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Isolating Bioactive Compound from Marine Prosobranch *Purpura persica* from Tuticorin Coast

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Abstract: Antibiotics have revolutionized life saving medicine by providing cure for many number of life threatening diseases in human history, unexpectedly, many pathogenic microorganisms have developed resistant towards current antibiotic and this trend has become more and more serious. Hence the present study has been aimed to find out new marine derived antibiotic from Prosobranch mollusc *Purpura persica.* The whole body crude extract of methanol was partially purified by normal phase silica gel 160-120 mesh (Glaxo, Bombay) column chromatography with low polar to high polar solvent Hexane: Chloroform (F1); Chloroform (F2); Benzene (F3); Benzene: Methanol (F4), and Methanol F5). The antimicrobial activity of crude and eluted fractions were assayed against ten bacterial pathogens viz *Aeromonas hydrophila, Bacillus cereus, Escherichia coli, Pseudomonas aerogenosa, Salmnella typhi, Shigella flexneri, Vibrio cholera 0139, Vibrio cholera classical, Vibrio cholerae 01790 and Vibrio cholerae EITOR* and nine fungal pathogens viz. *Aspergillus flavus, Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Fusarium moniliforme, Trichoderma sp. Penicillium citrinum, Penicillium oxallicum and Rhizopus sp.* respectively using the agar disc diffusion method. To find out the most probable antibiotic compound HPLC and GC-MS studies were carried out. Among the tested bacterial pathogens *S. typhi, P. aerogenosa, S. flexneri* and *B. cereus* and fungal pathogens *A. fumigatus, A.* terreus, *F. moniliforme* and *Trichoderma sp.* showed inhibition in growth by crude, F2, F3 and F5 fractions of *P. persica* respectively. The GC-MS and HPLC analysis revealed the presence chloridate cholest-5-en-3-01(3a) - carbano chloridate, a chloride compound 9, 12-octadecadienoyn chloride (z, z), and a cholest-5-en-3-ol(3)-carbonochloridate, eugenol, dibutyl phthalate, 1,2-Benzenedicarboxylic acid, diisooctyl ester and Phthalic acid, bis (7-methyloctyl) ester, and a steroid cholest-5-ene, 3-bromo-(3a), 2-piperidinone a monoterpene azulene, a fluro compound acetic acid, tri fluro- tetradecyl which were responsible for inhibiting the growth of microbes tested and the present test organism *P. persica* have great potential for developing useful drugs.

Keywords: Antibacterial Activity, Solvents, Inhibitory Zone, GC-MS Analysis, Test Pathogens

1. Introduction

The knowledge acquired in the past two decades and the discovery of new groups of antimicrobial compounds makes natural antibiotics the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections [1, 2]. Different therapeutic applications of these compounds, from topical administration to systematic treatment of infections, have been developed by several biotechnological companies (http://www.inimaxpharma.com; http://biotech .deep13.com//alpha/alpha.html)

It is widely accepted among clinician medical workers,

microbiologist and pharmacologist that antibiotic resistant will, in the near future, leave health care professional without effective therapies for bacterial and fungal infections. The critical events are the emergence of *Staphylococcus aureus* with decreased sensitivity to methicilin [3], worldwide resistance to penicillin in *Staphylococcus pneumonia* and multiple resistances to *Mycobacterium tuberculosis*. The emergence among enterococci of resistance to another useful and widely effective antibiotic, vancomycin [4], might accelerate the spread of vancomycin- resistant genes via plasmids; throughout other species eventually limiting the efficacy of this drug. Consequently the priority for the next decades should be focused in the development of alternate drugs and as the recovery of natural molecules should be as natural as possible with a wide range of action over several pathogens easy to produce and not prone to induce resistant.

A very different kind of substances have been obtained from marine organism among other reasons because they are living in a very exigent, competitive and aggressive surroundings very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules. The knowledge of the physiological and bio chemical features of marine organism might contribute to the identification of natural products of bio medical importance. Over the last 30 years many structurally novel antimicrobial metabolites have been isolated from marine organisms.

Marine invertebrates offer good source of potential antimicrobial drugs [5, 6]. Studies on antimicrobial mechanisms and compounds of marine invertebrates may provide valuable information for new antibiotic discoveries and give new insights into bioactive compounds in molluscs. Among the invertebrates, the molluscs are very good source for biomedical important products [7]. Many classes of molluscs with bioactive compounds like antitumour, antileukemic, antibacterial, cytotoxic, anti inflammatory and antiviral properties have been reported [8,9]. These reports suggest that molluscs are the rich source for discovering novel lead compounds for the possible development of new types of antibiotics for pharmaceutical use. Keeping the importance of gastropods in terms of bioactive compounds with antibacterial properties, the present study has been undertaken to determine the antimicrobial activity of extracts from *P. persica* against various human pathogenic microorganisms.

2. Materials and Methods

2.1. Collection and Preparation of Samples

The mollusc *P. persica* was collected from intertidal rocky shore of harbour area of Gulf of Mannar, nearby Theraspuram Tuticorin, situated in the South east coast of India, during April 2015 to December 2015. The collected samples were rinsed with sterile sea water and carefully removed from their shells. The flesh was cut into small pieces and air-dried. The air-dried flesh was immersed in 100% A. R. Grade methanol for 10 days at room temperature. The extract from the solvents was filtered by using Whatman no. 1 filter paper and evaporated to dryness in rotary evaporator and the dried extract was stored at 0°C for further use.

2.2. Microbial Strains Used

Antimicrobial activity of tissue extracts were determined against 10 different bacterial pathogens, viz., *Aeromonas hydrophila, Bacillus cereus, Escherichia coli, Pseudomonas aerogenosa, Salomnella typhi, Shigella flexneri, Vibrio cholera* 0139, *Vibrio cholera classical, Vibrio cholerae 01790 and Vibrio cholerae* EITOR. These clinical strains were obtained from Basic Biomedical sciences, Bharathidasan University, Trichy.

2.3. Antimicrobial Susceptibility Assay

In vitro anti bacterial activity was assayed by the disc diffusion method of Bauer *et al.*, [10], Antibacterial activity was expressed in diameter zone of inhibition which was measured with the outer side of the disc to inner side of the inhibition zone. Each active extract was tested thrice for confirmation of activity.

Invitro antifungal activity was determined using the techniques of Kelman *et al.*, [11]. The fungi pathogens *Aspergillus flavus, Aspergillus terreus, Aspergillus niger, Aspergillus fumigatus, Fusarium moniliforme, Trichoderma sp. Penicillium citrinum, Penicillium oxallicum and Rhizopus sp.* were obtained from TNAU, Coimbatore. Crude extract was assayed for anti fungal activity following the above mentioned disc diffusion method.

After initial screening the extracts showing broad spectrum were fractionated using normal phase silica gel 160-120 mesh (Glaxo, Bombay) column chromatography with low polar to high polar solvent Hexane: Chloroform (F1); Chloroform (F2); Benzene (F3); Benzene: Methanol (F4), and Methanol F5). The fractions thus obtained were once again evaporated, concentrated, and assayed for antibacterial and antifungal activity. The extracts showing broad spectrum activity was examined for MIC by testing at different concentrations viz; 1mg/ml, 10mg/ml, and 100mg/ml.

