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Separation and Characterization of Antibacterial Compounds from *Aegle marmelos* Correa and *Thuja orientalis* L. Against Silkworm Pathogens

S. Manimegalai[1], R. Adhithya[2], S. Vellaikumar[3], M. Paramasivam[4] and S. Chandrasekaran[5]

[1]Associate Professor, [2]Senior Research Fellow, Department of Sericulture.
[3]Assistant Professor, Department of Plant Molecular Biology & Biotechnology.
[4]Research Associate, [5]Professor, Pesticide and Toxicology Laboratory, Department of Agricultural Entomology.
Tamil Nadu Agricultural University, Coimbatore- 641 003, Tamil Nadu, India.
[1]Author for Correspondence E- mail: megalai_siva@yahoo.com

Abstract

Antibacterial compounds from *Aegle marmelos and Thuja orientalis* extracted with chloroform proved effective against two gram positive pathogenic bacteria, *Staphylococcus aureus* and *Bacillus thuringiensis* infecting mulberry silkworm, *Bombyx mori* L. The chloroform extracts produced maximum inhibition against the pathogens. Thin layer chromatography separation and further analysis using GCMS revealed the presence of stigmast - 3- 5n-ol in leaf extract of *Aegle marmelos* and phenanthrene carboxylic acid in the bark extract of *Thuja orientalis*. Our results confer the utility of antibacterial compounds thus characterized for developing a novel drug for management of *S. aureus* and *B. thuringiensis*.

Keywords: silkworm bacterial pathogens, antibacterial fractions, *Aegle marmelos, Thuja orientalis,* thin layer chromatography, Gas chromatography and Mass Spectroscopy.

Introduction

The mulberry silkworm, *Bombyx mori* L. known for producing silk cocoons are affected by viral, fungal and protozoan pathogens (Das Gupta, 1950) among which bacterial pathogens independently cause cocoon loss to the tune of 75 per cent (Sidhu and Singh, 1968). The efficacy of antibiotics against bacterial pathogens of *B. mori*

was proved by several authors (Radha *et al.,* 1981; Samson, 1987; Baig *et al.,* 1990; Manimegalai and Chandramohan, 2008). Though bacteria is well managed by antibiotics, the activity of bacteria to acquire antibiotic resistance to drugs is a well known fact and hence limited attempts were made for the use of plant compounds especially the crude aqueous extracts of plants against silkworm bacterial pathogens (Selvakumar *et al.,*2001; Manimegalai and Chandramohan, 2005; Priyadharshini, 2006).

Though India has a very rich diversity of flora, its potential is not tapped to a greater extent. However, by using the science of ethnobotany and ethnopharmacognosy as a guide, the chemists came out with different sources and classes of compounds (Gurib- Fakim, 2006). Bioactive compounds currently extracted from plants serve as healing compounds for several ailments.

This paper deals with separation of antibacterial compounds from *Aegle marmelos and Thuja orientalis* against silkworm pathogens, *Staphylococcus aureus* and *Bacillus thuringiensis*. This is the first report on characterization of antibacterial compounds from plants against bacterial pathogens of *B. mori*.

Preliminary studies conducted to identify the effective solvent for extraction of antibacterial compounds from *A. marmelos and T. orientalis* revealed the superiority of chloroform over hexane, petroleum ether, dichloromethane, acetonitrile and ethanol.

Materials and methods

Collection of plant samples

The samples of leaf from *Aegle marmelos* and *bark* from *Thuja orientalis* were collected from Coimbatore, Western zone of Tamil Nadu, India. The collected samples were washed thoroughly in running tap water followed by rinsing twice with sterile distilled water and shade dried. The shade dried plant samples were powdered with a blender for the purpose of extraction.

Preparation of plant extracts

25g of air dried powder was filled in thimble and soaked overnight in chloroform and then extracted with chloroform in soxhlet extractor for six hours (Khatune, 2000). The residual extract was collected in a flask and transferred to a rotary flash vaccum evaporator for evaporation of the solvent. The residue thus obtained was dissolved in acetone and stored at 5°c in air tight bottles until further use.

Culture of silkworm pathogens

The bacterial pathogens maintained in the insect pathology laboratory, Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore was utilized for the study. The cultures of the silkworm pathogens, *Staphylococcus aureus* and *Bacillus thuringiensis* were frequently sub cultured in fresh slants and stored for future works.

