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ANALYSIS OF COMPOUND FROM METHANOLIC LEAF EXTRACT OF *MARTYNIA ANNUA* AND ITS ANTI BACTERIAL, ANTIBIOFILM, ANTIOXIDANT ACTIVITY

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Abstract:

Medicines derived from plants have made immense contribution towards the betterment of human health and act as a source of motivation for novel drug compounds. *M. annua* has numerous phytochemicals like tannins, alkaloids, phenols, carbohydrates that have potential to be used in the area of pharmacology. Thin layer chromatography was performed to separate the active compounds present in the methanolic extracts of *M. annua*. Plant extract, MA1 and MA2 were tested for antibacterial, antibiofilm and antioxidant activity. In the antibacterial activity of both liquid and solid medium, MA2 showed higher inhibition rate in the gram negative bacteria. In the antioxidant activity, MA2 showed maximum inhibitory concentration at 49.46µg/ml while compared with crude and MA1. A spectrum of GC-MS analysis revealed that compounds from methanolic extract of plant showed strong antibacterial, antioxidant, and anti inflammatory activities. Moreover, the separated fractions from methanolic leaf extract of *M. annua* were also characterized by GC-MS and FTIR analysis. GC-MS analysis of MA1 and MA2 showed different compounds which has antibacterial and antioxidant activity as reported earlier and results of FT-IR also confirmed the presence of functional groups. Due to the presence of these bioactive compounds in the isolated fraction of MA1 and MA2 may be responsible for the antioxidant, antibiofilm, and antioxidant activity. As the complex of compound has been present in fractions, further purification and structure of the fraction will be elucidated for the potential compound in future.

Keywords: *M. annua,* FTIR, Antibacterial, Antioxidant, Antibiofilm

1. Introduction:

Medicinal plants are the richest bio-resource of synthetic and traditional herbal medicine. It has been estimated that 14–28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno medicinal use of the plants. Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today, plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries. Plants still continue to be almost the exclusive source of drugs for the majority of the world's population.

Martynia annua Linn. belongs to the family Martyniaceae (or pedaliaceae) and is commonly known as Kaakanassikaa in Ayurveda, is well known herbaceous annual plant, distributed throughout India and it native of Mexico. The leaves of this plant are used for wound healing and tuberculosis glands of camel's neck. The juice of the leaves used as a gargle for sore throat. The leaf paste has the beneficial effect when applied to the bits of venomous insects and wounds of domestic animal. Fruits are used as anti-inflammatory and fruit ash mixed with coconut oil applied on burns and also treating of itching (Lodhi et al., 2013). *M. annua* plant reveals the presence of glycosides, tannins, carbohydrates, phenols, flavonoids and anthocyanins. Flowers contain cyanidin 3 galactoside, Whilst p-hydroxy benzoic acid are present in leaves and fruit, respectively (Mail et al., 2002). The leaf contain chlorogeic acid and fatty acids. palmitic acid, stearic acid and arachidic acid are present in seeds. According to the literature survey the whole plant used by santal tribals (India) for fever, hair loss, scabies, sores and carbuncles on the back.

Though microbial infections are common ailments, their treatment is a serious problem due to continuous drug resistance that microorganisms soon develop against antibiotic drugs (Walkty et al., 2014).Efforts to discover new remedies are, therefore, essential. Great hope rests with plants as they contain different types of primary and secondary metabolites with antimicrobial Pharmacophores (Frey et al., 2014). Over the past few decades, numerous studies have been conducted on plants to explore possible candidates for antibiotic drugs (Luciano-Montalvo, Goji et al., 2006). Work has been done which aim at knowing the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become

resistant (Arunkumar et al., 2009). Microbial biofilm formation of microorganisms is associated with persistent tissue and foreign body infections. Up to 80% of human bacterial infections are biofilm-associated; with infections most frequently being caused by *Staphylococcus aureus, S. epidermidis, Pseudomonas aeruginosa*, and *Escherichia coli* (Romling et al., 2012). Antioxidants as protective agents may reduce oxidative damage in humans are considered. Antioxidants occur naturally in many fruits and are able to neutralize free radicals by donating an electron and convert them into harmless molecules (Zahra et al., 2014).

2. Review of Literature:

Literature review has been done to collect information regarding the plant and previous research on the bioactive compounds. Objective for the study have been framed by considering the literature review.