The most potent fractions of test mollusc were subjected to HPLC and GC/MS to characterize the possible compounds responsible for antimicrobial activity.

GC-MS analysis: GC-MS analysis was carried out on a GC Clarus 500 Perkin Elmer system comprising a AOC 20i auto sampler and gas chromatography interfaced to a mass spectrometer (GC-MS).

2.4. Identification of Compounds

Interpretation on mass spectrum GC-MS was conducted using the data base of National Institute Standard and Technology (NIST) from Dr. Duke's phytochemical and Ethnobotanical databases.

2.5. Confirmation of Some of the Characterized Compounds by HPLC

In order to prepare the correct solvent system for the most potent fraction of test animal (F3 fraction of *P. persica*) the silica gel column purified extracts of test animals were repurified by alumina column and TLC for HPLC analysis. The isolated compounds were analyzed by HPLC (Shimadzu, Advanced VP binary series) using a C18 Hhypersil (ODS) column. Standards were run to confirm the presence of the same in the isolated compounds of the test animals.

3. Results and Discussion

3.1. Antibacterial Activity of Extracts from Purpura persica

The crude extract inhibitory zone of *P. persica* range varied from 2mm (*P. aerogenosa*) to 7mm (*S. typhi*) (Figure 9). The crude extract was partially purified through silica gel column chromatography. Of the five fractions F2, F3and F5 of *P. persica* exhibited wide spectral antibacterial activity in most of the bacterial strains tested (Figure 10, 11 & 12). F2 fraction was highly active against *S. typhi* (19mm) and it also showed inhibitory activity against *P. aerogenosa* (13mm and *S. flexneri* (12mm). F3 fraction the maximum inhibitory zone was obtained in *V. cholerae* classical and *B. cereus* (11mm). Maximum spectrum antibacterial activity was attained at 100mg concentration against *S. typhi* (13mm) and minimum against *V. cholerae* 0179 (3mm) in F2 fraction.

Maximum inhibitory zone was exhibited against *B. cereus* (9mm) and minimum against *E. coli* (4mm) by F3 fraction. Several marine molluscan extracts possessed broad spectrum antibacterial activities affecting the growth of bacteria, fungi and yeasts [12]. The crude whole body methanol extracts of *P. persica* showed wide spectrum antibacterial activity against seven pathogens out of ten pathogens tested. Rajaganapathy [13] also reported that the methanol extract from the whole body of *Hemifuses pugilinus* exhibited activity against *Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumonia.* Similarly Santhanaramasamy and Murugan [14] experimentally analyzed the methanolic extract of *C. virgineus* and *C. ramosus* and they also observed the broad spectrum antibacterial activity of body tissue extract. Thilaga [15] screened the antibacterial activity *of* a marine mollusc *Babylonia spirata* against *bacterial* pathogens.

Similar result was reported by Chellam *et al.*, [16] in chloroform extract of *Pterai chinensis* which inhibited eight fish pathogens and the acetone extract in the same animal showed broad spectral activity against all the fish pathogens tested. Abraham *et al* [17] studied the antibacterial activity in alcoholic extracts of holothurians species inhibited *S. typhi.* The present study with test animal P. persica also corroborates the earlier findings of suppressing the activity of S. typhi. Even then also difference in antibacterial activity found in the molluscan extracts may depend on the solvents used for extracts and the compounds extracted [18]. Gnanamhal *et al.*, [19] found that 0.07 mg of *Trochus radiatus* inhibited *Proteus mirabilis* and 0.15 mg of extract inhibited *Serratia marcescens*. Some of the peptides obtained from oysters inhibited *E. coli* at 330 mg/ml and *Vibrio alginolyticus* at 162 mg/ml [20].

3.2. Antifungal Activity of Extracts from Purpura persica

Crude methanol extract of *P. persica's* inhibitory range varied in between 2mm (*A. flavus*) and (*A. fumigatus*) 6mm (Figure 1) (Figure 13). Among the silica gel fractions, maximum activity was observed in fraction F2 (Figure 14). Maximum inhibition zone was obtained against *A. fumigatus* (24mm) (Figure 2) and minimum against *A. niger* (5mm) in fraction F2. It also showed higher degree of inhibition zone against *A.* terreus (23mm), (Figure 3), F. moniliforme (22mm) (Figure 4), *Trichoderma sp.* (20mm) (Figure 5), *P. citrinum* (19mm) (Figure 6). *A. flavus* (18mm) and *P. oxallicum* (17mm) (Figure 7) Fractions F1 and F5 (Figure 15) also shown inhibition zone against *A. terreus* (21mm) and *Trichoderma sp.* (16 mm) respectively. In MIC maximum antifungal activity was exhibited against *F. moniliforme* (12 mm), (Figure 8) *A. terreus* (12 mm) and minimum against *Rhizopus* sp. (2 mm) at 1 mg level and maximum against *A. fumigatus* (!9mm) and minimum against *A. niger* (1mm) at 100mg level in F2 fraction (Figure 16). F1 fraction exhibited a maximum antifungal spectrum against *Aspergillus terreus* (10mm) even at 1 mg.

As a result potential activity was found in *P. persica's extract* against *A. fumigatus, A.* terreus, *F. moniliforme* and *Trichoderma sp.* Prem Anand and Patterson Edward [21] reported moderate antifungal activity from the extract of various bivalve molluscs. Highest inhibitory activity was observed against *Aspergillus niger* with methanol extract of *Microcosmus curvus* by Karthikeyan *et al.*, [22]. Similar result was obtained by Chandran *et al.*, [23, 24, 25] in *Perna viridis methanol* extract against *Aspergillus flavus* and *Mucor* sp. Comparable work was that of Mohamed Hussain & Ananthan [26, 27] who observed that the chloroform extract of *Didemnum psammathodes and Didemnum candidum* inhibited *Penicillium* sp. whereas no antifungal activity was noticed against *A. flavus, A. fumigatus* and *A. niger*. Several marine natural products showed significant antifungal activity. Callipeltins J and K, MIC at 1 µm [28]; the triterpene glycoside holothurin B, MIC at 1.56 µg/ml [29]; the macrolids neopeltolide MIC at 0.62 µg/ml [30]); AP, a polypeptide type AMP isolated from the Chilean scallop *Argopecten purpuratus*, showed antifungal activity against *F. oxisporum* and *Saprolegnia parasitica* [31].

3.3. Characterization of Most Potent Fractions of Test Mollusc

As the F2 & F3 of *P. persica* showed more antimicrobial activity these fractions were subjected to HPLC and GC-MS to characterize the complete carbon skeleton responsible for probable antimicrobial derivative from the test molluscs.

