28	0	16	27	0	10	18	0.90	9.98	86

### CONCLUSION

In short, results from findings suggest that mixed inoculation of microbial biofertilizers with different beneficial properties would be the future trends of bio-fertilizer application for sustainable crop production. Even though, the combinations of *Azotobacter sp.*, *Rhizobium sp.* and *Pseudomonas sp.* are promising for the growth promotion of French bean in the present investigation, further large scale field application of these isolates at different agro climatic condition is necessary to confirm their potentiality for formulation as an effective biofertilizers for sustainable crop production.

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# GREEN MEDIATED SYNTHESIS OF ZINC OXIDE NANOPARTICLE USING *IPOMOEA QUAMOCLIT* TO EXPLORE THE POTENTIAL OF ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY AGAINST PATHOGENS

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## ABSTRACT

*Ipomoea quamoclit*, a medicinal plant has been used for the synthesis of zinc oxide nanoparticles in this study. The present study has demonstrated an ecofriendly and efficient method for production of zinc oxide nanoparticles from flower, leaf and seed extracts of the plant, *Ipomoea quamoclit*. The phytochemical analysis of this plant revealed that it is rich in phenolics, flavonoids, steroids, saponins, carbohydrates, triterpenes compounds. The zinc oxide nanoparticle has shown remarkable antimicrobial activity against various tested microorganisms such as *Pseudomonas aeruginosa* PAO1, *Salmonella typhimurium*, *Serratia marcescens* and *Micrococcus luteus* by conventional agar well diffusion method. Among the various nanoparticles, the zinc oxide nanoparticles synthesized from flower extract of *Ipomoea quamoclit* have notably marked reduction in the biofilm formation in all the tested strains. This study provides a preliminary data about antibiofilm activity of zinc oxide nanoparticle synthesized from the leaf, flower and seed extract of plant *Ipomoea quamoclit*. This may pave the way in designing the new alternative therapeutic management of biofilm associated infections in future. The observations of the present investigation appear to be promising and valuable source for antimicrobial and antibiofilm principles against the bacterial strains.

**KEYWORDS:** *nanoparticle, biofilm, antimicrobial, zinc oxide, SEM*

## INTRODUCTION

Nanoparticle is of huge choice of interest due to its potential biological properties of antimicrobial and antibiofilm activity. Zinc oxide is considered as GRAS (generally recognized as safe) metal oxide nanoparticles for its application in medicinal use. It possesses anti-cancer, anti-diabetic, antibacterial and antifungal properties.<sup>1</sup> ZnO nanoparticles has been synthesized from plant extracts such as *Azadirachta indica*,<sup>2</sup> *Aloe vera*<sup>3</sup> and from bacteria such as *B. licheniformis*<sup>4</sup>, *Rhodococcus pyridinivorans*<sup>5</sup>, *A. hydrophilla*,<sup>6</sup> from microalgae and macroalgae such as *Sargassum muticum*, *Chlamydomonas reinhardtii*<sup>7</sup> and from fungus such as *Aspergillus fumigatus* TFR-8,<sup>4</sup> *Candida albicans*<sup>8</sup> and from other protein sources such as egg albumin<sup>9</sup>, soluble starch, L-alanine.<sup>10</sup> *Ipomoea quamoclit*, popularly called as mayil mannikkam, cypress vine, star glory has been widely used as conventional and alternative medicine, to treat various kind of ailments<sup>11</sup> such as rheumatoid arthritis and hemorrhoids.<sup>12</sup> The therapeutic properties of these medicinal plants are due to the presence of bioactive substances such as flavonoids, alkaloids, glycosides, tannins, terpenoids, saponins, protein, carbohydrate and other phenolic compounds, but the capability of synthesizing nanoparticles is not yet explored. Hence the synthesis of zinc oxide nanoparticles was carried out using the biobased aqueous extract of *Ipomoea quamoclit* flower, leaf, seed to study the antibacterial and antibiofilm activity against *Salmonella typhimurium*, *Pseudomonas aeruginosa* PAO1, *Serratia marcescens* and *Micrococcus luteus*. These pathogenic microorganisms are commonly found on contaminated food and water which leads to diarrhoea, fever and gastric enteritis, medical devices which enhances the growth and forms biofilm on the surfaces, pulmonary infections. Hence, the present study illustrated using *Ipomoea quamoclit*, a nontoxic and eco-friendly bioresource for synthesizing zinc oxide nanoparticles and evaluating the effect of synthesized nanoparticle against various pathogenic microorganisms. It also focused on the determination of size and shape of biosynthesized nanoparticles. This may act as a tool for preventing

colonization and dissemination of microbes on the medical associated devices and its infection. Thus, the study was carried out to investigate the potential properties of zinc oxide nanoparticles on antimicrobial and antibiofilm using phytosynthesized strategy, to overcome biofilm associated infections.

## MATERIALS AND METHODS

### **Sample collection**

Fresh and healthy parts of leaves/seeds/flower of the plant *Ipomoea quamoclit* (SKC-Herbarium-0374) was collected from local garden of Sivakasi, Tamilnadu washed with water, air dried and prepared powder.

### **Preparation of plant extracts**

10 g of *Ipomoea quamoclit* flower/leaf/seeds extract powder was weighed and dissolved in 100 ml of double distilled water. The *Ipomoea quamoclit* leaves/seeds/flower extract was kept in hot plate at 70°C for 15 minutes. The boiled plant extract was filtered using Whatmann paper. Then 90 ml of 1mM concentration of Zinc acetate was mixed with 10 ml of aqueous plant extract and incubated for 24 hours and observed the color change.

### **Synthesis and Characterization of nanoparticles using different parts of plant extracts**

Different concentrations of aqueous Zinc acetate were mixed with different ratios of phyto-extracts (Flower/leaf/seeds), to improve the efficiency and yield of ZnO NPs. The bioreduction of ZnO in aqueous solution was monitored at regular time intervals by taking small aliquots (1.0 ml) and subsequently measuring UV-Vis spectra at 200 to 800 nm of the colloidal solution. The maximum absorbance of synthesized nanoparticle was noted on UV-Vis spectrophotometer. The colloidal solution was centrifuged at 5,000 rpm for 30-60 min to collect the nanoparticles and the pellet obtained was air dried to obtain powder and dispersed in 1ml of deionized water/DMSO. Finally, the dried nanoparticles were analysed by Fourier Transform Infrared spectroscopy (FT-IR), X-ray diffraction (XRD) and Scanning Electron Microscope (SEM).<sup>13</sup>

### **Analysis of antimicrobial activity by well diffusion method**

The ZnO nanoparticles synthesized from plant *Ipomoea quamoclit* leaf, flower and seed extract were tested for their antimicrobial activity against pathogenic organisms like *Pseudomonas aeruginosa* PAO1, *Salmonella typhimurium*, *Serratia marescens*, *Micrococcus luteus* by agar well diffusion method. The pure cultures of the organism were subcultured on Muller–Hinton broth at 35°C on a rotary shaker at 200 rpm. 10<sup>9</sup> cells of each strain were added on the Muller–Hinton agar plates and wells were made by using gel puncture. 100 mg of phytosynthesized nanoparticles was dissolved in 1ml of DMSO. Different concentrations of nanoparticles such as 25 µl, 50 µl, 75 µl of the samples were added onto the wells, incubated at 37°C for 18-24 h and the different levels of zone of inhibition were measured.

### **Analysis of antibiofilm activity by Zinc oxide nanoparticles**

The antibiofilm activity of phyto-synthesized ZnO nanoparticles was studied using 96- well microtitre plate with modifications. Biofilm production by pathogenic strains grown in Luria broth (LB) was treated/untreated with nanoparticles and studied using a semi-quantitative adherence assay. Briefly, for control experiment, the equal number of cells was seeded in a 96-well microtitre plate and after 24- 48 hrs of incubation at 37°C, the biofilm plates were washed thrice with phosphate buffered saline to remove free floating cells. The adhered cells were stained with 1% crystal violet for 15 min, washed and air dried and resuspended in 200 µl of 33% glacial acetic acid/ethanol. Biofilm formation was quantified by measuring at OD<sub>570</sub>. In another set, 100 µg of zinc oxide nanoparticle solution was added to the culture inoculums and the culture was grown overnight. The treated sample was then seeded onto the microtiter plate and followed the same procedure as mentioned above. The assays were performed in triplicate manner to tested biofilm inhibition.<sup>14</sup> Statistical analyses were performed using ANOVA tools.

## RESULTS AND DISCUSSION

*Ipomoea quamoclit*, a medicinal plant is used for various bioactivities around the world for various illnesses which includes antioxidant, antimicrobial, anticancer, antidiabetic activities as well as insecticidal



activity.<sup>11,12</sup> Hence this medicinal plant parts (flower, leaf and seed) are used for the bioreduction, capping and stabilization for the synthesis of zinc oxide nanoparticles.

### **Visible color change and UV-Vis spectra analysis of formation of zinc oxide nanoparticles**

The formation of zinc oxide nanoparticles was monitored with color change and UV-Vis spectroscopy. The color of the reaction mixture started changing to light purple within 10 min and to dark purple after 1 h, indicating the generation of zinc oxide nanoparticles due to the reduction of zinc metal ions  $Zn^{+}$  into zinc oxide nanoparticles by the active compounds present in the flower extracts (Fig.1A). The UV-Vis spectrum of zinc oxide nanoparticles from *Ipomoea quamoclit* has maximum absorbance at 393 nm, which indicated the presence of zinc oxide nanoparticles (Fig.2A). Similarly, the generation of zinc oxide nanoparticles with leaf extracts, indicated the color change from pale yellow to dark yellow after 1 h, with the help of the active molecules present in the leaf extracts (Fig.1B). This color is due to the excitation of SPR. The UV-Vis spectrum showed the maximum absorbance was observed at 420 nm, which further confirmed the presence of zinc oxide nanoparticles (Fig.2B). The synthesis of zinc oxide nanoparticle with seed extract showed that the color changes to brownish black after 16 h of incubation (Fig.1B) and has maximum absorbance 370 nm in the UV-Vis spectrum which further confirmed the presence of zinc oxide nanoparticles (Fig.2C).

### **Antimicrobial activity of synthesized Zinc oxide nanoparticles**

Antimicrobial property of synthesized ZnO nanoparticles were tested against four strains of bacteria *Pseudomonas aeruginosa* PAOI, *Salmonella typhimurium*, *Serratia marcescens* and *Micrococcus luteus* by conventional agar well diffusion procedure. 100 mg of freshly prepared nanoparticles is dissolved in 1 ml of DMSO and tested different concentration such as 25  $\mu$ l, 50  $\mu$ l and 75  $\mu$ l for each experiment. The results indicated that the nanoparticles synthesized using biological source have antimicrobial activity against each pathogenic microorganisms (Fig.3). The maximum zone of inhibition against *S. typhimurium* and *S. marcescens* was observed for ZnO NP from flower extract whereas the ZnO NP from leaf extract showed maximum activity against *P. aeruginosa*, *S. typhimurium* and *M. luteus* and ZnO NP from seed extract showed maximum activity against *P. aeruginosa* and *S. marcescens* (Table 1). The finding suggested that the antibacterial activity of ZnO NPs is most likely due to the attachment and interaction of the ZnO NPs to the bacterial cell membrane, DNA, RNA, proteins and ribosome thereby affecting the metabolic activity and viability of the cell leading to death.<sup>15</sup> Studies reported that ZnO NPs have antibacterial activity against multidrug-resistant strains such as *S. aureus* and *E. coli*.<sup>15</sup>

### **Antibiofilm activity of Zinc oxide nanoparticles**

The efficacy of ZnO-NPs on biofilm inhibition was investigated with the aim of finding on their biomedical applications.<sup>14</sup> Antibiofilm activity of Zinc oxide nanoparticles synthesized from flower, leaf and seed extract of *Ipomoea quamoclit* were studied against human pathogenic strain *Pseudomonas aeruginosa* PAOI, *Salmonella typhimurium*, *Serratia marcescens* and *Micrococcus luteus*. Biofilm formation of the control organisms (untreated) and test (treated with nanoparticles) was studied in microtitre plate and stained with crystal violet and measured the absorbance at 570 nm. Among the nanoparticles, the Zinc oxide nanoparticles synthesized from flower extract of *Ipomoea quamoclit* have notably marked reduction in the biofilm formation in all the tested strains (Fig.4), inferring their potential antibiofilm activity.

### **Phytochemical analysis**

Plant contains rich in natural compounds such as alkaloids, flavonoids, saponins, steroids, tannins present in leaves, stems, roots shoots, flowers, barks and seeds which serve as bioreduction and stabilization agents to synthesize the nanoparticles<sup>13</sup>. The results in our study showed that *Ipomoea quamoclit* contains the presence of phenolic compounds and other metabolites that helped in the reduction, capping and stabilization of zinc oxide nanoparticles.

### **Characterization studies**

#### **SEM image of Zinc oxide nanoparticles synthesized from *Ipomoea quamoclit***

SEM technique was employed to visualize the size and shape of zinc oxide nanoparticles. The typical SEM showed that the product mainly consists of particles like zinc oxide nanoclusters with panoramic view and the size range from 150 to 200 nm. However, further observation with high magnification revealed that these zinc oxide nanoclusters are assembled by smaller nanoparticles which exhibited good uniformity and the average size of these nanoparticles is about 40 to 45 nm, 60 to 65 nm, 15 to 20 nm synthesized from flower,

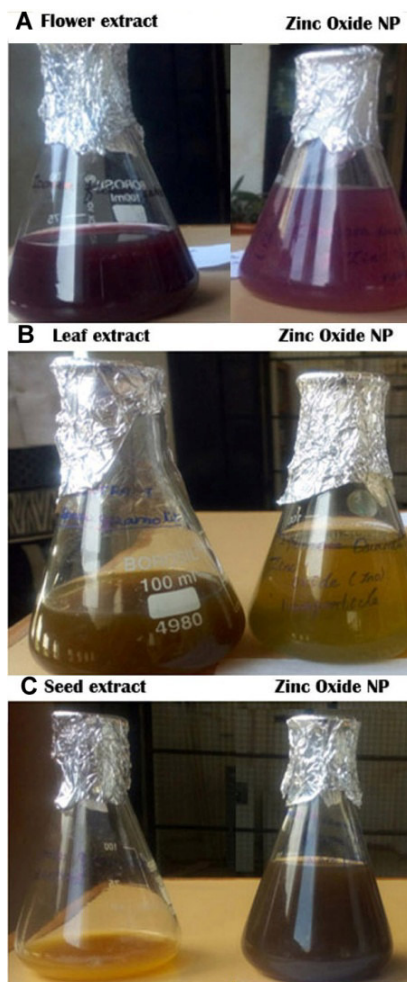


leaf and seed extracts respectively, almost in accordance with SEM analysis and confirmed the development of zinc oxide nanostructures (Fig.5).

### ***X-ray diffraction of Zinc oxide nanoparticles***

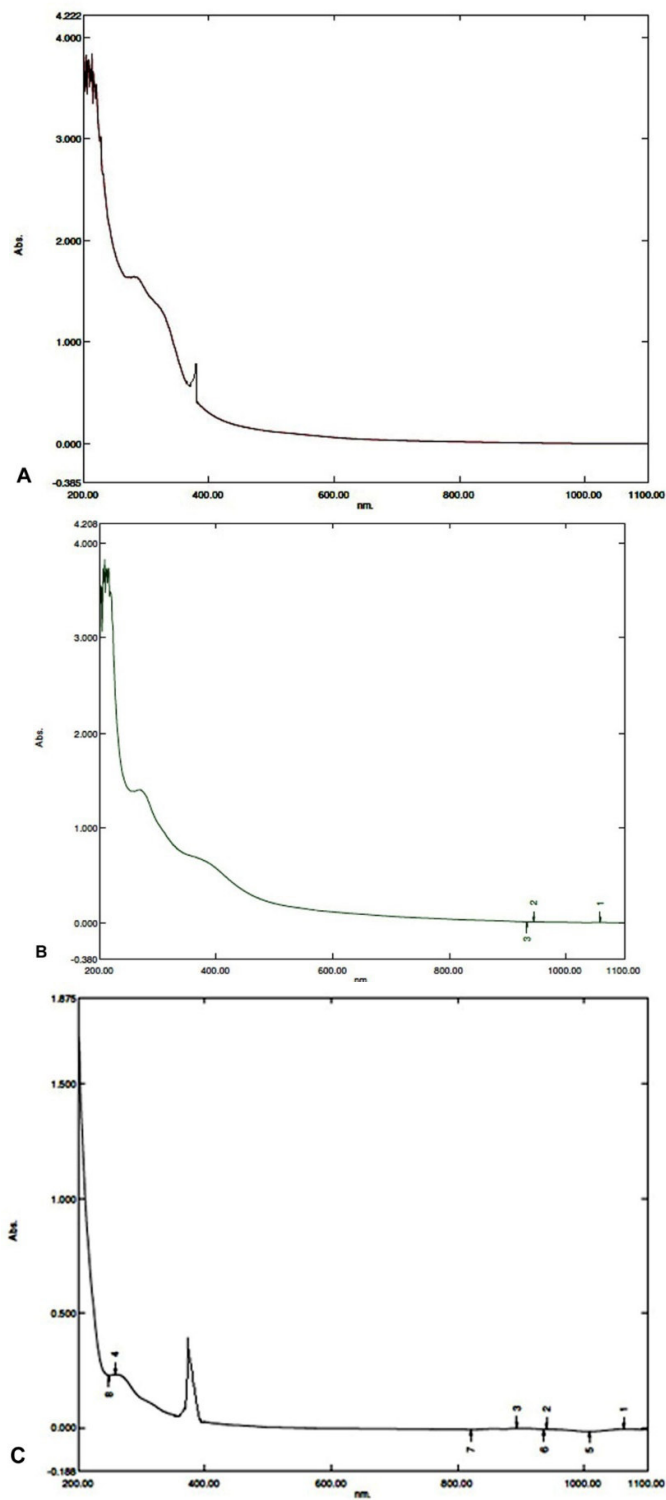
XRD pattern of zinc oxide nanoparticles synthesized by treating *Ipomoea quamoclit* flower extract with 1mM aqueous zinc acetate solution. The characteristic peaks were observed at 18.67°, 28.73°, 40.98° from flower extract, 28.20°, 32.07°, 38.02° from leaf extract, at 44.01°, 64.39°, 77.51° from seed extract, which confirms the formation of zinc oxide nanoparticles synthesized by using extracts of *Ipomoea quamoclit* (Table 2).

**Figure1**  
***Visual observation of color change upon formation of Zinc oxide nanoparticles***  
***(a) Flower (b) Leaf (c) Seed NPs***



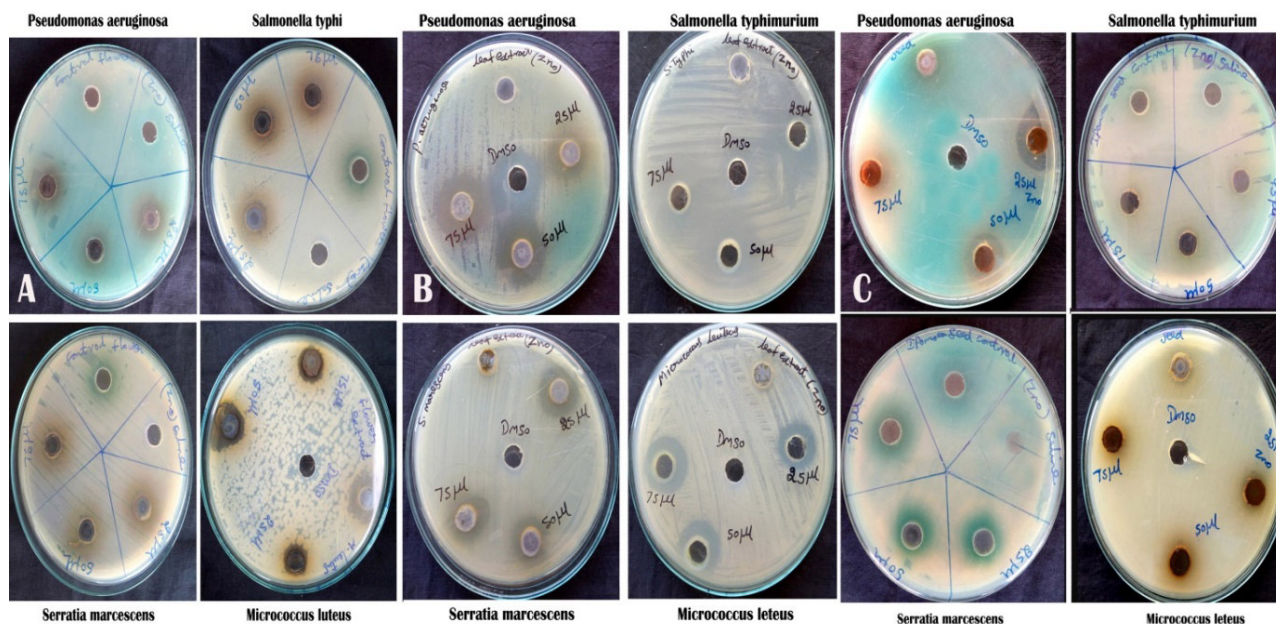
**Figure 2**

**UV Visible absorption spectra of synthesized Zinc oxide nanoparticles from (a) Flower; (b) Leaf (c) Seed of *Ipomoea quamoclit***