- **Ganesan et al. (2014),** described the antimicrobial activity of some flowers of Eastern Ghats, Tamil Nadu. He had taken flowers of ten ethanomedicinal plants including *M. annua*. Flowers were extracted using ethanol. Among the ten medicinal plants *M. annua* shows the maximum level of inhibition compared with other flower extract. *Bacillus subtilis* (28 mm), *Candida albicans* (27 mm), *Klebsiella pneumonia* (13 mm), *Pseudomonas aeruginosa* (24 mm), *Staphylococcus aureus* (28 mm) and *Streptococcus mutans* (16 mm), respectively. *M. annua*, showed (MIC 2.31 mg/ml) maximum level of inhibition against *B. subtilis* (10 mm), followed by *K. pneumonia* (8mm), *P. aeruginosa* and *C. albicans* showed (7 mm) in each. For controlling *S. mutans* (10 mm) the MIC is 3.47 mg/ml and for *S. aureus* (10 mm) the MIC is 5.79 mg/ml. Hence it will be the good source for antibacterial activity.
- **Ahmed et al. (2015)** was described the antibacterial activity of the methanolic extract of *Carissa opaca* and its compound isolated from fraction of ethyl acetate. He concluded that less polar fractions exhibited stronger efficacy than polar ones, and ethyl acetate fraction proved to be the most toxic to the tested microorganisms with minimum inhibitory concentrations of 8.0, 7.8 and 7.78 mg/mL against *P. aeruginosa, C. albicans* and *B. subtilis*, respectively.
- **Al-dhabi et al. (2015)** was isolated novel isosteviol compound from *Pittosporum tetraspermum*. The compound exhibited significant activity against bacteria such as *Staphylococcus epidermidis* (125 μg/mL), *Staphylococcus aureus* (125 μg/mL), and *Klebsiella pneumoniae* (62.5 μg/mL). The MIC of the compound against *Candida albicans, Aspergillus niger*, and *Trichophyton mentagrophytes* was

62.5, 125, and 500 μg/mL, respectively. The compound showed comparatively better antibiofilm activity against *E. coli*, *S. typhi*, and *P. aeruginosa* also, it exhibited good antioxidant properties. Anticancer properties of the compound against Vero and MCF7 cell lines were its advantage. Novel isosteviol would be useful to reduce the infectious diseases caused by pathogenic microorganisms or slow the progress of various oxidative stress-related diseases.

- **Mostafa et al. (2011)** described the antibacterial and antioxidant activity of different plant part of the *Rumex vesicarius L*. Among that Ether extract of roots was found to be the most effective against *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* and *Streptococcus pyogenes,* methanol extract of roots was found to be the most effective against *Streptococcus pneumonia* and ethanol extract of flowers was found to be the most effective one against *Escherichia coli*. DPPH scavenging activity stem found to have the highest antioxidant activity, fruits were found to be the most effective plant part.

3. Significance of the study:

The chemical components present in plants have various biological roles and hence it has therapeutic value. They are known to have various biological activities such as antimicrobial, antifungal, antioxidant, etc. alkaloids, flavonoids, tannins, and other phenolic compounds are the important bioactive compound in plants, which are generally called as secondary metabolites. To develop active molecules, Pharmaceutical research is now broadly focusing on natural compounds of plant origin. In the present study, Isolation of compound from leaf extract of *Martynia annua* was performed to know about the biological activity.

4. Scope of the study:

The main scope of this study was to know the biologically active compounds present in the plant.

5. Objectives of the study:

- To analyse phytochemicals in M.annua leaf using various solvent.
- To characterize the *M. annua* methanolic leaf extract (PE) by Gas Chromatography and Mass Spectrometry (GC-MS) and Analytical High Performance Liquid Chromatography (HPLC).
- To separate and isolate the fractions using partial Thin Layer Chromatography (TLC) techniques.
- To observe the various activities viz., antibacterial, antibiofilm and antioxidant activity of methanolic leaf extract and separated fractions.

- To find the functional organic group vibration of the compounds by Fourier Transform Infrared (FTIR).
- To identify the compounds from the isolated fractions by GC-MS analysis.

6. Research Methodology:

6.1 Preparation of the Plant Extracts

To remove the surface contaminants the leaves were washed thrice with running tap water and the leaves were dried under shade for 2–3 weeks and ground into fine powder, placed in air tight polythene bags and stored in refrigerator at 4°C for future use.