3.4. GC/MS and HPLC

When F2 fraction was subjected to GC/MS analysis a steroid compound with chloridate cholest-5-en-3-01(3a) - carbano chloridate with maximum percentage of 72.31% and a chloride compound 9, 12-octadecadienoyn chloride (z, z), were identified as antimicrobial compounds. In F3 fraction (Figure 17, 18, 19 & 20) of *P. persica* only six antimicrobial compounds were identified such as a phenolic compound eugenol, three Plasticizer compounds, dibutyl phthalate, 1,2-Benzenedicarboxylic acid, diisooctyl ester and Phthalic acid, bis (7-methyloctyl) ester, and a steroid cholest-5-ene, 3-bromo-(3a)-. Among the anti microbial compounds present, 79.95% was the 1, 2-Benzenedicarboxylic acid, diisooctyl ester. An alkaloid compound 2-piperidinone was found to be in maximum (12.89%) when F5 fraction (Figure 21, 22, 23, 24 & 25) of *P. persica* was subjected to GC/MS analysis. Apart from that this fraction also had a monoterpene azulene, a fluro compound acetic acid, tri fluro- tetradecyl ester (4.88%) and an alcoholic compound 1-Octanol, 2, 7-dimethyl (5.57%).

Various characterized compounds were confirmed in accordance with the merging of retention time shown by the standard retention time in HPLC. The retention factor of 12.018min, 7.603min, 14.20min and 19.275 of the isolated compounds from F3 fraction of *P. Persica* on HPLC were completely identical to that of standard azulene (Figure 26) diethyl phthalate (Figure 27), oleic acid (Figure 28) and eugenol (Figure 29). Marine molluscan extract are usually complex mixtures of bioactive molecules mainly proteins, peptides and sterols.

The HPLC and GC/MS spectra from *P. persica's extract* provided a complete carbon skeleton of chloridate cholest-5-en-3-01(3a) - carbano chloridate, a chloride compound 9, 12-octadecadienoyn chloride (z, z), and a cholest-5-en-3-ol(3)-carbonochloridate, a phenolic compound eugenol, three Plasticizer compounds, dibutyl phthalate, 1,2-Benzenedicarboxylic acid, diisooctyl ester and Phthalic acid, bis (7-methyloctyl) ester, and a steroid cholest-5-ene, 3-bromo-(3a) alkaloid compound 2-piperidinone a monoterpene azulene, a fluro compound acetic acid, tri fluro-tetradecyl ester and an alcoholic compound 1-Octanol, 2, 7-dimethyl which might be responsible for the inhibition of *S. typhi, P. aerogenosa, S. flexneri, B. subtilis, A. fumigatus, A. terreus* and *F. moniliforme* in the present study.

Similar finding was reported by Way On Mudianta *et al.*, [32] in an Indonesian sponge Halichondria sp. has provided 3- alkyl piperidine alkaloids tetradehydrohaliclonacyclamine A, the mono-N- oxide and a C-2 epimer. Brominated indoles 6- bromo 2-methylthioindolin-3-one extracted from Australian Muricid *Dicathais orbita* has been identified as anticancer drug indole derivatives of 6, 6′ -dibromoindigo have been antimicrobial activity [33]. An alkaloid Batzelladine L and M isolated by Hua *et al.*, [34] inhibits *S. aureus*.

Antifungal activity of crude and various fractions of *Purpura persica* against different pathogens

Fractionated extract (F2) of *Purpura persica* against pathogens

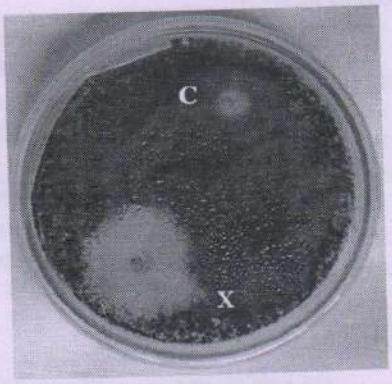

Figure 1. *Aspergillus fumigatus.*

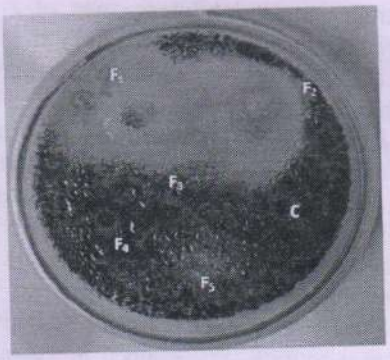

Figure 2. *Aspergillus fumigatus.*

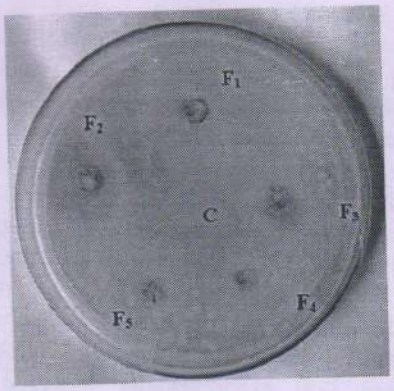

Figure 3. *Aspergillus terreus.*

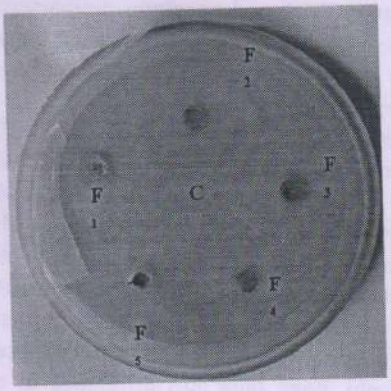

Figure 4. *Fusarium moniliforme.*

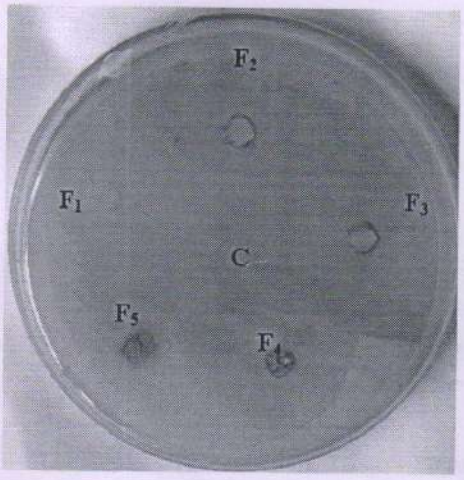

Figure 5. Trichoderma sp.

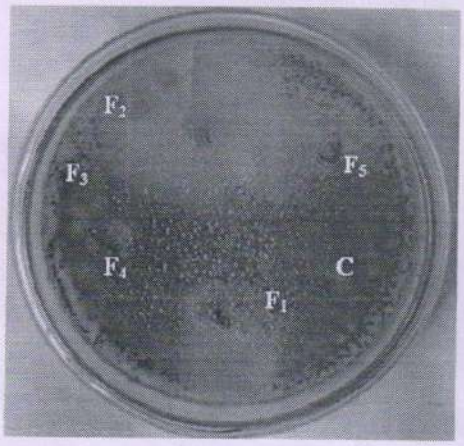

Figure 6. Pencillium citrinum.