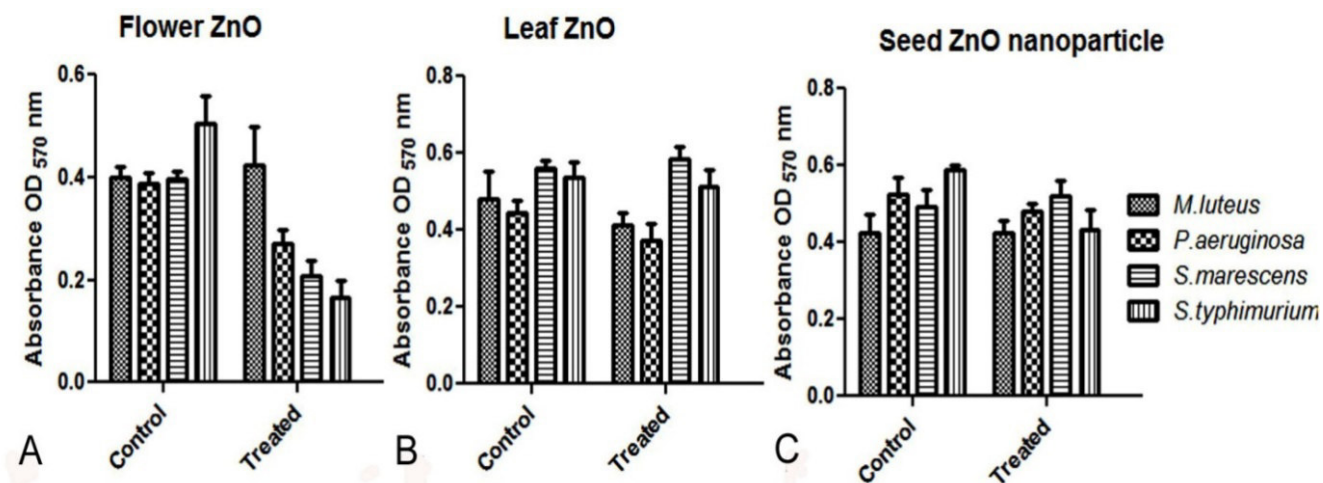
**Figure 3**

**Antimicrobial activity of Zinc oxide nanoparticles from (a) Flower; (b) Leaf; (c) Seed of *Ipomoea quamoclit* against tested pathogens using agar-well diffusion assay**

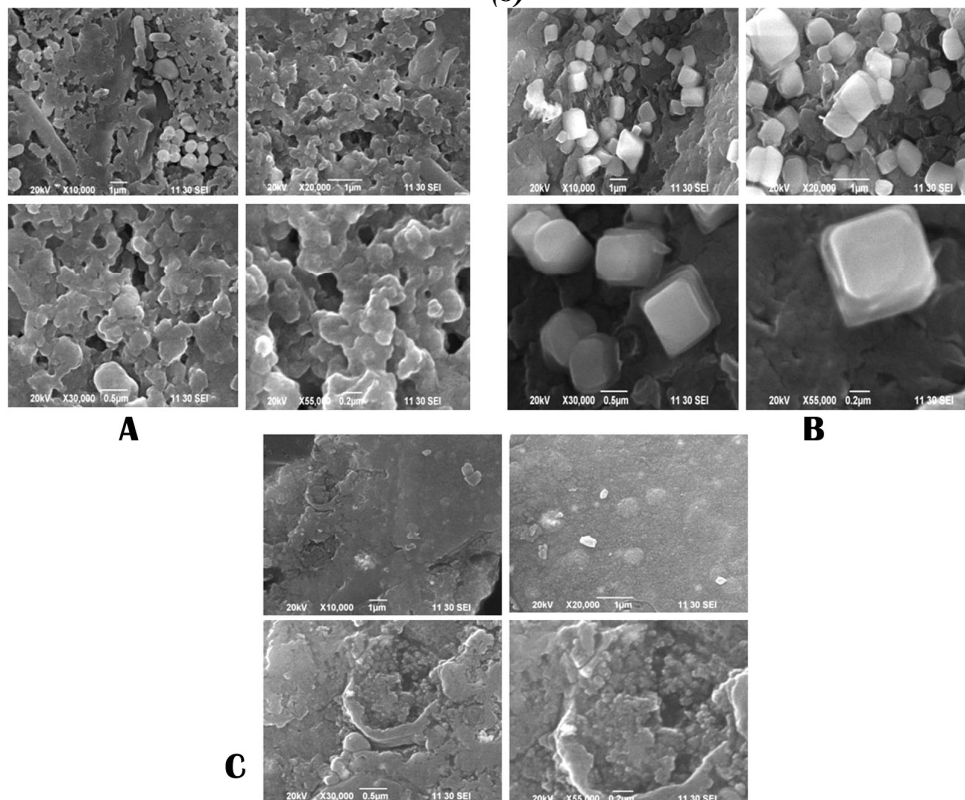


**Figure 4**

**Antibiofilm activity of Zinc oxide nanoparticles from (a) Flower; (b) Leaf; (c) Seed of *Ipomoea quamoclit* against tested pathogens. The results are represented as mean  $\pm$  SD of 4 replicates with  $P$  value  $<0.001$**



**Figure 5**  
**SEM micrograph of Zinc oxide nanoparticles synthesised from**  
**(a) Flower; (b) Leaf; C) Seed of *Ipomoea quamoclit***  
**(b)**



**Table 1**  
**Antibacterial activity of Zinc oxide nanoparticles by agar well diffusion method**

S.No	Microorganisms	Zone of inhibition				
		Plant extract	Negative control	25µl	50µl	75µl
Flower ZnO NPs						
1.	<i>P. aeruginosa</i>	-	-	7 mm	5 mm	10 mm
2.	<i>S. typhimurium</i>	6 mm	-	8 mm	10 mm	10 mm
3.	<i>S. marcescens</i>	6 mm	-	8 mm	9 mm	7 mm
4.	<i>M. luteus</i>	7 mm	-	2 mm	5 mm	2 mm
Leaf ZnO NPs						
1.	<i>P. aeruginosa</i>	-	-	9 mm	14 mm	16 mm
2.	<i>S. typhimurium</i>	-	-	7 mm	10 mm	12 mm
3.	<i>S. marcescens</i>	-	-	6 mm	5 mm	7 mm
4.	<i>M. luteus</i>	-	-	8 mm	10 mm	14 mm
Seed ZnO NPs						
1.	<i>P. aeruginosa</i>	4 mm	-	9 mm	8 mm	8 mm
2.	<i>S. typhimurium</i>	3 mm	-	-	-	-
3.	<i>S. marcescens</i>	6 mm	-	5 mm	6 mm	9 mm
4.	<i>M. luteus</i>	3 mm	-	6 mm	8 mm	6 mm



**Table 2**  
**X-ray Diffraction of zinc oxide nanoparticles from (a) Flower; (b) Leaf; (c) Seed extracts of *Ipomoea quamoclit* showing strongest 3 peaks**

Peak	2 Theta (deg)	D (Zn)	I/ II	FWHM (deg)	Intensity (counts)	Integrated Int (counts)
<b>Flower ZnO NP</b>						
12	28.7333	3.10441	100	1.46670	27	417
1	18.6750	10.18493	89	3.05000	24	708
18	40.9833	2.20041	59	1.63330	16	244
<b>Leaf ZnO NP</b>						
20	29.0230	3.07414	100	0.73180	70	557
29	41.0900	2.19494	70	0.70000	49	394
5	19.5250	9.27786	37	1.25000	26	317
<b>Seed ZnO NP</b>						
60	77.5187	1.23041	100	0.33320	257	972
53	64.3978	1.44560	96	0.30430	247	829
43	44.0147	2.05564	90	0.27940	232	746

## CONCLUSION

The major problem posed by the pathogenic bacteria is that it becomes resistant to the classes of antibiotics and generates new strategy to evade from the susceptibility process. Therefore, there is an immediate necessitate to discover the alternative solutions as antibiotic resistant microbes is rising internationally and insist the introduction of new drug which shows potential antimicrobial agents against various antibiotic resistant strains. Nanoparticles biosynthesized with plant compounds have yielded promising findings in biomedicine. The synthesized NPs from plants, are capped with phytochemical compounds, disturb the bacterial cell wall and causes damage and toxicity to the bacterial cell. The present study demonstrated that ZnO NPs synthesised from *Ipomoea quamoclit* are capable of providing antibacterial activity against the various tested pathogens and can have great potential for the preparation of antibacterial drugs and its applications in their clinical and drug formulation needs further investigation.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# APPLICATION OF BIOTECHNOLOGY IN THE PRODUCTION OF NATURALLY COLORED COCOONS IN *SAMIA CYNTHIA RICINI* : A STUDY ON THE EFFECT OF LIGHT EXPOSURE ON THE COLOUR DEVELOPMENT IN COCOONS.

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## ABSTRACT

Eri silkworm or *Samia Cynthia ricini* is a type non- mulberry polyphagous silkworm that feeds majorly on castor leaves. In this study, we have selected *Samia Cynthia ricini* to conduct the experiments to study the nutritive value and morphological differences of Eri silkworm by feeding different leaves (*Ricinus communis*- Castor leaf, *Carica papaya*-papaya leaf and physiological differences of Eri silkworm under different light exposure *Terminalia catappa* – Indian Almond leaf), to study the morphological changes and cocoon colour of Eri Silkworm by exposing them to different conditions. Study of nutritive value and morphological differences of Eri – Silkworm to different food habits. Effect of light on morphological and physiological differences of EriSilkworm was estimated. Coloured cocoon formation was studied in different food habits. Pink colored cocoons were developed using vital stains in the lab. This study proves that colored cocoons can be produced naturally using vital stains. This work is commercially very beneficial for the development of the nation since it reduces the cost of dying and man power in sericulture industry.

**Keywords:** Ericulture, Nutritive value, light exposure, colored cocoons, Janus green.

## INTRODUCTION

Ericulture is practiced in India since rearing of Eri silkworm is easier than mulberry silkworm, due to its adaptability for the changing environment and food habit. Eri silkworm produces yellow colour silk which is called as Ahimsa silk or Gandhi silk as the process of silk extraction is done, without killing the pupae. Since the filament in the cocoon is not continuous and uneven thickness, these cocoons are used for spinning to produce the Eri silk. Dyeing of silk produces harmful toxins which can cause hazardous effect which can severely affect human health. Here in this study we have tried to produce naturally colored Eri silkworm by altering the food sources <sup>1,2</sup>. This reduces the cost of dying and produces eco-friendly colored silk. This method is very economical since the dye used for feeding silkworms is not expensive and did not harm the silkworm's lifecycle <sup>3,4,5</sup>. Feeding worms with the leaves sprayed with janus green in mulberry silk worms has already shown good result in producing pink cocoons <sup>6,7,8</sup>. Here in this paper we are concentrating on the methods to produce colored cocoons in eri silk worm. An attempt is been made to see the effect of light exposure on the cocoon formation and quality of the eri silk.

## METHODOLOGY

### Estimation of nutritive value and morphological differences of Eri silkworm to different leaves:

The three different types of leaves, castor leaf- *Ricinus communis*, papaya leaf- *Carica papaya* and Indian almond leaf- *Terminalia catappa* were fed twice a day and the number of feeding time was increased when the instar level elevates.

### Study of the morphological and physiological differences of Eri silkworm under different light exposure environment:

Five Eri silkworms (2<sup>nd</sup> instar larvae) were isolated in each chamber. Measurement of weight of length of the five larvae was taken each day and the average was calculated. Same procedure was repeated until the larvae started spinning cocoon

### Evaluation of morphological changes and cocoon colour of Eri silkworm by feeding janus green dyed castor leaf.

The leaves were washed and air dried without exposure to the sun and fed to the isolated fifth instar larvae thrice a day.

## RESULTS AND DISCUSSION

Eri silk worms which were fed with castor and almond leaves showed a very good improvement in the body weight (**Table 1**). The worms fed with papaya showed mortality by 10 days, indicating that nutritive value of the papaya is not supportive for the growth of the silk worm.



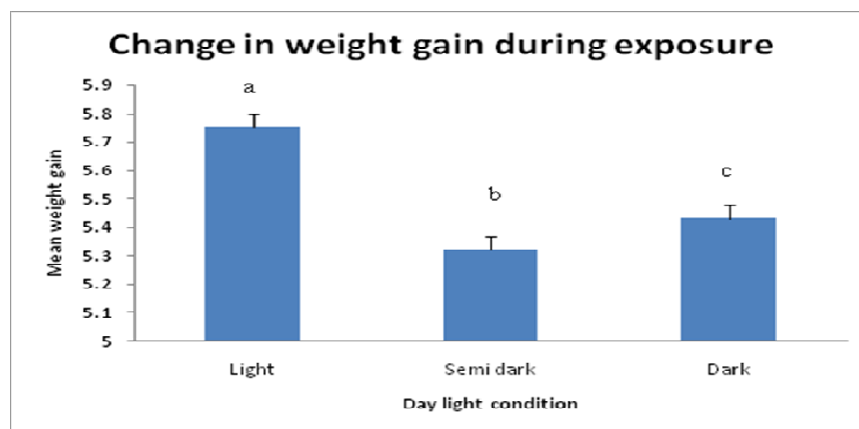
**Fig.1. Eri silkworms feeding on castor (a), papaya (b) and almond leaves (c)**

The difference in the body weight showed significant change in the castor and almond group compared to the ones in the papaya tray. Eri silkworms showed better appetite for castor leaves compared to almond leaves. Papaya leaves were not preferred and many worms were dead before they even started spinning the cocoons (**Fig.1**).

**Table 1. Change in the body weight of silkworms**

Type of feed	Initial weight in grams	Weight after 5 days	Weight after 10 days
Castor	0.944±0.02 <sup>a</sup>	2.13±0.005 <sup>a</sup>	4.97±0.01 <sup>a</sup>
Papaya	0.91±0.01 <sup>a</sup>	0.78±0.002 <sup>b</sup>	0 <sup>b</sup>
Almond	0.89±0.02 <sup>a</sup>	2.17±0.01 <sup>a</sup>	5.01±0.02 <sup>a</sup>

One way ANOVA was applied with boneferroni post hoc test and significance was considered at  $p < 0.05$ . Identical letters shows insignificant difference among the group and different alphabets shows significance between the groups. Change in the body weight and length of silkworms to different exposure periods showed significant decrease in the semi dark condition compared to light exposure. Light exposure also played an important role in the cocoon formation (**Fig 2**).

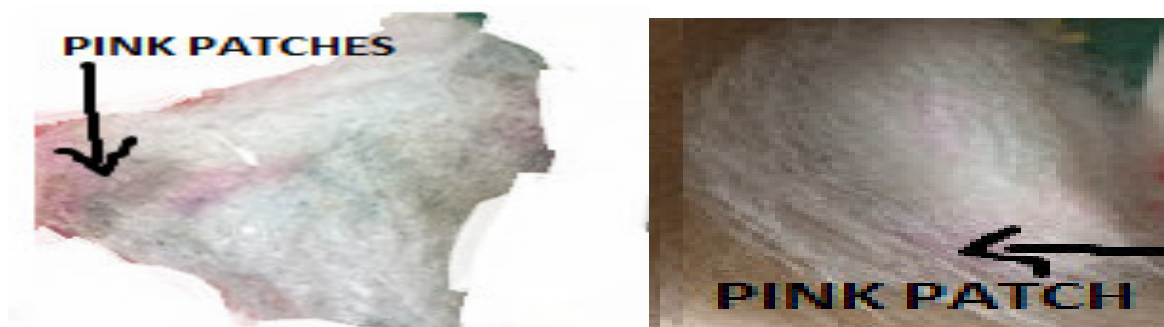


**Fig.2. change in the body temperature in the exposure.**

One way ANOVA was applied with boneferroni post hoc test and significance was considered at  $p < 0.05$ .

### Development of colored cocoons:

Pink colored cocoons were developed due to Janus green fed along with the castor leaves. Patches of pink color was observed. Some larva had entered pupa stage without spinning the cocoon. Body weight showed significant in the silkworms fed with the leaves sprayed with janus green. Our results showed there were pink patches in the cocoons and in future if the concentration of the stain is increased we should be able to produce pink colored cocoons. Janus green is used since it is a vital stain and does not harm the larva. Pink patches ( Fig.3) is shown in the following figure.



**Fig.3. Pink colored cocoons formed due to exposure to Janus green.**

As the objective of this experiment is to compare the nutritive value between two variety of silkworms, it is evident that the nutritive value of Eri Silkworm is more compared to *Bombyx Mori* according to <sup>9, 10</sup>. The larvae kept in Dark condition expressed significantly better result compared to larvae in Light and Semi Dark conditions in respect to weight, length of larvae, weight of cocoon, rate of mortality and rate of fecundity. The experiment was performed to produce coloured silk naturally by feeding the Eri silkworm with dyed leaves after observing the different procedures to produce colored cocoons which will be economically beneficial <sup>11-16</sup>.

### CONCLUSION

Food habits of Eri silkworm indicates that the castor leave fed silkworms were growing at an optimum rate and it has given a better yield (cocoon weight). Indian Almond leave fed silkworm has also given similar result so this clearly shows that Indian Almond can be used as a substitute feed for Eri Silkworm. Papaya leave fed silkworms died due to more latex content in the leave that caused them to release haemolymph prior to death. The pink spots were observed in this particular experiment when the larve were fed from the 5<sup>th</sup> instar stage. The complete cocoon colour may be changed completely when the dyed leaf are fed from the beginning. This work is commercially very beneficial for the development of the nation since it reduces the cost of dying and man power in sericulture industry.

## ACKNOWLEDMENT

NBAIR Bangalore

## CONFLICT OF INTEREST

There is no conflict of interest

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## ISOLATION AND FUSION OF SUNFLOWER (*Helianthus annuus*) AND SOYBEAN (*Glycine max*) PROTOPLASTS

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### ABSTRACT

Sunflower (*Helianthus annuus*) and soybean (*Glycine max*) are common edible oils used around the world. Sunflower and groundnut usually has high oil content while soybean yields comparatively less oil whereas rich in protein. Somatic hybridization between the two plants, sunflower and soybean to create an intergeneric hybrid was attempted using PEG mediated method. The intergeneric somatic hybridization is aim to produce a novel hybrid with higher oil content. Mesophyll protoplasts of sunflower and soybean plants grown in pots were used in for the isolation of protoplasts using a modified isolation solution (Cellulase and pectinase mixed in sorbitol, SDS, CaCl<sub>2</sub> and KOH solution). Fused protoplasts were cultured in a modified Pelletier B liquid media with different combinations of 2, 4-dichlorophenoxy acetic acid and  $\alpha$ -Naphthalene acetic acid and 6-benzyl amino purine for callus induction. The size of induced micro callus was observed in 1 mgL-1NAA, 0.5 mgL-1 2, 4-D after 17 days of culturing in cavity slide. Micro callus induction was highly significant in different growth hormone combinations against callusing parameter under 17 day's dark condition

**KEY WORDS:** Sun flower, Soybean, Protoplast, Micro callus, Fusion, PEG

### INTRODUCTION

Sunflower (*Helianthus annuus*) and Soybean (*Glycine max*) are major economic crops of the world. Sunflower is cultivated primarily for its seeds which yield one of the world's most important edible oil. The oil is considered premium oil because of its light colour, high level of unsaturated fatty acids, negligible linolenic acid, lack of trans fat, bland flavour and high oxidative stability. Soybean (*Glycine max*) is a native of eastern Asia and has become the most important plant source of edible bean and protein in the world. Soybean is also rich in nutrient content with carbohydrates, minerals, saponins and isoflavonoids such as genistein and daidzein. Isoflavones are non-nutritive substances that possess health protective benefits<sup>1, 2</sup>. Processed soybean oil, is used as a base for printing inks (soy ink) and oil paints due to its quality as a drying oil. There are many plant breeding techniques adopted for crop improvement and producing genetically modified plants<sup>3</sup>. These techniques have proven particularly useful in introgression of desired genes into new plants. However, there are certain barriers to the production of hybrid plants due to sexual and species incompatibility. These barriers can be overcome through the use of protoplast fusion to produce new hybrid plants. Protoplasts are promising tools to carry out para-sexual hybridization by fusing two distantly related species which otherwise cannot fertilize and form new plants through normal sexual hybridization<sup>4,5</sup>. Protoplast fusion is an efficient method for genetic modification by fusing two different plant species<sup>6,7</sup> or genera<sup>8,9,10</sup> for producing a novel Ahybrid<sup>11,12</sup>. Advancement in protoplast fusion and culture techniques has increased the utility of plants in biochemical and genetic research and also in genetic improvement of medicinal plants<sup>13</sup>. Plant breeding and improvement in productivity in soybean have been achieved through biotechnology, genetic engineering and tissue culture. These technologies allow rapid incorporation of specific improvements within a short period, especially by the transfer of specific genes into plants<sup>14,15,16</sup>. Plant protoplast techniques have been used since the 1960s and they are now repeatedly used for genetic modification of economically important plants. By mid-1980s, important cereals such as wheat, rice, maize, and barley have been successfully regenerated from protoplasts<sup>17,18</sup>. Isolation and regeneration of protoplast were successfully carried out in various plants of importance<sup>19, 20, 21, 22</sup>. Protoplast fusion and culture provide a good approach towards somatic hybridization by aiding conventional plant



breeding techniques. The application of protoplast fusion in *Helianthus* species has previously been reported for the transfer of *Sclerotinia sclerotiorum* resistance genes from wild *Helianthus* species to cultivated sunflowers to produce resistance sunflower hybrids<sup>23</sup>. Asymmetric fusion of protoplast under an optimal condition resulted in producing more colonies and micro calli in PEG mediated hypocotyl protoplast fusion in sunflower<sup>24</sup>. Nevertheless, successful development of protoplast-to-plant systems was still limited in many plants because it usually depends on various factors especially genotypes<sup>25</sup>. The effects of genotypes on plant development have been demonstrated in several in vitro systems including protoplast isolation and regeneration<sup>26, 27, 28</sup>. Reports on the fusion of sunflower and soybean protoplasts is almost rare. Soybean plants are of potential value through hybridization with sunflower as these are the valuable source for disease resistance, superior oil quality and elevated protein content. The study aimed to isolate protoplasts from *Helianthus annuus* and *Glycine max* derived from different plant tissues using modified methods, fusion of the isolated protoplasts using Polyethylene glycol 6000 (PEG) and Induction of callus.