Agar well diffusion assay

Sterilized nutrient agar medium for *S. aureus* and T 3 medium for *B. thuringiensis*

was poured into sterile petriplates and allowed to solidify. Each petriplate was divided into four equal quarters using a marker pen and wells of 6 mm in diameter were made in each quadrat of the plate using sterile cork borer. For each organism, 20µl (1mg of extract concentration) of the prepared plant sample was loaded in each well using sterilized dropping pipette. Three replications were maintained for each treatment. The antibiotics, streptomycin sulphate and erythromycin each at 100 µg/ml served as positive control for *S. aureus* and *B. thuringiensis* respectively. 100 per cent acetone served as negative control since plant extracts were diluted in acetone.

Separation of antibacterial compounds through thin layer chromatography

Different solvent systems (ranging from low polar to high polar) were tested for the effective separation of antimicrobial compounds from chloroform extract of *Aegle marmelos and Thuja orientalis.* A suitable mobile phase (chloroform) was standardized based on the separation of antibacterial compounds. Chloroform was poured in the TLC tank and the TLC plate spotted with plant extract was kept in the tank with approximately 0.5 mm immersed in solvent at the bottom. The tank was closed with a glass lid so as to have the chamber completely filled with the solvent vapours. The plates were kept in the TLC tank till the solvent front reached the top of TLC plate. Then, the plate was removed from the tank and kept in open air at room temperature for evaporation of the solvent.

The TLC run plates were observed under bright light and the separated spots were marked and the respective Relative front values were calculated using the formula.

$$\text{Rf value} = \frac{\text{Distance moved by the solute from the origin}}{\text{Distance moved by the solvent from the origin}}$$

Minimum inhibitory concentration (MIC) of plant extracts

The minimum inhibitory concentration of active bands from the two plant extracts were determined by tube dilution method (Claeys *et al*., 1988). Four sets of four eppendorf tubes were taken to which 900 µl of 24 h old bacterial inoculum at 10^{-4} dilution was added. To the first tube of first set, 0.1 ml of plant extract was added and serially diluted till the fourth tube. To the second set, 0.1 ml of the negative control (acetone) was added and serially diluted. To the third set, 0.1 ml of antibiotics solution was added and serially diluted. Fourth set contained microbial inoculum only. The eppendorf tubes were then incubated for an hour. 50 µl of the suspension was taken from each tube and spotted on the medium in a petridish divided already into four equal quadrats. The plates were incubated for 24 hours at 37 ˚c. Observations were made for the visible growth of the organism. The tube containing the lowest concentration of the extract which when streaked on the plate did not show any visible growth of organism after 24 h was considered as minimum inhibitory concentration.

Gas Chromatography – Mass Spectrometer analysis

Analysis was conducted using Shimadzu (QP 2010. plus) GC MS with autosampler (AOC- ZOS) and auto injector (AOC- ZOI). MS conditions were as follows: Detector mass spectrometer voltage 70 ev and its source temperature was 200˚c. The injector temperature was 250˚c and split mode (1:3) 1 µl injection. The DB -1 column was

performed with length 30 m x 0.25 mm and coating thickness film, 0.25 µm. The oven was adjusted at 150˚c for 1 min and increased at the rate of 3˚c/min upto 200˚c for 2 min. The final temperature of 220˚c was achieved with increment of 4˚c/min and holding for 7 min. The total running time was 31.67 min.

The components were identified by comparing their retention times with those of authentic samples as well as by comparing their mass spectra with those of Wiley 275 library.

Results

Susceptibility of silkworm pathogens to plant extracts

Diameter of Inhibition zone (DIZ) produced by plant extracts were comparable to positive control (Table 1). From the results, it is inferred that bark extract of *Thuja orientalis* proved effective against both *Bacillus thuringiensis* and *Staphylococcus aureus* with DIZ of 23.00 and 16.00 mm respectively and leaf extract of *Aegle marmelos* was effective only against *S. aureus* (16.00 mm).

Table 1: Diameter of inhibition zone of *Thuja orientalis* and *Aegle marmelos* against silkworm pathogens.

