CHAPTER 6

IN VITRO CONTROL OF *Pseudomonas aeruginosa* PB112 STRAIN ISOLATED FROM INFECTED FISH BY SOME PLANT EXTRACTS AND THEIR QUALITATIVE PHYTOCHEMICAL SCREENING AND GC-MS ANALYSIS

6.1Introduction

In developing countries, microorganisms are frequently a cause of prevailing diseases, presenting a serious public health issue. In aquaculture several infectious diseases are reported in twentieth century mainly belongs to *Pseudomonas, Aeromonas, Streptococcus* and *Vibrio* species and few parasitic origins like protozoan, monogenean and arthropods. Bacterial species causes high mortality and severe economic loss during its outbreak (Raa, 1996; Wedemeyer, 1996; Toranzo *et al.*, 2005). Skin ulceration and epidermal damage are the most common symptoms that appear on diseased fishes. The isolated strain *Pseudomonas aeruginosa* PB112 infection cause high mortality and loss for a period of time. Antibacterial therapy will provide only short-term relief. As use of antibiotic to control microbial pathogens such as Pseudomonas leads to multidrug resistance, antibiotic residues in environment, transmission of antibiotic in the food chain leads to several problems. So it is inevitable to probe for alternative methods of controlling the pathogen. One such alternative is the use of herbal medicinal plant-extracts. There are a huge number of medicinal plants in West Bengal. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. All medicinal, plant contains certain active constituent, it responsible to some pharmacological activity. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct. The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steriods, phenols glycosides and tannins. The information obtained from extracts of medicinal plants makes pharmacological studies possible. The mode of action of plants producing therapeutic effects can also be better investigated if the active ingredients are characterized. Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines. GC-MC Plays a key role in the analysis of unknown components of plant origin. GC-MS ionizes compound and measures their mass numbers.

Hence in present study we have evaluated the efficiency of three selected medicinal plant extracts *Andrographis paniculata*, *Datura metel* and *Aegle marmelos* were used against the isolated *P.aeruginosa* PB112 strain in vitro condition.

6.1.1 Description of plants

Andrographis paniculata

Systematic position:

Kingdom: Plantae

Order: Lamiales

Family: Acanthaceae

Genus: *Andrographis*

Species: *A.paniculata*

Andrographis paniculata (Wall), commonly known as Kalmegh is a herbaceous plant native to India and Sri Lanka. It is an erect branched annual, 0.3-0.9 meters high, branches sharply quadrangular winged in the upper part; leave - lanceolate, acute, undulate, pale beneath; Flowers small, solitary distant, in axillary or terminal racemes or panicles, bracts lanceolate.

Datura metel

Systematic Position:

Kingdom: Plantae

Order: solanales

Family: Solanaceae

Genus: *Datura*

Species: *D.metel*

Datura metel (Linn.) is a shrub-like perennial herb. It grows in the wild in all the warmer parts of the world, and is cultivated worldwide for its chemical and ornamental properties. The plant is an annual herb growing up to 3 ft. high. It is slightly furry, with dark violet shoots and oval to broad oval leaves that are often dark violet as well. The pleasantly-scented 6-8 in. flowers are

immensely varied, and can be single or double. Colours range from white to cream.

Aegle Marmelos

Systematic Position:

Kingdom: Plantae

Order: Sapindales

Family: Rutaceae

Subfamily: Aurantioideae

Genus: Aegle

Species: *A.marmelos*

Aegle Marmelos (Linn.), is also known as Bael, grows well in the dry forests on hilly and plain areas. *A. marmelos* is a widely distributed plant and found in India, China, Sri Lanka, Myanmar, Pakistan, Bangladesh and Nepal. It is a slow-growing, medium sized tree, twenty five to thirty feet tall. The stem is short, thick, soft, flaking bark, and spreading, sometimes spiny branches, the lower ones drooping. The leaflets are oval or lancet shaped, four to ten cm long, two to five cm wide. Leaves composed of three to five leaflets in it. The flowers are greenish white in colour with a peculiar fragrant. Fruit is spherical or oval in shape with a diameter of two to four inch.

6.2 Materials and methods:

6.2.1 Collection of plant materials

Fresh leaves of three locally available medicinal plants namely *Andrographis paniculata* (Kalmegh), *Datura metel* (Datura), *Aegle marmelos* (Bael) were collected from the garden of the Department of Botany, University of Kalyani, Kalyani and Faculty of agriculture, Bidhan Chandra Krishi Viswavidyalaya, Kalyani Campus. The plants were identified based on their physical characteristics.

6.2.2 Preparation of plant extracts

Materials

1) Leaves of selected plants
2) Distilled water
3) Mixer grinder
4) Solvent (methanol, acetone, chloroform: methanol (1: 1), benzene, petroleum ether)
5) Rotary evaporator
6) Separating funnel

Procedure

1) The leaves of the plants were separated, washed several times in running tap water and once rinsed in distilled water.
2) The washed materials were dried in dark using blotting paper for about a month.
3) The dried materials were powdered in mixer grinder.
4) The leaf powder was extracted in methanol. 10gm of leaf powder was soaked in 100ml of methanol and macerated at room temperature for forty eight hours.
5) The extract was then filtered with Whatman filter paper No.1. The filtrate was then concentrated with a rotary evaporator under reduced pressure at 60°C to afford crude methanol extract. The dried extract was kept at 4°C until use.
6) The crude methanol extract was fractionated into benzene, chloroform, chloroform: methanol (1:1), acetone and methanol fractions by solvent-solvent partitioning using separating funnel (Bahl and Bahl, 1992).
7) Each solvent fraction was again concentrated using the rotary evaporator by the same procedure.
8) Each concentrated fraction was finally resuspended in respective solvent with a concentration gradient of 100mg/ml, 50mg/ml and 25mg/ml.

