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Phytochemical Investigation of the antibacterial activity of essential oils of *Premna latifolia* & *Lantana camara*

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Abstract
Eomenacanthus (menopone) or bird lice is a serious problem affecting birds and it can severely effect the egg production in poultry. Although many chemicals such as DDT and pyrethrin can effectively act against menopone, they could impart serious health problems. In Kerala, the leaves of the plants, *Premna latifolia* Roxb. and *Lantana camara* were found to be used by the villagers as an effective bio insecticide against bird lice. The pharmacological activity of the medicinal plants mainly lies in the secondary metabolities which are comparatively smaller molecules in contrast to primary metabolites such as peptides, proteins and carbohydrates. So our aim was to investigate the chemical compounds present in the leaf extract of these two plants which are effective in acting against menopone. The revolutionary advances achieved in the field of chromatography and spectroscopy had made the isolation and structure elucidation more simple and reliable.

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1. Introduction

In Kerala, ayurvedic system uses enormous medicinal plants in the wild and semi wild state for the treatment of various diseases. Over 400 species of plants are used for the commercial preparation of beneficial drugs and the use of various parts of these plants to cure specific ailments has been in vogue from ancient times in our indigenous medicine. Even after the emergence of more sophisticated “Allopathic” field of medicine, the folk medicine and the herbalism has not lost their importance yet and about 75% of people in the third world countries still rely on herbal preparations and the isolation and characterization of the plant extracts requires special attention.

1.1 Materials and Methods

Eomenacanthus (menopone), the lice attacking chicken, is a common problem suffered by the villagers whose livelihood depends on poultry. Bird lice eggs are laid on the shaft of bird’s feathers and will hatch after a few days. The lice were not able to survive for more than a few days without their hosts. Even though the usuage of insecticides such as DDT, sodium fluoride, 10% pyrethrins and chlordane are effective in their treatment (Warren et al., 1948; Fowler et al.,1983; Warren et al., 1945; Edgar et al., 1949; Fairchild et al., 1955), they can induce serious health hazards not only to birds but also to humans who handle these birds. Silver nano particles which are also effective against bird lice are too unaffordable to the common village men.

1.2 Results and Discussion

As an effective bio insecticide against menopone, the leaves of plants *premna latifolia* & *lantana camara* were macerated by villagers and kept them in cages of birds for drinking. So in this paper, we emphasis on the usefulness of the leaves of these two plants against the treatment of bird lice.

2. Review of Literature

The anti microbial and anti insecticidal activity (Renjana et al., 2013; Kumari et al., 2011; Mahire et al., 2009; Rajendran et al., 2010; Dua et al., 2010; Milton Helio et al., 1999; Oluwadayo et al., 2008; Baars et al., 1999; N Priyanka et al., 2013) of the leaves of these two plants of the family verbanaceae under study arouse a curiosity in us to probe in detail about the chemical constituents present in their leaf extracts. It is strongly felt that both these plants contain chemical compounds of same class or of same functional groups which makes them effective against the menopone disease. The leaves of these two plants were macerated by villagers in boiling water and kept in birds cage as an effective medicine against menopone. So it was our desire to probe in to the fact that whether the compounds present in the leaf extract of these two plants responsible for the cure of this disease are of same or different type.