Plant	Pathogen	Mean diameter of Inhibition in mm*		
		Chloroform	Negative control	Positive control
Thuja orientalis (Bark)	*Bacillus thuringiensis*	23.0	-	24.0 (Erythromycin)
	Staphylococcus aureus	16.0	-	16.0 (Streptomycin sulphate)
Aegle marmelos (leaf)	*B. thuringiensis*	-	-	-
	S. aureus	16.0	-	17.0 (Streptomycin sulphate)

"_" indicates no zone of inhibition

*Mean of two strains of same pathogens replicated thrice

Thin layer chromatography

Separation of compounds by thin layer chromatograph revealed the presence of five bands from bark extract of *T. orientalis* and six bands from leaf extract of *A. marmelos* under bright light. (Plate 1 and 2). The rf values ranged from 0.06 to 0.81 for five fractions from *T. orientalis*. Similarly, six different fractions from *A. marmelos* exhibited rf values that ranged from 0.03 to 0.78 (Table 2).

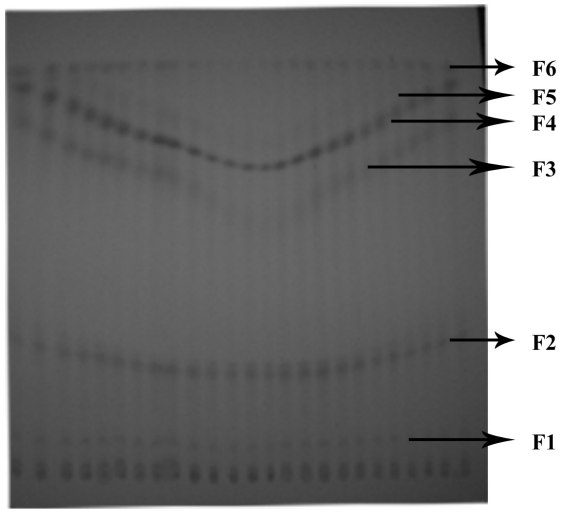

Plate 1: TLC Fractions from chloroform leaf extract of *Aegle marmelos* visualized under bright light

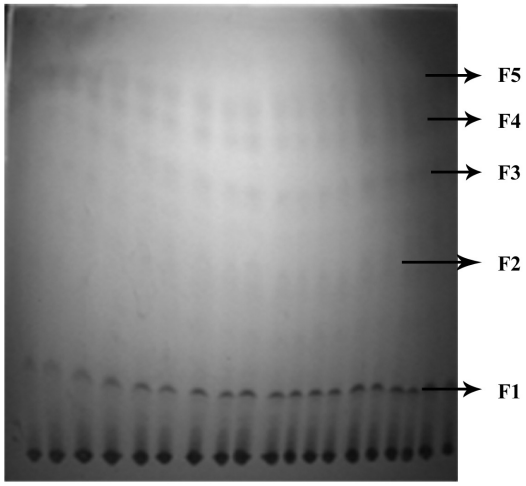

Plate 2: TLC Fractions from chloroform bark extract of *Thuja orientalis* visualized under bright light.

Determination of Minimum inhibitory concentration

Minimum inhibitory concentration studies carried out revealed that all the visible bands showed potent antibacterial property (Table 2). MIC was found to be 250 µg/ml for all the fractions of *T. orientalis* against both the pathogens, *B. thuringiensis* and *S. aureus*. In case of *A. marmelos*, fraction 1 and fraction 5 were found effective against *S. aureus* at 250 µg/ml and fraction 2 and 3 exhibited the activity at MIC of 500 µg/ml.

Table 2: Minimum Inhibitory Concentration of *Thuja orientalis* and *Aegle marmelos* fractions on *B. thuringiensis* and *S. aureus.*

Plant	TLC studies		MIC in µg/ml	
	Fractions	Rf value	*B. thuringiensis*	*S. aureus*
Thuja orientalis (bark)	F1	0.06	250	250
	F2	0.28	250	250
	F3	0.41	250	250
	F4	0.53	250	250
	F5	0.81	250	250
Aegle marmelos (leaf)	F1	0.03	_	250
	F2	0.06	_	500
	F3	0.28	_	500
	F4	0.66	_	250
	F5	0.74	_	250
	F6	0.78	_	250

"-" not effective

GC MS Characterization of antibacterial compounds

The compounds characterized from various TLC fractions obtained from *T. orientalis* and *A. marmelos*, their molecular formula and molecular weight are presented in Table 3 and Table 4 respectively.