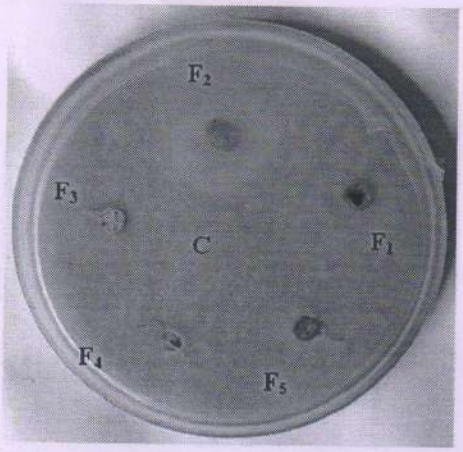

Figure 7. Penicillium oxalicum.

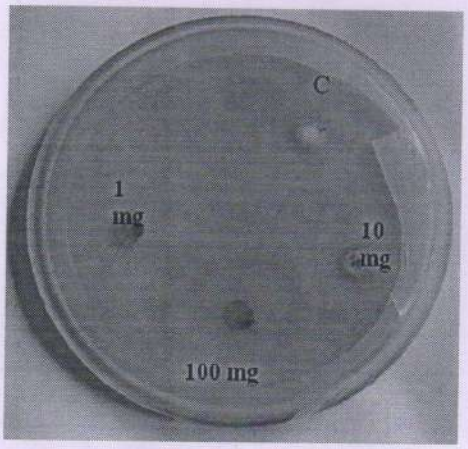

Figure 8. Fusarium moniliforme.

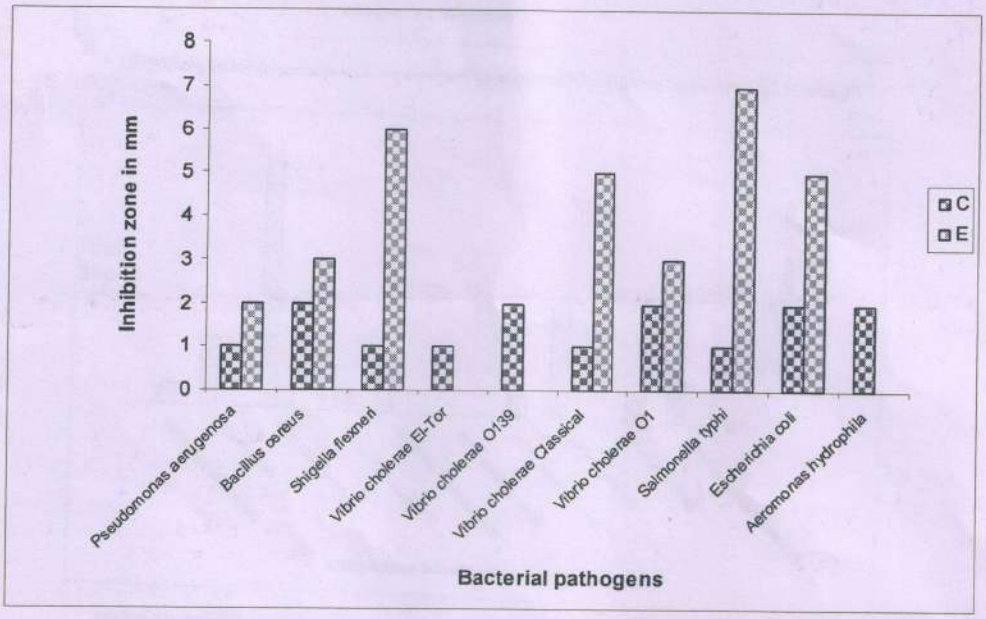

Figure 9. Antibacterial activity of crude extract of Purpura persica against different pathogens.

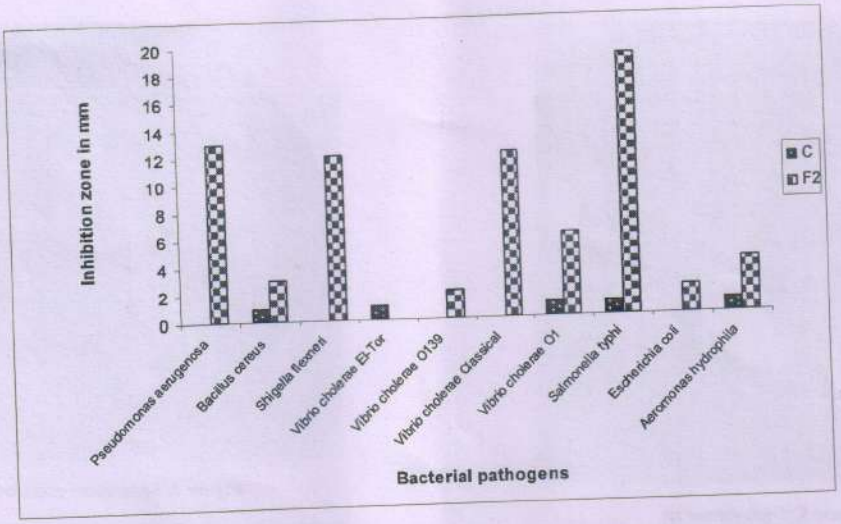

Figure 10. *Antibacterial activity of F2fraction of Purpura persica against different pathogens.*

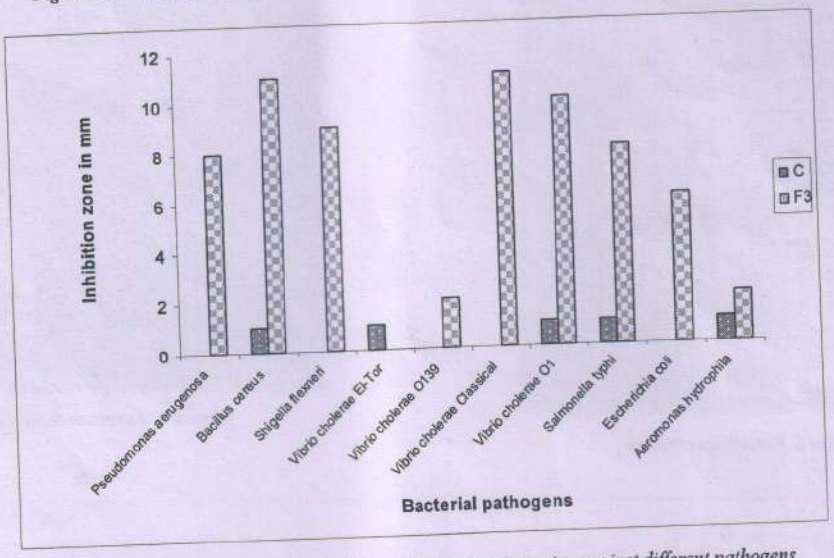

Figure 11. *Antibacterial activity of F3fraction of Purpura persica against different pathogens.*