6.2.3 Antibacterial activity test

The Antimicrobial susceptibilities were tested by the disc diffusion test, the agar dilution method for determining the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) according to the guidelines recommended by the Clinical and Laboratory Standards Institute (Wikler, 2008).

Disc diffusion method

1) Disc diffusion test was performed using sterile 6 mm-diameter filter paper antibiotic discs (Whatman, Maidstone, UK).
2) The discs were prepared using 20 µL of extract of each concentration, which is 100mg/mL, 50mg/mL, and 25mg/mL respectively. Thus each disc contained 2mg, 1mg, and 0.5mg respectively.
3) All discs were dried at room temperature overnight.
4) An isolated colony from previous cultured fish pathogenic *Pseudomonas aeruginosa* strain PB112 was touched with a wire loop and the growth transferred to a tube containing 5 mL of nutrient broth (Hi Media). The broth culture was incubated at 37°C for overnight until the turbidity of the 0.5 McFarland standards; this resulted in a suspension containing approximately 1 to 2 x 10^8 cfu/mL.
5) The 0.5 McFarland suspension was transferred to nutrient agar (Hi Media) by spread plate technique, the final inoculum on the agar would be approximately 10^6 CFU/mL.
6) The discs were placed on the surface of the inoculum nutrient agar and incubated at 37 °C for eighteen to twenty four hours.
7) The disc diffusion test was determined by measuring the diameter of the inhibition zone.
8) Experiments were performed in triplicate and the mean of the diameters of the inhibition zones calculated.

6.2.4 Study of microbial growth and survival dynamics in the presence of plant extracts

For more accurate determination of antimicrobial activity, liquid culture experiments were performed. The growth curve of the test culture was compared with methanolic leaf extracts of these plants and non-treated culture as control. 5ml of bacterial strain was inoculated in those plant extract containing broth. It was incubated at 37^0C for twenty four hours. Optical density was measured at 600 nm for every two hours for both plant extracts treated and non-treated cultures using UV spectrophotometer. The growth curves of the treated cultures were plotted against the non-treated culture and were compared to know the significant inhibitory efficiency of these plant extracts to test pathogen.

6.2.5 Phytochemical analysis

Phytochemical tests were done to find the presence of the active chemical constituents such as alkaloid, glycosides, terpenoids and steroids, flavonoids, reducing sugars, triterpenes, phenolic compounds and tannins by the following procedure.

Test for alkaloids (Meyer's Test)

The extract of these plants were evaporated to dryness and the residue was heated on a boiling water bath with 2% Hydrochloric acid. After cooling, the mixture was filtered and treated with a few drops of Meyer's reagent (Siddiq and Ali, 1997). The samples were then observed for the presence of turbidity or yellow precipitation (Evans, 2002).

Test for glycoside

To the solution of the extract in Glacial acetic acid, few drops of Ferric chloride and concentrated sulphuric acid are added, and observed for reddish brown colouration at the junction of two layers and the bluish green colour in the upper layer (Siddiq and Ali, 1997).

Test for terpenoid and Steroid

4 mg of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet colour was observed for terpenoid and green bluish colour for steroids (Siddiq and Ali, 1997).

Test for flavonoid

4 mg of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, five to six drops of concentrated hydrochloric acid was added and red colour was observed for flavonoids and orange colour for flavones (Siddiq and Ali, 1997).

Test for reducing sugars

To 0.5 ml of extract solution, 1 ml of water and five to eight drops of Fehling's solution was added at hot and observed for brick red precipitate.

Test for triterpenes

300 mg of extract was mixed with 5 ml of chloroform and warmed at 80^0C for thirty minutes. Few drops of concentrated sulphuric acid was added and mixed well and observed for red colour formation.

Test for phenolic compounds (Ferric chloride test)

300 mg of extract was diluted in 5 ml of distilled water and filtered. To the filtrate, 5% Ferric chloride was added and observed for dark green colour formation.

Test for tannins

To 0.5 ml of extract solution, 1 ml of water and one to two drops of ferric chloride solution wad added. Blue colour was observed for gallic tannins and green black for catecholic tannins (Iyengar, 1995).

6.2.6 GC-MS analysis

GC-MS technique was used in this study to identify the phytocomponents present in the extracts. This technique was carried out at the Indian Institute of Chemical Biology, Kolkata, West Bengal. This analysis was performed using GC SHIMADZU – QP5050A system and gas chromatograph interfaced to a mass spectrometer equipped with Elite-I fused silica capillary column. The constituents were identified after comparison with those available in the computer library (NIST and WILEY) attached to the GC-MS instrument and documented.

Identification of components

Interpretation of mass spectrum GC-MS was conducted using the database using the NIST and WILEY library having more patterns. The spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST107 and WILEY229 library. The Name, Molecular Weight, Molecular formula and Structure of the components of the test materials were ascertained.