Figure 1: Images of the plants (A) *Premna latifolia* and (B) *Lantana camara*

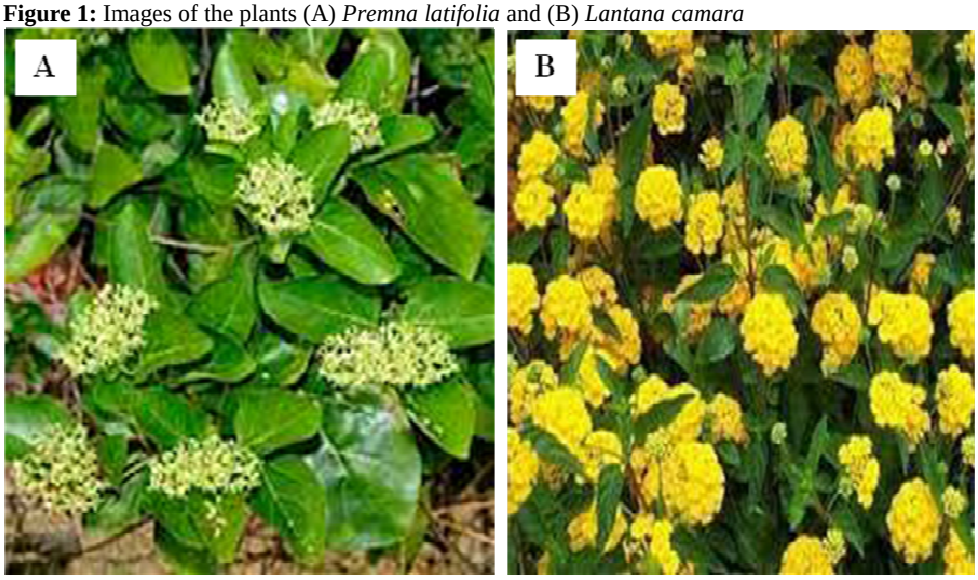


Figure 2: Image of menopone attacking birds

Figure 3: Gas chromatogram of the leaf extract of *Premna latifolia*

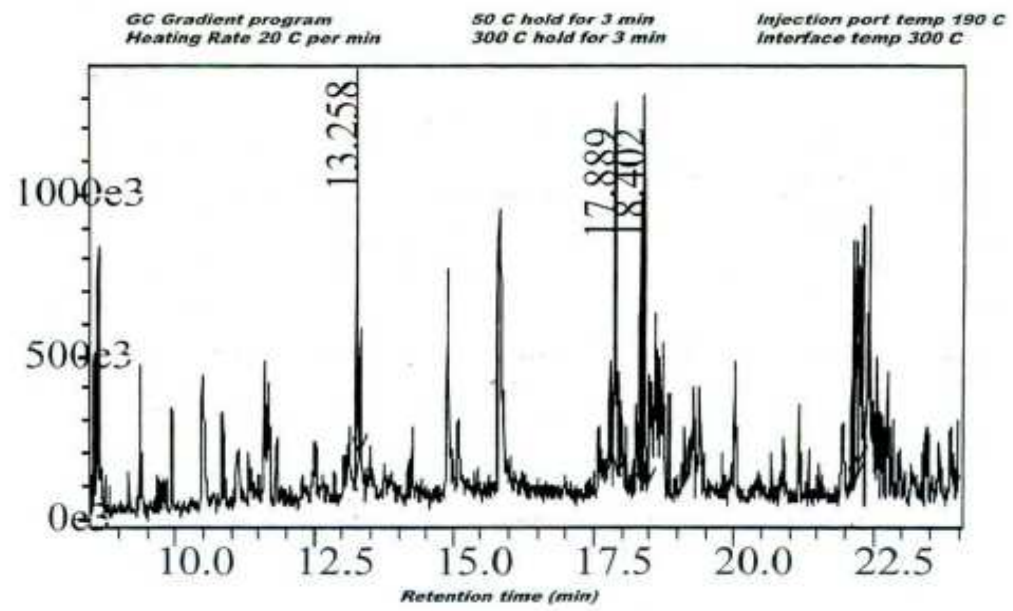
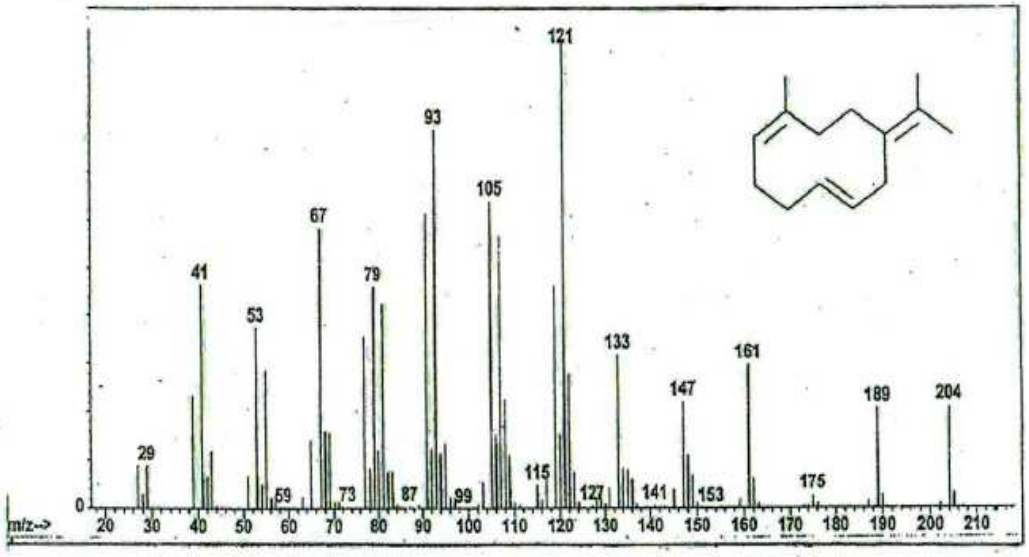


