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Study on Antimicrobial Activity and Chemical Profiling of *Pamburus missionis* (Wight) Swingle



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**Jaya Sree S.^{1*}, Vijayakumar N.²,
Suseela Gomathi K.³, Mary Helen P. A.⁴**

¹Research Scholar, Department of Botany and
Research Centre, S.T. Hindu College, Nagercoil,
Tamilnadu, India.

²Associate Professor, Department of Botany and
Research Centre, S. T. Hindu College, Nagercoil,
Tamilnadu, India.

³Lecturer, Department of Biotechnology, Malankara
Catholic College, Mariagiri, Kaliakkavilai- 629 153,
Tamilnadu, India.

⁴Assistant Professor, Department of Biotechnology,
Malankara Catholic College, Mariagiri,
Kaliakkavilai- 629 153, Tamilnadu, India.

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ABSTRACT

The antimicrobial properties of acetone leaf and stem extracts of *Pamburus missionis* (Wight) Swingle was determined against eleven bacterial and eight fungal pathogens as test organisms using the agar disc diffusion method. The acetone leaf and stem extract of plant showed antimicrobial activity in varying proportion. The study focuses on the analysis of the acetone extract of *Pamburus missionis* leaves and stem by GC-MS analysis. The phytocomponents of the acetone extracts of *Pamburus missionis* were investigated by using Gas Chromatography–Mass Spectrometry, while the mass spectra of the compounds found in the extract was matched with the National Institute of Standards and Technology (NIST) Library. Fifteen components from leaves and ten components from stem of *Pamburus missionis* were identified. The active principles with their retention time, molecular formula, molecular weight and concentration (%) in the acetone extracts of leaf and stem of *P. missionis* were obtained.



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INTRODUCTION

Medicinal plants throughout the world have been used since ancient times for healing purposes. Various types of plants i.e. trees, shrubs, herbs and grasses etc. have been used extensively to cure illnesses and diseases, (Lambert *et al.*, 1997) for therapeutic purposes, and for relieving stress, fevers, pains and infections (National Research Council, 1992). For a long period of time, plants have been a valuable source of natural products for maintaining human health. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments (Seenivasan *et al.*, 2006). *Pamburus missionis*, (Rutaceae). Kattu Naranthi (Tamil) is a branched shrub or small tree, armed with stout straight spines, these are 2-3 cm long, arising singly on the side of the bud in the axils of the leaves. Leaves are oval, oblong-obovate or elliptical, 6-10 cm long, 2-4 cm broad, very thick, coriaceous, glandular-punctate, the tip is rounded, sometimes slightly emarginate, the base is narrowed rather abruptly into the petiole, the margin is entire, becoming gray and apparently crenate in drying; lateral veins are inconspicuous, tertiary ones are not apparent, the two faces are very similar in appearance, on drying these are velvety gray-green unlike those of any other member of the subfamily Citratae (Aurantioideae). Flowers are 12-20 mm in diameter, fragrant, with small pointed sepals and 5 or 4 white obovate caducous petals about 1 cm long. Pistil is about 1 cm long. Fruit is about 2.5 cm in diameter, orange-colored when ripe, with a thick peel dotted with oil glands, 4-5 celled, the cells containing 1 or 2 seeds surrounded by a sticky gum. The fruits of *Pamburus missionis* yield fragrant oil which is traditionally used in the treatment of chronic rheumatism and paralysis (Somasundaram, 1967). The leaves of this tree are traditionally used for the treatment of swellings, fractures, piles and fistula. Decoction of leaves is given internally for phlegm and puerperal diseases (Jayaweera, 1982). *Pamburus missionis* (Wight) Swingle, a small thorny shrub with thick leaves, Family Rutaceae, a popular Indian tree which also has the medicinal claims, was selected for the current research work. It was planned to carry out the taxonomic identification of these plants. The aim and objectives of the present study is to evaluate the acetone extract for antimicrobial activity, as well as the characterization of phytoconstituents from leaves and stem of *Pamburus missionis*.

MATERIALS AND METHODS

Plant materials

The plant parts such as leaf and stem of *Pamburus missionis* (Wight) Swingle for the proposed study were collected from Colachel, Kanyakumari District, Tamil Nadu, India. The taxonomic identification of the plant was confirmed by Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, India. (Certificate No. BSI/SRC/5/23/2014-15/ Tech/1139).