6.2 Cold Extraction

Ten gram of powdered sample was soaked in distilled water and methanol, ethanol, petroleum ether, chloroform in 100ml separately for 12hrs at room temperature. The extracts were then filtered through whatmann no:1 filter paper and concentrated to final volume of 50ml and subjected to phytochemical analysis.

6.3 Phytochemical Screening of Extracts

Methanol, ethanol, petroleum ether, chloroform and distilled water extracts were used for preliminary phytochemical analyses using standard procedures (Harborne, 1973). The following qualitative tests for both the metabolites were done as follows:

a) Test for Alkaloids

***Mayer's test*:** About 0.5 ml filtrate was taken and few drops of Mayer's reagent was added and the formation of yellow color precipitation indicates the presence of alkaloids.

Wagner's test: About 0.5 ml filtrate was taken and few drops of Wagner's reagent was added and the formation of a reddish brown precipitate indicates the presence of alkaloids.

b) Test for Flavonoids and Phenols

Ferric chloride test: Dark bluish black color appearance was found while adding 0.5 ml of filtrate along with few drops of ferric chloride which indicates the presence of flavonoids.

Lead acetate test: 0.5 ml of filtrate was taken and few drops of 10% lead acetate solution was added. Appearance of yellow colour precipitate indicates the presence of flavonoids.

c) Test for Tannins

Gelatin test: 0.5 ml of filtrate was taken and few drops of 1% gelatin along with few drops of sodium chloride. White color precipitation was formed which indicates the presence of tannins.

Ferric chloride test: Dark bluish black color appearance was found while adding 0.5 ml of filtrate along with few drops of ferric chloride which indicates the presence of tannins.

d) Test for Saponins

Foam test: 0.5 ml of filtrate was taken and it is diluted with 5 ml distilled water and shaken vigorously. The formation of foam to a length of 1cm indicated the presence of saponins and steroids.

Lead acetate test: 0.5 ml of filtrate was taken and few drops of 10% lead acetate solution was added. Appearance of yellow colour precipitate indicates the presence of saponins.

e) Test for Steroids and Sterols

Salkowski's test: 0.5 ml filtrate was dissolved in 2 ml of chloroform and equal volume of concentrated sulphuric acid was added along the sides of the test tube. The upper layer turns red and lower layer turns yellow with green fluorescence, indicating the presence of the steroids and sterols.

f) Test for Glycosides

***Keller Kilani test*:** 0.5 ml of filtrate was taken with that 2 ml glacial acetic acid and 1 drop of ferric chloride was added where 1 ml conc. Sulphuric acid was added along the sides of the test tube. Violet ring was formed which is followed by brown ring formation which indicates the presence of glycosides.

Legal's test: Add 0.5 ml filtrate in 0.5 ml pyridine to that 2% sodium nitropruside and few drops of 20% sodium hydroxide was added. Appearance of pink to blood red color indicates the presence of glycosides.

g)Test for Carbohydrates

Fehling's test: 0.5 ml of filtrate was taken to that few drops of Fehling's solution was added and boiled in a water bath. The formation of yellow or red precipitate indicates the presence of reducing sugars.

Benedict's test: 0.5 ml of filtrate was taken to that few drops of Benedict's solution was added and boiled in a water bath. The appearance of red or yellow or green precipitate indicates the presence of reducing sugars.

h) Test for Protein & Amino Acids

Ninhydrin test: About 0.5 ml of filtrate was taken and 2 drops of freshly prepared 0.2% ninhydrin

reagent was added and heated. The appearance of pink or purple colour indicates the presence of proteins, peptides or amino acids.

6.4 TLC of Plant Extract

Analytical TLC (Awa et al., 2012)

Thin layer chromatography (TLC) was performed on a silica gel plate (TLC Aluminium Sheets Silica Gel 60 F_{254}, 0.2 mm, 20×20 cm,) were cut with ordinary household scissors. Plate markings were made with soft pencil. An aliquot of *M.annua* methanolic extract was spotted on the silica gel plate using glass capillary tube. Then plates were allowed for 2 minutes of air dry. The solvent system (mobile phase) of chloroform:methanol (9:1 v/v) was prepared and poured in a TLC chamber. Air dried spotted silica plates were introduced in to the TLC chamber and closed with lid for avoiding solvent evaporation. The entire setup was kept undisturbed till the mobile phase develops chromatogram. The plates were run in the above solvent system and air dried. The spots were visualized under ultraviolet (UV) lamp. The movement of the analyte was expressed by its retention factor (R_f). Different bands were observed and corresponding R_f value were calculated as (Lim et al., 2002).

$$\mathbf{R_f} = \frac{\textbf{Distance travelled by the solute}}{\textbf{Distance travelled by the solvent front}}$$

The separated bands were scrapped using sterile scalpel and scrapped bands were dissolved with considerable volume of methanol. Then the dissolved mixture were allowed to stir continuously for 4-5 hours using magnetic stirrer. After 4-5 hours, the stirred compounds were filtered with Whatmann No. 1 filter paper. Then the solvent evaporation occurs in the filtrate. The condensed powder was scrapped out and taken for further investigation.