Figure 4: Mass spectrum of Germacrene B



Mass spectrum of Germacrene B

Figure 5: Mass spectral fragmentation pattern of Germacrene B

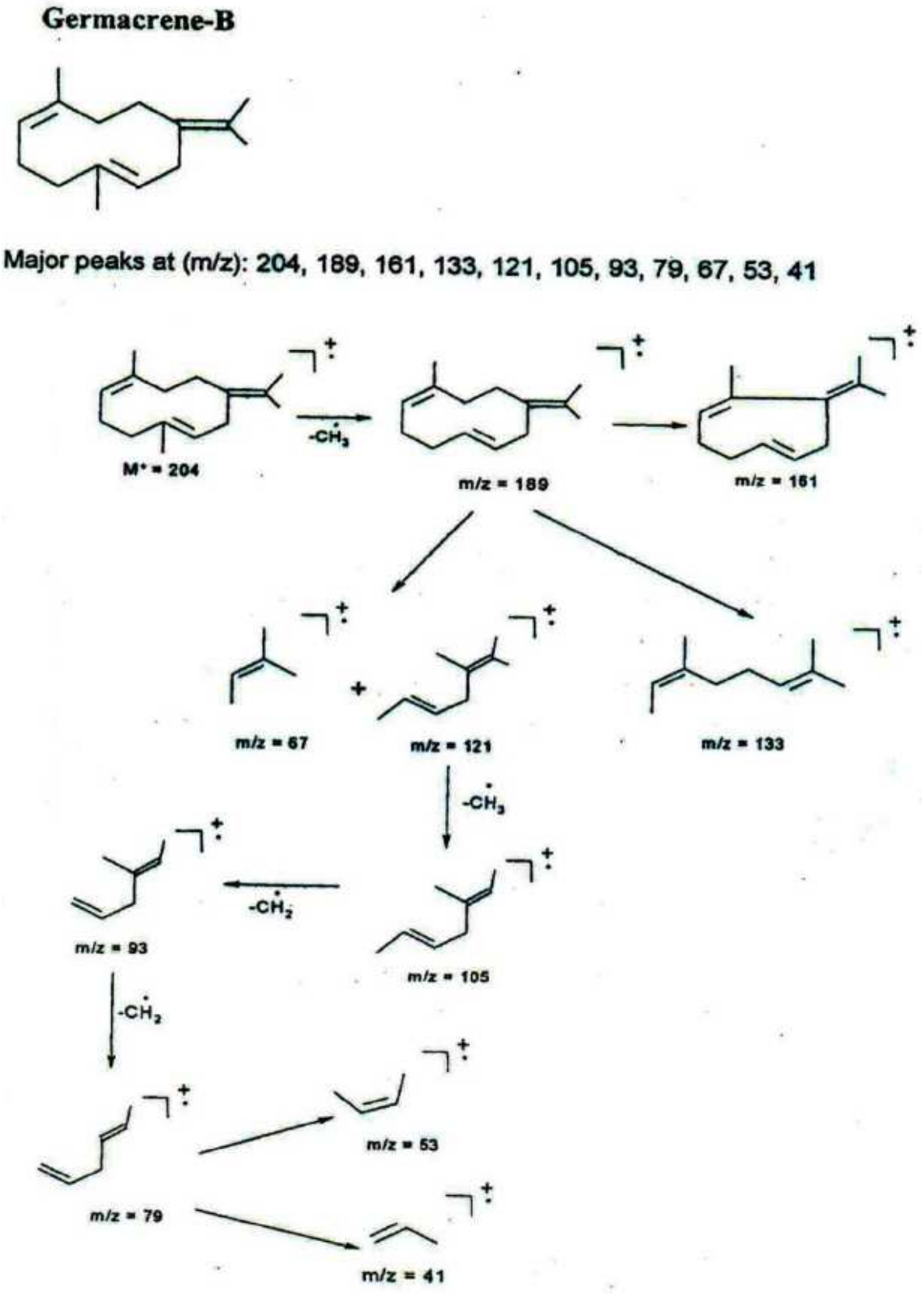