Preparation of Plant Extract

The collected fresh plant leaves and stem were rinsed with distilled water and air dried in shade. The dried plant material was then homogenized by electric mixer grinder to obtain coarse powder and stored in air-tight bottles for further analysis. The shade dried, powdered leaf and stem was extracted (Mukherjee 2002) with acetone solvent by hot extraction method using Soxhlet apparatus. The extracts were evaporated in a rotary vacuum evaporator at 40°C to dryness and stored at 4°C in an air tight bottle for further analysis (Ragavendra, 2011).

Antimicrobial Activity of Acetone Extract of *Pamburus missionis*

Antimicrobial activity of acetone extracts of leaf and stem of *Pamburus missionis* were carried out by disc diffusion method (Bauer *et al.*, 1966; Murrey *et al.*, 1995).

Culture Media

The media used for antibacterial test was Nutrient agar and for antifungal test was Potato dextrose agar.

Inoculum

The bacteria were inoculated into nutrient broth and incubated at 37°C for 4 hours. Similar procedure was done for fungal strains by inoculating in Potato dextrose broth for 6 hours.

Microorganisms used

The microorganisms used for antimicrobial studies were obtained from Microbial Type Culture Collection (MTCC), Chandigarh. The different bacterial strains (Gram positive and Gram negative) used are *Staphylococcus epidermis* (MTCC 6810), *Vibrio vulnificus* (MTCC 1145), *Enterobacter aerogens* (MTCC 111), *Clostridium perfringens* (MTCC 450), *Bacillus subtilis*

(MTCC 121), *Klebsiella pneumonia* (MTCC 4030), *Enterococcus faecalis* (MTCC 439), *Pseudomonas putida* (MTCC 4910), *Pseudomonas aeruginosa* (MTCC 4676), *Actinomadura atramentaria* (MTCC 2560), *Bacillus megaterium* (MTCC 453). The different fungal strains used for study were *Candida glabrata* (MTCC 3984), *Ashbya gossypii* (MTCC 358), *Rhizomucor miehei* (MTCC 546), *Candida tropicalis* (MTCC 184), *Aspergillus fumigatus* (MTCC 4333), *Aspergillus niger* (MTCC 961), *Aspergillus aculeatus* (MTCC 1331), *Candida albicans* (MTCC 183).

Antimicrobial activity

Antimicrobial activity of acetone extracts of leaves and stem were determined by disc diffusion method. Briefly, Petri plates containing 20 ml of nutrient agar (for bacteria) potato dextrose agar (PDA) medium (for fungi) were allowed to dry in sterile chamber. The sterile filter paper discs (Whatman No.1 paper, 5 mm diameter) were impregnated with 10 µg of acetone extracts was placed on the inoculated agar surface. Gentamycin (50 mcg/disc) was used as positive control for bacteria and for fungi Nystatin was used (10 µg) and acetone solvent were placed as negative controls. The plates were incubated at 37°C for 24 hours for antibacterial activity. The inoculated plates were incubated for 48–72 hours at 28°C for antifungal activity. The antimicrobial activity against each test organism was quantified by determining the zone of inhibition around the paper discs in millimeters. Each assay was replicated three times and results expressed as the mean of three replicates. All results were expressed as Mean ±Standard Deviation (SD) of three replicates.

GC-MS analysis

Acetone extracts of leaf and stem of *Pamburus missionis* were analyzed for the presence of different compounds by GC-MS. GC-MS analysis of some of the potent volatile constituents present in the extracts was performed at Vellore Institute of Technology (VIT), Vellore (Tamil Nadu), India. The clarus 680 GC used in the analysis employed a fused silica column packed with Elite-5MS (30.0 m, 0.25 mmID, 250 µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min with a split ratio of 10:1. The 1 µl sample extract injected into the instrument was detected by the Turbo gold mass detector (Perkin Elmer) with the aid of the Turbo mass 5.4.2 software.

During the 32.00 minute GC extraction process, the oven was maintained at an initial temp 60°C for 2 min, ramp 10°C/min to 300°C, hold 6 min. The injector temperature was set at 250°C (mass analyzer). The different parameters involved in the operation of the clarus 600 MS, were also standardized (Inlet line temperature: 240°C; Source temperature: 240°C). Mass spectra were taken at 70eV; a scan interval of 0.5s and fragments from 50 to 600 Da. The MS detection was completed in 32 minutes. The relative percentage of the extract constituents was expressed as percentage with peak area normalization.

Identification of components

The identity of the components in the extract was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. National Institute of Standards and Technology library sources were also used for matching the identified components from the plant material (Mc Lafferly, 1989; Stein, 1990).