Table 3: GC MS characterization of antibacterial fractions from the chloroform leaf extract of *Aegle marmelos.*

Fractions	Compounds characterized	Retention time (min)	Area (%)	Molecular formula	Molecular weight (gm/mol)
1	Stigmast-3-5n-ol	28.70	70.44	$C_{29}H_{50}C$	414.00
2	1-Octadecene	14.95	15.72	CH_2=CH $(CH_2)_{15}$ CH_3	254.48
3	1-Octadecene	9.87	13.59	CH_2= CH $(CH_2)_{15}$ CH_3	254.48
4	1,2-benzene dicarboxylic acid	29.87	19.94	$C_8H_6O_4$	390.00

Table 4: GC MS characterization of antibacterial fractions from the chloroform bark extract of *Thuja orientalis.*

Fractions	Compounds characterized	Retention time (min)	Area (%)	Molecular formula	Molecular weight (gm/mol)
1	Tetratricontane	23.34	24.92	$C_{34}H_{70}$	478.91
2	Dioctyl phthalate	30.08	30.05	$C_6H_4(COOC_8H_{17})_2$	390.56
3	1,2-Benzene dicarboxylic acid	30.06	24.88	$C_8H_6O_4$	390.00
4	1,2-Benzene dicarboxylic acid	29.84	36.39	$C_8H_6O_4$	390.00
5	1-Phenanthrene carboxylic acid	22.10	48.11	$C_{20}H_{28}O_2$	316.00

Stigmast -3-5n-ol with molecular weight of 414. 00 g/mol was the major compound characterized from *A. marmelos* followed by 1, 2 benzene di carboxylic acid. (Fig. 1 and Fig.2)

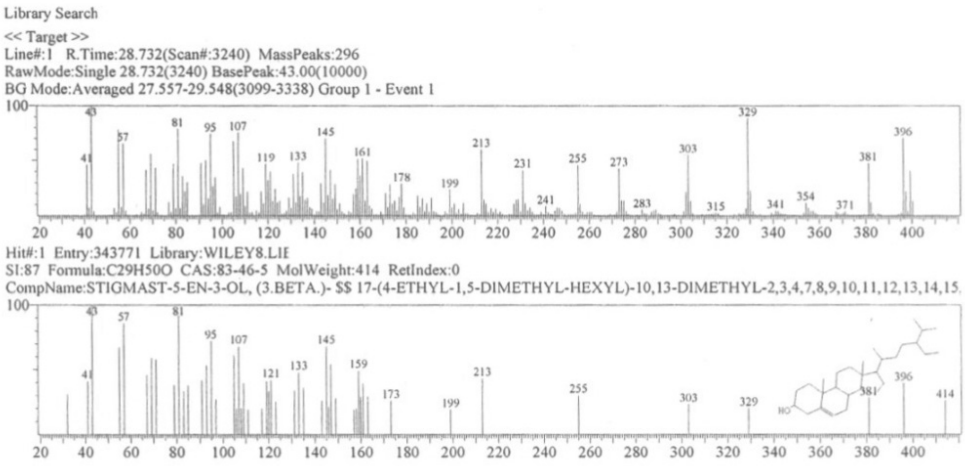

Figure 1: Mass spectrum of twelfth peak in fraction I of *Aegle marmelos*, retention time of 28.732 min with molecular weight 414 matched with stigmast-5-en-3-ol.

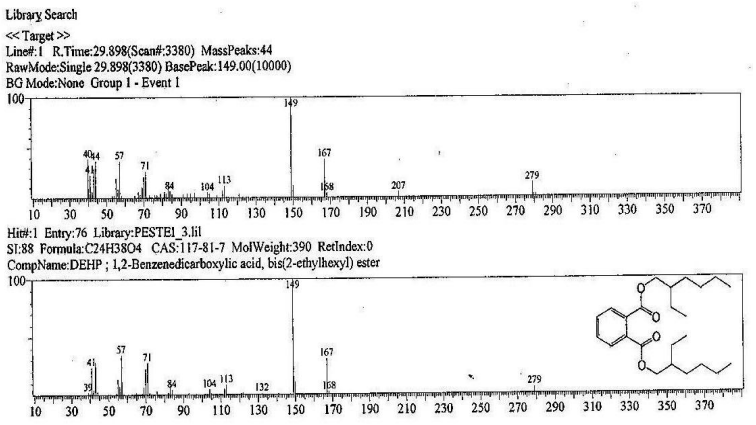

Figure 2 & 4: Mass spectrum of ninth peak in fraction 3 of *Thuja orientalis*, retention time of 29.898 min with molecular weight 390 matched with 1, 2- Benzene dicarboxylic acid.