Figure 6: Gas chromatogram of the leaf extract of *Lantana camara*

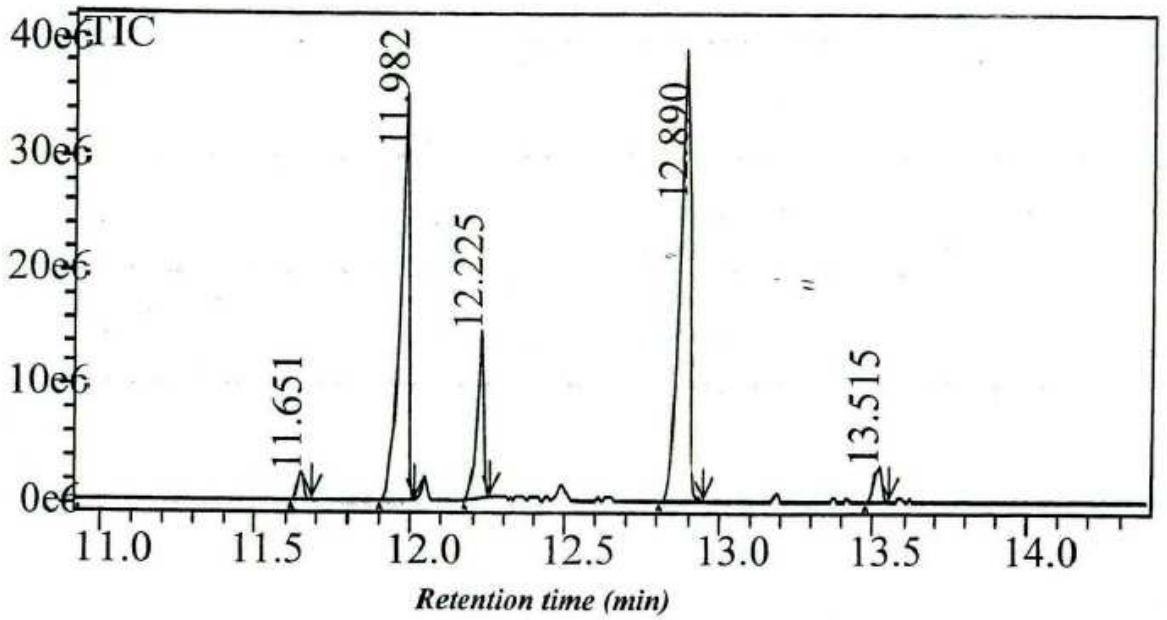


Figure 7: Mass spectrum of β-caryophyllene