RESULTS AND DISCUSSION

Antimicrobial activity of acetone extracts of leaves and stem extracts of *Pamburus missionis*

The antimicrobial activity of acetone extracts of leaves and stem of *Pamburus missionis* were investigated by agar disc diffusion method. All the used parts of the plant showed antimicrobial activity against bacterial and fungal strains but in varying proportion.

Antibacterial activity

The antibacterial activity of acetone extracts of leaves and stem of *Pamburus missionis* were explained as follows: The highest zone of inhibition was exhibited by *Enterobacter aerogens* (20±0.90 mm), *Enterococcus faecalis* (20±0.75 mm) while the lowest inhibitory activity was showed by *Clostridium perfringens* (10±0.2 mm), *Actinomadura atramentaria* (10±0.5 mm). The extract showed activity against all tested bacterial pathogens. In acetone stem extract, the extract showed potent activity against *Actinomadura atramentaria* (18±0.2 mm) and showed moderate activity against *Enterobacter aerogens* (6.3±0.28 mm). The extract showed no activity against *Clostridium perfringens* and *Pseudomonas putida*. A positive control (Gentamycin) showed activity ranging from 19.6±0.57 mm to 28.6±1.15 mm whereas negative control showed no activity against the bacteria. The results are displayed in Table 1. Samundeeswari *et al.*, 2012

reported that the antibacterial property of *Naringi crenulata* investigated by well diffusion assay infers that Leaf Methanolic Extract (LME) exhibits higher activity against the test pathogens when compared to other solvent extracts. The maximum zone of inhibition of LME (1000 µg/mL) was found to be 22 and 21 mm respectively against *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Table 1. Antibacterial activity of acetone extracts of *Pamburus missionis*

Sl. No	Bacterial Pathogens	Zone of inhibition (mm) mean±SD		
		Leaf extract (10µg)	Stem extract (10µg)	Gentamycin (50mcg/disc)
1	<i>Actinomadura atramentaria</i>	10±0.5	18±0.2	28.6±1.15
2	<i>Bacillus megaterium</i>	17±0.90	15±0.45	22.6±0.57
3	<i>Bacillus subtilis</i>	16±0.2	15±0.2	23.5±0.5
4	<i>Clostridium perfringens</i>	10±0.2	0	22.8±1.04
5	<i>Enterobacter aerogens</i>	20±0.90	6.3±0.28	22.8±0.76
6	<i>Enterococcus faecalis</i>	20±0.75	15±0.8	23.3±0.57
7	<i>Klebsiella pneumoniae</i>	15±0.7	11.3±1.15	26.2±0.52
8	<i>Pseudomonas aeruginosa</i>	12±0.5	8±0.55	24.5±0.86
9	<i>Pseudomonas putida</i>	15±0.8	0	19.6±0.57
10	<i>Staphylococcus epidermis</i>	15±0.70	8±0.1	26.9±0.90
11	<i>Vibrio vulnificus</i>	14.3±0.41	14±0.2	27±0.5

Antifungal activity

The antifungal activity of acetone extracts of *Pamburus missionis* were explained as follows: In leaf extract, the extract showed good inhibitory activity against *Rhizomucor miehei* (17±0.55 mm) and weak activity was shown against *Aspergillus fumigatus* and *Candida albicans* with an inhibition zone of 11±0.2 mm and 11±0.15 mm respectively. The extract showed no antifungal

activity against *Candida tropicalis* and *Aspergillus niger*. In stem extract, the extract showed maximum inhibition zone of 19 ± 0.70 mm against *Candida glabrata* and showed minimum activity against *Aspergillus fumigatus* (12 ± 0.8 mm). *Aspergillus niger* showed no activity for the extracts. A positive control (Nystatin) showed zone of inhibition in the range of 8.3 ± 0.57 mm to 17.5 ± 0.86 mm. Negative control showed no activity for fungi. The results are depicted in the Table 2. Manisha Vats *et al.*, (2011) reported that petroleum ether and chloroform extracts exhibited prominent antibacterial and antifungal activity. The chloroform extract showed good inhibitory properties for *A. niger*>*P. aeruginosa*>*C. albicans*. Zachariah *et al.*, 2008 studied the antibacterial activity and antifungal activity of *Murraya koenigii* against *Staphylococcus aureus* and *Candida albicans* respectively. The maximum inhibitory zone of inhibition of coumarin was against (22 mm) *Bacillus subtilus* and *Candida albicans* (21 mm).