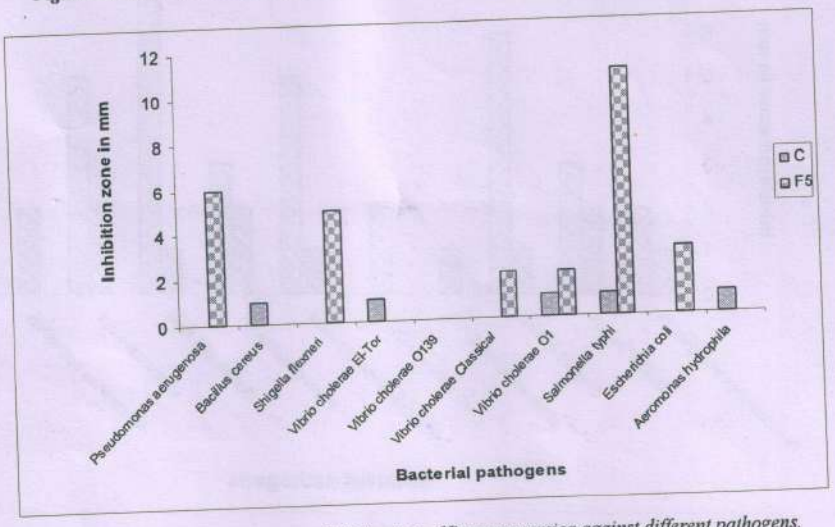

Figure 12. *Antibacterial activity of F5 fraction of Purpura persica against different pathogens.*

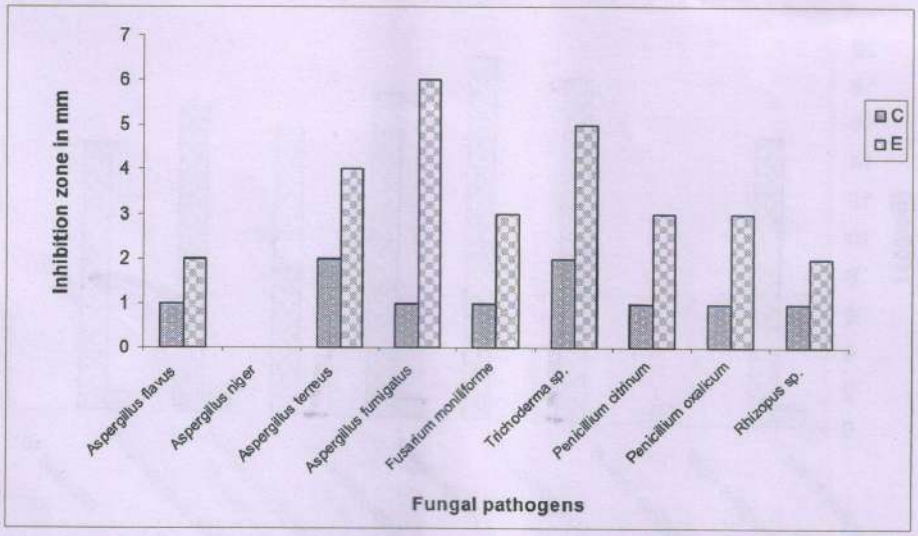

Figure 13. Antifungal activities of crude extract of Purpura persica against different pathogens.

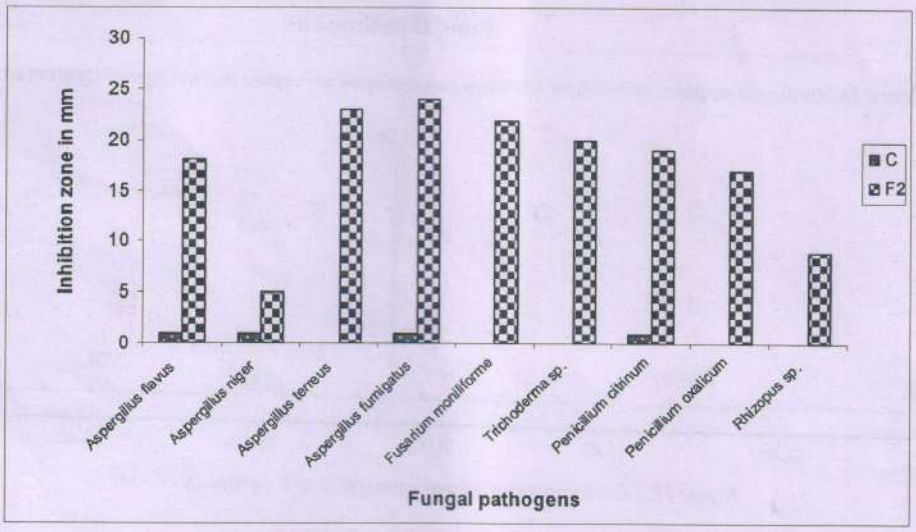

Figure 14. Antifungal activities of F2 fraction of Purpura persica against different pathogens.

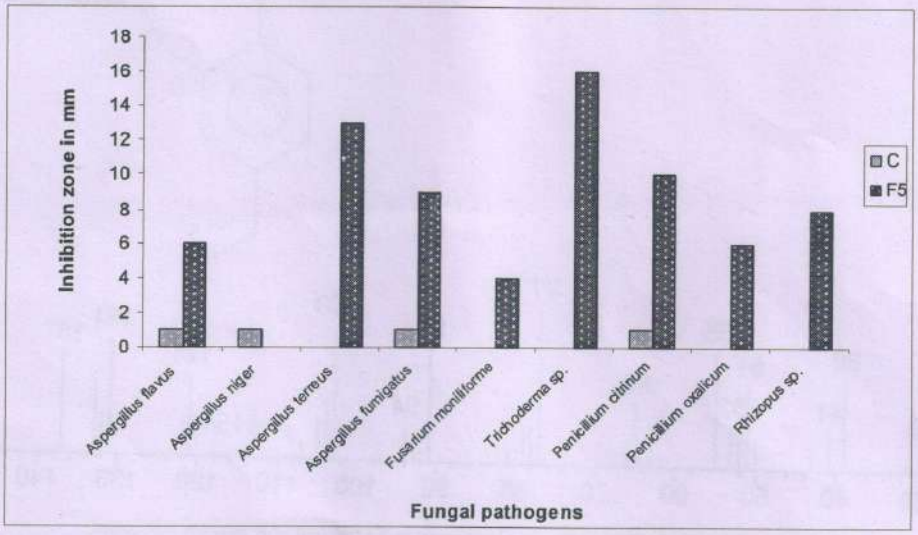

Figure 15. Antifungal activities of F5 fraction of Purpura persica against different pathogens.