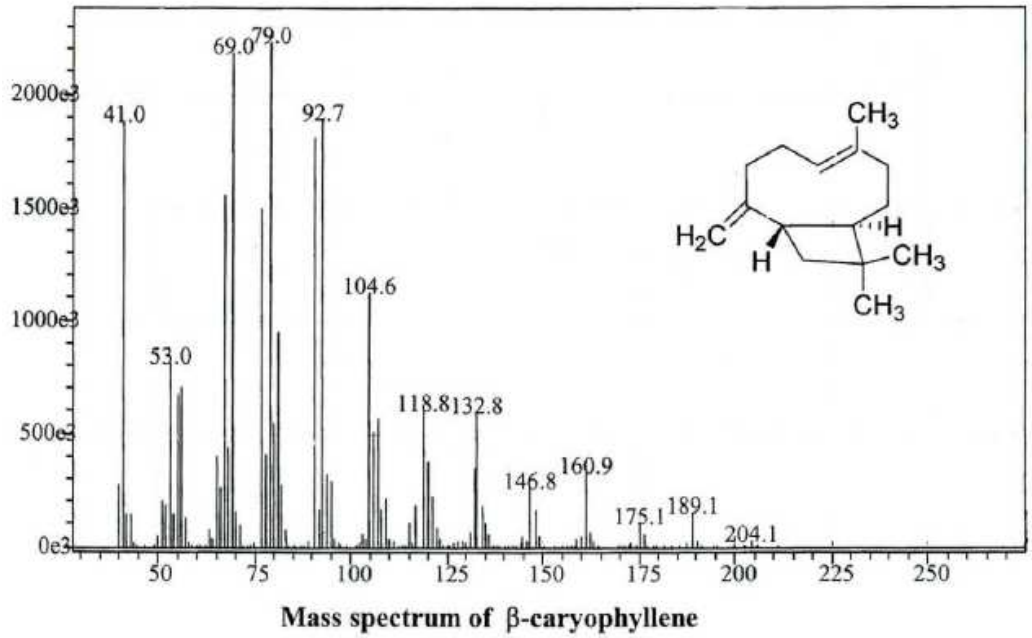


Figure 8: Mass spectrum of Germacrone

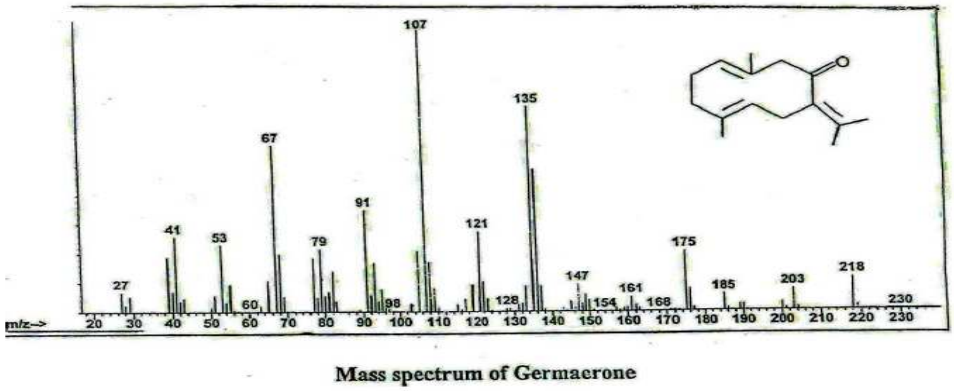
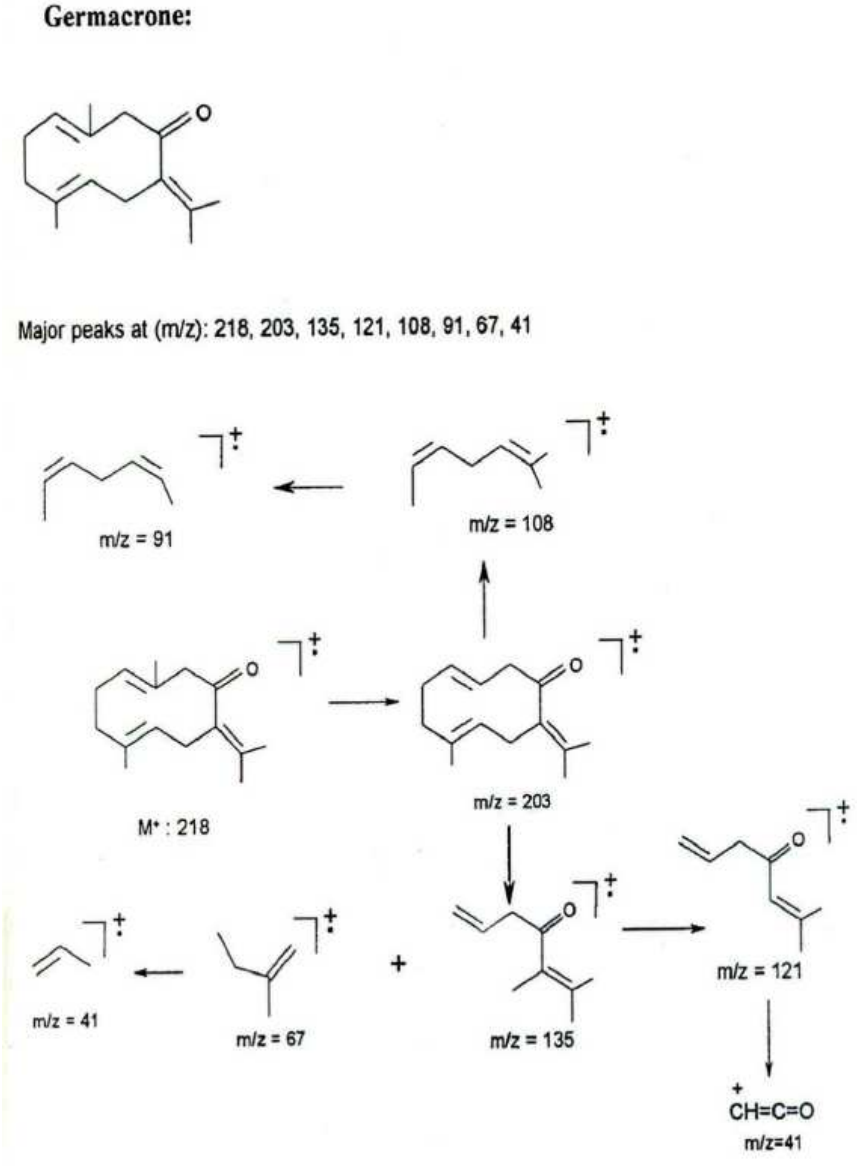