6.5 Semi Preparative TLC (Fuentes et al., 2012)

An aliquot of fractions were spotted on the silica gel plate using glass capillary tube. Then plates were allowed for 2 minutes of air dry. The solvent system (mobile phase) of chloroform: methanol (9:1) v/v was prepared and poured in a TLC chamber. Air dried spotted silica plates were introduced in to the TLC chamber and closed with lid for avoiding solvent evaporation. The entire setup was kept undisturbed till the mobile phase develops chromatogram. The plates were run in the above solvent system and air dried. The plate under UV light (254 nm) was developed. The distances travelled by each spot were measured with a ruler and recorded.

6.6 Characterization

6.6.1 Gas-Chromatography-Mass Spectrometry (GC-MS)

Samples of leaves were ground with liquid nitrogen and extracted using methanol. The extracted samples were analyzed using Gas-Chromatography-Mass Spectrometer (GC-MS). GC-MS analysis was carried out at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU), New Delhi on a Shimadzu GCMS-QP2010 PLUS GC-MS equipped with an AOC-20i auto injector and AOC-20s auto-sampler units. Separation of metabolites was performed on an RTX-5MS GC column (Restek Corporation) with 0.25mm thickness, 30m length, and 0.25mm diameter. One micro liter of sample was injected in split mode in a 1:10 split ratio by the auto sampler attached to the instrument. The injection temperature was set at 230°C and column oven temperature was 70°C. Helium was used as the carrier gas at a constant linear velocity of 40.3 cm/s. Mass spectrometry settings were adjusted as follows: ion source temperature, 230°C; interface temperature, 280°C; and solvent cut time, 3.5 min. The temperature program used was: 1 min hold at 70°C, followed by 9°C/min ramp to reach a temperature of 310°C held for 5 min, and final ramp of 20 °C/min to reach a final temperature of 320°C held for 2 min.

6.6.2 High Performance Liquid Chromatography (HPLC) analysis

High-performance liquid chromatography is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase (s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The detector may also provide other characteristic information. HPLC analysis of MLE of *M.annua* was performed using the methodology of Anjum et al., (2011). About 1 mg of concentrated sample was dissolved in 1 ml of Methanol and 20 µl was injected to determine the phytocontituents.

6.7 Antibacterial Assay

In vitro antibacterial activities of methanolic extracts of *M.annua,* and its fractions were determined by standard agar well diffusion assay (Nostro et al., 2000). The multidrug resistant organism *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus epidermidis* were collected from Government Mohan Kumaramangalam Medical College Hospital, Salem. Petri dishes (100 mm) containing 25 ml of Mueller–Hinton Agar (Merck) seeded with 100 µl inoculum of

bacterial strain (Inoculum size was adjusted so as to deliver a final inoculum of approximately 10^6 CFU/ml). Media was allowed to solidify and then individual petridishes were marked for the bacteria inoculated. Wells of 6 mm diameter were cut into solidified agar media with the help of sterilized cup-borer. 100 µl of extract, fractions and antibiotic were poured in the respective wells and the plates were incubated at 37°C for overnight. Cefotaxime antibiotic (1U strength) was used as standard. The experiment was performed in triplicate under strict aseptic conditions and the antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by the respective extract at the end of incubation period.