Table 2. Antifungal activity of acetone extracts of *Pamburus missionis*

Sl. No	Fungal Pathogens	Zone of inhibition (mm) (Mean±SD)		
		Leaf extract (10µg)	Stem extract (10µg)	Nystatin (10µg)
1	<i>Ashbya gossypii</i>	13±0.6	17±0.11	14.5±0.5
2	<i>Aspergillus aculeatus</i>	12±0.11	17±0.2	8.3±0.57
3	<i>Aspergillus fumigatus</i>	11±0.2	12±0.8	14.3±1.15
4	<i>Aspergillus niger</i>	0	0	9.5±0.5
5	<i>Candida albicans</i>	11±0.15	18±0.15	17.1±0.76
6	<i>Candida glabrata</i>	15±0.40	19±0.70	17.5±0.86
7	<i>Candida tropicalis</i>	0	16±0.85	14.6±0.57
8	<i>Rhizomucor miehei</i>	17±0.55	17±0.50	9.6±0.57

GC-MS analysis of acetone leaf extract of *Pamburus missionis* using GC-MS

The crude acetone extract of *Pamburus missionis* leaf was analysed using GC-MS Table 3 and Figure 1. The extract had the following major composition: Ethanol, Pentamethyl (24.652%), n-Hexadecanoic acid (15.593 %). Minor components like 1-Methylene-2B-Hydroxymethyl-3,3-

Dimethyl-4B-(3-Methylbut-2-Enyl)-Cyclohexane (2.488%), 3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-(Trimethylsiloxy)Trisiloxane (2.486%), 2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- (2.305%). Among the identified phytochemicals, the fatty acid esters namely, hexadecanoic acid methyl ester have the property of antioxidant, hypocholesterolemic, nematicide, pesticide, flavouring agent, lubricant and anti-androgenic activities. The biological activities listed are based on Dr. Duke's phytochemical and ethnobotanical databases by Dr. Jim Duke of the Agricultural Research Service/USDA. Analogous to the present study, hexadecanoic acid methyl ester was identified in the methanol extract of *Spirulina platensis* (Kumar *et al.*, 2011). The ethanolic extract of *Mussaenda frondosa* was subjected to GC-MS analysis and 20 chemical constituents namely (-)-quinic acid, 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol, naphthalene, decahydro-2-methoxy, 1,2, 3-benzenetriol, hexadecanoic acid ethyl ester, linoleic acid ethyl ester, oleic acid, etc. were identified (Gopalakrishnan and Vadivel, 2011).