## MATERIALS AND METHODS

Seeds of high yielding sunflower and soybean were procured from University of Agricultural Science (UAS), Bengaluru. Seedlings were raised in plastic pots up to a height of 30 inches. Young leaves and shoot tips were used for the isolation of protoplasts. All plant materials were excised from 15 days old healthy plants grown under controlled environmental conditions. Glass wares, other necessary implements, solutions (except protoplast isolation solution) and media were sterilized (121°C for 20 min at 22 psi)<sup>28</sup>. The protoplast isolation solution was filter sterilized using a membrane filter. The excised young shoots, hypocotyls and leaves of the seedlings were taken and washed thoroughly in running tap water with teepol followed by immersing it in 70% ethanol for 1 min. and in 0.1% Mercuric chloride (HgCl<sub>2</sub>) for 2 min. After the treatment, the explants were washed aseptically 3-4 times with sterile double distilled water to remove the traces of HgCl<sub>2</sub>. These explants were transferred to Petri dishes for further process<sup>18</sup>.

### 1. Protoplast Isolation

The sterilized plant tissues were sliced to 1-2mm sizes using a sterilized blade. The sliced plant tissues were transferred to 14cm petri dishes containing an osmotic regime (13% Mannitol) to induce plasmolysis for an incubation period of 40 min. After incubation, mannitol solution was replaced by filter sterilized protoplast isolation solutions (Cellulase and pectinase mixed in sorbitol, SDS, CaCl<sub>2</sub> and KOH solution). The mixture was further incubated in a shaker incubator of 20rpm at room temperature for different incubation periods (3, 6 and 16h respectively) to evaluate the protoplast yield<sup>20, 29, 30</sup>.

### 2. PROTOPLAST PURIFICATION

Protoplasts were filtered through a nylon mesh (100µm pore size) and the coarse and un tissues were discarded. The filtrate was transferred in 15ml capacity screw-capped centrifuge tubes containing CPW (Cell and Protoplast Washing) solution and centrifuged twice (100rpm, 5 min) and the pellet was retained by discarding the supernatant. Flootation purification was carried out by sucrose density centrifugation (21 % sucrose), at 100rpm for 5 min. The floating protoplasts were then suspended in a washing solution for further assessment<sup>31</sup>. Counting of protoplast was carried out using a Neubauer hemocytometer. The protoplast sample was mixed uniformly and 2.0 µl of the sample was placed into the hemocytometer, were allowed to settle for 5 min. The purified protoplasts were counter under a light microscope (10X) and yield was calculated<sup>32</sup>.

### 3. PROTOPLAST VIABILITY

The viability of protoplast was examined after enzyme incubation by observing their photosynthetic activity. Spherical protoplasts with their intact cell membranes were considered to be viable while those with damaged cell membranes and irregularly shaped were considered to be non-viable. Protoplast suspension (2.0µl) was slowly loaded into the counting chamber of the hemocytometer. The protoplasts were allowed 5 min and counted under a microscope. Percent viability of protoplasts was calculated and tabulated<sup>32</sup>.

### 4. PROTOPLAST FUSION



Protoplast fusion was carried out using chemical fusogen PEG 6000 mixed in fusion solution [5% (v/v) DMSO, 90mM mannitol, 60mM CaCl<sub>2</sub> and 25mM glycine, pH 5.6-5.7)<sup>4,33</sup>. Polyethylene glycol 6000 1 ml, each of concentrations, 10, 20 and 30% were added to 1ml protoplast suspension and were incubated to 10, 20 and 30 min fusion periods. Following the incubation, 160μl of a solution containing 5% (w/v) glucose, 0.735% (w/v) CaCl<sub>2</sub>.2H<sub>2</sub>O, (pH 10.5) was overlaid on each drop<sup>4</sup>. The percentage of binary and multi fusions were observed under an inverted microscope and the fusion percentages were calculated using the following formulas<sup>3</sup>.

$$\text{Binary fusions (\%)} = \frac{\text{Number of fusion between two protoplasts} \times 100}{\text{Total number of protoplasts}}$$

$$\text{Multi fusions (\%)} = \frac{\text{Number of fusions between more than two protoplasts} \times 100}{\text{Total number of protoplasts}}$$

## 5. PROTOPLASTS CULTURE

Fused protoplasts of sunflower and soybean were aseptically inoculated in cavity slides containing Pelletier B media supplemented with different concentrations of 2,4-D, BAP and NAA (0.5mg/ml 2, 4-D, 0.5mg/ml NAA and 0.5 mg/ml BAP, 0.5mg/ml 2, 4-D, 0.5mg/ml NAA and 1mg/ml BAP. After 7 days 2ml Pelletier C medium was supplemented with three hormonal combinations followed by a consecutive addition of same medium on 11 day of incubation for further development of callus<sup>34</sup>. Cultures were maintained in dark condition under controlled temperature (25°C) for callus induction<sup>35,36</sup>.

## RESULT AND DISCUSSION

### Isolation of protoplast

Protoplasts were isolated from young leaves and shoot tips of 15 days old seedlings. In both the sunflower and soybean plants, young leaves gave the maximum number of protoplast compared to their shoots<sup>37,38</sup> (Table 1&2). It was observed that the protoplast yield in sunflower was significantly higher as compared to soybean. Maximum number of protoplasts were obtained at the incubation duration of 16h in a shaker incubator (Fig.1). Results also showed that incubation period above 16h causes a decrease in number of isolated protoplasts and their viability. This might be due to the exhausting of the enzyme in the mixture in addition to damage of the protoplast which enter in to the death phase. The type of tissues is often considered as an important factor that governs the release of viable protoplasts<sup>5, 28</sup>. Protoplast yields was higher with leaf tissues than hypocotyls and shoots<sup>7, 39, 40</sup>. Duration of incubation also had an impact on the release of protoplast<sup>27,41</sup>.

### Protoplast viability

The viability of protoplast was examined after 24h of enzyme incubation by observing their photosynthetic activity. The result shows that the maximum number of viable protoplast were obtained during the lower incubation period (Table 2). Tissue types, enzyme concentrations and incubation periods affected both yields and viability of protoplasts which finally determined the number of viable protoplasts. The differential responses in yields and viability may result from the differences in the extent of cell wall thickening and the physiological status of plant materials<sup>19, 25, 42, 43, 44</sup>.

### Protoplast fusion

Three different concentrations of PEG 6000 were used as chemical fusing agent to fuse the isolated protoplasts of sunflower and soybean. Maximum fused protoplasts were obtained when the concentration of 30% PEG 6000 was used as chemical fusing agent (fig 4). It was also observed that the binary fused protoplasts were maximum with 20 minutes incubation period compared to multi fused protoplasts (Table 3). From these results it was evident that the concentration of PEG is an important factor in relation to fusion. PEG is a water soluble compound whose other linkage make the molecule slightly negative in

charge. The high molecular weight of the polymer acts as abridges connecting the protoplasts together. A strong affinity of PEG for water causes local membrane dehydration and increased fluidity. This in combination with the reduction of an exclusion volume between adjacent protoplasts causes diminishing mutual membrane electrostatic repulsion. It was also observed that combination of PEG and  $\text{Ca}^{2+}$  at high pH results in better fusion because the addition of  $\text{Ca}^{2+}$  to the PEG causes the potential of the surface negative charge on protoplasts to be reduced, facilitating protoplast adhesion. Previous studies documented the use of PEG 30, 40 and 50% of 8000, 6000 and 1540 MW for protoplasts fusion<sup>34, 44, 45, 46, 47</sup>. The concentration and low molecular weight of PEG help to increase the rate of protoplast fusion<sup>13</sup>. Therefore identification of suitable MW of PEG is important for increasing yield and viability of fused protoplasts of sunflower and soybean. Fusion of the protoplasts highly induced by high alkalinity as the condition influence the formation of intramembranous lysophospholipids such as lysolecithin and lysophosphatidyl-ethanolamine that increase membrane fluidity results in fusion<sup>36</sup>.

**Table: 1: Protoplasts yield from leaves of *H. annuus* and *G. max* using different enzyme concentrations (pectinase and cellulase) and SDS.**

Enzyme concentration % (w/v)	Incubation time (h)	No. of isolated protoplast / ml		No. of isolated protoplast /ml at 20hr	
		<i>H. annuus</i>	<i>G. max</i>	<i>H. annuus</i>	<i>G. max</i>
Pectinase + cellulase+ SDS (0.5+0.8+0.1)	3	$8.0 \times 10^5$	$4.0 \times 10^5$	$5.0 \times 10^5$	$1.0 \times 10^5$
	6	$1.3 \times 10^6$	$7.0 \times 10^5$	$6.0 \times 10^5$	$3.0 \times 10^5$
	16	$1.9 \times 10^6$	$1.0 \times 10^6$	$9.0 \times 10^5$	$5.0 \times 10^5$
Pectinase + cellulase+ SDS (0.8+2+0.1)	3	$9.0 \times 10^5$	$5.0 \times 10^5$	$6.0 \times 10^5$	$2.0 \times 10^5$
	6	$1.3 \times 10^6$	$8.0 \times 10^5$	$7.0 \times 10^5$	$4.0 \times 10^5$
	16	$6.0 \times 10^5$	$1.3 \times 10^6$	$1.1 \times 10^6$	$7.0 \times 10^5$
Pectinase + cellulase+ SDS (2+2+0.1)	3	$1.0 \times 10^5$	$4.0 \times 10^5$	$7.0 \times 10^5$	$3.0 \times 10^5$
	6	$1.6 \times 10^6$	$6.0 \times 10^5$	$9.0 \times 10^5$	$5.0 \times 10^5$
	16	$2.6 \times 10^6$	$1.5 \times 10^6$	$1.2 \times 10^6$	$7.0 \times 10^5$

### Protoplast culture and induction of callus

Micro callus induction was highly significant in combinations of different growth hormone against callusing parameter during the incubation under dark condition for 17 days. It was found that micro calli production was increased by frequent addition of low concentration of 2, 4-D with high concentration of BAP at prolonged dark period. After 17 days of culturing the protoplasts under dark condition, development of green and light green colour callus were observed (fig 5). Significant changes in the colour of calli were not found in the present experiment.

Initiation of micro callus depend on the media supplemented with different concentrations of growth hormones on fused protoplasts. It was found that micro callus production was increased by frequent addition of low concentration of 2, 4-D with high concentration of BAP at prolonged dark period.

**Table: 2**  
**Protoplasts yield from leaves of *H. annuus* and *G. max* at different incubation periods and their viability.**

Incubation period (h)	No of isolated protoplasts/ml <i>H. annuus</i>	Viability (%)	No of isolated protoplasts/ml <i>G. max</i>	Viability (%)
3	$1.33 \times 10^5$	62	$6.67 \times 10^5$	56
6	$1.27 \times 10^6$	55	$8.0 \times 10^5$	54

16	$1.53 \times 10^6$	49	$5.33 \times 10^5$	51
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**Table: 3**  
**Number of binary and multi fused protoplasts of *H. annuus* and *G. max* at different concentrations of PEG 6000 and duration**

PEG 6000 % (w/v)	Fusion period (min)	Binary fusion (%)	Multiple fusion (%)
10	10	$14 \pm 2$	$11 \pm 3$
	20	$18 \pm 3$	$14 \pm 2$
	30	$22 \pm 3$	$9 \pm 4$
20	10	$24 \pm 3$	$16 \pm 3$
	20	$30 \pm 2$	$14 \pm 3$
	30	$24 \pm 3$	$12 \pm 2$
30	10	$29 \pm 2$	$17 \pm 3$
	20	$36 \pm 4$	$15 \pm 3$
	30	$28 \pm 4$	$19 \pm 2$

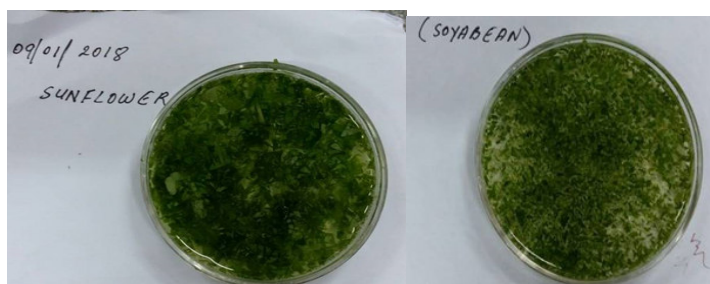
The dark period might be crucial for the stability of protoplasts and hence for the formation of micro callus<sup>13,48</sup>. In the initial culture media in which the visible calli were developed, low concentration of 2, 4-D and high concentration of NAA were found favorable<sup>31</sup>. But the callus proliferation may require a higher concentration of 2, 4-D<sup>49,50,51,52</sup>. No significant difference in the colour of micro calli was observed in the experiment which might be linked to the endogenous levels of auxins and cytokinins in the used plant material.

## CONCLUSION

Isolation and fusion of sunflower and soybean protoplasts was carried out with an aim to produce callus which can be further maintained and regenerated into a whole new plant. The hybrid plant might possess characteristics similar to both the parents and this may address the improvement of certain traits in both sunflower and soybean plants. Protoplasts were isolated from tender leaves and shoot tips of sunflower and soybean plants using different combination of pectinase and cellulase enzyme concentrations in combination with 0.1% SDS and different incubation periods. The viable protoplasts of the two different plants were suspended in chemical fusing agent i.e., PEG (6000 MW) and  $\text{Ca}^{2+}$  at high pH. Micro calli were induced with Pelletier B medium supplemented with varying concentrations of auxins and cytokines followed by the addition of Pelletier C medium at definite intervals.

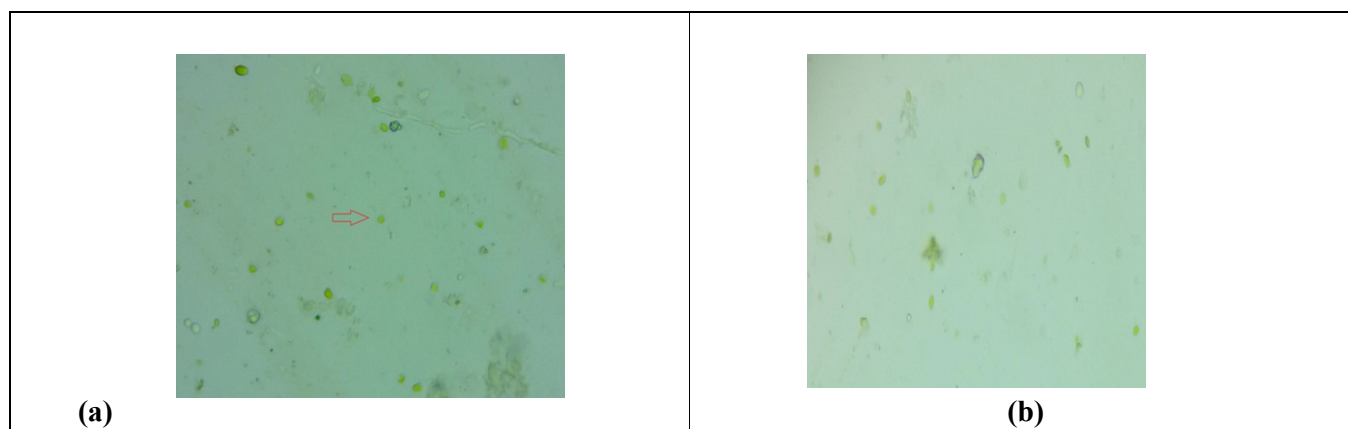


Fig1: Seedling (15 days old) *Helianthus annuus* and *Glycine max*

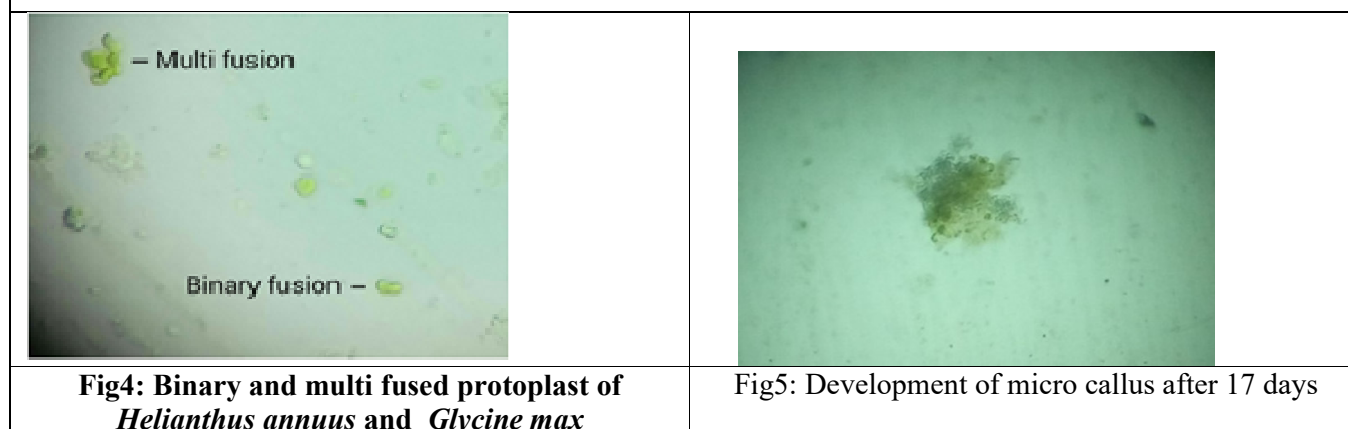


(a) (b)

Fig2: Plant tissues treated modified enzyme mixture for protoplast isolation (a) *Helianthus annuus* (b) *Glycine max*



**Fig3: Protoplast of (a) *Helianthus annuus* (b) *Glycine max***



## ACKNOWLEDGMENTS

We express our gratitude to the Principal, Kristu Jayanti College (Autonomous), Bengaluru for giving a platform to complete the present work

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## ANTI-OBESITY EFFECT OF DIETRY MUSROOMS *HYPsizyGUS ULMARIUS* AND *PLEUROTUS EOUS* ON HIGH FAT FED MICE

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### ABSTRACT

Obesity is one of the major threat to human health in 21<sup>st</sup> Century. It is also known to be a risk factor for the development of metabolic disorders, type 2 diabetes, systemic hypertension, cardiovascular disease, dyslipidemia and atherosclerosis. *Hypsizygyus ulmarius* (blue oyster mushroom) and *Pleurotus eous* (pink oyster mushroom) are edible oyster mushrooms that easy to cultivate and endemic to South India. Their fruiting bodies are pimented, large, thick and fleshy and has a unique flavour, and are well known for their nutrient value. The present study aimed at the eco-friendly cultivation of the mushrooms and the study of the anti-obesity effect of the edible oyster mushrooms on C57 BL/6 - high fat fed mice. A significant difference was observed in the mice group fed with higher dose of *Hypsizygyus ulmarius* (200-400mg/kg Body weight, orally, once daily) compared with *Pleurotus eous* (200-400mg/kg Body weight, orally, once daily) in the parameters body weight ( $37.5 \pm 0.57$ ), triglycerides ( $162.51 \pm 9.47$ ), total cholesterol ( $162.51 \pm 3.71$ ), mesenteric fat weight (visceral adipose:  $0.85 \pm 0.16$ ). Histopathological analysis of the liver also suggests a significant difference in steatosis. The results suggest that *Hypsizygyus ulmarius* can control obesity to a greater extend if consumed regularly compared to *Pleurotus eous*. This is the first report on the anti-obesity activity of these oyster mushrooms.

**Keywords:** *Oyster mushrooms, Hypsizygyus ulmarius, Pleurotus eous*., *Anti-obesity effect, Triglycerides, Mesenteric fat.*

### 1. INTRODUCTION

Obesity is a major health concern today because, half or more of the adult population is now identified as overweight (body mass index or BMI > 25 kg/m<sup>2</sup> - 30 kg/m<sup>2</sup>) or obese (BMI 30kg/m<sup>2</sup>) in no less than 11 member countries of the Organization for Economic Cooperation and Development<sup>1</sup>. Globally, obesity is a serious health problem due to its strong association with increased dyslipidemia, cardiovascular disease (including hypertension, stroke, myocardial infarction), insulin resistance, glucose metabolism disorders, osteoarthritis and some cancers<sup>2</sup>. Obesity is becoming a worldwide epidemic, resulting in a major risk factor for coronary heart diseases including diabetes mellitus, metabolic syndrome, stroke, and some cancers<sup>3</sup> and obesity has become a global health issue. A genetic predisposition towards obesity, a lack of physical activity and increased caloric intake are the primary underlying causes in its development. Excess caloric energy is stored as triacylglycerol (TGs) in adipose tissue, which contribute to the hypertrophy of adipocytes. Dysfunction of adipose tissue in obese individuals results in the disruption of metabolic homeostasis such as elevated plasma glucose, high serum TGs and high LDL-C levels. Therefore, obesity is highly associated with cardiovascular disease, fatty liver, cancer, type-2 diabetes, stroke and osteoarthritis. The World Obesity Federation estimates a rise in the number of obese and overweight adults by 2025. The figures show that 2.7 billion adults worldwide will be overweight and 177 million adults will be obese by 2025<sup>4</sup>. The most common methods for preventing and treating obesity are maintaining an optimal weight by calibrating li festyle habits by introducing well-structured calorie restriction and exercise programs<sup>5</sup>. Lifestyle corrections are very challenging for obese patients, who subsequently resort to the use of medications as an adjunct. However, factors such as the cost and potential side effects are the main causes for failure of weight and obesity management with medication. Mushrooms are considered as a functional food, which can provide health benefits beyond the traditional nutrients they contain<sup>6</sup>. Mushrooms are foods with a high satiating power and low energy density, i.e. the relation between the calories and the volume of a food and have a very high percentage of moisture (81.8-94.8%). According to bibliographical data,

mushrooms contain a greater quantity of insoluble fibre (2.28–8.99 g/100g edible portion) than soluble (0.32–2.20 g/100g edible portion)<sup>7</sup>. Mushrooms are unique foods abundant in essential macro and micronutrients, highest in dietary fiber and water, less calorie producing, low carbohydrate content and good sources of protein and fat. Mushroom species release various bioactive secondary metabolites such as terpenoids, flavonoids, tannins, alkaloids, and polysaccharides which are of great therapeutic applications<sup>8</sup>. Existing reports suggest that secondary metabolites produced by the mushrooms are characterised by high antioxidant and anti-inflammatory capacity. However, little is known about the anti-obesity effect of edible oyster mushrooms *Hypsizygus ulmarius* and *Pleurotus eous*. These two oyster mushrooms being endemic to South India and very economical to cultivate, there is a vast need to spread alertness about the most important health benefits of these mushrooms. Therefore, introduction of cost-effective and side effect free weight loss treatments is an important field of study for the prevention and control of many diseases that occur associated With obesity. Usage of mice as a model for dietinduced obesity (DIO) has risen significantly with the increased incidence of obesity. While numerous mouse strains are susceptible to DIO, sensitivity among strains varies greatly. SWR/ J and CAST / EiJ, for example, appear to be DIO tolerant while the C57BL/6 strain is highly susceptible. Hence, C57BL/6 mice are widely used in obesity research with HFD feeding. In the light of these findings, the present study was conducted to cultivate the mushrooms using a vegetable waste based substrate and determine the anti-obesity effect of the mushroom samples by feeding them to experimentally induced C57BL /6 obese mice and by the preliminary examination of the clinical chemistry data such as body weight and organ weight, serum triglyceride, total cholesterol, visceral adipose etc. to compare the obesity reducing effects of *Hypsizygus ulmarius* and *Pleurotus eous*.