6.7.1 Bactericidal Studies in Broth

The isolated fractions, crude and antibiotic were tested against 4 human-pathogenic bacteria including one Gram positive *Staphylococcus epidermidis* and three Gram negative *Pseudomonas aeruginosa, Klebsiella pneumonia and Escherichia coli.* Bacterial strains were maintained on Mueller–Hinton agar slants and incubated at 37°C for 24–48 h (Muller and Hinton, 1941). The inoculum were spread over Mueller–Hinton agar plates (10^7 cfu). Each tube was inoculated with 250 µl of the 4 tested bacterial suspensions. The Crude, fractions and antibiotic (Cefotaxime) were added into test tubes containing 4.5 ml of Mueller–Hinton broth medium and incubated at 37° C for 24 h. Three replicates were set up for each treatment (Salem et al., 2014). Control tubes were prepared by adding 250 µl of each bacterial suspension without the extract. Antibiotic (Cefotaxime) and crude treatments (as above) were prepared for comparison. Bacterial growth was measured as optical density (OD) at 600 nm using Systronics Double beam Spectrophotometer 2203. The percentage of bacterial growth inhibition was calculated as indicated earlier (Banjara et al., 2012) as follows:

Percentage of growth inhibition = OD of control − OD of test / OD of control × 100

6.7.2 Anti-Biofilm Assay

To study the anti-biofilm activity *Pseudomonas aeruginosa* (Gram negative), *Staphylococcus epidermidis* (Gram positive) bacterial strains were selected. The cultures were grown overnight and diluted to 1:100 in 4ml fresh nutrient medium in the test tube containing different concentrations (20, 40, 60, 80, 100 µg/ml) of crude, fractions and antibiotic (cefotaxime) were added to the bacterial cells and incubated at 37°C for 24 h. After incubation, the medium was removed, the side walls of the test tubes were thoroughly washed with distilled water, and 0.1% (w/v) crystal violet was added

and incubated for 45 min. The crystal violet was removed and washed thoroughly with distilled water. For quantification of the attached cells, the crystal violet was dissolved in 90% ethanol, and the absorbance was measured at 595 nm (Mohanty et al., 2012).

6.9. *In vitro* Antioxidant activity

6.9.1. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging method was used to evaluate the antioxidant property. DPPH radical scavenging activity of the extract was measured by the method described by (Barros et al, 2008). The concentrations of the plant extracts and fractions required to scavenge DPPH showed a dose dependent response (Hsu et al., 2007). The antioxidant activity of each sample was expressed in terms of IC_{50}, and was calculated from the graph after plotting inhibition percentage against various concentrations. For this, different concentrations (20, 40, 60, 80, 100 µg/ml) of extract, fractions and ascorbic acid (standard) were prepared with methanol (Sigma Aldrich) as the test solutions. About 1 ml of each prepared concentrations were placed into test tubes and 0.5 ml of 1 mmol/l DPPH solution in methanol was added. The test tubes were incubated for 15 min and the absorbance was read at 517 nm. A blank solution consisted of DPPH dissolved in same amount of methanol. Ascorbic acid was used as reference compound. The DPPH radical scavenging activity percentage was calculated by using the following formula:

DPPH radical scavenging activity (%) = A control – A Treated / A control*100

Where A control is the absorbance of a DPPH solution without extract; A Treated is the absorbance of the tested extract. All measurements were performed in triplicate.

6.9.2. IC_{50} Value

Inhibition Concentration (IC_{50}) parameter was used for the interpretation of the results from DPPH method. The discoloration of sample was plotted against the sample concentration in order to calculate the IC_{50} value. It is defined as the amount of sample necessary to decrease the absorbance of DPPH by 50% (Williams et al., 1995).

6.10. Fourier Transform Infrared Spectroscopy (FTIR)

The fraction was subjected to spectroscopic analysis by Fourier Transform Infrared Spectroscopy (FTIR). Fourier transforms infrared spectroscopy is a technique which is used to obtain an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gas (Griffiths et al., 2007).

ART (Attenuated Total Reflectance) model FT-IR Spectrophotometer (Bruker, United States) was used for the analysis of the fractions. Sample (Fractions) were mixed with KBr (spectroscopy grade) and then compressed, in order to prepare translucent salt discs (3 mm diameter). The disc was immediately kept in the sample holder and FT-IR spectra were recorded in the absorption range between 4000 and 400 cm^{-1} at 4 cm^{-1} resolution.