JAYARAJ ANNAPACKIAM COLLEGE FOR WOMEN (AUTONOMOUS), PERIYAKULAM

Report of the Field Visit

Name of the student	K. ASWINI SUBHASHRI
Class	II - B.Sc ZOOLOGY [R]
Year	2018 - 2019
Place	ANGLADE INSTITUTE OF NATURAL HISTORY, SHC, KODAIKANAL

REPORT:

I went for Environmental studies trip to Anglade Institute of Natural history, Sacred Heart College, Shenbaganoor, Kodaikanal. It was a three days trip from 25.02.2019 - 27.02.2019. I had a garden visit and saw various colourful flowers, giant trees and humming birds. At the meuseum I saw many extinct and endangered specimens. The trek to upper Palani hills and Lower Palani hills in two batches was very adventerous. And at the night we had a cultural programmes relating with environment. and the treck towards Silver Cascade, was very intresting. It was a heart breaking moment to saw the unhygenic polluted water, it shows how careless we are polluting our nature. I return back to our College by carrying beautiful memories

Signature of the Staff

PG and Research Centre of Zoology
Jayaraj Annapackiam College
for Women (A)
Periyakulam-625 601
Theni (Dt) Tamilnadu

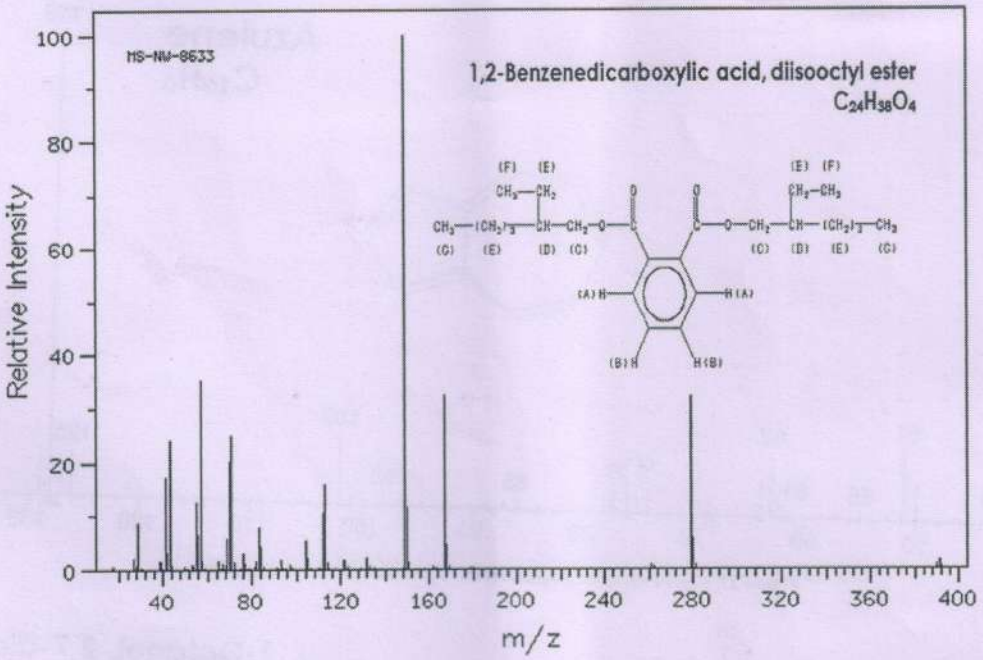

Figure 19. Chromatogram Component in F3 fraction of P. persica - 1,2-Benzenedicarboxlic acid, diisooctyl ester.

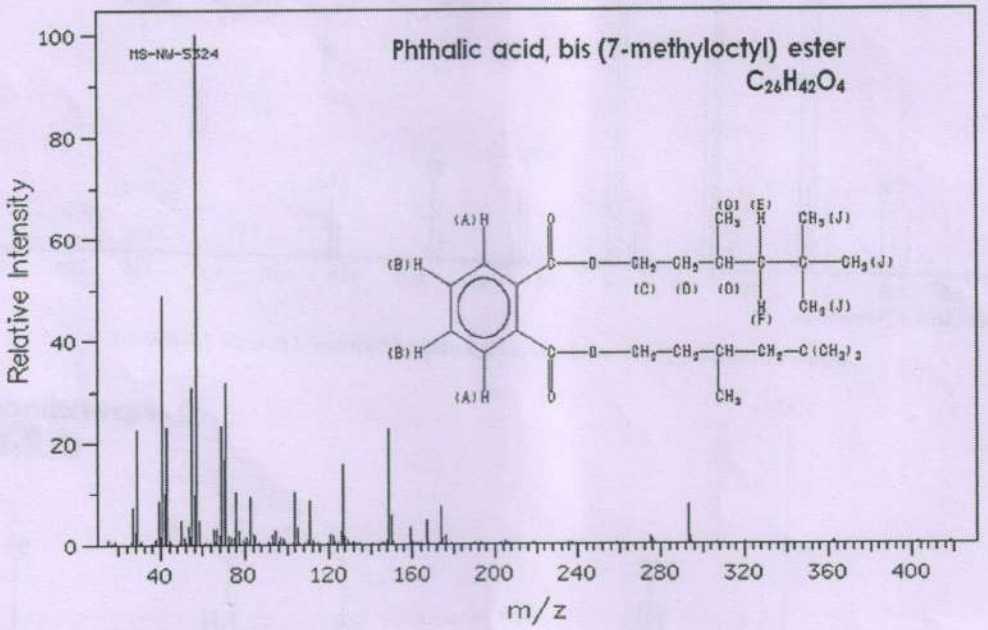

Figure 20. Chromatogram Component in F3 fraction of P. persica Phthalic acid, bis (7- methyloctyl) ester.

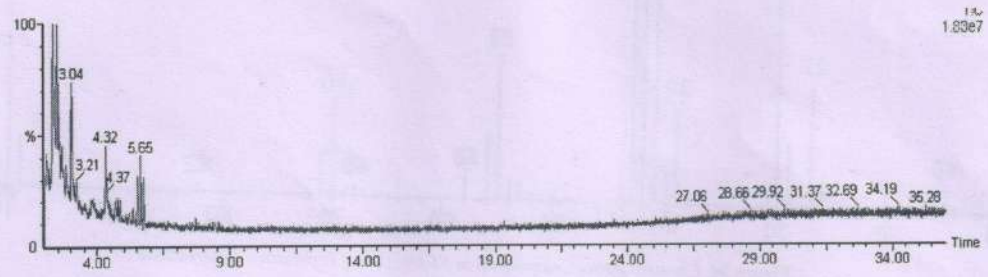

Figure 21. Chromatogram of column extract (F5) of P. persica by GC-MS.