6.3 Results

The antibacterial activities of acetone, benzene, chloroform, chloroform: methanol, methanol extracts of *A. paniculata*, *D. metel*, *A. marmelos* against *P. Aeruginosa* PB112 strain were examined in this study and their potency were qualitatively assessed by the presence or absence of inhibition zones and zone diameter. The extracts were prepared using different solvents. The inhibitory zone around the antibiotic discs indicated absence of bacterial growth and it was reported as positive and absence of zone as negative. The diameters of the zones were measured using diameter measurement scale. The solvents, without plant extract, were used as negative controls and did not inhibit bacterial growth. Antibiotic – Tetracycline (100µg/ml) and Levofloxacin (100µg/ml) was showed inhibition

zone of 25 mm to 35 mm against this pathogen. It was found that levofloxacin standard showed higher activity than tetracycline standard. Acetone extract was non-effective against the bacterial pathogen. Thus the antibacterial activity of plant extracts prepared by solvent was nonsignificant. Benzene extract of *Andrographis paniculata* produced less significant result (0.5-1.5mm) whereas no effects for the extracts of *Datura metel* and *Aegle marmelos*. Chloroform extracts of *Datura metel* and *Andrographis paniculata* show significant result but in case of *Aegle marmelos*, it shows little inhibition activity only in highest concentration. Chloroform: methanol (1:1) solvent extract of these three plants also showed considerable zone of inhibition against this test organism. But methanol extract of all the three plants was the most potent against this bacterium with largest diameter of zone of inhibition. Among the three plants, methanol extract of *Datura metel* showed highest activity, *Aegle marmelos* exhibited low activity and *Andrographis paniculata* showed moderate result. The highest inhibitory effect was observed in highest concentration of methanolic extract (100mg/ml) i.e. 20mm in *Datura metel,* 15mm in *Andrographis paniculata* and 7mm in *Aegle marmelos* whereas lowest concentration of 10mg/ml is effective in both the plant *D.metel* and *A. paniculata* and 20mg/ml in *A.marmelos* for the development of minimum zone of inhibition. Detailed results are given in table6.1 and table 6.2.

Table 6.1: Antibacterial activities of these plant extracts (mg/ml) and antibiotic (100 µg /ml) against *Pseudomonas aeruginosa* PB112 strain tested by disc diffusion assay. "–" indicates absence.

Name of Plants	Solvents Used	Zone of inhibition in different conc. (Diameter in mm)					
		100mg/ml	50mg/ml	25mg/ml	Control	Tetracyclin	Levofloxacin
Andrographis paniculata	Acetone	-	-	-	-	25± 1.2	35± 1.2
	Benzene	1.5±0.01	0.5±0.01	-	-		
	Chloroform	3±0.1	2.7±0.1	2±0.1	-		
	Chloroform: Methanol	5±0.12	3.5±0.1	1.5±0.01	-		
	Methanol	15±0.5	12.5±0.5	10±0.5	-		
Datura metel	Acetone	-	-	-	-	25± 0.5	35± 0.5
	Benzene	-	-	-	-		
	Chloroform	5±0.15	3±0.1	1.3±0.01	-		
	Chloroform: Methanol	8±0.25	5.3±0.25	2.5±0.1	-		
	Methanol	20±0.5	15±0.5	9.5±0.5	-		
Aegles marmelos	Acetone	-	-	-		25± 1.2	35± 1.2
	Benzene	-	-	-	-		
	Chloroform	1.2±0.01	-	-	-		
	Chloroform: Methanol	3±0.01	2±0.01	0.7±0.01	-		
	Methanol	7±0.5	4.5±0.2	3±0.013	-		