7. RESULTS AND DISCUSSION

In this study, qualitative estimation of phytoconstituents in the leaves of *Martynia annua* was analysed using five different solvents namely petroleum ether, chloroform, ethanol, methanol and distilled water.All the extracts were subjected into various tests and methods. The changes of colour conforms the presence of various phytochemicals(Fig:1). All the extracts showed the various phytochemicals such as tannins, saponins, flavonoids, alkaloids, cardiac glycosides and reducing sugars. The presence/absence of phytoconstituents were discussed in the (Table 1)

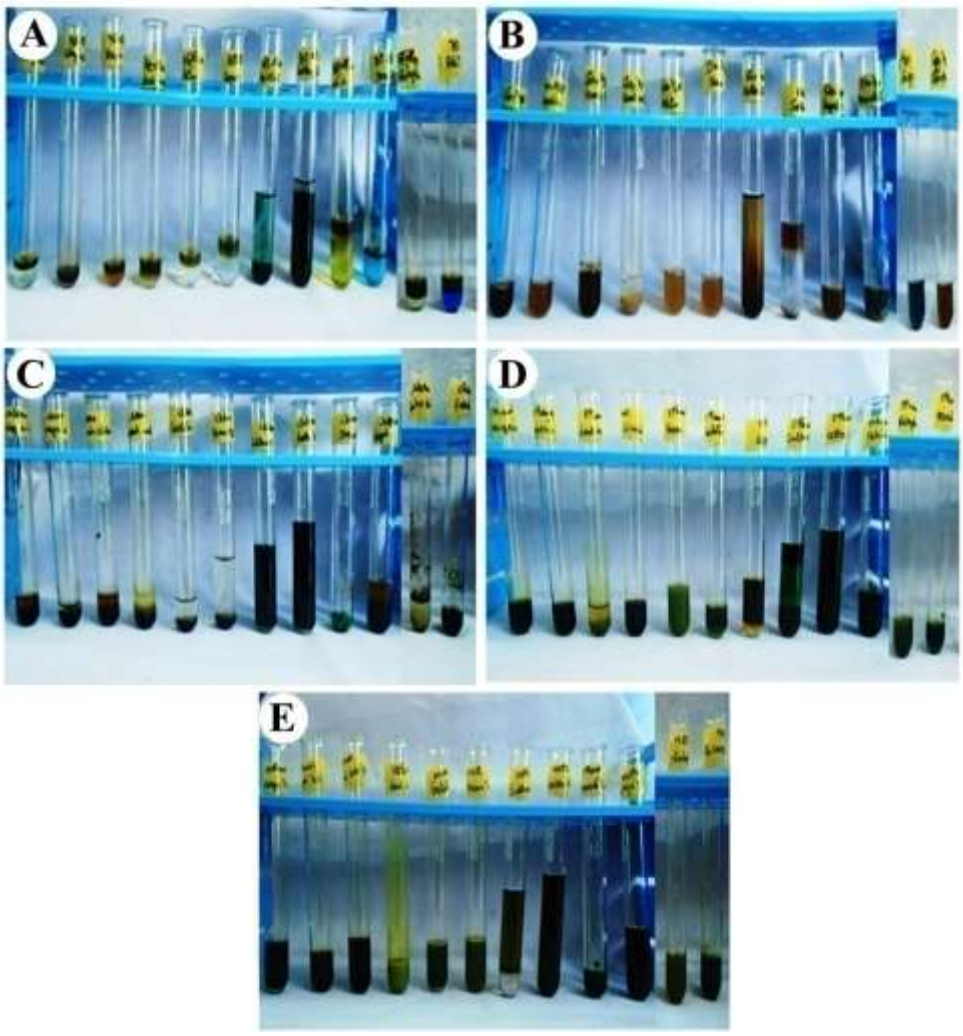


Fig. 1 Phytochemical screening of extracts from various solvents **A**) Petroleum ether, **B**) Distilled water, **C**) Chloroform, **D**) Ethanol and **E**) Methanol

Table 1: Preliminary phytochemical screening of *Martynia annua* leaf extracts

Phytochemicals		**Methanol**	**Ethanol**	**Petroleum Ether**	**Chloroform**	**Distilled water**
Alkaloids	Mayer's	-	-	-	-	
	Wagner's	+	-	+	+	+
Phenols	Ferric chloride	+	+	+	+	
	Lead	-	-	-	-	

	Acetate				
Flavonoids	Ferric chloride	+	+	+	+
	Lead Acetate	-	-	-	-
Tannins	Ferric chloride	+	+	+	+
	Gelatin Test	-	-	-	-
Saponins	Foam Test	-	-	-	-
	Lead Acetate	-	-	-	-
Cardiac glycosides	Legal's Test	-	-	-	-
	Keller Kilani	-	+	+	-
Phytosterols	Salkowski's	-	-	-	-
Carbohydrates	Benedict's	+	-	-	+
	Fehling's	+	-	-	-
Proteins	Ninhydrin	-	-	-	-

As the above results methanolic leaf extract showed the major phytoconstituent. Hence methanolic leaf extract was selected and extracted with soxhlet apparatus and were subjected to further characterizations and investigations.