## 2. MATERIALS AND METHODS

### 2.1 Mushroom Cultivation and Preparation of Extracts

In the current study, *Hypsizygus ulamrius* and *Pleurotus eous* cultures obtained from Centre for Biotechnology (Biocenter), Hulimavu, Bangalore was used as the parental strain. The pure cultures were maintained on Potato Dextrose Broth. After four weeks, growth of the fungus in the liquid medium was observed. Mushrooms *Hypsizygus ulamrius* and *Pleurotus eous* were cultivated and harvested in the laboratory according to standard method<sup>9</sup> with slight modifications. Mushroom cultivation was carried out through standard methods by using a vegetable waste based substrate. Vegetable waste was boiled (60–80°C) in water bath for 10–15 min in the proportion of 1:1:1 (Vegetable waste: rice husk: water) and blended with 4% CaCO<sub>3</sub> and 2% CaSO<sub>4</sub>. This was then packed (250g) in polythene bags (200x300 mm) and sterilized in an autoclave at 121°C for 30 minutes. Following this, the bags were inoculated with actively growing mycelium of *Hysizygus ulmarius* and *Pleurotus eous*. The yield was recorded periodically. The Ethanolic, Acetone and Water extracts were prepared according to standard methods<sup>10</sup>.

### 2.2 Design of animal experiment

The animal experiment was planned and carried out referring to the available methodology<sup>2,11</sup>. Six to Seven weeks old C57 BL/6 healthy male mice purchased from Biogen, Bangalore were selected for the study. All C57 BL/6 mice were handled at the animal house facility at Vittarthaa Life Sciences, Bangalore accredited by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Govt. of India). The Constitution of Institutional Animals Ethics Committee (IAEC) approved the protocol. All Animals were maintained at standard husbandry condition as per the CPCSEA, regulations, Govt. of India. All animals were acclimatised on a normal diet (Research Diets, Inc. USA- Table 1) before the start of the experiment and observed for clinical signs. After 1 week of acclimatization, the mice were fed on High Fat Diet (Research Diets, Inc. USA) containing 45 kcal% fat. Lean controls were fed on control diet - 10 kcal% fat. Humane procedures were used during the study, and the animals did not show any abnormality – morbidity or mortality during the study.

### 2.3 High Fat Diet (HFD) Mice Model Development

The C57BL/6 mice were then divided into 7 study groups (5 mice/group): (1) Lean Control group fed with a normal diet (No treatment group), (2) group fed with a HFD (high fat diet control), (3) group fed with HFD plus 4mg/kg body weight Atorvastatin (orally, once daily (HFD + Atorvastatin treated group) (4) group fed with HFD plus *Hypsizygus ulmarius* extract – 200mg/kg body weight orally once daily (HFD+ *Hu*-200mg/kg treated group) (5) group fed with HFD plus *Hypsizygus ulmarius* extract – 400mg/kg body weight

orally once daily (HFD+ *Hu*-400mg/kg treated group) (6) group fed with HFD plus *Pleurotus eous* extract – 200mg/kg body weight orally once daily (HFD+ *Po*-200mg/kg treated group) (7) group fed with HFD plus *Pleurotus eous* extract – 400mg/kg body weight orally once daily (HFD+ *Po*-400mg/kg treated group). For 12 weeks, mice of all HFD treatment groups consumed HFD containing 45% kcal fat purchased from Research Diets (cat. no. D12492; Research Diets, Inc., New Brunswick, USA). Lean control were fed on control diet - 10 kcal% fat. Obese mice were selected for the study based on the body weight (>35g) and serum total cholesterol (> 200mg/dl). Lean control were selected based on body weight (>24g - <27g) and total cholesterol (<100mg/dl). Obese mice were continued to be fed with High Fat Diet and were treated with; *Hypsizugus ulmarius* mushroom extract and *Pleurotus oeus* mushroom extract at 2 dose levels 200 and 400mg/kg body weight. Lean control was continued with low fat diet and positive control was Atorvastatin - 4mg/kg Bwt. The study was conducted for a period of 4 weeks. During the treatment period, the following parameters were measured; Change in Body weight, Serum Total Cholesterol, Serum Triglycerides, Mesenteric fat weight and GSH reduced value.

#### 2.4 Measurement of body weight and organ weight

At the end of 4 weeks of treatment, along with the above mentioned parameters, the following were recorded after necropsy - Organ weights, Adipose morphometry and Histopathology of the liver. Throughout the experimental period, the body weight of mice belonged to all 7 treatment groups were measured weekly using a calibrated weighing balance according to CPCSEA regulations (Wensar weighing scales limited.- Model No: ECB600). Feed intake was measured at the same time once a week during the study period. After necropsy, Liver and adipose tissue organ weights were taken using a calibrated weighing balance.

#### 2.5 Serum biochemical analysis

From all animals initial and final whole blood was collected for measuring the total cholesterol and triglycerides in fed state and checked for the change in Body weight and weight of Mesenteric fat weight. The levels of total cholesterol (TC) and triglyceride (TG) in rat plasma were determined using colorimetric method as described previously<sup>12</sup> with slight modifications. 5ml of blood was collected in a test tube. The blood was centrifuged at 6000rpm for 5-6 minutes. At the end of the centrifugation, the pellet would contain the red blood cells and the supernatant would contain serum. The supernatant i.e., serum was separated and used for triglyceride estimation. For the estimation of triglycerides, 20µl of distilled water was used as blank. For standard, 20µl of standard trical U solution (Kit-DiaSys Diagnostic Systems Germany) was used. The sample was prepared by taking 20µl of serum sample followed by the addition of 100µl of triglyceride solution to each of the wells (DiaSys Diagnostic Systems Germany). The plates were incubated for 20 minutes at room temperature. The absorbance was read at 500nm using BMG LABTEC FLUO Star Omega plate reader instrument and the total triglycerides were calculated. For the estimation of total cholesterol, 20µl of distilled water was used as blank. For standard, 20µl of standard trical U solution (Kit-DiaSys Diagnostic Systems Germany) was used. The sample was prepared by taking 20µl of serum sample followed by the addition of 100µl of Cholesterol solution to each of the wells (DiaSys Diagnostic Systems Germany). The plates were incubated for 20 minutes at room temperature. The absorbance was read at 500nm using BMG LABTEC FLUO Star Omega plate reader instrument and the total cholesterol was calculated.

#### 2.6 Estimation of GSH level

GSH reduced value was measured using Ellman's reagent (DTNB method) in all the mice sera at initial and final bleeding. The assay was carried out based on the reaction of GSH with 5,5'-Dithiobis-2-Nitrobenzoic acid (DTNB - also known as Ellman's reagent) that undergoes autooxidation and produces the TNB chromophore and oxidized glutathione-TNB adduct (GS-TNB). The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH in the sample. Whole blood was collected without EDTA from mice which is kept in room temperature for 30mins. The collected whole blood was centrifuged at 6000 rpm for 6 mins and separate the clear portion (serum) from plasma using a syringe or micro pipette. The experiment was performed in duplicates in 96 well microtiter plate as follows: The wells were labelled as water blank, blank, standard and sample. The water blank well was filled with 200µl distilled water. Blank well was filled with 150µl of 0.1M KPE Buffer, pH 7, followed by 50µl of 5,5'-Dithiobis-2-Nitrobenzoic acid (DTNB). The standard well was filled with 120µl of 0.1M KPE Buffer, pH 7, followed by 30µl GSH standards and 50µl of DTNB. The sample well was filled with 120µl of 0.1M KPE Buffer pH 7, followed by 30µl of unknown sample and 50µl of DTNB. The mixture was mixed well and incubated at room temperature (25°C) for 20 minutes. Absorbance was measured at 412nm using a microplate reader.

## 2.7 Measurement of Fat Deposition

Mesentery adipose tissues were collected from each animal at necropsy. The adipose tissues were excised and uniformly collected as much as tissue possible from each animal. The entire collected tissues were weighed and fixed in 10% buffered neutral formalin for 48h. The Formalin-fixed tissues were processed in alcohol and paraffin-embedded. 4 $\mu$  sections were cut and stained with Haematoxylin and eosin. Images were captured using microscope pro 2.1 camera. For adipocytes morphometry, ten random microscopic fields having intact adipocytes were selected. Microscopic images were captured at 400X magnification. Adipocytes with intact cell membrane and showing whole cell area were only measured. Cells lying at boundary of field with incomplete area were not measured. Quantitative evaluation of adipocytes was performed using computer-assisted image analysis “Image J” software. The data was analyzed using Graph pad prism software and area was expressed in  $\mu\text{m}^2$ .

## 2.8 Histopathology of Liver

Liver were collected and weighed from each animal at necropsy. The entire collected tissues were weighed and fixed in 10% buffered neutral formalin for 48h. The Formalin-fixed tissues were processed in alcohol and paraffin-embedded. 4 $\mu$  sections were cut and stained with Haematoxylin and eosin. The stained slides were evaluated for pathological findings.

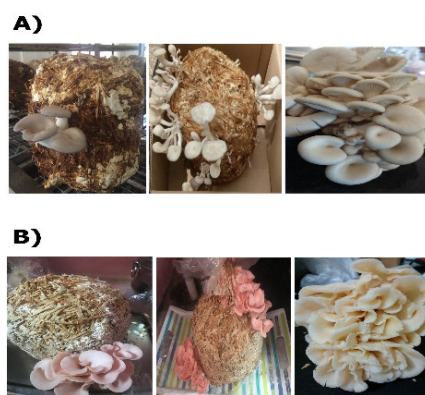
## 2.9 Statistical significance analysis

Statistical analysis was performed using GraphPad Prism software V5.00. All values are presented as the mean  $\pm$  standard deviation. Statistical analysis using ‘two-way-ANNOVA’ followed by Bonferroni post-tests.  $P < 0.05$  was considered to indicate a statistically significant difference compared with HFD control and Lean control.

## 3. RESULTS AND DISCUSSION

### 3.1 Specimen Collection and Mushroom Cultivation

The specimen were collected and cultured as described earlier. Mushroom cultivation was carried out on the vegetable waste based substrate, the fruiting bodies were harvested and the yield was recorded (Figure 1). The fruiting bodies were dried in a Hot Air Oven, powdered and preserved under sterile conditions for feeding to the animals.



**Figure 1. A) *Hypsizygus ulmarius* fruiting bodies before and after harvest, B) *Pleurotus eous* fruiting bodies before and after harvest.**

### 3.2 HFD Mice Model Development

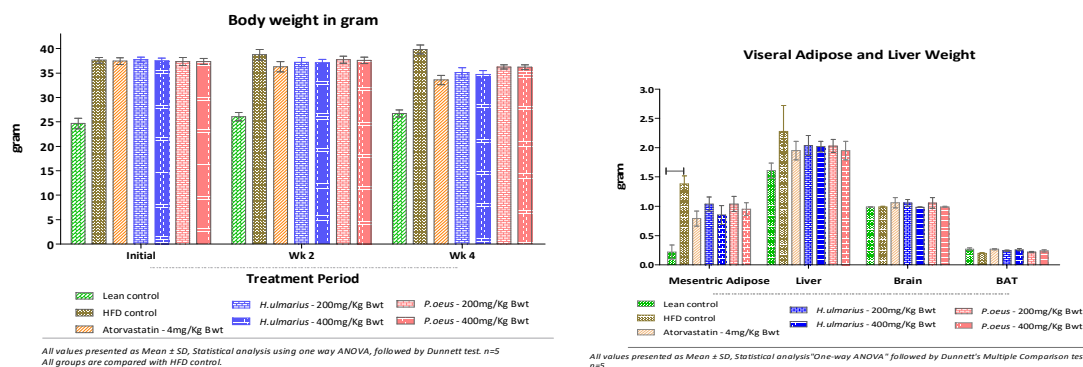
Young animals of age 6-7 weeks C57 BL/6 mice were selected for the study. After 1 week of acclimatization all animals except control group were fed on High Fat Diet (Research Diet, USA) containing 60 kcal% fat. Lean controls were fed on control diet - 10 kcal% fat. All animals were fed on High fat diet for a period of 12 weeks. Animals on high fat diet showed a significant increase in lipid profile where Total Cholesterol for all animals was  $\sim 235\text{mg/dl}$ ; and Triglycerides for all animals was  $\sim 216\text{mg/dl}$ . Total cholesterol and Triglycerides on high cholesterol diet increased by 2.5 times the baseline. Whereas the animal fed with chow diet the lipid profile remained constant comparable to the baseline data. During the



model development period body weight, feed consumption and cage side observations were recorded. All animals fed with high cholesterol diet gained 40% more body weight compared with animals fed on chow diet. All animals remained healthy during the entire model development period, there were no clinical signs of morbidity or mortality. This confirmed the development of obesity in all animals that were selected for the study.

### 3.3 Effect of *Hu* and *Pe* on body weight and organ weight

To evaluate the anti-obesity effects of the mushroom extracts, the changes in body weight and serum lipid profile of HFD-induced obesity mice were recorded after the treatment period of 16 weeks. The High Fat Diet fed animals showed a significant increase in body weight when compared with lean control. The treatment has shown non statistical significant decrease in body weight when compared with HFD control at end of week 4 of treatment. The body weight of HFD control group ranged between  $37.62\pm 0.58$  to  $39.82\pm 0.93$  and Lean control ranged between  $24.66\pm 1.08$  to  $26.7\pm 0.71$ g as shown in Figure 2. In the group treated with Atorvastatin -4mg/kg body weight, the body weight from initial week to week 4 ranged between  $37.44\pm 0.68$  to  $33.58\pm 0.95$ . The body weight decreased from 37.5g to 34.72g in mice treated with HFD plus *Hypsizygus ulmarius* powder – 400mg/kg body weight (figure 2). In the mice treated with HFD plus *Hypsizygus ulmarius* powder – 200mg/kg body weight, the body weight decreased from 37.72g to 35.08g. Whereas in case of *Pleurotus eous*, the mice treated with HFD plus *Pleurotus eous* powder – 400mg/kg body weight, there was a significant decrease in the body weight of 37.36g to 36.2g (figure 2). In the mice treated with HFD plus *Pleurotus eous* powder – 200mg/kg body weight, the body weight decreased from 37.36g to 36.24g. In similar researches observations shows that feeding of *P. (Pleurotus) ostreatus*, *P. sajor-caju* and *P. florida* mushroom reduced body weight significantly in hypercholesterolemic rats by 17.36%, 23.37% and 24.13% respectively. Other mushroom like *Hericium erinaceus* also reduce body weight significantly in high-fat diet fed mice. Whereas, *P. tuber-regium* and *Termitomycetes chypeatus* feeding reduced body weight. in hypercholesterolemic rats slightly but not significantly<sup>11</sup>. Thus *Hypsizygus ulmarius* extracts have a better effect in decreasing body weight compared to *Pleurotus eous*. This can be due to the higher amounts of Phenolics and Flavanoids produced by *Hypsizygus ulmarius*, as these secondary metabolites suppress the increase in body weight and fat storage, serum levels of total cholesterol and triacylglycerols, lowers the activity of fatty acid synthase in animal liver and inhibits fatty acid synthase activity and suppresses adipogenesis in adipocytes<sup>13</sup>. The decreased in bodyweight in mice treated with HFD and *Pleurotus eous* can be due to the alkaloids and tannins produced by this mushroom. Alkaloids attenuates obesity-induced inflammation, obesity related metabolic disorders, and liver diseases. Some of the alkaloids reduces food intake and increases energy expenditure and lipid oxidation<sup>14</sup>. They produce thermogenic effect (increase basal metabolic rate) and energy expenditure<sup>15</sup>. Caffeine, which is a tannin stimulates fat breakdown, potentiates the anorectic and thermogenic effects in addition to its diuretic effect<sup>16,17</sup>. The results also suggests that the consumption of *Hypsizygus ulmarius* and *Pleurotus eous* in higher, regular amounts can aid in weight loss.



**Figure 2.**  
**Effect of *Hypsizygus ulmarius* and *Pleurotus eous* on body weight and organ weight reduction in different rat groups.**

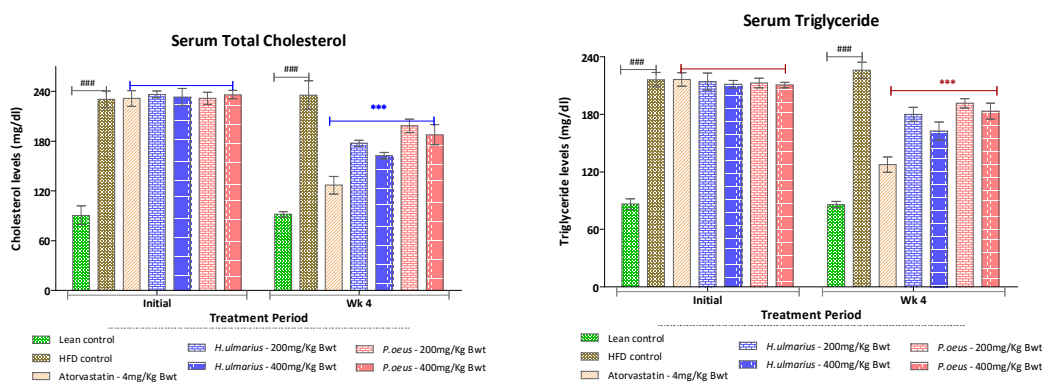


### 3.4 Effect of Hu and Pe on lipid profile of serum

To evaluate the anti-obesity effects of the mushroom samples, we analysed the changes in serum lipid profile of HFD-induced obesity mice treated with *Hypsizygus ulmarius* and *Pleurotus eous* for 12 weeks. As shown in the figure 3 (B), the treatment has shown statistical significant decrease in serum triglyceride levels when compared with HFD control at end of week 4 of treatment. The triglyceride values on Lean control decreased from 86.38mg/dl to 85.76mg/dl, HFD control ranged between 216.07mg/dl to 225.99mg/dl, Atorvastatin treated group ranged between 216.25mg/dl to 127.56mg/dl. In the mice group treated with HFD plus *Hypsizygus ulmarius* – 200mg/kg body weight, the serum triglyceride value lowered from 213.96mg/dl to 179.85mg/dl. In case of the group treated with 400mg/kg body weight of *Hypsizygus ulmarius* powder, the serum triglyceride value decreased from 211.24mg/dl to 162.51mg/dl as shown in figure 3. The serum triglyceride levels of mice treated with HFD plus 200mg/kg body weight *Pleurotus eous* decreased from 212.66mg/dl to 191.47mg/dl. In the mice group treated with HFD plus *Pleurotus eous* – 400mg/kg body weight, the values decreased from 210.55mg/dl to 183.3mg/dl (figure 3 - B). *Hypsizygus ulmarius* is observed to have greater effect on lowering serum triglycerides because of its high nutrient value, lovastatin content and secondary metabolite production. The secondary metabolites regulate energy metabolism and decrease level of intracellular lipids<sup>17</sup>. The HFD fed animals have shown significant increase in serum total cholesterol levels when compared with lean control. The treatment has shown statistical significant decrease in serum total cholesterol levels when compared with HFD control at end of week 4 of treatment. The serum cholesterol levels of Lean Control, HFD Control and Atorvastatin ranged between 90.63-91.76mg/dl, 230.53-235.6mg/dl and 231.37-126.99mg/dl respectively (figure 3 - A). In Group 4, which was treated with HFD plus *Hypsizygus ulmarius* 200mg/kg body weight, the serum total cholesterol levels decreased from 236.31mg/dl to 177.53mg/dl. In the group treated with HFD plus *Hypsizygus ulmarius* 400mg/kg body weight, the serum total cholesterol levels decreased from 233.37 mg/dl to 162.51 mg/dl. In the mice group treated with HFD plus *Pleurotus eous* – 200mg/kg body weight, the total cholesterol levels decreased from 231.78mg/dl to 198.48.78 mg/dl and in the group treated with 400mg/kg body weight of HFD plus *Pleurotus eous* (Figure 3).

### 3.5 Effect of Hu and Pe on abdominal fat deposition

HFD fed animals have shown significant increase in mesenteric adipose when compared with lean control. Treatment has shown statistical significant decrease in mesenteric adipose when compared with HFD control at end of week 4 of treatment as shown in figure 3 – (C,D). The visceral adipose weight ranged between 0.85 to 1.04 in mice treated with *Hypsizygus ulmarius*. In the mice groups treated with *Pleurotus eous*, the value ranged between 0.95 to 1.04 (figure 3 – C). This shows the positive effect of the mushroom extracts in bringing down the mesenteric fat weight. The morphometry of mesenteric adipose fat deposition was observed and recorded. The significant difference in the mesenteric fat deposition upon feeding the mice with different concentrations of *Hypsizygus ulmarius* and *Pleurotus eous* is shown in figure 4. The mesenteric adipose fat weight was less in groups fed with higher concentrations of the mushrooms.