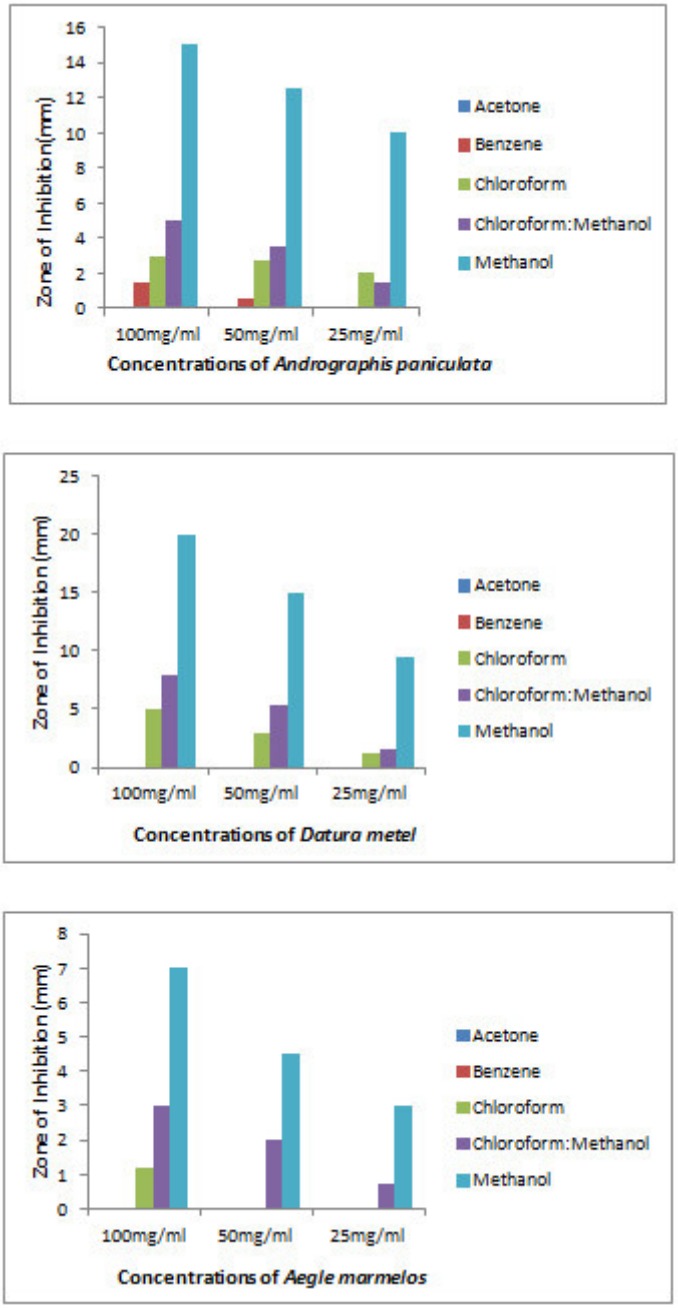

Fig 6.1: Variation in the inhibitory potential of different plant extracts using different solvent.

Table 6.2: Minimum Inhibitory concentration of the plant extracts

Sl.No.	Plants	Solvents used	Minimum Inhibitory Concentration
1.	*Andrographis paniculata*	Benzene	50mg/ml
		Chloroform	20mg/ml
		Chloroform:Methanol (1:1)	15mg/ml
		Methanol	10mg/ml
2.	*Datura metel*	Chloroform	20mg/ml
		Chloroform:Methanol (1:1)	15mg/ml
		Methanol	10mg/ml
3.	*Aegle marmelos*	Chloroform	75mg/ml
		Chloroform:Methanol (1:1)	25mg/ml
		Methanol	20mg/ml

The phases of culture growth, lag (initial adjustment), log (exponential growth in plentiful nutrients), stationary (nutrient limit met, growth ceases), and death (waste accumulates, cells die), are commonly monitored by measuring the turbidity or OD (600nm) of the culture. Growth profile of *P.aeruginosa* PB112 strain (Figure 6.2) in the presence of methanol leaf extracts of *A. paniculata* and *D. metel* are lower than the control all through the growth period. It may be anecdotal that the active metabolites having mild growth reducing capability but are powerless to stop the growth. It may also be the case that the concentration of active metabolites is inadequate to stop the growth completely. It can be noticed that sufficient inhibition of the tested bacteria was observed at higher concentrations, indicating that under more realistic testing conditions, higher concentrations of plant extract antimicrobials are required. Figure6.2 shows a plot of OD vs time for culture.

After two hour culturing period overall *D.metel* extract had shown the highest growth reduction capability. Divergence of the two plots illustrates that different optical configurations will have different dynamic ranges with respect to optical density measurements.

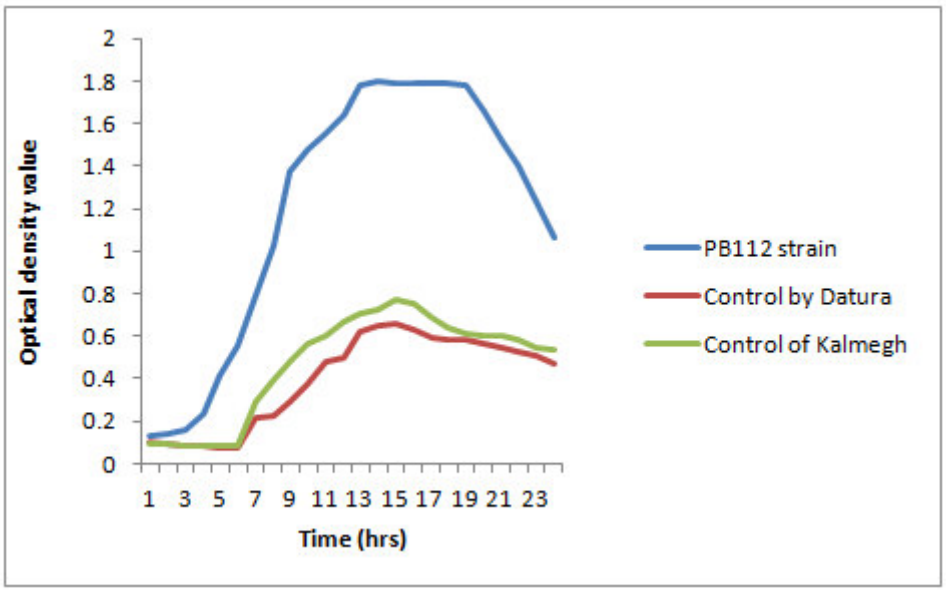

Fig 6.2: Growth curve measurement of PB112 strain and control by Datura and Kalmegh.

The phytochemical active compounds of these three plants were qualitatively analysed and the results are presented in (Table 6.3, Table 6.4 and Table 6.5).The phytochemical analysis of *Datura metel* showed the presence of alkaloids, tripenoid, steroids, flavonoid, triterpenes, phenolic compounds and tannins. *Andrographis paniculata* have tannin, flavonoid, diterpenes, and starch and *Aegle marmelos* have tannin, saponin, flavonoid, phenol, sterols and triterpenoid.