Figure 9: Mass spectral fragmentation of Germacrone



3. Objective of Research

The main objective of our work was to minimize the use of persistent pesticides and chemicals in the treatment of diseases and hence a move to eco-friendly, reliable and non hazardous 'ayurvedic system of medicine'. Our piece of work will actually be a milestone for the whole village community whose main source of income is poultry and hence the use of such medicines will surely help the public to produce eggs and meat without being infected by chemicals and pesticides.

4. Material and Methods

4.1 Collection of plant leaves

Firstly we contacted villagers to get Fresh leaf samples of *Premna latifolia* and *Lantana camara* and as per their instructions, fresh leaves from nearby areas of Mattom and Kunnamkulam in Thrissur district, Kerala, India were collected.

4.2 Extraction of oil from plant leaf

Literature data regarding the presence of flavones, glycosides and aromatic terpenes from the leaves of *Premna latifolia Roxb*. was already available (Bheemasankara Rao et al. 1981, Bheemasankara Rao et al. 1978, Suresh et al., 2011). In our work, Fresh leaves of the plant was collected and dried in shade. It is then powdered and steam distilled for 6 hours. After taking the leaf extract in a separating funnel, it is shaken well with petroleum ether. The combined ether extract is dried with anhydrous Na_2SO_4. The ether extract was concentrated under reduced pressure and the distillate is again collected.

5. Thin Layer Chromatography (TLC)

A primary screening of the leaf extract was done using thin layer chromatography as it was already proven to be suitable for separating steroids, lipids, carotenoids and quinines. Previous reports were already available about the utility of thin layer chromatography in analyzing the organic compound constituents [Gupta et al., 1963, Wollish et al., 1961, Nigam et al., 1965). Silica gel was used as the adsorbent and is applied on the glass plate by pouring method. After activating the plate, it was developed using the solvents ethyl acetate and benzene in the ratio 9:1.

5.1 Spray reagents for TLC
(a) Conc. H_2SO_4
50% Conc. H_2SO_4 was used.

(b) Anisaldehyde-H_2SO_4 reagent
0.5 ml anisaldehyde was mixed with 10 ml glacial acetic acid and 85 ml methanol and 5 ml Conc. H_2SO_4 in the corresponding order. The plate is sprayed, heated to 100^0 C for 5-10 min and then evaluated.

(c) Vanillin-Sulphuric acid reagent
The reagent was prepared by dissolving 1 g vanillin in ethanol (100 ml) and 5 ml sulphuric acid in 100 ml ethanol. The chromatogram (TLC) was sprayed first with 5% H_2SO_4, followed immediately by 1% ethanolic vanillin. The sprayed plate is then heated at 110^0C for 5-10 minutes until maximum visualization of the spot.

(d) Baeyer's reagent
Alkaline potassium permanganate solution was used as the Baeyer's reagent. After spraying the plate with the reagent, unsaturation is indicated by the discharge of pink colour.

(e) Liebermann-Burchard reagent
5 ml acetic anhydride was added carefully to 5 ml Conc. H_2SO_4 and this mixture was added to 50 ml absolute alcohol, while cooling in ice. The sprayed plate is heated to 110^0C until maximum visualization of spots.