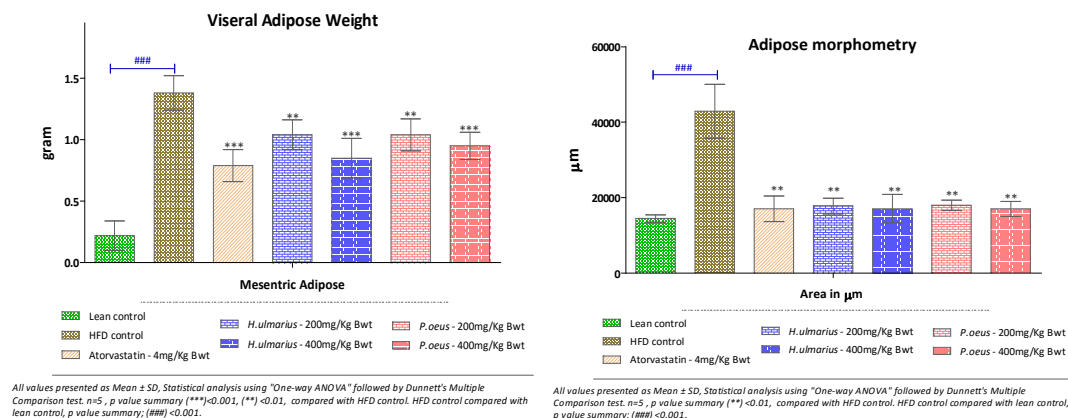


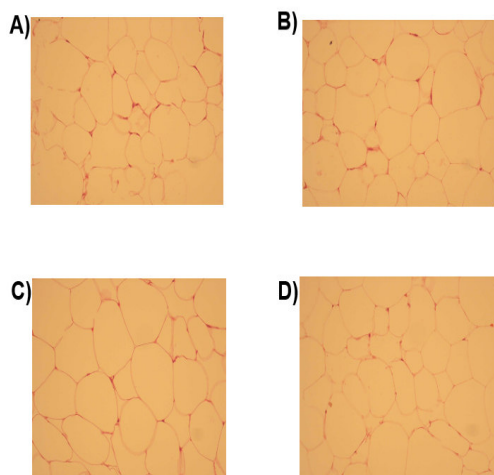
Figure 3.

**Serum Triglyceride, Total Cholesterol content, Visceral adipose weight; Mesentery fat deposition and Adipose Morphometry measured in all in different mice groups.**

### 3.6 Effect of Hu and Pe on hepatic steatosis of HFD-induced obesity mice

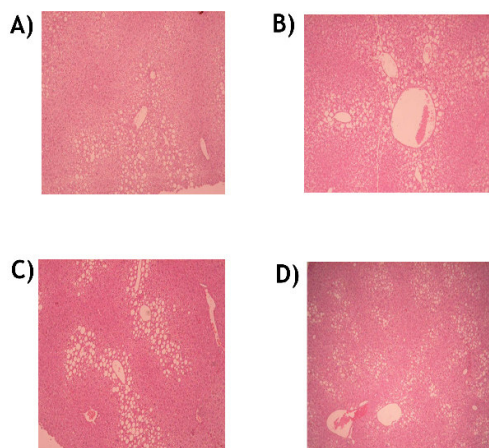
Liver weight alterations and hepatic steatosis are common obesity-related phenomena. Therefore, we measured the changes in weight and fat accumulation of liver in HFD-induced obesity mice treated with *Hypsizygus ulmarius* and *Pleurotus eous* and compared the observations with the groups treated with Atorvastatin and Normal diet. The group fed with a HFD (high fat diet control), showed increased liver weight compared to the No treatment group (Lean Control group fed with a normal diet) (figure 5). There was a significant difference in the number of lipid droplet in the liver tissues of the groups stained with Hematoxylin & Eosin (figure 5). The liver section of the HFD treated group showed dramatic increase in the number of lipid drops compared to the No treatment group. In comparison, only a few lipid drops were seen in the HFD+ *Hypsizygus ulmarius* – 400mg/kg body weight than in HFD+*Pleurotus eous* – 400mg/kg body weight treated groups ( $P < 0.005$ ; Figure 5 – C,D). These results indicate that higher dosage of *Hypsizygus ulmarius* significantly inhibits the liver hypertrophy, fat accumulation and hepatic steatosis in HFD-induced obesity mice. In this study, we found that HFD feeding significantly increased liver weight (Figure 2), hepatic lipid droplet accumulation (Figure 5) and steatosis (Table 1). When treated with higher doses of *Hypsizygus ulmarius*, we observed a significant decrease in HFD increased liver weight and steatosis (Table 1). However, in the mice groups treated with *Pleurotus eous*, there was mild to moderate steatosis in up to 4 animals. Whereas in groups treated with different doses of *Hypsizygus ulmarius*, the steatosis was observed to be HFD feeding has been shown to induce hepatosteatosis in mice and in rats<sup>2</sup>. The treatment period was for 4 weeks, animals in all groups had free access to fed and water, *ad libitum*. All animals remained healthy during the entire 4-week period, with no clinical signs of morbidity or mortality. There were no statistically significant changes in the body weight gain within the groups during the period during the treatment period. The feed consumption in all the groups including the chow diet control animals remained normal. A significant body weight decrease was observed in all treated groups with Atorvastatin and mushroom treated animals compared with the High Fat fed animals. At the end of treatment period in Atorvastatin treated animals 15% decrease in body weight was observed and animals treated with both variant of mushroom showed a decrease in body weight by 10 – 13%. Animals treated with *H. ulmarius* mushroom powder showed a greater decrease in body weight compared with that of *P. oeus*. HFD fed animals have shown significant increase in body weight when compared with lean control. Treatment has shown non statistically significant decrease in body weight when compared with HFD control at end of week 4 of treatment (Figure 1). HFD fed animals have shown significant increase in serum triglyceride and cholesterol levels when compared with lean control. Treatment has shown statistical significant decrease in serum triglyceride and cholesterol levels when compared with HFD control at end of week 4 of treatment. (Figure 2,3). Animals treated with *H. ulmarius* mushroom powder showed a greater decrease in serum triglyceride and cholesterol levels compared with that of *P. oeus*. HFD fed animals have shown significant increase in serum total cholesterol levels when compared with lean control. Treatment has shown statistically significant decrease in serum total cholesterol levels when compared with HFD control at end of week 4 of treatment (Figure 3). HFD fed animals have shown significant increase in mesenteric adipose weight and size when compared with lean control. Treatment has shown statistically significant decrease in mesenteric adipose weight and

size when compared with HFD control at end of week 4 of treatment (Figure 4,6). Animals treated with *H.ulmarius* mushroom powder showed a greater decrease in mesenteric adipose weight and size compared with that of *P.oeus*. The morphometry of adipose tissue was clear with Haematoxylin and eosin staining (Figure 4). At the end of observation period, all surviving animals were subjected for necropsy and gross pathological changes. All animals showed no signs of gross pathological changes externally and in any visceral organs. Histopathology of liver showed a significant development of steatosis in all High fat fed animals. Treatment has shown statistically significant decrease in steatosis when compared with HFD control at end of week 4 of treatment . Animals treated with *H.ulmarius* mushroom powder showed a decrease in steatosis compared with that of *P.oeus*.



**Figure 4.**  
*Adipose morphometry observed at 10X stained with Hematoxylin & Eosin;*

**A) Mesenteric Adipose fat in G4; HFD + *Hypsizugus ulmarius* powder (200mg/kg Body weight, oral, once daily); B) Mesenteric Adipose fat in G5; HFD + *Hypsizugus ulmarius* powder (400mg/kg Body weight); C) Mesenteric Adipose fat in G6; HFD + *Pleurotus eous* powder (200mg/kg Body weight) ; D) Mesenteric Adipose fat in G7; HFD + *Pleurotus eous* powder (400mg/kg Body weight).**



**Figure 5.**  
*Liver sections*

**A). HFD + *Hypsizugus ulmarius* (200mg/kg Bwt, oral, once daily) at 10X stained with Hematoxylin & Eosin; ; B). HFD + *Hypsizugus ulmarius* (400mg/kg Bwt, oral, once daily) at 10X stained with Hematoxylin & Eosin; C). HFD + *Pleurotus oeus* (200mg/kg Bwt, oral, once daily) at 10X stained with Hematoxylin & Eosin; D). HFD + *Pleurotus oeus* (400mg/kg Bwt, oral, once daily) at 10X stained with Hematoxylin & Eosin.**

Table 1.

*Effects of Hypsizygus ulmarius and Pleurotus eous extracts on steatosis in liver tissue in high-fat diet (HFD)-fed rats compared with the control groups.*

Group	G1	G2	G3	G4	G5	G6	G7
Number of animals	5	5	5	5	5	5	5
Treatment	Lean control	HFD control	Atorvastatin 4mg/Kg Bwt	<i>H.ulmarius</i> 200mg/Kg Bwt	<i>H.ulmarius</i> 400mg/Kg Bwt	<i>P.oeus</i> 200mg/Kg Bwt	<i>P.oeus</i> 400mg/Kg Bwt
Vacuolation	0	2	1	2	1	1	2
Sinusoidal congestion	0	2	0	1	2	0	1
Steatosis Minimal	0	0	2	0	1	0	0
Steatosis Mild	0	1	3	3	4	1	3
Steatosis Moderate	0	3	0	1	0	4	2
Steatosis Severe	0	1	0	0	0	0	0

#### 4. CONCLUSION

Today, obesity is a common disease that affects a large proportion of people and is leading to chronic conditions and even death. Since the commercially available anti-obesity drugs have been proved to have some side effects, it is required to switch to nutraceutical based, cost-effective measures to overcome this situation. Oyster mushrooms are well known for their explicit taste and nutrient value. The present study suggests that, *Hypsizygus ulmarius* and *Pleurotus eous* are oyster mushrooms that produce bioactive compounds which can reduce fat accumulation and obesity. The reduction in parameters of body weight, organ weights, mesentery fat deposition and liver steatosis shows that *Hypsizygus ulmarius* and *Pleurotus eous* produces bioactive compounds that can control obesity. This is the first report on the anti-obesity activity of *Hypsizygus ulmarius* and *Pleurotus eous*. Since these mushrooms are highly economical and endemic to South India, a majority of obesity related health issues of people belonging to this area can be cured by making these oyster mushrooms a part of their regular diet.

#### ACKNOWLEDGEMENT

Grateful acknowledgement is made to the authorities of Vittarthaa Life Sciences Pvt.Ltd., Bangalore; The Management and Faculty of Department of Microbiology, Jain University, Bangalore for the tutelage and provision of facilities to carry out this research work.

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## ISOLATION AND IDENTIFICATION OF TANNIC ACID DEGRADERS FROM TANNERY EFFLUENT

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### ABSTRACT

Tannery effluent is a serious environmental threat to soil and water pollution especially in the developing countries. A number of reviews on tannin degradation have appeared in the past most of which concentrated on fungi like *Aspergillus* sp. or *Penicillium* sp. Few strains of bacteria have been found to degrade tannic acid. Many studies have reported isolation of anaerobic or facultative anaerobic bacteria capable of degrading tannins. Aerobic microorganisms capable of degrading tannic acid have been comparatively studied in scarce and so the search is persistent for organisms which are best degraders of tannic acid and better source of tannase. The present study aimed at isolating bacteria capable of utilizing tannic acid as sole source of carbon aerobically, screen and characterize them in order to identify efficient strains that can be used for tannin bioremediation. Nine bacterial strains capable of utilizing tannic acid as sole carbon source in minimal agar medium was isolated from the effluent of a tannery and was identified as members of genera *Klebsiella*, *Bacillus*, *Staphylococcus*, *Corynebacterium*, and *Pseudomonas*. The isolates grew at concentration as high as 1% (w/v) of tannic acid and produced extracellular tannase to hydrolyze the same. When grown in minimal medium containing 0.2% and 1% tannic acid (w/v) at 37°C, the strains produced tannase at 24 hours. Maximum growth was observed in 0.4% (w/v) tannic acid. Tannic acid degradation product, glucose was analysed qualitatively and quantitatively.

**KEYWORDS:** *Tannery effluent, Tannic acid, Tannase, Bacillus, Staphylococcus, Corynebacterium.*

### INTRODUCTION

Environmental pollution has become a major concern in developing countries in the last few decades. Major sources of water pollution are the untreated or partially treated industrial effluents. The toxic pollutants include acids, alkali, oils, fats, floating dissolved organic matter, toxic acids and colouring agents. There are industries which contribute to this pollution, such as the tannery, steel, iron, dye, sugar cane, distillery, pulp and paper mill etc. Tannery industry is one among the major industries of our country and there are 2161 major tanneries in India. Discharged effluents from tanneries are one the major sources of water pollution. A tannery discharges waste from 21,500 – 21,950L a day, corresponding to 85–88 L/Kg of leather processed. Tannery effluents contains tannins and large amounts of waste like protein, chlorides, trivalent chromium, nitrogen, sulphate, sulphide, COD, BOD and suspended solids. The term “Tannins” was first introduced by Seguin in 1976 to describe the chemical constituent present in galls which are used to convert putricible hides to stable leather.<sup>1</sup> Tannins are defined as naturally occurring water soluble poly-phenolic compounds of varying molecular weight which differ from most other natural phenolic compounds in their ability to precipitate proteins in solutions. This property is the basis for their wide spread use in Tannery industries. Tannins are wide spread in plant kingdom and are found in leaves, fruits, bark and can be accumulated in large amount in particular organs or tissues of plants. After lignins, they are the second most abundant group of plant poly-phenols and are toxic to plants, animals and soil microorganisms. Tannery effluent has been found to cause stunted growth, chlorosis and reduction in plant yields and cause deleterious effect on animals, humans and even soil microorganisms.<sup>2-3</sup> If the environmental pollution by discharge of tannery effluents is ignored, it can lead to drastic damage to the environment. A number of reviews on tannin degradation have appeared in the past most of which

concentrated on fungi like *Aspergillus sp.* or *Penicillium sp.* Few strains of bacteria have been found to degrade tannic acid. Many studies have reported isolation of anaerobic or facultative anaerobic bacteria capable of degrading tannins. Aerobic microorganisms capable of degrading tannic acid have been comparatively studied in scarce and so the search is persistent for organisms which are best degraders of tannic acid and better source of tannase.<sup>5-9</sup> The present study was an effort in identifying aerobic bacterial communities that can break down tannins and pave way to alleviate pollution of water bodies by tannery effluent.

## MATERIALS AND METHODS

### *Collection of Tannery effluent and physicochemical analysis of the sample*

The tannery effluent was collected in sterile bottles from the effluent discharging site of M.S. Tanneries, Dindigul, Tamil Nadu, India. The effluent was stored at 4°C until further use. The effluent sample was analysed for the physico-chemical parameters like colour, odour, pH, Total Solids (TS), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD).<sup>10</sup>

### *Enrichment, isolation and identification of tannic acid degraders*

1ml of tannery effluent was suspended in two conical flasks containing 20ml of minimal broth medium<sup>11</sup> supplemented with 0.2% and 1% (w/v) tannic acid as sole carbon source. The flasks were incubated in shaker condition at 37°C for 5 days. The growth of the microbial consortia was observed with respect to reduction in colour of effluent and increase in turbidity. From enrichment flasks containing (0.2% and 1%) tannic acid as sole carbon source, serial dilution was performed in sterile saline (0.89% NaCl) and spread plated onto the nutrient agar plates. The nutrient agar plates were incubated at 37°C for 24 hours. After 24 hours, grown colonies on nutrient agar plate were studied for their colony morphology. Each of the different colonies was purified by quadrant streak method on nutrient agar and pure cultures were maintained in nutrient agar slants. The pure cultures were appropriately labeled and sub cultured once in fifteen days throughout the completion of the project. Tentative identification of the isolates up to genera level was carried out by standard staining, morphological and biochemical tests by Bergey's Manual of Determinative Bacteriology.<sup>12-13</sup>

### *Screening test for tannic acid degradation ability of the isolates*

The identified organisms were rechecked for their tannic acid degrading ability by streaking them on minimal agar medium containing 0.2% tannic acid as sole carbon source.<sup>14</sup>

### *Swarm plate assay for chemotaxis utilization of tannic acid*

The ability of the identified isolates to utilize tannic acid as a sole carbon source through chemotaxis was checked by swarm plate assay.<sup>13</sup> Minimal medium agar plates containing tannic acid as sole carbon source (0.2%) were prepared. 2ml of log phase cultures of each isolate was harvested by centrifugation and pellets were resuspended in 0.85% saline. 20µl of each isolates was spot inoculated at the the centre of the each plate. Plates were then incubated at 37°C for 24hr.

### *Tannin-protein complex degrading activity*

5µl of a freshly prepared filter sterilized tannic acid (20% w/v) was poured on nutrient agar plates and allowed to stand at room temperature for 20 minutes. The excess tannic acid was then discarded and the plates were washed three times with sterile quarter strength Ringers solution. Overnight grown cultures of each isolate were then streaked onto nutrient agar plates and incubated at 37°C for 18-24 h.<sup>14</sup>

### *Demonstration of tannase*

Tannase activity was studied by visual reading method.<sup>15</sup> 1 ml of fresh nutrient broth cultures of each isolate were harvested and suspended in 5ml of substrate medium (pH 5.0) containing NaH<sub>2</sub>PO<sub>4</sub> (33 mmol<sup>-1</sup>) and gallic acid methyl ester (20 mmol<sup>-1</sup>) to prepare a dense suspension. The substrate medium was incubated aerobically at 37°C for 24 hours. After incubation, the sample was alkalized with equal amount of saturated NaHCO<sub>3</sub> solution (pH 8.6) and exposed to the atmosphere at room temperature for 1 hour observe the colour change Green to brown coloration of the medium was judged as positive indicator of tannase.

***Effect of tannic acid concentration on the growth of the isolates***

Minimal broth medium containing various concentration of tannic acid (0.2%, 0.4%, 0.8%, and 1%) prepared and sterilized. 1ml of fresh culture (SV3, SV4, SV8) was inoculated in the respective flasks.<sup>14</sup> The flasks were incubated in shaker condition at 37°C for 5 days. Absorbance at 540nm, pH and estimation of residual tannic acid was carried out.

***Estimation of residual tannic acid***

Residual tannic acid in the culture broth was estimated spectrophotometrically.<sup>14</sup> Cells were grown overnight in minimal medium containing 0.2%, 0.4%, 0.8% and 1% tannic acid respectively. To 1ml for culture filtrate, 2ml of bovine serum albumin (1 mgml<sup>-1</sup>) and 0.17mol-l NaCl in 0.2M acetate buffer (pH 5.0) was added. After 10 min, the tubes were centrifuged at 2200 rpm for 10 min. The pellet was washed with acetate buffer and 4 ml of SDS/TEA solution (1% sodium dodecyl sulphate, w/v and 5% triethanolamine, v/v) and 1 ml of FeCl<sub>3</sub> was added. An uninoculated medium was kept as a control. The tubes were then incubated at 30°C for 15 min. Absorbance was measured at 510nm in spectrophotometer for each concentration and for each isolate. Residual tannic acid of the effluent was also determined.

***Detection of glucose as breakdown product******Qualitative analysis***

Presence of glucose was detected by thin layer chromatography.<sup>16</sup> Silica gel slurry was prepared and then poured on to the glass plate and spread evenly. The plates were then dried at 40°C in hot air oven. Culture supernatant of each isolate was applied using a capillary tube as a spot on TLC plate and was allowed to dry. The plate was then placed in a chamber saturated with mobile phase solvent mixture (ethyl acetate: isopropanol: water: pyridine - 26:14:7:2) and allowed to run by ascending chromatography. Once the solvent had travelled 3/4<sup>th</sup> of the TLC plate, the plate was dried in hot air oven at 110°C and was developed by spraying alkaline permanganate. Spots were observed and the R<sub>f</sub> value was calculated and compared with the standard.

***Quantitative analysis***

The culture supernatant was also tested for presence of glucose by anthrone method.<sup>16</sup>

**RESULTS AND DISCUSSIONS*****Physicochemical analysis of the sample***

Colour of the effluent was observed as dark brown and the odour was that of rotten egg. The pH of the tannery effluent was found to be 7.9. The effluent was expected to be acidic because of the presence of tannic acid in the effluent, but it was found to be near the neutral pH which can be probably due to the presence of chromium sulphate, NaCl used in tannin process.<sup>17-18</sup> The amount of TS, TSS, TDS, BOD and COD was determined in the tannery effluent and were found to be well above the standard range of value as shown in Table 1.<sup>10</sup>

**Table 1**  
***Analysis of physico-chemical parameters of treated and untreated tannery effluent***

Physico-chemical parameters	Value
pH	7.90
Total Solids (mg/L)	1,97,50,000
Total Suspended Solids (mg/L)	1,760
Total Dissolved Solids (mg/L)	24,900
BOD (mg/L)	48
COD (mg/L)	262
Colour	Dark brown

***Enrichment, isolation and identification of tannic acid degraders***

Turbidity accompanied by the decreasing dark brown colouration of the medium to light brown colour was observed in minimal broth medium containing 0.2% tannic acid as sole carbon source when enriched with tannery effluent. Comparatively scanty growth was observed in minimal broth medium containing 1% tannic acid sole carbon source. Serial dilution and spread plating of aliquots in nutrient agar plates from the

enriched medium produced nine isolated colonies. The isolated colonies were purified by quadrant streak method and were named as SV1, SV2, SV3, SV4, SV5, SV6, SV7, SV8 and SV9. The isolates were studied for their colony morphology, staining reactions and biochemical tests. Based on staining reactions, motility test and biochemical characters, the isolates were identified as members of the genera *Staphylococcus*, *Bacillus*, *Klebsiella*, *Corynebacterium* and *Pseudomonas* as presented in Table 2.