The findings of the present investigation suggests that the organic solvent extraction was suitable to verify the antimicrobial properties of medicinal plants and they supported by many investigation. The present study justifies the claimed uses of leaves in the traditional system of medicine to treat various infectious diseases caused by the microbes. This study also encourages cultivation of the highly valuable plant in large scale to increase the economic status of the cultivators in the country. Based on this further chemical and pharmacological investigations can be done to isolate and identify minor chemical constituents in the seeds and to screen other

potential bioactivities may be recommended. Sixteen compounds were identified in methanolic fraction of *Andrographis paniculata* leaf extract, twelve compounds were identified in methanolic fraction of *Datura metel* leaf extract and thirty three compounds were identified in methanolic leaf extract of *Aegle marmelos* by GC-MS analysis. The chromatogram obtained by methanol fraction of *Andrographis paniculata* and *Datura metel and Aegle marmelos* leaf were shown in Fig 6.3, Fig 6.4 and Fig 6.5 respectively. The active Principle, area of the peak, Concentration (%), Retention Time (RT), Molecular formula and Molecular weight were presented in Table 6.6, Table 6.7 and Table 6.8. Among the sixteen compound of *Andrographis paniculata* the Cyclopentadecanone, 2- Hydroxy (12.84%), Naphthalene (9.14%), among the twelve compounds of *Datura metel* the Phytol (13.37%), 9,12,15-Octadecatrien-1- ol (13.33%) and in *Aegle marmelos* among the thirty three compounds Phenol, 2-methoxy-3-(2-propenyl)- [Eugenol] (70.88%), Caryophyllene (8.31%) and Naphthalene (1.44%) were represented in high percentage.

Table 6.3: Phytochemical analysis of methanolic leaf extract of *Datura metel*

Sl. No.	Tests	Result
1.	Alkaloids	+
2.	Glycosides	-
3.	Tripenoid and steroid	+
4.	Flavonoid	+
5.	Reducing sugar	-
6.	Triterpenes	+
7.	Phenolic compounds	+
8.	Tannins	+

Table 6.4: Phytochemical screening of methanol extract from leaves of *Aegle marmelos*

Sl. No.	Tests	Result
1.	Tannins	+
2.	Flavonoids	+
3.	Saponins	+
4.	Phenols	+
5.	Sterols	+
6.	Triterpenoid	+
7.	Reducing sugar	-

Table 6.5: Phytochemical screening of methanol extract from leaves of *Andrographis paniculata*

Sl. No.	Tests	Result
1.	Tannins	+
2.	Flavonoids	+
3.	Steroid	+
4.	Starch	+
5.	Saponin	-
6.	Alkaloid	-
7.	Aminoacid	-
8.	Diterpenoid	+
9.	Reducing Sugar	-