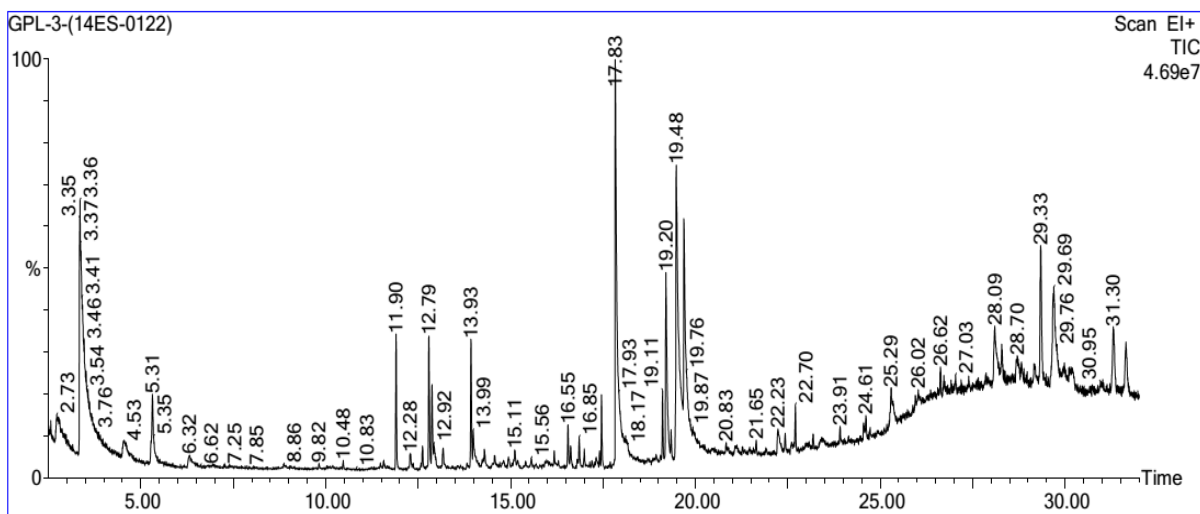


Figure 1. GC-MS chromatogram of acetone extract of *Pamburus missionis* leaf

Table 3. GC-MS analysis of the acetone extract of leaf of *Pamburus missionis*

Sl. No	Retention Time (RT)	Constituents	Molecular Formula	MW	Peak Area (%)
1	3.359	Ethanol, Pentamethyl	C ₇ H ₁₆ O	116	24.652
2	5.314	Cyclohexene, 4-Methylene-1-(1-Methylethyl)-	C ₁₀ H ₁₆	136	2.749
3	11.902	1-Methylene-2B-Hydroxymethyl-3,3-Dimethyl-4B-(3-Methylbut-2-Enyl)-Cyclohexane	C ₁₅ H ₂₆ O	222	2.488
4	12.792	Naphthalene, Decahydro-4A-Methyl-1-Methylene-7-(1-Methylethenyl)-	C ₁₅ H ₂₄	204	2.629
5	13.928	2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)-	C ₂₁ H ₃₀ O ₃ S	362	2.305
6	17.829	N-Hexadecanoic Acid	C ₁₆ H ₃₂ O ₂	256	15.593
7	19.200	Phytol	C ₂₀ H ₄₀ O	296	5.591
8	19.480	Z,Z-6,28-Heptatriactontadien-2-One	C ₃₇ H ₇₀ O	530	14.205
9	19.685	Oleic Acid	C ₁₈ H ₃₄ O ₂	282	6.379
10	25.297	Methyl 2-Hydroxy-Eicosanoate	C ₂₁ H ₄₂ O ₃	342	2.753
11	28.094	Methyl 3-Bromo-1-Adamantaneacetate	C ₁₃ H ₁₉ O ₂ Br	286	3.305
12	29.334	2,4,4-Trimethyl-3-Hydroxymethyl-5A-(3-Methyl-But-2-Enyl)-Cyclohexene	C ₁₅ H ₂₆ O	222	4.657
13	29.690	2R-Acetoxymethyl-1,3,3-Trimethyl-4T-(3-Methyl-2-Buten-1-Yl)-1T-Cyclohexanol	C ₁₇ H ₃₀ O ₃	282	7.143
14	31.295	3,7,11,15-Tetramethyl-2-Hexadecen-1-Ol	C ₂₀ H ₄₀ O	296	3.106
15	31.640	3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-(Trimethylsiloxy)Trisiloxane	C ₁₃ H ₃₆ O ₄ Si ₄	368	2.486

GC-MS analysis of acetone stem extract of *Pamburus missionis* using GC-MS

The GC-MS analysis of acetone extracts of stem of *Pamburus missionis* were depicted in Figure 2 and Table 4. Some of the identified components were Ethanol, Pentamethyl (57.837 %), Spiro[2.4]Hepta-4,6-Diene (15.135 %), 1-Ethyl-2 Phenylpyrazolium Bromide (6.198 %), n-Hexadecanoic acid (6.034 %), 3-Methyl-2-(2-Oxopropyl)Furan (1.936 %), 13-Docosenamide, (Z)- (1.463 %), Hentriacontane (1.341 %). Most of these compounds have not been reported in the *Pamburus missionis*. The GC-MS analysis of ethanol extract of *Naringi crenulata* stem revealed the presence of 47 bioactive phytochemicals, major constituents along with minor constituents. The major constituents were hexadecanoic acid 19.65 %, Lupeol 13.70 %, lup-20(30)-en-3-one 12.75 %, Stigmast-4-EN-3-one 8.2 %, Tetratetracontane 5.97 %, (7Z)-7-Tetradecenal 5.58 % etc., other major and minor constituents were also present. There are presence of many secondary metabolites and bioactive phytochemicals in the stem of *Naringi crenulata* (Sampathkumar and Ramakrishnan, 2011).