### Screening test for tannic acid degradation ability of the isolates

On inoculation of the pure cultures in minimal agar medium with tannic acid (0.2%) as sole carbon source, it was observed that only *Klebsiella* sp., *Bacillus* sp., and *Pseudomonas* sp. were able to show significant growth. Other remaining six isolates showed very poor growth even after 8 days. It is possible that these isolates may have utilized the breakdown product excreted or secreted in the enrichment medium as result of tannic acid breakdown but not tannic acid as such. So when screened for tannic acid degrading ability, they were unable to utilize it as single cultures.

**Table 2**  
**Identification of the Isolates**

Isolate	Colony Morphology	Gram staining	Motility	Endospore	C	O	I	MR	VP	CU	U	SH	G	TSI	Identified Organisms
SV1	Round White	+, cocci clusters	-	-	+	-	-	+	+	-	+	+	+	A/A	<i>Staphylococcus</i> sp.
SV2	Round Yellow	+, rod.	+	+	+	+	-	-	-	-	-	+	+	A/K	<i>Bacillus</i> sp.
SV3	Round Milky White.	-, Rod.	-	-	+	-	-	-	+	+	+	+	+	A/A	<i>Klebsiella</i> sp.
SV4	Irregular Watery Transparent	+, Rod.	+	+	+	+	-	-	-	-	-	+	+	A/K	<i>Bacillus</i> sp.
SV5	Round White	+, cocci clusters	-	-	+	-	-	+	+	-	+	+	+	A/A	<i>Staphylococcus</i> sp.
SV6	Round White	-, rod.	+	-	+	+	-	-	-	+	-	-	+	K/K	<i>Pseudomonas</i> sp.
SV7	Round Yellow	+, rod.	-	+	+	-	-	-	-	-	+	+	-	K/K	<i>Corynebacterium</i> sp.
SV8	Round Yellow Transparent.	-, rod.	+	-	+	+	-	-	-	+	-	-	+	K/K	<i>Pseudomonas</i> sp.
SV9	Irregular brown	-, rod.	+	-	+	+	-	-	-	+	-	-	+	K/K	<i>Pseudomonas</i> sp.

**Key:** S- Sangeetha, V- Vasuki, C-Catalase test, O-Oxidase test, I-Indole production test, MR-Methyl Red test, VP- Voges Proskauer test, CU-Citrate Utilization test, U-Urease test, SH-Starch Hydrolysis test, G-Gelatin liquefaction test, TSI-Triple Sugar Iron Agar test,

**Legends:** ‘+’ - positive, ‘-’ negative, A - acid production, K - alkaline end product

### Swarm plate assay for chemotaxis utilization of tannic acid

Utilization of the tannic acid by *Klebsiella* sp., *Bacillus* sp., and *Pseudomonas* sp. was further confirmed by chemotaxis in swarm plate assay method. It was observed that after 20hour of incubation, the cultures swarmed from the center of the plate (carbon deficient zone) towards the edge of the plate (carbon sufficient zone). This was evident from a clear zone formed at the center of the plate increasing outwards indicating tannic acid utilization.<sup>21</sup>

### Tannin-protein complex degrading activity

Tannin protein complex degrading activity of the isolates was checked on plates containing nutrient agar coated with tannic acid forming Tannin Protein Complex (T-PC) which gives an opaque shiny colour to the medium. Zone of clearing was produced by *Klebsiella* sp., *Bacillus* sp., and *Pseudomonas* sp. due to reduction of opacity around the colonies of the isolates, indicating hydrolysis of the tannin protein complex. The clearing zone turned brown after 24 hours, which may be because of the oxidation of tannic acid released after hydrolysis of tannin protein complex.<sup>15</sup>

### Demonstration of tannase

The tannase activity of *Klebsiella* sp., *Bacillus* sp., and *Pseudomonas* sp. was demonstrated by visual reading method and all the three isolates showed positive results for tannase activity as confirmed by the change in colour of the alkalized medium from green to brown on exposure to atmosphere.<sup>22-23</sup>

### Effect of tannic acid concentration on the growth of the isolates

The tolerance level of the isolates of *Klebsiella* sp., *Bacillus* sp., and *Pseudomonas* sp. was checked in minimal medium containing varying concentrations of tannic acid. As presented in Figure 1, it was observed that *Klebsiella* sp., and *Bacillus* sp., could grow well within 2 days till 0.4% concentration above which the lag phase continued till 4 days. The isolate *Pseudomonas* sp. reached log phase within 2 days till 0.8% concentration of tannic acid it could also grow at 1% concentration tannic acid. But the growth was properly observed only after 3 days.<sup>14</sup> Figure 2 indicated that the pH of the medium decreased in all the medium flasks. This indicated that the selected isolates reduced tannic acid present in the medium to acidic products resulting in decrease in pH within 2 days and the pH continued to decrease. This was evident in all the flasks. This may be due to the fact that tannic acid is broken down into glucose and gallic acid, thus reducing the pH which themselves get broken down into other acidic by products.<sup>24</sup>

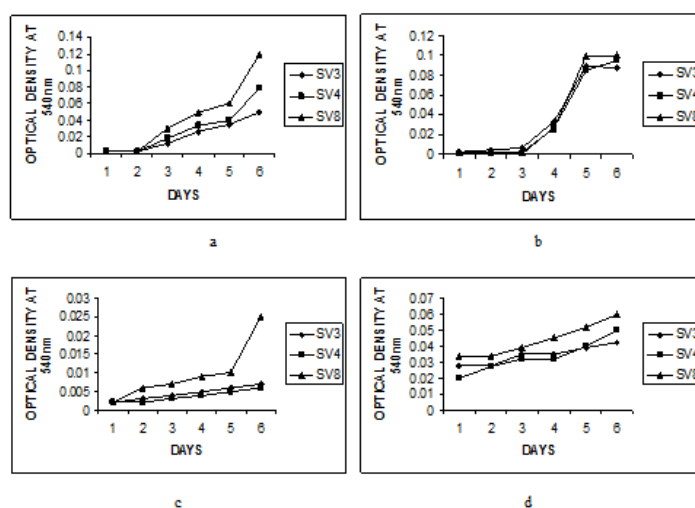
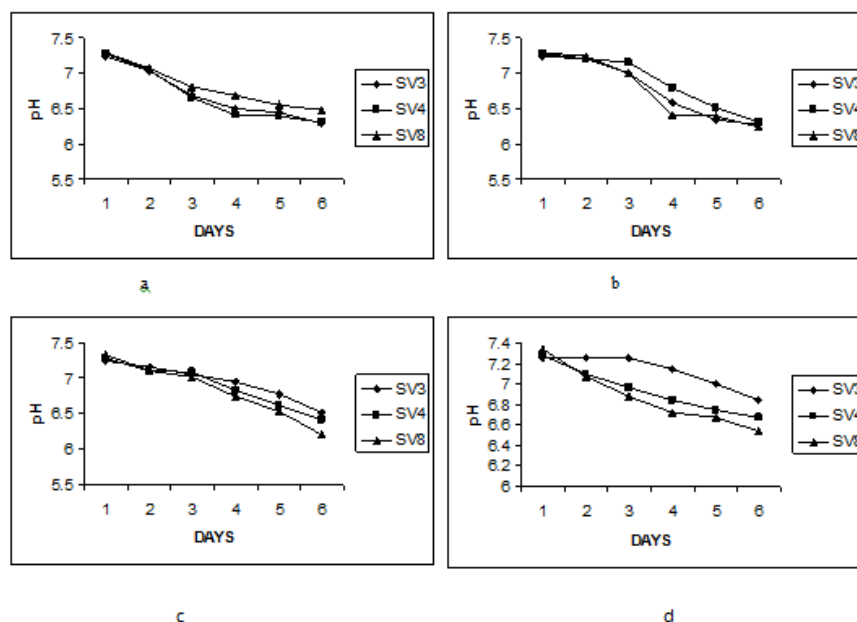


Figure 1

Growth curve of isolates in minimal broth medium with varying concentrations of tannic acid  
(a) 0.2% (b) 0.4% (c) 0.8% (d) 1%

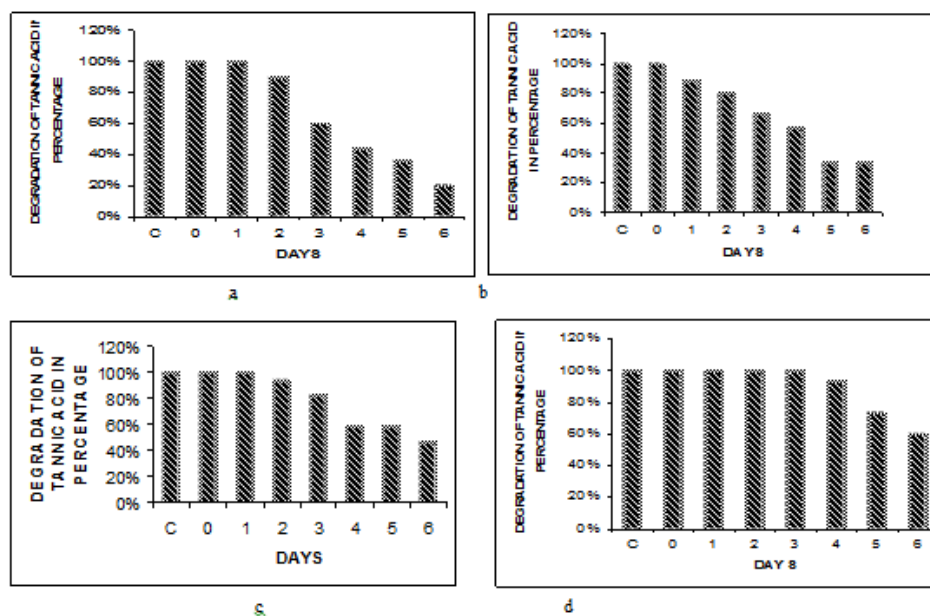




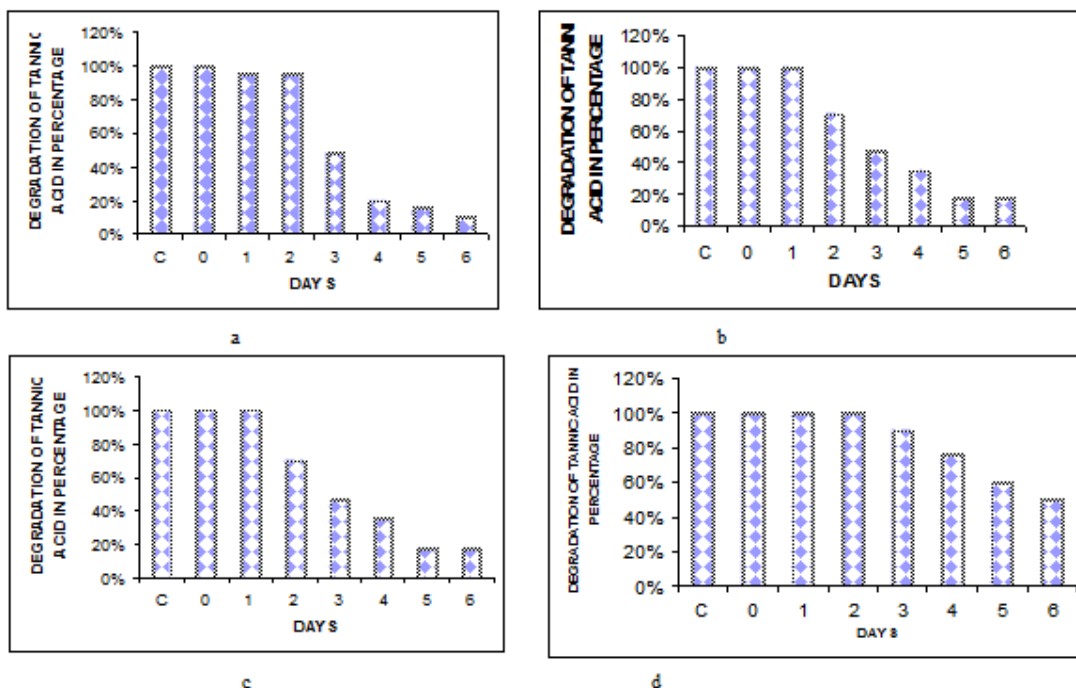
**Figure 2**  
**Change in pH of minimal broth medium on inoculation with the isolates**  
 (a) 0.2% (b) 0.4% (c) 0.8% (d) 1%

#### Estimation of residual tannic acid

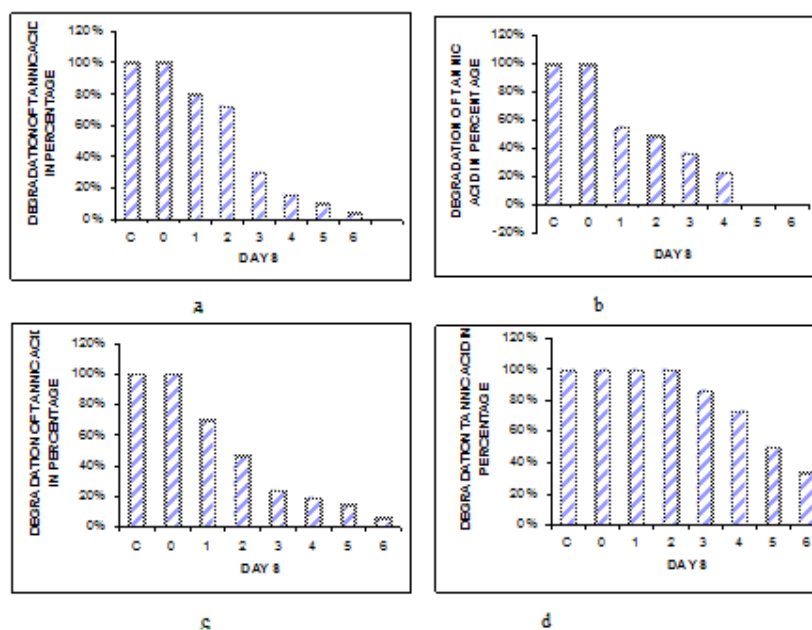
The utilization of tannic acid by the isolates was also determined by spectrophotometric analysis of the residual tannic acid in the medium. Incubation of an uninoculated medium as control under the same conditions showed that a small part of tannic acid also underwent auto degradation and formed precipitate. The amount of residual tannic acid in the medium expressed in terms of percentage of the uninoculated control showed maximum utilization of tannic acid by the isolate *Pseudomonas* sp. till 0.8% concentration within 2 days. *Klebsiella* sp. and *Bacillus* sp., showed maximum utilization of tannic acid in 0.4% tannic acid (Figure 3, 4 and 5).<sup>15</sup>



**Figure 3**  
**Percentage of residual tannic acid in minimal broth medium inoculated with *Klebsiella* sp.**  
 (a) 0.2% (b) 0.4% (c) 0.8% (d) 1%



**Figure 4**  
**Percentage of residual tannic acid in minimal broth medium inoculated with *Bacillus* sp.**  
 (a) 0.2% (b) 0.4% (c) 0.8% (d) 1%



**Figure 5**  
**Percentage of residual tannic acid in minimal broth medium inoculated with *Pseudomonas* sp.**  
 (a) 0.2% (b) 0.4% (c) 0.8% (d) 1%

#### Detection of glucose as breakdown product

Qualitative analysis of tannic acid breakdown product viz., glucose was by thin layer chromatography was carried out. The  $R_f$  of the three samples were found to be consistent with the standard  $R_f$  value of glucose.<sup>25</sup> Quantitative analysis of the glucose as breakdown product of tannic acid was also carried out and the concentration of glucose was estimated between 7.6-7.8  $\mu\text{g/ml}$  (Table 3).

**Table 3**  
**Qualitative and Quantitative analysis of glucose**

Sample	Qualitative analysis		Quantitative analysis
	Rf	Inference	Concentration (µg/ml)
Standard	0.64	Glucose	-
SV3	0.64	Glucose	7.8
SV4	0.63	Glucose	7.6
SV8	0.66	Glucose	7.7

## CONCLUSION

The isolates obtained in this study have been observed for aerobic utilization of tannic acid as sole carbon source and further can be used for bioremediation of tannin from tannery effluents and tannin-contaminated soils. The ability of all the isolates to utilize tannic acid and produce tannase exhibited their habitat-specific adaptation. Some of these bacterial isolates can have applications in reduction of antinutritional quality of tannin rich food and feed and for the large-scale production of the enzyme tannase with widespread applications in food processing, brewing, pharmaceuticals, medicine, textiles and detergents.

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# COMPARATIVE STUDY OF THREE INVASIVE PLANT SPECIES *VIZ. MIKANIA MICRANTHA, CHROMOLEANA ORDORATA, AND SPHAGNETICOLA TRILOBATA* ON GERMINATION OF CROP SEEDS

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## ABSTRACT

*Mikania micrantha*, *Sphagneticola trilobata* and *Chromoleana ordata* are three important invasive alien plant species. Alien plant species have caused serious damage to natural ecosystems and plantation crops in many parts of the world. Presently their invasion was observed in agriculture land of Kerala also. The successful invasion of these plants depends on their wide eco-physiological tolerance, strong reproductive ability and allelopathic effects. The study mainly focuses to compare these three invasive plants based their inhibitory effects on crop seeds. Bioassay on the allelopathic effects of leaf extract of these invasive plants showed significant inhibition on crop seeds namely *Capsicum annuum*, *Amaranthus* sp and *Oryza sativa*. Phytochemical screening of the aqueous leaf extract revealed the presence of a variety of secondary metabolites such as phenolic, flavonoids, alkaloids and terpenes. Quinone was observed only in the extract of *Sphagneticola trilobata*. Allelopathic potential of these plants may facilitate their successful invasion.

**KEYWORDS:** *Allelopathy, Invasive, Inhibitory effects, Crop seeds.*

## 1. INTRODUCTION

An invasive species are species that is non-native (or alien) to the ecosystem under consideration and its introduction causes economic, environmental and human health hazard. Some plant species produce compounds in their leaves or root systems which constrain the growth of other plants around them which are called as allelochemicals. Not all plants introduced from other ecosystem are harmful, but only a small percentage of them having a dynamic reproductive and proliferative potential become invasive. Plant invasions have been recognized as one of the most serious global processes impacting the structure, composition and function of natural and semi-natural ecosystems<sup>1,2</sup> Invasive alien plant species intensify poverty and threaten development through their impact on agriculture, forestry, fisheries and natural systems, which are an important basis of people livelihoods in developing countries. This damage was aggravated by climate change, pollution, habitat loss and human-induced disturbance<sup>3</sup>. For effective management of invasive species, knowledge about their ecology, morphology, phenology, reproductive biology, physiology and photochemistry is essential for the biological invasion<sup>4</sup>. A comparative study of different invasive plant species on common background features is not observed as a common practice and also family wise study on invasive plant species is rare. The present study is an attempt to compare three invasive plant species in the same family Asteracea viz. *Mikania micrantha*, *Chromolaena odorata* and *Sphagneticola trilobata*. Bioassay conducted to study their inhibitory effect on crop seeds. Secondary metabolites were detected using phytochemical screening. The study will be useful in future study and the management of invasive plants.

## 2. MATERIALS AND METHODS

### 2.1. Collection of invasive plants and preservation

Fresh leaves of all the three invasive plant species were collected from the agriculture area of Pallassana (10°38'9"N 76°39'38"E) in Palakkad district in February 2018 and air dried. The identity of the plant material was verified by Dr. T.V. Sajeev, Kerala Forest Research Institute.



## 2.2. Preparation of Crude Extract

Aqueous Extraction was prepared using 15g of leaves were air dried and grind using a mortar and pestle. The aqueous extract prepared in different concentration in 100%, 50% and 25%.

## 2.3. Bioassay

The aqueous extract of leaves of invasive plant species was assayed by using the standard sponge bioassay<sup>5</sup>; 20 seeds of test plants: *Oryza sativa*, *Amaranthus* sp and *Capsicum annuum* were distributed. Germinating seeds were counted after 3 days of experiment. Radicle length was measured after 4 days treatment.

## 2.4. Phytochemical Screening

Crude extracts of leaves of all the three plants both in aqueous and methanol extraction were phytochemically evaluated to determine the presence of secondary metabolites according to standard methods.<sup>6,7</sup>

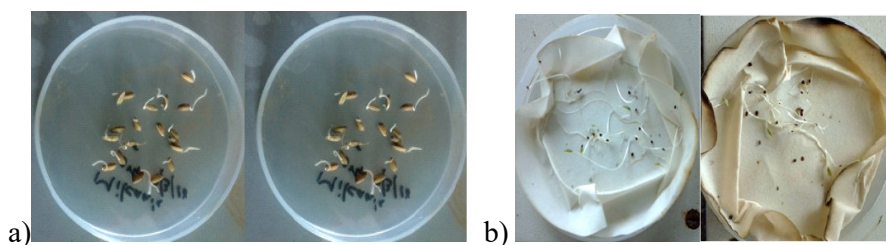
# 3. RESULT AND DISCUSSION

## 3.1. Bioassay

The germination rate decreased with the increase in the extract concentration. There is significant difference observed between the inhibitory effects of different plant extracts on germination of *Amaranthus* sp seeds. *Mikania* extract shows the highest inhibitory effect and the *S. trilobata* shows lowest inhibition on *Amaranthus* seed germination. (Fig 1b) Concentration of plant extracts has significant difference in inhibitory effect on *Amaranthus* sp seeds (Fig 2b). Highest concentration (100%) of all the three plants extract shows lowest mean germination (12%) and lowest concentration (25%) showed highest mean germination (31%). There is no significant difference in the inhibitory effect between the three of plant extracts on *Oryza sativa* seed. (Fig 1a and Fig 2c) Concentration of plant extracts showed significant difference. In the case of *C. annum* both concentration and type of plant extract are non-significant in inhibitory effects (Fig 2a). There is no significant inhibition difference observed between plant extracts on radicle length.