7.1. Characterization

7.1.1. GC-MS analysis

The compounds recognized from the GC-MS analysis of methanolic extract of *Martynia annua* leaves exhibiting various phytochemicals (**Fig. 2**) and they were predominantly responsible for the antibacterial and antioxidant activity of the extracts. The retention time, percentage peak of various bioactive compounds and its activity was presented in the (**Table 2**).

Fig.2 The peaks shows the various phytoconstituents of methanolic leaf extract of *Martynia annua*.

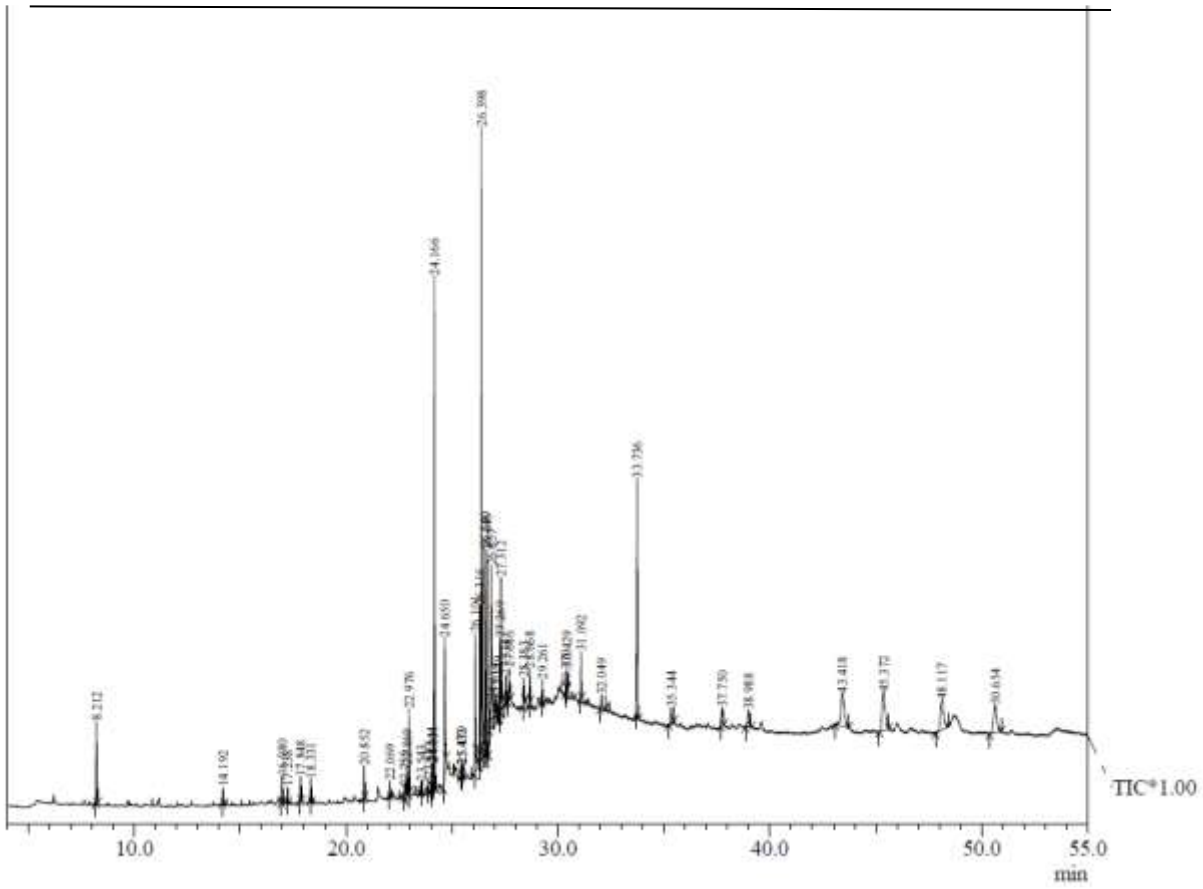


Table 2 The retention time, percentage peak of various bioactive compounds and its activity