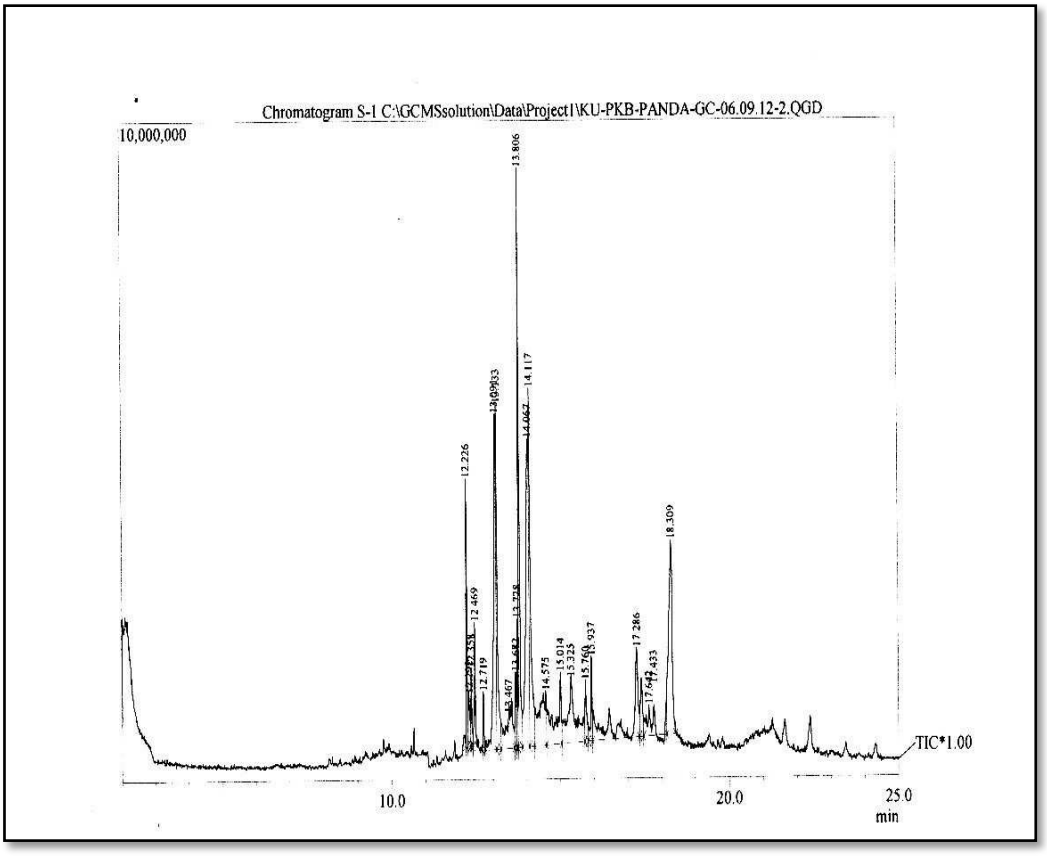

Fig 6.3: Chromatogram of *Andrographis paniculata*

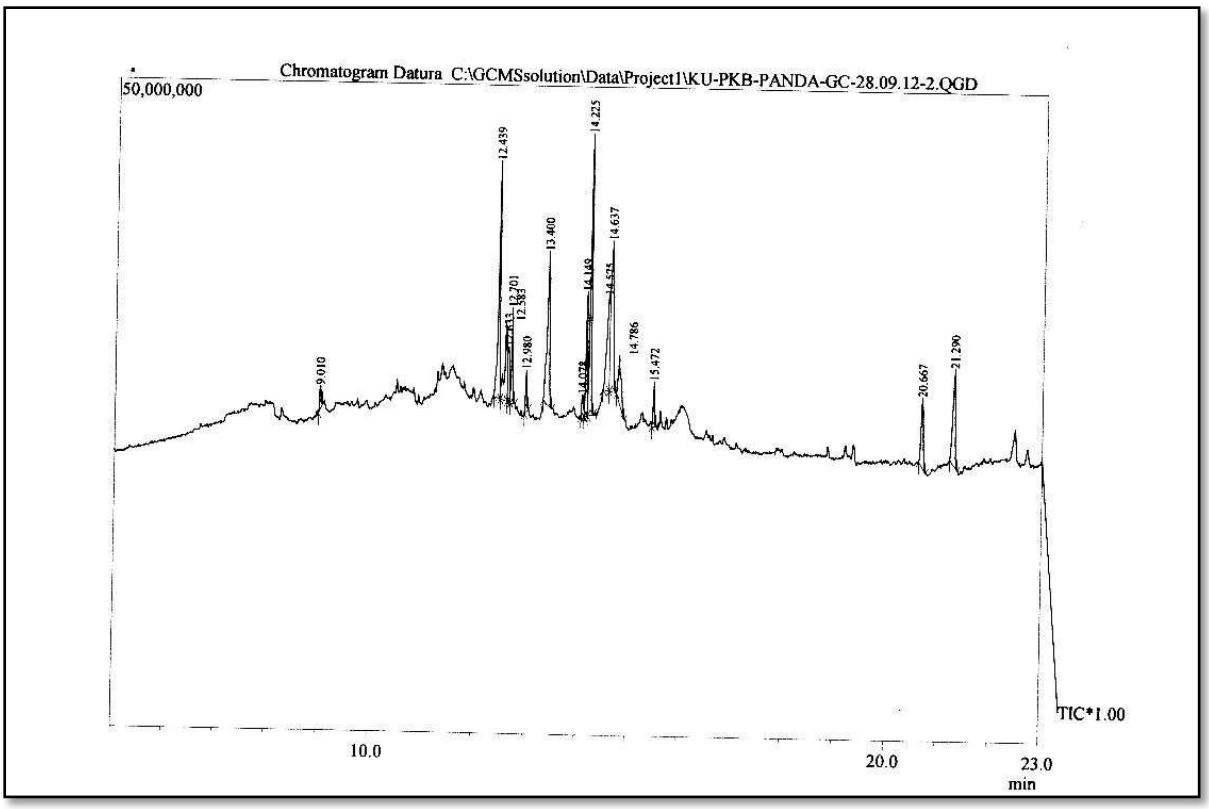

Fig 6.4: Chromatogram of *D.metel*

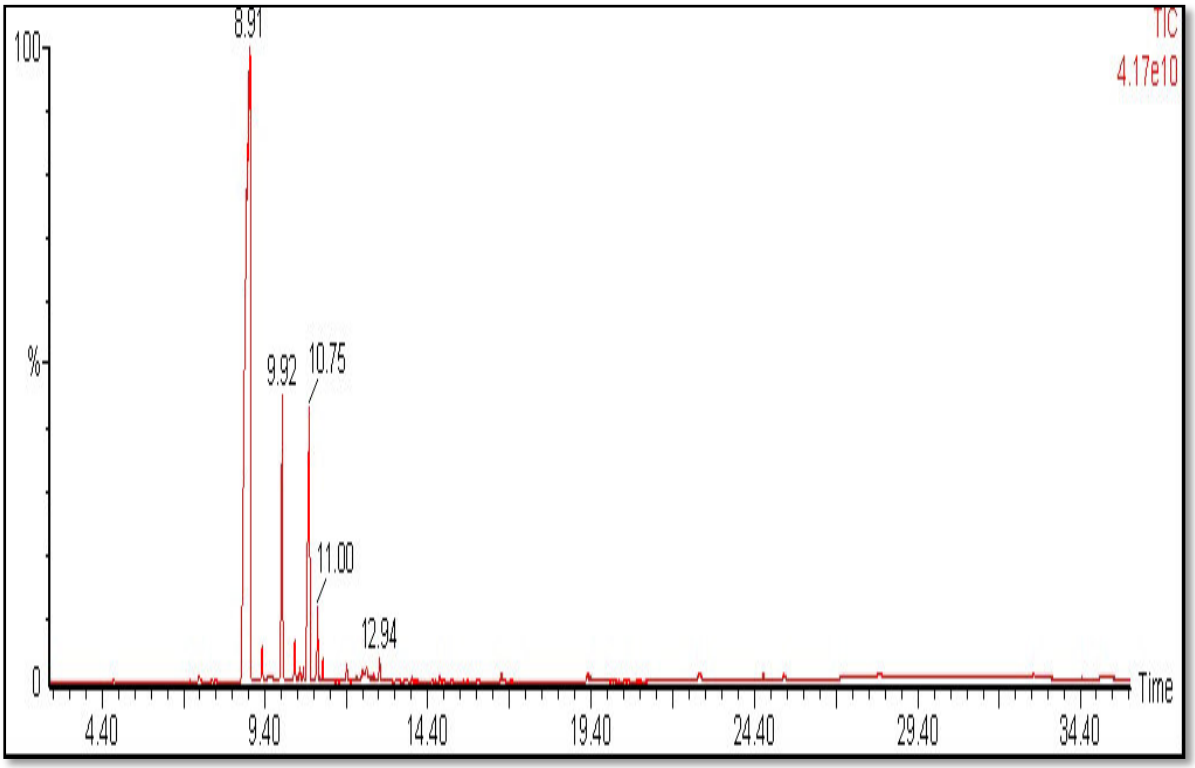

Fig 6.5: Chromatogram of *Aegle marmelos*

Table 6.6: Characterization of peaks obtained from GC-MS studies of methanol leaf extract of *Andrographis paniculata*

R.time	Area	Area%	Name	Mol. Formula	Mol.wt	Mass peak	Base peak
12.226	7389167	3.2	Nephytadiene	$C_{20}H_{38}$	278	40	68.15
12.292	2642932	1.14	Androstan-11-amine	$C_{21}H_{37}N$	303	81	124.20
12.469	3929488	1.7	2- Hexadecan-1-ol	$C_{20}H_{40}O$	296	55	81.15
13.133	14139347	6.12	n-Hexadeconoic acid	$C_{16}H_{32}O_2$	256	76	73.10
13.467	9177351	3.97	Cyclodecanol	$C_{10}H_{20}O$	156	176	81.15
13.728	3664754	1.59	9,12,15-Octadecatrienoic acid	$C_{19}H_{32}O_2$	292	68	79.15
13.806	21103468	9.13	Phytol	$C_{20}H_{40}O$	296	33	71.15
14.067	29666172	12.84	Cyclopentadecanone, 2-Hydroxy	$C_{15}H_{28}O_2$	240	96	67.15
14.117	17394697	7.53	8,11,14-Eicosatrienoic acid	$C_{20}H_{34}O_2$	306	98	79.15
14.575	12236087	5.29	E-8-Methyl-9-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	268	157	55.15
15.014	11272851	4.88	Eicosanoic acid	$C_{20}H_{40}O_2$	312	99	99.15
15.325	15840094	6.85	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	414	214	414
15.760	3031763	1.31	Hexadeconoic acid	$C_{19}H_{38}O_4$	330	146	98.10