## 3.2. Preliminary Phytochemical Analysis of Leaves

Phytochemical screening of leaves of all the invasive plants revealed the presence of different kind of secondary metabolites (Fig 3). Table 1 tabulates the phytochemicals present in aqueous and methanolic extract of the three invasive species. Aqueous leaf extract of *Chromolaena odorata* showed the presence of flavonoids, terpenoid, phenol, saponin and cardiac glycoside. Negative results were observed for quinone, anthraquinone and coumarin. The aqueous leaf extract of *Mikania micrantha* showed the presence of flavonoids, terpenoid, phenol, saponin and cardiac glycoside. In the case of *Sphagneticola trilobata* the aqueous leaf extract the presence of flavonoids, terpenoid, phenol, quinone, saponin, cardiac glycoside and coumarin were observed. Secondary metabolites revealed from aqueous extraction are different from methanol extraction. Secondary metabolites that exert allelopathy can be released in the form of volatile compounds, root exudates, above-ground plant leachates or plant litter.<sup>8</sup> Released allelochemicals are indeed subject to sorption on soil particles as well as chemical and microbial decomposition.<sup>9,10</sup>



**Figure 1**  
Bioassay of leaves extract of invasive plants on a) *Oryza sativa* b) *Amaranthus* sp

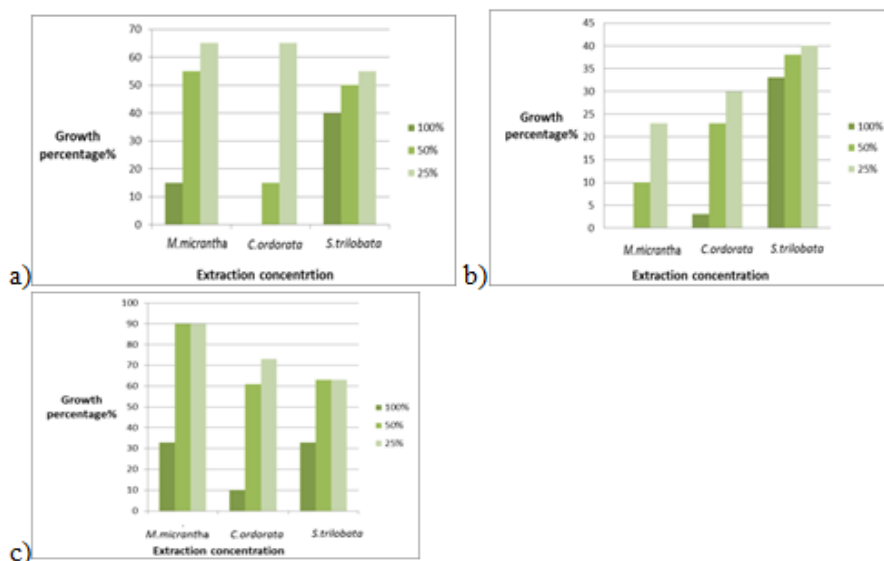


Figure 2.

Effect of leaf extraction of the invasive plant on germination percentage of a) *Capsicum annum* b) *Amaranthus* and c) *Oryzasativa* seeds after 4 days of incubation.



Figure 3.

Phytochemical Analysis of Leaves extract of Invasive plants.

Table 1.

Phytochemical screening of Aqueous and methanol extraction of the plant species.

Extraction type	Plant species	Compounds								
		Flavonoid	Terpinoid	Phenol	Quinone	Antra-quinone	Alkaloid	Saponin	Cardiac glycoside	Coumarin
Aqueous	<i>C. odorata</i>	+	+	+	-	-	-	+	+	-
	<i>M. micrantha</i>	+	+	+	-	-	-	+	+	-
	<i>S. trilobata</i>	+	+	+	+	-	-	+	+	+
Methanol	<i>C. odorata</i>	+	-	+	-	-	-	+	+	+
	<i>M. micrantha</i>	-	-	+	-	-	-	-	+	+
	<i>S. trilobata</i>	-	-	+	+	-	-	-	-	+

#### 4. CONCLUSION

Present work focuses on comparative study of the invasive behaviour of three prominent invasive plants *M. micrantha*, *S. trilobata* and *C. odorata* of Asteraceae family. Aqueous extraction of all the three plants shows significant inhibitory effect on native crop seeds viz. *Amaranthus* sp and *Oryza sativa*. In the case of *Capsicum annum* both concentration and type of plant extract showed no significant inhibitory effects.

Phytochemistry screening revealed that secondary metabolites are different in three of the plant leaves. Certain secondary metabolites may act as allelochemical that inhibited the germination of crop seeds. To identify the allelochemicals present in the leaf samples further evaluation is needed. Secondary metabolites obtained are dependent on the type of extraction.<sup>10</sup> All the three plant species are threat to agriculture sector and thus to economy. Thus proper management is essential to protect the agricultural sector from the invasive plants.

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# INSECTICIDAL ACTIVITY OF *HYDROCOTYLE LEUCOCEPHALA* AND *HEDYCHUM CORONARIUM* PLANT EXTRACTS AGAINST *HELICOVERPA ARMIGERA*

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## ABSTRACT

Agriculture plagued by insect pests is a major threat for food security as well as economy. Getting rid of insects by complete eradication is impossible and not advisable as it will affect the delicate ecological balance that has been in the case of chemical control programs. Pest management using chemical agents also resulted in more problematic effects on environment, animal and human health due to their toxic effects. Need of the hour, therefore, is an alternate concept of insect management strategies that reduce the number of pests to a level that do not compromise with the yield or economy and even maintain ecological balance. Many plant extracts and essential oils are known to possess ovicidal, repellent and insecticidal activities against various stored-product insects. The insect's rigid exoskeleton cannot expand much; it must be shed and replaced with a newer one as the insect grows. This process is called moulting which is governed by hormones. Higher plants can be used to develop environmental safe methods for insect control by using the plant extracts that can inhibit the growth of the insect by preventing molting. In the present work, *Hydrocotyle leucocephala* (Hl) and *Hedychium coronarium* (Hc) plant extracts was treated against *Helicoverpa armigera*, an insect feeding on more than 182 plant species. Plant extract injections to the larvae of *Helicoverpa armigera* showed mortality of larvae in case of both of Hl and Hc plant extracts. Topical application of both the plant extracts also showed mortality. The percentage of mortality of larvae increased with increase in concentration of plant extracts. The results obtained suggest that plant extracts can be used as a formulation in integrated pest management, which are a good alternative to conventional synthetic insecticides.

**KEYWORDS :** *Insecticides, moulting, Helicoverpa armigera, plant extract, mortality*

## INTRODUCTION:

Worlds' increasing population and attack of insects on the crops has left for demand in increase of food. One fifth of the worlds total crops are destroyed by insects. The loss due to pests and diseases is about 35% on the field and 14% in storage giving a total loss of about 50% agricultural crops annually.<sup>1</sup> Damage by the insects' pave way for bacterial and fungal infections and thereby considerable loss of food crops. To eliminate the pests, world uses around 2 million tonnes of pesticides.<sup>2</sup> Use of insecticides has contributed immensely to the increase in agricultural productivity and to the improvement in human health, particularly the eradication of malaria in the developed countries of the world in the 20th century and beyond. However, it has been established that use of synthetic organic pesticides, particularly the chlorinated hydrocarbons such as DDT and derivatives has led to serious environmental pollution (water, air and soil), affecting human health and causing death of non-target organisms. And also the control of agricultural pests and crop diseases is threatened by the evolution of pesticide resistance.<sup>3</sup> This has accelerated the search for more environmentally and toxicologically safe and more selective and efficacious pesticides. Plants have been much more successfully exploited as sources of pesticides for pests because of the production of secondary metabolites.<sup>4</sup> Higher plants are used to develop environmental safe methods for insect control.<sup>5</sup> Plants may provide potential alternatives to currently used insect-control agents because they constitute a rich source of bioactive chemicals.<sup>6</sup> Insecticidal activity of many plants against several insect pests has been demonstrated.<sup>7,8,9</sup> The toxic effects of plant extracts or pure compound on insects can be manifested in

several manners including toxicity, mortality, antifeedant growth inhibitor, suppression of reproductive behaviour and reduction of fecundity and fertility. The plant used for the study were *Hedychium coronarium*, popularly called ginger lily is a rhizomatous flowering plant belonging to family Zingiberaceae and *Hydrocotyle leucocephala* that is popularly called as Brazilian Pennywort. Both of the plants have been reported to have medicinal properties.<sup>10</sup> In this work, insecticidal study was carried out using *Helicoverpa armigera* as the pest species as it is host to more than 200 plant crops.<sup>11</sup> Frequent outbreak of *H. armigera* has led to severe social disturbances with several farmers losing their life because of crop failure especially in regard to the cotton.<sup>12</sup>

## MATERIALS AND METHODS

### 1. Plant material

Leaves of *Hedychium coronarium* (Hc) and *Hydrocotyle leucocephala* (Hl) were obtained from the Foundation of Revitalization of Local Health Traditions (FRLHT), Yelahanka, Bangalore. The leaves were rinsed with distilled water, cut into small pieces and air dried.

#### 1.1 Preparation of extracts

Each plant sample (14 g) was extracted with methanol using soxhlet extractor. The extraction was continued until the solvent in the thimble became clear. After complete extraction, the extract was filtered and solvent was distilled off in a distillation assembly at 65°C. The extract was then further evaporated and the residue was weighed.

The percentage yield was calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Weight of the extract}}{\text{Weight of the plant leaves}} \times 100$$

### 2. Phytochemical analysis (*Hedychium coronarium* and *Hydrocotyle leucocephala*)

The extracts were subjected to preliminary qualitative phytochemical investigation. The various tests and reagents used are given below.

#### 2.1. Test for Carbohydrates –

- 2.1.1. Molisch's Test (General Test): - To 2- 3 ml. of the extract, add few drops of alpha-naphthol solution in alcohol, shake and add conc. H<sub>2</sub>SO<sub>4</sub> from sides of the test tube. Violet ring is formed at the junction of two liquids.
- 2.1.2. Fehling's Test: - Mix 1 ml. Fehling's A and 1 ml. Fehling's B solution, boil for one minute. Add equal volume of test solution. Heat in boiling water bath for 5-10 min. First a yellow, then brick red ppt is observed.
- 2.1.3. Benedict's test: - Mix equal volume of Benedict's reagent and test solution in test tube. Heat in boiling water bath for 5 min. Solution appears green, yellow or red depending on amount of reducing sugar present in test solution.

#### 2.2. Test for Proteins

- 2.2.1. Biuret Test (General Test): - To 3 ml test solutions add 4% NaOH and few drops of 1% CuSO<sub>4</sub> solution. Violet or pink colour appears.
- 2.2.2. Xanthoprotein Test: - Mix 3 ml test solution with 1 ml. Conc H<sub>2</sub>SO<sub>4</sub>. White ppt is formed. Boiled precipitate turns yellow. On adding NH<sub>4</sub>OH, ppt turns orange.

#### 2.3. Test for Amino Acids

- 2.3.1. Ninhydrin Test (General Test):- Heat 3 ml. Test solution and 3 drops 5% Ninhydrin solution in boiling water bath for 10 min. Purple or bluish colour appears.
- 2.3.2. Test for Tyrosine: - Heat 3 ml test solution and 3 drops Million's reagent. Solution shows dark red colour.

#### 2.4. Test for Fats and Oils - Filter paper gets permanently stained with oils.

- 2.4.1. Solubility Test: - The extract is soluble in ether, benzene and chloroform, but insoluble in 90% ethanol and in water. (Exception: castor oil, soluble in alcohol).



- 2.5. Tests for Steroid
  - 2.5.1. Salkowski Reaction: - To 2 ml of extract add 2 ml. of chloroform and 2 ml. conc. H<sub>2</sub>SO<sub>4</sub>. Shake well. Chloroform layers appears red and acid layer shows yellow fluorescence.
  - 2.5.2. Liebermann- Burchard Reaction:- Mix 2 ml extracts wit chloroform. Add 1-2 ml. acetic anhydride and 2 drops conc. H<sub>2</sub>SO<sub>4</sub> from the side of test tube. First red, then blue and finally green colour appears.
- 2.6. Test for Glycosides - Preparation of test solution: - The test solution was prepared by dissolving extract in the alcohol or hydro-alcoholic solution.
  - 2.6.1. Legal's Test: - To aqueous or alcoholic extract, add 1 ml. pyridine and 1 ml. sodium nitroprusside. Pink to red colour appears.
- 2.7. Test for Flavonoids: - To small quantity of residue. Add lead acetate solution. Yellow coloured precipitate is formed.
- 2.8. Test for Alkaloids:- Evaporate the aqueous, alcoholic and chloroform extracts separately. To residue, add dilute HCl. Shake well and filter. With filtrate, perform following test
  - 2.8.1. Mayer's Test: - Mayer's reagent with 2-3 ml of the filtrate produces precipitate
  - 2.8.2. Wagner's Test: - 2-3 ml filtrate with few drops Wagner's reagent gives brown ppt.
  - 2.8.3. Hager's Test: - 2-3 ml filtrate with Hager's reagent gives yellow ppt.
- 2.9. Test for Tannins and Phenolic Compound - To 2-3 ml. of aqueous or alcoholic extracts, add few drops of following reagents:
  - 2.9.1. 5% FeCl<sub>3</sub> Solution: - Deep blue –black colour.
  - 2.9.2. Lead Acetate Solution: - White ppt.
- 2.10. Test for Triterpenoids - Preparation of test extracts solution: - The test extract solution was prepared by dissolving extract in the chloroform.
  1. Salkowski Test: - Few drops of concentrated sulphuric acid were added to the test solution, shaken on standing lower layer turned golden yellow.

### 3. Preparation of stock solutions and working standards for bioassay

100 mg of the plant extracts was weighed and made upto 1ml with methanol. This serves as stock solution (100 mg/ml). From this working standards of 20µg, 40µg, 60µg, 80µg and 100µg were prepared for each plant extract by diluting them in distilled water.

### 4. Rearing of larva

#### 4.1. Requirements for insect rearing

The equipment required for rearing were electronic balance, microwave, liquid blender, autoclave and apparatus were polythene bags (autoclavable), plastic trays, rearing cups, absorbent and non-absorbent cotton, forceps (blunt end), watch-glass and camel hair brush, ocular dissection microscope and needles. The parental stock of *Helicoverpa armigera* (NBAIL-MP-NOC-001) of about late first instar and second instar larvae was obtained from NBAIL–National Bureau of Agricultural Insect Resource (formerly known as NBAIL), Bengaluru, India.

#### 4.2. Conditions for insect rearing

The rearing was carried out at 25±2°C, 65±5% R.H. and 12L: 12D (Light: Dark) photoperiod. The larvae were group reared on Chickpea based semi-synthetic media with slight modification in individual cups as they exhibit cannibalism.<sup>13</sup> Freshly prepared semi-synthetic diet in the semi-solid form was transferred to rearing cups (4mL in each). After solidification at room temperature, to each cup larvae were transferred using a camel hair brush and the cups mouth was covered with cloth.

### 5. Bioassay of plant extract for Toxicity

Plant extracts was assayed for its toxicity on *Helicoverpa armigera* larvae by injection method and topical application. Freshly moulted third (80-110mg) instar larvae were used for injection and topical application. Different concentrations of plant extracts were used for assessment of insecticidal activity and for each concentration 30 larvae were used and the tests were carried out in triplicates.

### 5.1. Injection method

Different concentrations varying from 20µg/10µL to 100µg/10µL were injected in to the hemolymph of the larvae. For each concentration, effects upon survival were monitored over the next 48 hours. Methanol was used as control. Mortality rate was monitored.

### 5.2. Topical Application

Different concentrations varying from 20µg/10µL to 100µg/10µL was applied topically on the dorsal side of the thorax of the third instar larvae using a brush and reared on a semi-synthetic diet. Topical application was continued for 3 days after which the larvae were left undisturbed. Application of methanol served as control. Number of larvae pupated, adult emergence and death were recorded at the end of 14<sup>th</sup> day.

## RESULTS AND DISCUSSION

### 1. Preparation of extracts –

- 1.1. Plant extract of *Hedychium coronarium* was prepared in methanol and the extract after evaporation was found to be a dark green, sticky, paste with a characteristic odour with the percentage yield of 7.51%
- 1.2. Plant extract of *Hydrocotyle leucocephala* was prepared in methanol and the extract was blackish green, semi-solid paste, with a distinct smell. The yield percentage was found to be 7.57%.
2. **Phytochemical analysis** - Results obtained from qualitative analysis of phytochemicals of the total methanolic extracts of both plants under investigation (*Hc* and *Hl*) are presented in Table 1.

**Table 1: Phytochemical Analysis of methanolic extracts of *Hedychium coronarium* and *Hydrocotyle leucocephala***

SI.No	Phytochemical constituent	Tests	<i>Hedychium coronarium</i>	<i>Hydrocotyle leucocephala</i>
1	Carbohydrates	Molisch's Test	-	+
		Benedicts Test	-	+
		Fehlings Test	-	+
2	Proteins	Biuret Test	-	-
		Xanthoprotein Test	-	-
3	Amino Acid	Ninhydrin Test	-	-
		Tyrosine Test	-	-
4	Fats and Oils	Filter Paper Stain Test	-	-
		Solubility Test	-	-
5	Glycosides	Legals Test	-	+
		Keller – Killiani Test	-	+
6	Saponins	Foams Test	+	-
7	Flavonoids	Lead Acetate Test	-	+
		Sodium Hydroxide test	-	+
8	Alkaloids	Mayers Test	-	+
		Wagners Test	-	+
9	Phenolic Compounds	Lead Acetate Test	-	+
		Acetic Acid Test	-	+
10	Triterpenoids	Salkowski Test	+	+

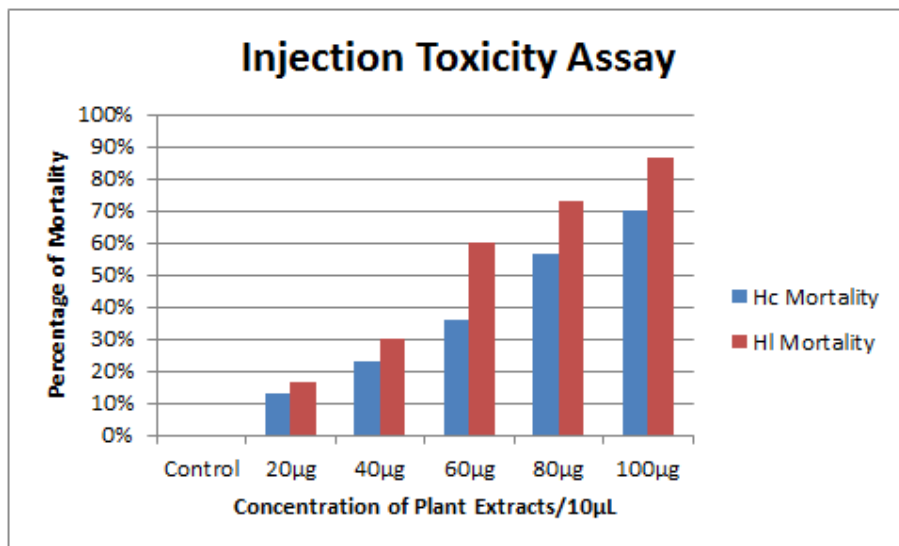
**3. Bioassay of plant extract for Toxicity** - Plant extracts of *Hedychium coronarium* (Hc) and *Hydrocotyle leucocephala* (Hl) were used to assess their toxicity on the *Helicoverpa armigera* larvae.

### 3.1 Injection Method:

Plant extract injections to the larvae of *Helicoverpa armigera* showed mortality of larvae in case of both of Hl and Hc plant extracts. The percentage of mortality of larvae increased with increase in concentrations of plant extracts. For the highest concentration used, Hc extract showed 70% and Hl extract showed 86.6% mortality as tabulated in Table 2 and in Figure 1.