6. Materials and Methods for Anti Bacterial and Anti Insecticidal Activity

Bacterial strainsHuman pathogens such as Escheria coli and Salmonella typhimurium were collected from the Dept. Of Aquaculture and Fishery microbiology, M.E.S. College, Ponnani. Cultures were serotyped at National Salmonella and Eischerichia centre, Kasauli, Himachal Pradesh. Cultures were maintained on nutrient agar slants and acetone was used as the solvent for preparing solutions (Goran Kronvall et al., 2011). The antibacterial activities of compounds under study against the two selected bacterial stains were done using Cup-Plate method. The medium for testing anti bacterial activity was nutrient agar containing 5g peptone, 15g agar, 4g D-glucose, 5g Nacl, 1.5g beef extract, 1.5g yeast extract and 100 ml distilled water. The pH was adjusted at about 7.4 ± 0.2. The two bacterial stains were enriched in nutrient for 16-18 hours and these young cultures were used as inoculums. To prepare a mat growth of bacteria on petri dishes, young cultures of Eischerichia Coli and Salmonella typhimurium were swabbed over sterile nutrient agar plates using sterile cotton swabs. Using a sterile cork boarer, wells (10 mm) were made on these plates. In each well 100µl (100 ppm) of the compound in acetone was added and the results were interpreted. The results of antibacterial activity are being interpreted as:
5mm-15mm as resistant, 15-25 mm as moderately sensitive and beyond 25 mm zones of inhibition are regarded as very sensitive.

7. Gas Chromatography - Mass Spectrometry (GC-MS)

Gas chromatography is one of the most useful instrumental tools for separating and analyzing organic compounds that can be vapourised without decomposition. For volatile compounds, fatty acids, mono and sesquiterpenes and hydrocarbons, gas chromatography is generally used (Carmen W Huie et al., 2002). Gas chromatography in combination with mass spectroscopy has revolutioned the analytical field. The GC-MS combination can deal with a few nanograms of the material and can generate large amount of information. Capillary GC columns can be connected directly to the mass spectrometer without an enriching device and this allows MS scans to be carried out rapidly to provide several analyses at different points of a GC peak. Thus partially overlapping chromatography peaks can be easily resolved. GC-MS was performed on a 6890-series GC system (HEWLETT PACKWARD) equipped with 5973 mass selective detector. The column used was a DB-5 column of length 30 cm and diameter 0.32 mm. The carrier gas was helium and the heating rate was 20^0C per minute. Injection port temperature was 190^0C and interface temperature about 300^0C. Quantification of the compounds was made by percentage-peak-area calculations.

8. Results

The results obtained can be summarized as

Table 1: TLC characterization of class of compounds present in leaves of *Premna latifolia*

Sl.No.	Spray reagent	Observation (Colour)	Inference
1	Conc.H_2SO_4	Black spots	Presence of organic compounds
2	Anisaldehyde-sulphuric acid reagent	Red violet	Terpenoids present in essential oil
3	Vanillin-sulphuric acid reagent	Blue	Terpenoids
4	Baeyer's reagent	Decolourised	Presence of unsaturated compounds
5	Liebermann-Burchard reagent	Pink	Presence of triterpenoids

Table 2: TLC characterization of class of compounds present in leaves of *Lantana camara*.

Sl. No.	Spray reagent	Observation (Colour)	Inference
1	Conc.H_2SO_4	Black spots	Presence of organic compounds
2	Anisaldehyde-sulphuric acid reagent	Blue	Mono terpenoids
3	Vanillin-sulphuric acid reagent	Blue black	Terpenoids
4	Baeyer's reagent	Decolourised	Unsaturated compounds
5	Liebermann-Burchard reagent	Pink	Presence of triterpenoids

The antibacterial activity was detected by measuring the diameter of the inhibitory zone around each well. The diameters of inhibition zones were measured and recorded.

Table 3: Anti bacterial activity of essential oil of *Premna latifolia*

Sl. No.	Name of Bacteria	Zone of Inhibition (mm)	Interpretation
1	Escherichia coli	27	Sensitive
2	Salmonella typhimurium	20	Moderately active

Table 4: Anti bacterial activity of essential oil of *Lantana camara*

Sl. No.	Name of Bacteria	Zone of Inhibition (mm)	Interpretation
1	Escherichia coli	25	Sensitive
2	Salmonella typhimurium	18	Moderately active

Table 5: Effectiveness of antibiotics towards bacteria (compared with Kirby Bayer chart)

Sl. No.	Name of Bacteria	Names of antibiotics		
		Gentamycin	Penicillin	Ampicillin
1	Escherichia coli	S	R	R
2	Salmonella typhimurium	R	S	S

S - Sensitive, R – Resistant

By comparing tables 3, 4 and 5, it was observed that Escherichia coli and Salmonella typhimurium were more susceptible towards essential oils obtained from the leaves of the two plants in comparison to that of antibiotics. Since the leaves of the plants *Premna latifolia* and *Lantana camara* were sensitive towards bacteria's Escherichia coli and Salmonella typhimurium, the leaves of such plants can be used as anti bacterial and anti insecticidal agents.