15.937	3499129	1.51	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390	21	149.15
17.286	17085515	7.39	2-beta-hydroxy-9-oxoverrucosane	$C_{20}H_{32}O_2$	304	162	217.20
18.309	21114364	9.14	Naphthalene	$C_{15}H_{24}$	204	169	107.15

Table 6.7: Characterization of peaks obtained from GC-MS studies of methanol leaf extract of *Datura metel*

R.time	Area	Area%	Name	Mol. Formula	Mol.wt	Mass peak	Base peak
9.010	3703202	1.11	2-Methoxy-4-vinylphenol	$C_9 H_{10} O_2$	150	62	77.05
12.439	40014118	11.97	1-Octadecyne	$C_{18} H_{34}$	250	67	43.10
12.633	10550820	3.16	Alpha-[5-Ethyl-2-furyl]glycine	$C_8 H_{11} NO_3$	169	75	124.10
12.701	12910931	3.86	1-Eicosyne	$C_{20} H_{38}$	278	70	43.10
13.400	38923213	11.64	1,2-Benzenedicarboxylic acid	$C_{30} H_{50} O_4$	474	84	149.05
14.149	17989490	5.38	9,12,15-Octadecatrienoic acid	$C_{19} H_{32} O_2$	292	94	79.10
14.225	44699915	13.37	Phytol	$C_{20} H_{40} O$	296	54	71.10
14.575	29059496	8.69	Z-10-Pentadecenol	$C_{15} H_{30} O$	226	133	55.10
14.637	44545626	13.33	9,12,15-Octadecatrien-1-ol	$C_{18} H_{32} O$	264	100	79.10
14.786	14012328	4.19	Hyoscyamine	$C_{17} H_{23} NO_3$	289	78	124.10
20.667	16118571	4.82	17-(1,5-Dimethylhexyl)-2,3-dihydroxy-10	$C_{27} H_{44} O_3$	416	135	151.15
21.290	24754546	7.41	Vitamin e	$C_{29} H_{50} O_2$	430	90	165.15

Table 6.8: Characterization of peaks obtained from GC-MS studies of methanol leaf extract of *Aegle marmelos*