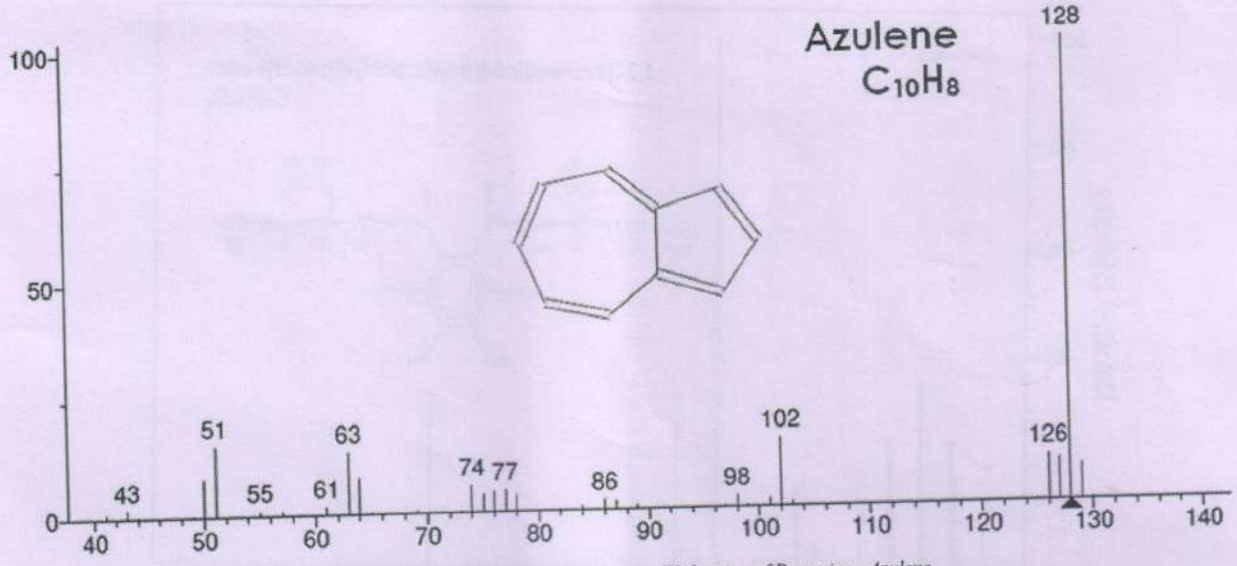

Figure 22. Chromatogram Components in F5 fraction of P. persica - Azulene.

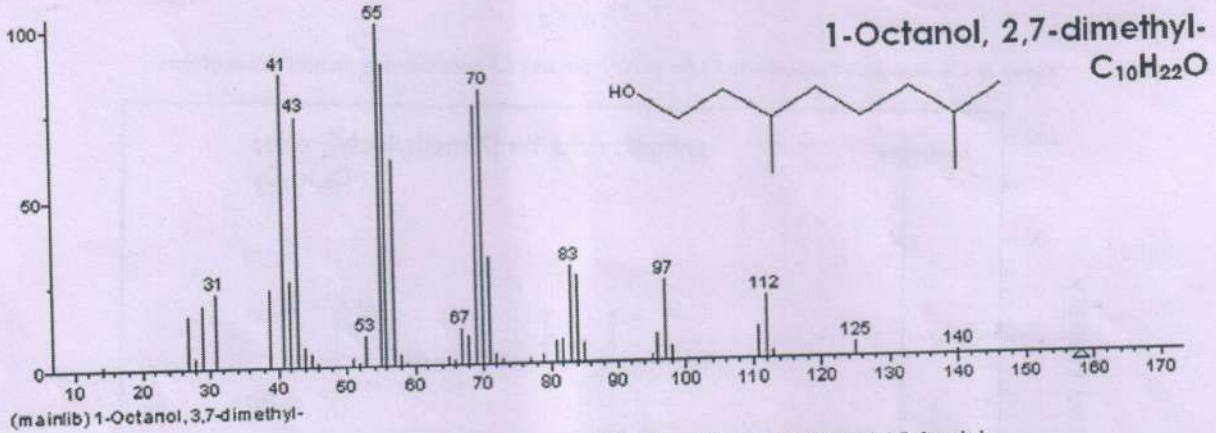

Figure 23. Chromatogram Components in F5 fraction of P. persica -1 Octanol, 2,7-dimethyl.

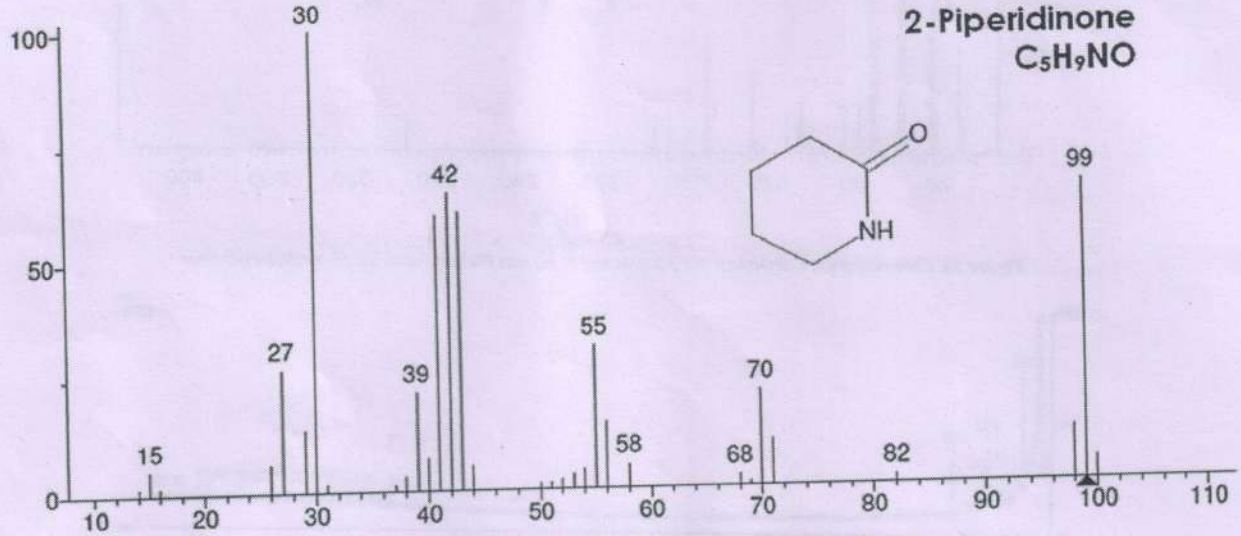

Figure 24. Chromatogram Components in F5 fraction of P. persica – Piperidinone.

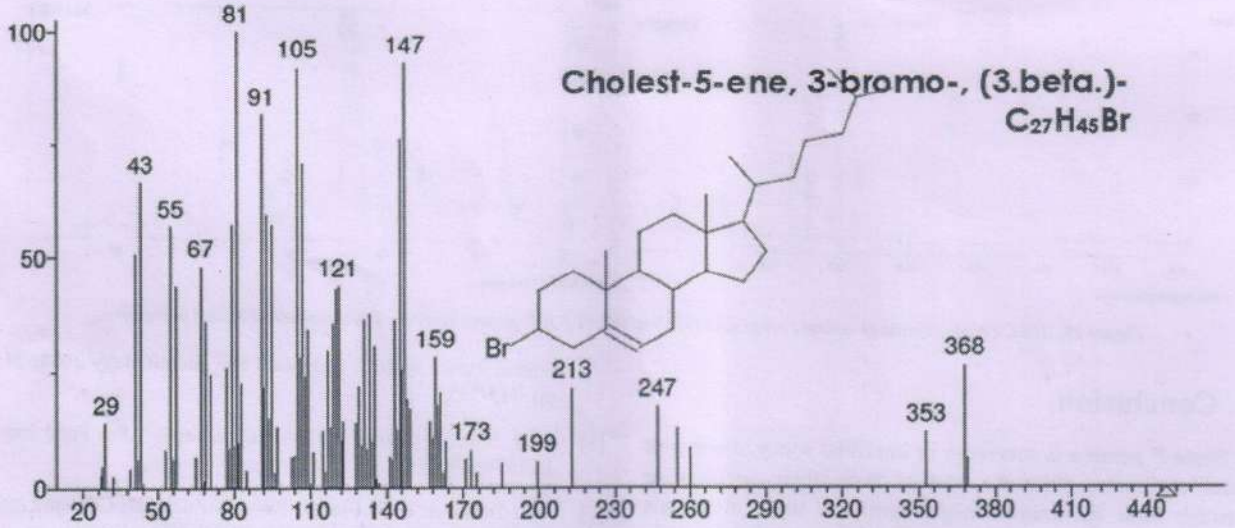

Figure 25. Chromatogram Component in F5 fraction of P. persica - Cholest-5-ene,3-bromo-,(3.beta.)-.