The GC-MS analysis (Figure 3) of the leaf extract of *Premna latifolia* showed the presence of α-humulene, camphene and germacrene B as the major chemical components. The compounds present and their retention times are given in Table 6.

Table 6: GC-MS analysis of leaves of *Premna latifolia*

Sl. No.	Components	Retention time
1	α-humulene	13.258
2	camphene	18.402
3	germacrene B	22.258
4	Unidentified	17.889

In the mass spectrum of germacrene B (the compound having the maximum retention time in Table 6), the molecular ion (M^+) peak appears at m/z 204. The other peaks at m/z values 189, 161, 133, 121 (base peak), 105, 93, 79, 67, 53, 41 ..... also are in agreement with the structure of germacrene B (Fig. 4). The mass spectral fragmentation patterns of Germacrene B is depicted in Fig. 5.

The GC-MS analysis of the leaf extract of *Lantana camara* showed the presence of α-terpinene, β-caryophyllene and germacrone as the major components. The compounds and their retention times are given in Table 7 and the GC is shown in Fig.6.

Table 7: GC-MS analysis of the leaves of *lantana camara*

Sl. No.	Components	Retention Time
1	α-terpinene	11.651
2	β-caryophyllene	11.982
3	germacrone	1 2.225
4	Unidentified	12.89
5	Unidentified	13.515

In the mass spectrum of β-caryophyllene, the molecular ion (M^+) peak appeared at m/z 204.1. The other peaks at m/z values 133, 105, 93, 91, 79, 69, 55, 41, 39.....etc are also quite consistent with the structure of β-caryophyllene. For germacrone, the peaks at m/z values 218, 203, 135, 121, 108, 91, 67, 41.....are in agreement with its structure.

Conclusion

Phytochemical investigation of essential oils of the leaves of *Premna latifolia* identified three components by GC-MS which were α-Humulene, camphene and Germacrene B. The GC-MS analysis of essential oils of leaves of *Lantana camara* identified α-terpinene, β-caryophyllene and Germacrone as the major components. TLC characterization of the leaves of both plants revealed the presence of terpenoids. The leaf extracts of both plants were found to exhibit both anti microbial and anti insecticidal activity. Also the leaf extracts were found to be sensitive against bacteria Escherichia coli and moderately active in Salmonella typhimurium by comparing with the Kirby Bayer chart. Hence the two plants, *Premna latifolia* and *Lantana camara* were actually a boon to the villagers since the villagers can avoid the use of persistent chemicals such as DDT and silver nano particles against the lice infecting their birds. Hence the development of low operational cost, eco friendly medicines should be encouraged rather than bioaccumulating chemicals in order to bring down environmental pollution and health hazards. With the aid of details revealed by phytochemical tools such as gas chromatography mass spectrometry (GC-MS), thin layer chromatography (TLC) and anti insecticidal activity, we came to the conclusion that it is not the same compound, but different compounds having the same property are responsible for the activity of the leaves of these two plants against bird lice.

To be more precise, mono and sesqui terpenoids present in the leaves of these plants are responsible for their anti bacterial and anti insectcidal activity. Monoterpenes like camphene, α-terpinene and sesquiterpenes such as α-Humulene, germacrene, β-caryophyllene, germacrone present in the leaves of these two plants are effective agents capable of controlling bird lice.

Research Highlights

1. Able to dependent on green plants rather than using hazardous chemicals.

2. Could reduce environmental pollution. Could help the whole community depending on poultry by making them aware of easily growing low cost medicinal plants against bird menopone.

Limitations

Only villagers in Kerala are mainly aware of the benefit of these two plants as effective medicine against bird lice. So it is necessary to educate the whole village community all over the world about the usefulness of the two plants *Premna latifolia* and *Lantana camara*. Also there is an ambiguity in the cultivation of these plants in different types of soil all over the world.

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