R.Time	Name of the compound	Mol.Wt	Area%
3.37	Pentane, 1,1-diethoxy-	$C_9H_{20}O_2$	0.04
3.8	Hexanoic acid, ethyl ester	$C_8H_{16}O_2$	0.04
4.77	Propane, 1,1,3-triethoxy-	$C_9H_{20}O_3$	0.10
6.03	Cyclohexanecarboxylic acid, 2-hydroxy-, ethyl ester	$C_9H_{16}O_3$	0.02
6.37	Benzoic Acid	$C_7H_6O_2$	0.02
6.61	Methyl Salicylate	$C_8H_8O_3$	0.03
7.1	6-Acetyl-á-d-mannose	$C_8H_{14}O_7$	0.1
7.37	Phenol, 4-(2-propenyl)-	$C_9H_{10}O$	0.29
7.76	Anisole, p-allyl-	$C_{10}H_{12}O$	0.04
7.87	Butanedioic acid, hydroxy-, (S)-	$C_4H_6O_5$	0.12
8.91	Phenol, 2-methoxy-3-(2-propenyl)- [Eugenol]	$C_{10}H_{12}O_2$	70.88
9.31	Copaene	$C_{15}H_{24}$	0.94
9.92	Caryophyllene	$C_{15}H_{24}$	8.31
10.3	à-Caryophyllene	$C_{15}H_{24}$	1.06
10.75	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	$C_{12}H_{14}O_3$	11.98
11	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	$C_{15}H_{24}$	1.55
11.89	Caryophyllene oxide	$C_{15}H_{24}O$	0.47
12.94	2',3',4' Trimethoxyacetophenone	$C_{11}H_{14}O_4$	0.54
13.93	2,5-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	0.09
14.78	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, acetate, (E,E)-	$C_{17}H_{28}O_2$	0.14
14.91	1-Naphthalenol, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(2-propenyl)-	$C_{15}H_{24}O$	0.08

15.14	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	0.06
15.93	4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	$C_{15}H_{26}O_2$	0.11
16.67	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	0.3
16.99	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	0.02
19.3	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	0.27
19.71	Octadecanoic acid	$C_{18}H_{36}O_2$	0.1
21.67	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	0.1
22.75	Tetradecanoic acid, hexadecyl ester	$C_{30}H_{60}O_2$	0.63
24.68	Pregn-5-en-20-one, 3-(acetyloxy)-16,17-epoxy-6-methyl-, (3á,16à)-	$C_{24}H_{34}O_4$	0.21
25.31	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	0.24
28.25	Hexadecanoic acid, octadecyl ester	$C_{34}H_{68}O_2$	0.59
31.74	9-Octadecenoic acid (Z)-, 9-octadecenyl ester, (Z)-	$C_{36}H_{68}O_2$	0.17
32.96	Hexadecanoic acid, hexadecyl ester	$C_{32}H_{64}O_2$	0.44

6.4 Discussion

One of the ongoing problems scientist and medical workers face in the fight against infectious diseases is the development of resistance to the agents used to control them. There has been a remarkable progress in the prevention, control and even eradication of infectious diseases with improved hygiene and development of antimicrobials and vaccines. In the modern world multiple drug resistance has developed against many microbial infections due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientists to search for new antimicrobial substances.

Plants are known to have beneficial therapeutic effects in traditional Indian system of medicine. It has been estimated by the World Health Organization that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care. The use of data on traditional medicine can provide a very valuable short cut by indicating plants with specific folk medicinal uses, which might be likely sources of biologically active compounds. Recent investigations on medicinal plants used in traditional medicine have led to the discovery of many new drugs and hundreds of pharmacologically active substances for synthetic modifications (Wang, 2008).The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world. Plants have been reported to possess antimicrobial, antifungal and other activities. This has been elucidated by various workers (Ramya *et al.*, 2008b; Sasidharan *et al.,* 1998; Sudharameshwari and Radhika, 2007). Pandey and Mishra (2011) reported that methanolic extract of *Aegle marmelos* has antibacterial activity against *P.aeruginosa* and the MIC values were obtained 11.90 mg/ml in methanolic extract. Britto and Gracelin (2011) investigated the phytochemicals present in leaves, stem, flowers and fruits of *Datura metel* which have some medicinal applications. Saranraj *et al.,* 2010 studied

that ethanol extract of *Datura metel* showed effective results against different test organisms like *P.aeruginosa, E.coli, S.aureus, K. pneumoniae, S.typhi*. Crude aqueous extract of *A.paniculata* leaves exhibit significant antimicrobial activity against gram-positive *S. aureus*, methicillin-resistant *S. aureus*, and gram-negative *Pseudomonas aeruginosa* (Zaidan *et al.*, 2005).

Significant activity against enterohemorrhagic strains of *E. coli* was found in the ethanol extract of *A. paniculata* (Voravuthikunchai and Limsuwan, 2006). According to Venkateson *et al.*, (2009) the antibacterial activity of *Aegle marmelos* extract of both solvents (Aqueous and ethanolic) against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aure*us and *Bacillus subtilis* were tested. The ethanolic extract showed considerably more activity than the aqueous extract. Maximum antibacterial activity was shown against *Bacillus subtilis* followed by *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Sundaram Ravikumar *et al.*, (2010) screened the *in vitro* antibacterial and antifungal activity of the chloroform extracts of the seventeen different coastal medicinal plants against different gram positive and gram negative and fungal ornamental fish pathogens. Of the selected plants *Datura metel* showed wide range of antimicrobial activity against many of the fish pathogens.

These plants could serve as useful source of new antimicrobial agents. The present study justifies the claimed uses of leaves in the traditional system of medicine to treat various infectious diseases caused by the microbes. This study also encourages cultivation of the highly valuable plant in large scale to increase the economic status of the cultivators in the country. The obtained results may provide a support to use of the plant in traditional medicine.