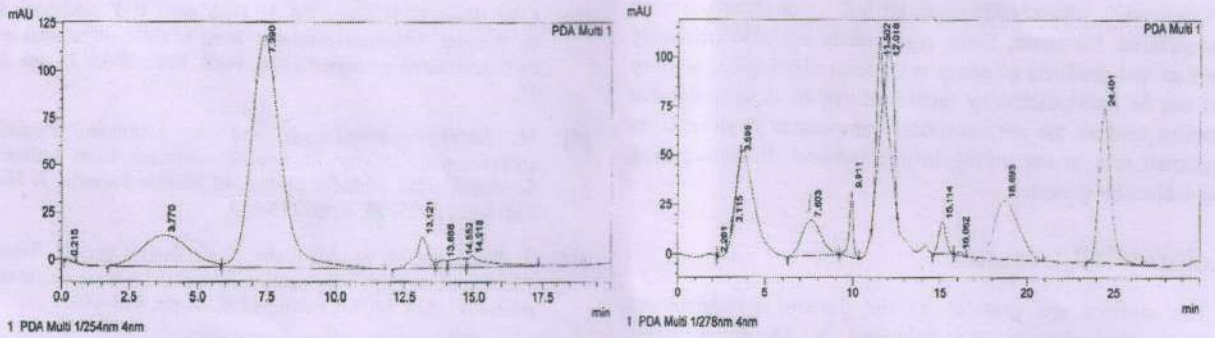

Figure 26. HPLC chromatogram of isolated compound (TLC) of (F5 S1 F3) P. persica compared to the standard azulene.

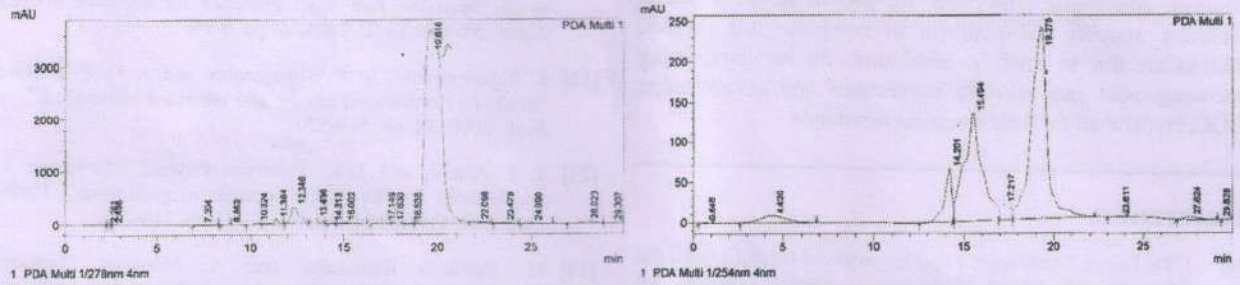

Figure 27. HPLC chromatogram of isolated compound (TLC) of (F3 S1 F4) P. persica compared to the standard eugenol.

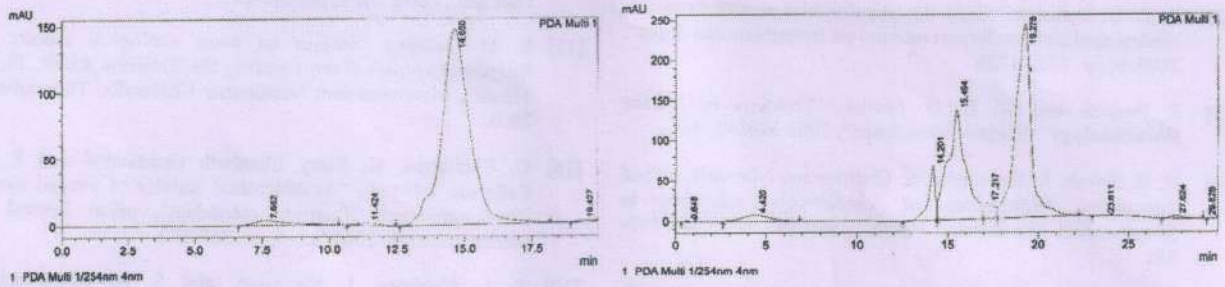

Figure 28. HPLC chromatogram of isolated compound (TLC) of (F5 S1 F5) P. persica compared to the standard oleic acid.

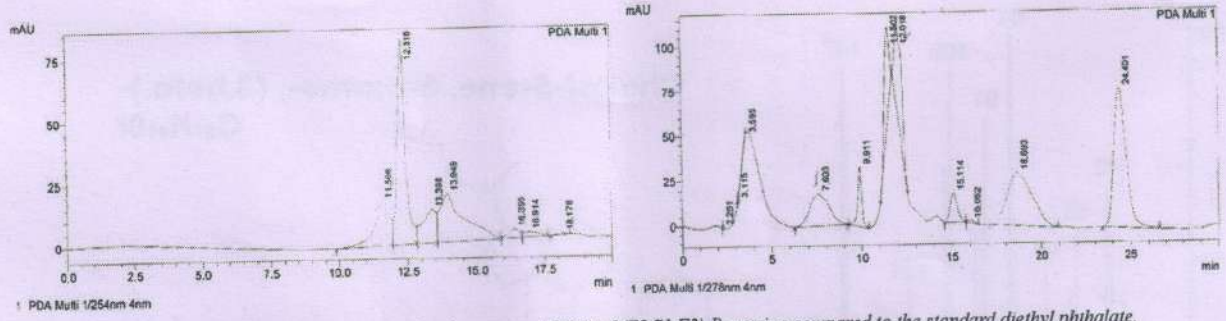

Figure 29. *HPLC chromatogram of isolated compound (TLC) of (F5 S1 F3) P. persica compared to the standard diethyl phthalate.*

4. Conclusion

Since *P. persica* is surviving in intertidal rocky shore with coral reef area, either to protect from their enemies or through food the animal might synthesis these chemicals which might be responsible for the inhibition of both bacteria and fungi. These antimicrobial compounds can be relatively synthesized, chemically modified, analyzed, and manipulated. However, these compounds are also primarily translational products of genes with potent biological activity and can be manipulated by techniques of modern molecular genetics confers the antimicrobial compounds *P. persica* an important role in expanding bridge between bioactive drug and molecular genetics.

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