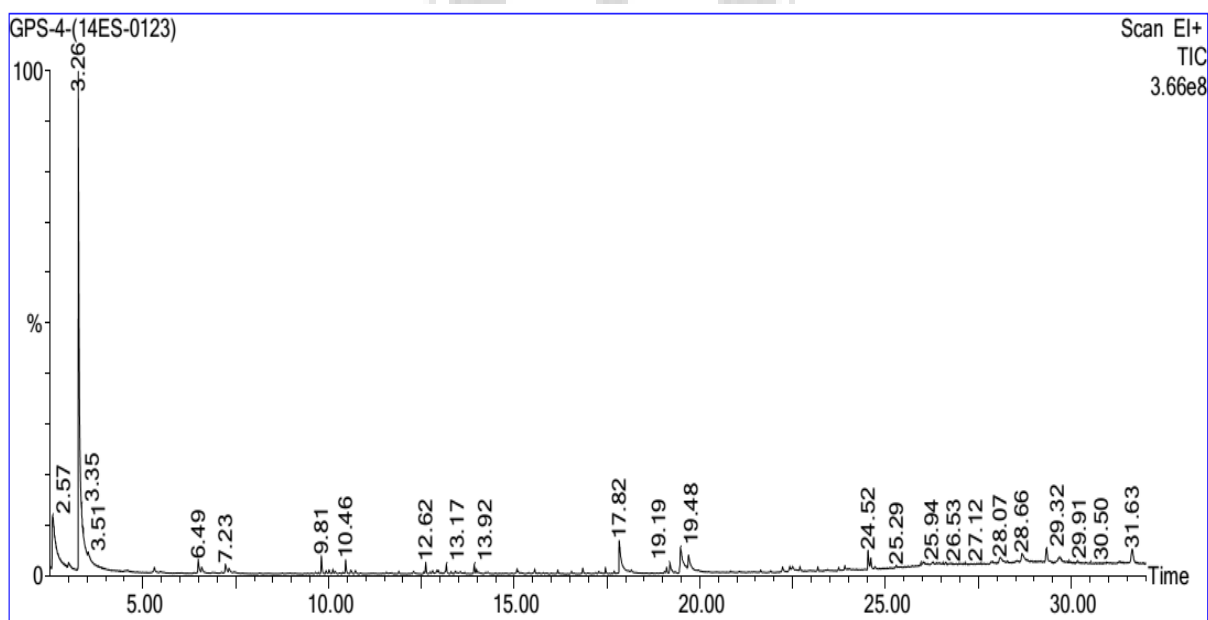


Figure 2. GC-MS chromatogram of acetone extract of *Pamburus missionis* stem

Table 4. GC-MS analysis of acetone stem extract of *Pamburus missionis*

Sl. No	Retention Time (RT)	Constituents	Molecular Formula	MW	Peak Area (%)
1	2.573	Spiro[2.4]Hepta-4,6-Diene	C ₇ H ₈	92	15.135
2	3.264	Ethanol, Pentamethyl	C ₇ H ₁₆ O	116	57.837
3	3.529	1-Ethyl-2-Phenylpyrazolium Bromide	C ₁₄ H ₂₄ O ₂ N ₂	252	6.198
4	9.806	Hentriacontane	C ₃₁ H ₆₄	436	1.341
5	17.824	N-Hexadecanoic Acid	C ₁₆ H ₃₂ O ₂	256	6.034
6	19.475	Z,Z-6,28-Heptatriactontadien-2-One	C ₃₇ H ₇₀ O	530	5.230
7	19.695	3-Methyl-2-(2-Oxopropyl)Furan	C ₈ H ₁₀ O ₂	138	1.936
8	24.522	13-Docosenamide, (Z)-	C ₂₂ H ₄₃ ON	337	1.463
9	29.329	N-Methyl-1-Adamantaneacetamide	C ₁₃ H ₂₁ ON	207	2.254
10	31.630	3-Butoxy-1,1,1,5,5,5-Hexamethyl-3-(Trimethylsiloxy)Trisiloxane	C ₁₃ H ₃₆ O ₄ Si ₄	368	2.570

CONCLUSION

Our systematic investigation reveals the potential of *P. missionis* leaves and stem as a good source of bioactive compounds such as fatty acid esters, alcohols, hydrocarbons, aldehydes, alkenes, fatty acids and amides that justify the use of this plant for its various ailments by traditional practitioners. The antimicrobial activity of acetone extracts of leaves and stem of *Pamburus missionis* were investigated by agar disc diffusion method. All the used parts of the plant showed antimicrobial activity against bacterial and fungal strains but in varying proportion. Further research interest in the study of these active bio compounds may yield nature friendly strong antioxidant, anti-microbial, anti-inflammatory agents and analgesic agents. The biological activities of each of the identified phytocomponents range from antimicrobial, antioxidant

and antitumoral activities. These findings have provided scientific basis to the ethnomedical usage of the plant. However, isolation of the individual phytochemical constituents, subjecting it to biological activity and toxicity profile will give fruitful results.

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