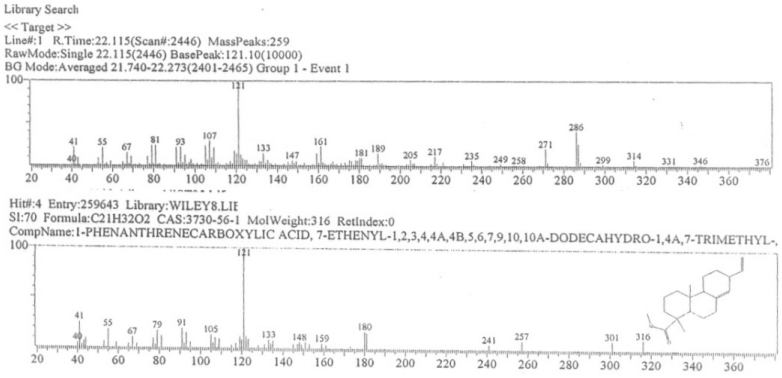

Figure 3: Mass spectrum of tenth peak in fraction 5 of *Thuja orientalis*, retention time of 22.115 min with molecular weight 316 matched with 1- phenanthrene carboxylic acid.

The major antibacterial compound characterized from *T. orientalis* was 1-phenanthrene carboxylic acid with molecular weight of 316. 44 g/mol followed by 1, 2 benzene di carboxylic acid with molecular weight of 390 g/mol. (Fig. 3 and Fig.4)

Discussion

Chloroform bark extracts of *Thuja orientalis* proved effective against *Staphylococcus aureus* and *Bacillus thuringiensis* infecting *Bombyx mori*. Similarly, chloroform leaf extract of *Aegle marmelos* was effecting in managing *B. thuringiensis*. Separation of compounds by thin layer chromatography and further analysis through GC MS revealed the presence of antibacterial compounds stigmast- 3- 5n-ol in leaf extract of *A. marmelos* and phenanthrene carboxylic acid in the bark extract of *T. orientalis*.

Manimegalai *et al*. (2009) characterized the diterpenoid, 20- de oxocarnosol and 6 β hydroxycarnosol from *Plectranthus amboinicus* against nuclear polyhedrosis virus of *B. mori*.

GC MS analysis of chloroform extract of *Aegle marmelos* leaves showed the presence of Stigmast – 3- 5n- ol as the major compound. This compound is a phytosterol and reported to be having inhibitory effects on growth, development and steroid metabolism in *B. mori* (Nario Awata *et al.*, 1976). The major compounds present in the bioactive fraction of leaf extract of *Aegle marmelos* were octadecene and benzene dicarboxylic acid. Benzene dicarboxylic acid was also reported to have antimicrobial action against bacterial pathogens.

The chloroform bark extract of *Thuja orientalis* yielded a number of compounds, among which phenanthrene carboxylic acid accounted for major proportion followed by 1, 2- benzene dicarboxylic acid and Tetratriacontane.

The moderate efficacy of essential oil extracted from fruit of *T. orientalis* (*Platycladus orientalis*) against *S. aureus* was reported by Hassanzadeh *et al*. (2001) and the compounds characterized from the oil were α pinene, sabinene, 3 carene, limonene and cedrol. The efficacy of leaf extract of *Thuja occidentalis* against human pathogen, *S. aureus* was reported by Jahan *et al*. (2006) and Zafar *et al*. (2009). Manimegalai and Chandramohan (2005) reported the efficacy of aqueous extract of *T. orientalis* against *B. thuringiensis* infecting *B. mori.*

Conclusion

In conclusion, chloroform extract of plants, *Thuja orientalis* and *Aegle marmelos* showed potent antibacterial effect against bacterial pathogens of mulberry silkworm, *Bombyx. mori*. This was evidenced by GC MS study which revealed the presence of stigmast 3-5-n-ol in leaf extract of *Aegle marmelos* and phenanthrene carboxylic acid in the bark extract of *Thuja orientalis.* These compounds could be further exploited for developing a formulation against bacterial disease of *B. mori*.

Acknowledgement

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