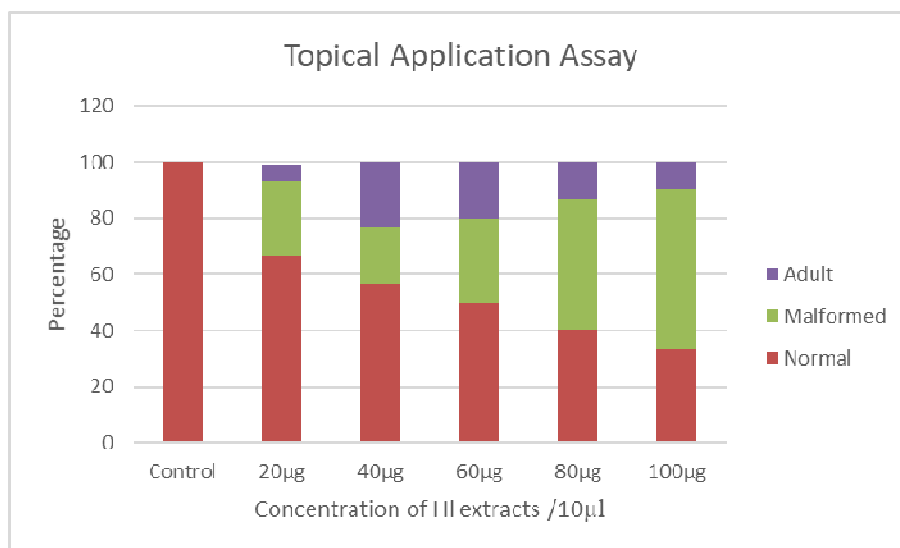
**Table 2 : Effect of injection of *Hedychium coronarium* and *Hydrocotyle leucocephala* plant extract on *H. armigera* larvae**

Concentration	<i>Hedychium coronarium</i>		<i>Hydrocotyle leucocephala</i>	
	Number of dead larvae	Mortality	Number of dead larvae	Mortality
Control	0	0%	0	0%
20µg	12	13.3%	15	16.6%
40µg	21	23%	27	30%
60µg	33	36%	54	60%
80µg	51	56.6%	66	73.3%
100µg	63	70%	78	86.6%

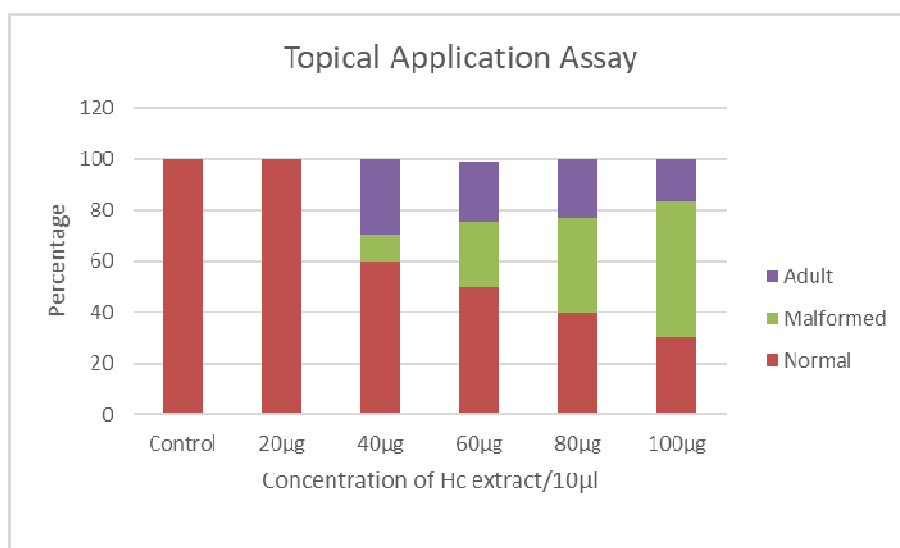


**Fig. 1: Effect of injection of *Hedychium coronarium* (Hc) and *Hydrocotyle leucocephala* (Hl) plant extract on *H. armigera* larvae**

**3.2 Topical Application:** The test insects showed malformed pupae at the end of 14<sup>th</sup> day. Percentage of adult decreased in proportion to the increased concentration of the plant extracts, indicating the plant extracts were responsible for the malformed pupae as in comparison with control. The percentage of malformed pupae was more (56.66%) for *Hydrocotyle leucocephala* extract as compared to *Hedychium coronarium* plant extract (53.33%) for the highest concentration used as shown in Figure 2 and 3. This result is promising as in control by the end of 14<sup>th</sup> day, all the larvae had emerged to adult, while the treated ones were in three different categories, such as adult, malformed pupae and normal pupae with adult form in lower percentage for the highest concentration used.



**Figure 2: Effect of topical application of *Hydrocotyle leucocephala* plant extract on *H. armigera* larvae**



**Figure 3: Effect of topical application of *Hedychium coronarium* plant extract on *H. armigera* larvae**

## CONCLUSION

Agriculture plagued by insect pests is a major threat for food security as well as economy. Getting rid of insect pests by complete eradication is impossible and not advisable as it will affect the delicate ecological balance that has been in the case of chemical control programs. Pest management strategies using chemical agents also resulted in more problematic effects on environment, animal and human health due to their toxic effects. Need of the hour, therefore, is an alternate concept of insect management strategies that reduce the number of pests to a level that do not compromise with the yield or economy and even maintain ecological balance. Many plant extracts and essential oils are known to possess ovicidal, repellent and insecticidal activities against various stored-product insects from ages.<sup>14, 15</sup> the results obtained in this study suggest that we can use plant extracts as a formulation of bioinsecticides for use in integrated pest management, which are a good alternative to conventional synthetic insecticides. In the present study, this lays a foundation to use *Hydrocotyle leucocephala* and *Hedychium coronarium* plant extract against *Helicoverpa armigera*. However, further more studies have to be carried out to analyze the component responsible for the insecticidal activity by LC- MS, FTIR and HPLC.

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## SYNTHESIS OF FLUORESCENT IRON OXIDE NANOPARTICLES

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### ABSTRACT

Nanoparticles have gained curiosity in technological advancements due to their property and enhanced performance. The use of fluorescent nanoparticles have become considerable in cancer research. The work was focused on synthesis of Iron oxide nanoparticle by co-precipitation method and coating with fluorescent dyes. Anionic and cationic detergents were utilized for coating of fluorescent dyes. They were characterized by SEM, UV-Vis spec, FTIR and Spectrofluorophotometer. SEM confirmed mean size of particles to be 20 nm. The characteristic peak of nanoparticle found to be at 210 nm was confirmed by UV-vis spec. FTIR confirmed the presence of iron and oxide. The fluorescent peak of SDS-rhodamine-coated nanoparticles and CTAB- Rhodamine-coated nanoparticles were found at 584 nm and 524 nm respectively. SDS-fluorescein-coated nanoparticles and CTAB-fluorescein-coated nanoparticles, peaks were at 530 nm and 520 nm respectively. SDS coated nanoparticles were more stable and bound effectively to the dye.

**KEYWORDS:** *Nanoparticles, fluorescent, rhodamine, fluorescein, co-precipitation*

### INTRODUCTION

Over the last decade, nanotechnology has developed to such an extent that it has become possible to fabricate, characterize and specially modify the functional properties of nanoparticles for biomedical applications which include but is not limited to diagnostics<sup>1-4</sup>. Iron oxide exhibits captivating physical properties especially in the nanometre range, not only from the viewpoint of basic science, but also for a variety of applied biomedical engineering. Magnetic nanomaterials have a range of distinctive properties and can offer attractive options<sup>5-9</sup>. The magnetic nanoparticles that have suitable surface characteristics, are highly used in a lot of *in vitro* and *in vivo* applications. For example, magnetic nanoparticles can be selectively targeted to a specific biological entity of interest using an external magnetic field. Moreover, an alternating magnetic field can be used to heat the area where magnetic particles are localized, and this serves as a basis for cancer hyperthermia therapy. These applications need special surface coating of the magnetic particles, which has to be non-toxic and biocompatible and should allow a targetable delivery with particle localization in a specific area. Iron oxide nanoparticles (IONPs) have a wide range of biomedical applications, such as magnetic resonance imaging contrast agents, drug delivery, specific cell labelling, cell tracking, diagnostics, and hyperthermia. Iron oxide particles shorten the effective transverse relaxation time (T<sub>2</sub>) of tissues that take up these particles by inducing magnetic field inhomogeneity around the Iron Oxides. These nanoparticles also have relatively low toxicity when handled in the usual metabolic pathways<sup>10</sup>. Nature of surface coatings and their subsequent geometric arrangement on the nanoparticles determine the overall size of the colloid and also play a significant role in biokinetics and biodistribution of nanoparticles in the body. Studies on different contrast agents have shown that the biodistribution depends on the size, charge and thickness of the coating of the particles<sup>11</sup>. In addition, the significant smaller size of nanoparticles potentially allows them to move across the biological membrane. Many Iron-oxide based formulations, based on bare nanoparticles and on nanoparticles coupled to different carriers like polymers, liposome, micelles matrix etc., are in clinical trials and few of them have reached the market<sup>12</sup>. To understand the basic biological functions in a living system one needs a sophisticated detection system. Luminescence technique has been widely used as a sensitive tool for many years in many areas of sciences and technologies. Fluorescence imaging, as opposed to white-light imaging, can be used to visualize beyond superficial tissue, and a high signal-to-background ratio can be achieved in the labelled tissue<sup>13</sup>. A challenge of fluorescence imaging is that the significant auto fluorescence of bodily tissues in the visible region of the

electromagnetic spectrum. Materials that fluorescence in the near-infrared (NIR) region (700–1000 nm) intended for use as imaging agents are of great interest, as they result in a low background signal and relatively deep penetration into biomatrices<sup>14–16</sup>. However, the wavelengths in the NIR (Near-infrared) region are beyond those visible to the human eye, and challenges of fluorescence imaging in this region include the need for specialized light sources and detection equipment. High sensitivity, poor hydrophilicity, rapid photobleaching, real-time monitoring impotency of organic fluorescent dyes such as fluorescein, Rhodamine-B makes it difficult to be employed for bioimaging. In an effort to synthesize highly stable, sensitive and uniform biomarkers, we have developed fluorescent nanoparticles for application as an efficient biomarker. In this study we have used Iron oxide nanoparticles to coat fluorescent dyes. The dyes used were Rhodamine B and Fluorescein individually. These two dyes were dissolved in two detergent, SDS and CTAB, both anionic and cationic detergents to study the best stable detergent, which can be used for functionalization. The Iron oxide nanoparticles were functionalized with detergents and finally coated with dyes and these were characterized spectroscopically and microscopically.

## Material and Methods

### *Synthesis of Iron oxide nanoparticles*

Iron oxide nanoparticles were synthesized using Iron chloride and Iron sulphate by co-precipitation method. The synthesized Iron oxide nanoparticles were stabilized by dissolving in N,N-dimethylhydrochloride. The complete procedure has been published elsewhere<sup>17</sup>.

### *Functionalization of nanoparticles*

Nanoparticles were functionalized with two separate detergents to compare the stability of these detergents on nanoparticles. Sodium dodecyl sulphate (SDS) and Cetyl trimethyl ammonium bromide (CTAB), 5mg each, were dissolved in 25 ml of distilled water and were saturated. Further, 10 ml supernatant solution was added with 5 ml of nanoparticles. The solution was incubated in cyclo-rotator for 3 days.

### *Fluorescent dye coating onto nanoparticles*

Rhodamine B and Fluorescein dyes were used for coating. Both the dyes, 1 mg each, was dissolved in 1 ml of N,N- dimethyl hydrochloride and mixed with the functionalized nanoparticles. The solution was incubated in cyclo-rotator for 24 hrs.

### *Characterization of nanoparticles*

#### *UV-Visible spectroscopy*

The synthesized Iron oxide nanoparticles were characterized using UV-Visible spectroscopy V-650 Jasco model. The samples were recorded between 200 and 800 nm.

#### *FTIR Spectroscopy*

The samples were characterized for their structural property by FT-IR spectra using Perkin Elmer Spectrum 1. The liquid form of samples were analysed and recorded with transmission mode scan in the spectral region of 4000-450 cm<sup>-1</sup>.

#### *Scanning Electron Microscopy*

The SEM images of Iron oxide nanoparticles were obtained by Zeiss Ultra 55 model SEM. The samples were drop casted onto silica grid and gold sputtered prior to imaging.

#### *Fluorescence Microscopy*

The fluorescent-coated nanoparticles were characterized using SHIMADZU RF 1501 model Spectrofluorophotometer to identify the fluorescent peaks. The scan was recorded from 350-750 nm range.

## RESULTS AND DISCUSSION

Iron oxide nanoparticles were synthesized by the co-precipitation method, which was found as the simplest and effective method of preparation. The obtained nanoparticles were found to be less aggregated and stable with good optical property.

**UV-Visible spectroscopy**

The synthesized naked nanoparticles showed a characteristic peak at 220 nm in UV-Visible spec, which is specific for Iron oxide nanoparticles<sup>18</sup>. The SDS and CTAB coated nanoparticles also showed similar characteristics peaks respectively. SDS functionalized nanoparticles were comparatively stable<sup>17</sup>.

**FT-IR Spectroscopy**

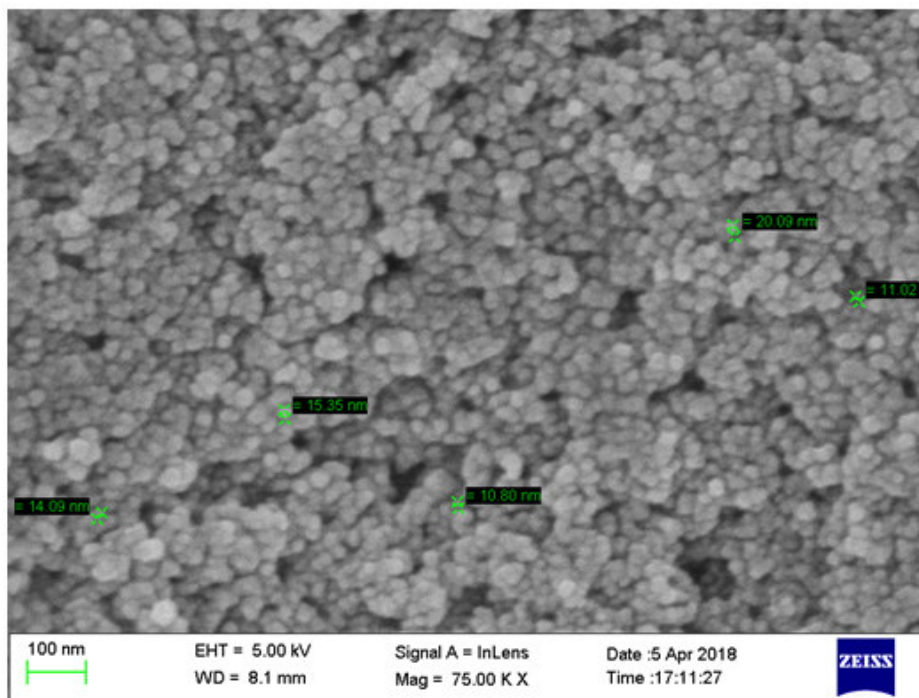
The FT-IR spectrum revealed the presence of Iron and oxide in the samples. Therefore it confirms the structural property of the synthesized Iron oxide nanoparticle, *ie.*, Iron and oxide as its principal elements. It also showed the presence of methyl group which is of N,N-dimethyl hydrochloride used for stabilization of nanoparticles<sup>19</sup>. The samples of SDS and CTAB coated nanoparticles also confirmed presence of their functional groups<sup>17</sup>.

**Scanning Electron Microscopy**

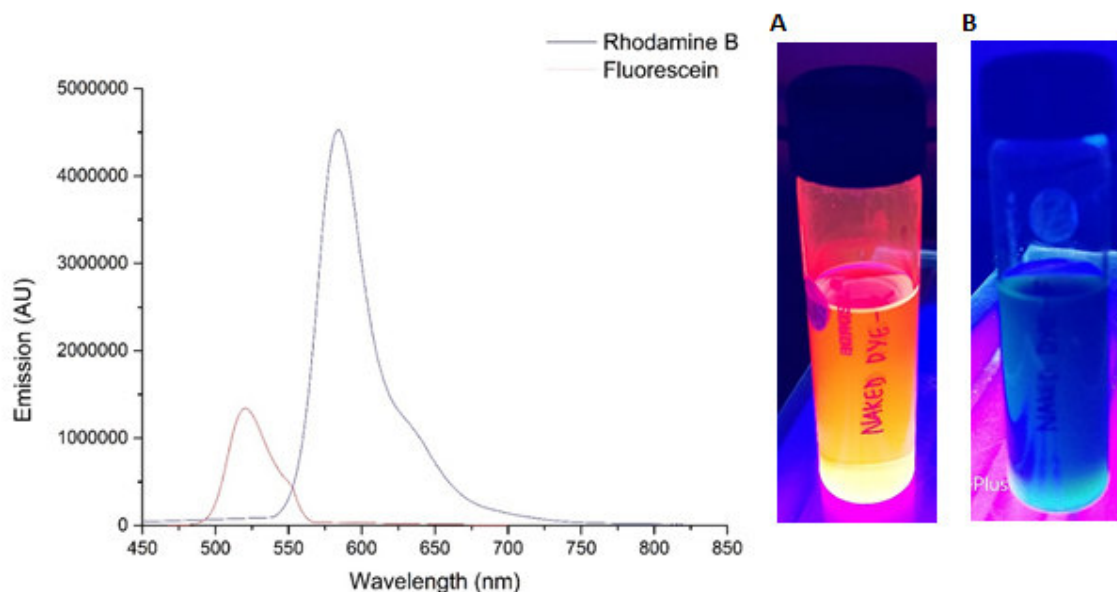
The SEM images of naked nanoparticles had showed a mean size of 20 nm (Fig. 1). The particles were spherical in shape, which is shown to be less toxic<sup>20</sup>.

**Fluorescent Spectroscopy**

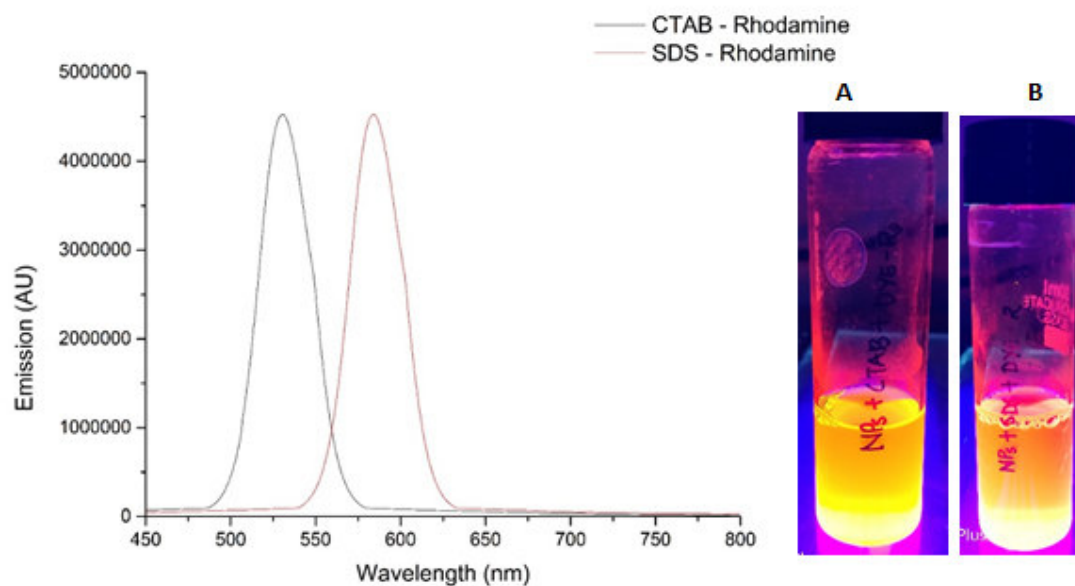
The Rhodamine b dye dissolved in N,N - dimethyl hydrochloride showed a peak at 580 nm range<sup>21</sup> which is in agreement with the previously reported around 575 nm<sup>22-23</sup> (Fig. 2). The fluorescein dye dissolved in N,N - dimethyl hydrochloride showed a maximum emission at 520 nm range which was also in conformation with previous reports<sup>23</sup>. The difference in band is due to chemical environment of the sample, which was dissolved in N,N - dimethyl hydrochloride. The SDS and CTAB coated Rhodamine b-Iron oxide nanoparticle showed maximum emission at 584 nm and 529 nm respectively (Fig. 3). The SDS coated Rhodamine-Iron oxide nanoparticle found more stable<sup>24</sup>. The SDS and CTAB coated fluorescein Iron oxide nanoparticles showed maximum at 530 nm and 520 nm respectively (Fig.4). In the fluorescein coated Iron oxide nanoparticles, SDS was found as more stable detergent than that of CTAB since peak in SDS samples maximum was in closer range than CTAB sample. The shift in the maximum is due to the nanoparticles and detergents complex.



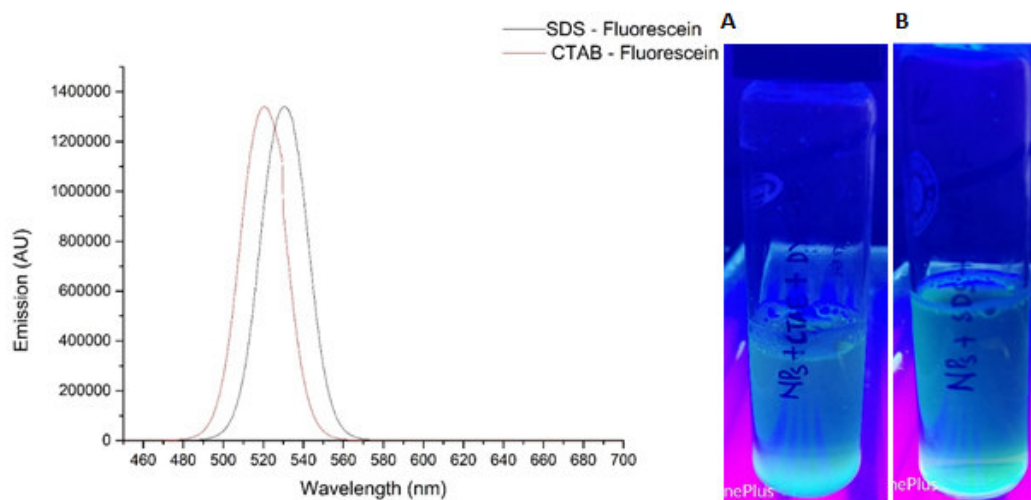
**Figure 1: SEM images of naked nanoparticles**



**Figure 2**  
Fluorescent spectra of dye, A- Naked Rhodamine b dye, B- Naked Fluorescein dye



**Figure 3**  
Fluorescent spectra of nanoparticles coated Rhodamine dye, A- Rhodamine b dye in CTAB, B- Rhodamine b dye in SDS



**Figure 4**  
**Fluorescent spectra of nanoparticles coated Fluorescein dye, A-Fluorescein dye in CTAB, B-Fluorescein dye in SDS**

## CONCLUSION

In the present study, Iron oxide nanoparticles were synthesized by co-precipitation from Ferrous sulphate and Ferrous chloride. Co-precipitation is most effective and simple method for synthesis of iron oxide nanoparticles. The synthesized nanoparticles were found to be stable and less aggregated. The nanoparticles were further functionalized with two detergents to find the most stable for the fluorescent dye coating. SDS and CTAB were used for functionalization of nanoparticles. SDS detergent were found to be more stable. The fluorescence dyes, Rhodamine (red) and fluorescein (green) were used for the coating, as they can be utilized for several purposes in molecular biology. Both dyes were effective in binding with nanoparticles and showed maximum emissions. The nanoparticles functionalized with SDS found to be more stable and bound effectively with both dyes. SDS functionalized nanoparticles coated with fluorescence dyes have good property and effective than the CTAB ones.

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