Peak#	R.time	Area	Area%	Compound name	Molecular weight	Formula	Nature of the compound	Activity
1.	26.398	12858699	15.00	9,12,15-octadecatrienoic acid, methyl ester, 9,12,15 octadecatrienoate, linolenic acid methyl ester	292	$C_{19}H_{32}O_2$	Linolenic acid ester	Antiinflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective Nematicide Insectifuge Antihistaminic, Antieczemic Antiacne, 5-Alpha reductase inhibitor Antiandrogenic Antiarthritic, Anticoronary Insectifuge
2.	24.166	9081782	10.60	Hexadecanoic acid, methy ester, palmitic acid methy ester, metholene	270	$C_{17}H_{34}O_2$	Palmitric acid (saturated fatty acids)	Anti bacterial activity,Ant fungal activity,Antitumo activity
3.	33.736	6349560	7.41	Squalene, squale 2,6,10,14,18,22-tetracosahexaene,	410	$C_{30}H_{50}$	Antioxidant	Neutralize differen xenobiotics, antiinflammatory, anti atherosclerotic and antineoplastic, role in skir aging and pathology, and

							Adjuvant activities
4.	26.857	5890997	6.87	Methyl octadeca-9,12-dienoate, 9,12-octadecadienoic acid methyl ester	294	$C_{19}H_{34}O_2$	Fatty acids	Antioxidant, anticancer
5.	26.540	4678467	5.46	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, (E)-phytol	296	$C_{20}H_{40}O$	Diterpene alcohol	Antimicrobial, anticancer anti-inflammatory, antidiuretic, immunostimulatory and anti-diabetic
6.	45.372	4049414	4.72	Gamma.-sitosterol,ethylcholest-5-en-3.beta.-ol, Clionasterol Fucosterol, .beta.-dihydro-	414	$C_{29}H_{50}O$	Sterol	Anti bacteria activity,cytotoxic activity (breast and colon carcinoma)
7.	43.418	3691215	4.31	Stigmasterol, Stigmasterin 5,22-Cholestadien-24-ethyl-3.beta.-ol	412	$C_{29}H_{48}O$	Lipid	Reduces plasma cholestero level, Inhibits hepatic synthesis
8.	24.650	3620838	4.22	Pentadecanoic acid Pentadecylic acid	242	$C_{15}H_{30}O_2$	Fatty acids	Anti bacterial activity,Ant fungal activity,Antitumo activity

9.	27.312	3498979	4.08	Methyl octadeca-9,12-dienoate, 9,12-octadecadienoic acid methyl ester	294	$C_{19}H_{34}O_2$	Fatty acids	Antioxidant, anticancer
10.	26.646	3250127	3.79	Methyl stearate Octadecanoic acid, methy ester	298	$C_{19}H_{38}O_2$	Fatty acids	Antibacterial activity
11.	48.117	3068336	3.58	4,22-Stigmastadiene-3-one Stigmasta-4,22-dien-3-one	410	$C_{29}H_{46}O$	Steroids	Antibacterial activity
12.	50.654	2996963	3.50	Stigmast-4-en-3-one, .delta.4-sitosterol-3-one	412	$C_{29}H_{48}O$	Steroids	Hypoglycemic
13.	26.101	2665769	3.11	13-Hexyloxacyclotridec-10-en-2-one	280	$C_{18}H_{32}O_2$	Triterpene	Antitumor
14.	26.316	2461230	2.87	9,12-octadecadienoic acid (z,z)-, methyl ester, linoleic acid,	294	$C_{19}H_{34}O_2$	Fatty acids	Antioxidant, anticancer
15.	22.976	1626502	1.90	2-Pentadecanone, 6,10,14-trimethyl-, Hexahydrofarnesyl acetone	268	$C_{18}H_{36}O$	Essential oil	Anti microbial activity
16.	27.269	1409129	1.64	Hexadecanamide, palmitic acid amide	255	$C_{16}H_{33}NO$	Fatty acid amide	Antinociception, Anticonvlsant activity

17.	27.686	918467	1.07	9,12-octadecadien-1-ol,linoleyl alcohol	266	$C_{18}H_{34}O$	Fatty alcohol	No activity reported
18.	31.092	906103	1.06	Phenol, 4,4'-methylenebis[2,6-bis(1,1-dimethyl	424	$C_{29}H_{44}O_2$	Phenols	Antimicrobial and antioxidan activity
19.	38.988	853797	1.00	Stigmast-5-en-3-ol, oleat,stigmast-5-en-3-yl octadecenoate	678	$C_{47}H_{82}O_2$	Steroids	Antioxidant,antimicrobial,anti inflammatory

7.2. HPLC Chromatogram

7.2.1. High Performance Liquid Chromatography (HPLC)

The preferred aim was achieved using polar compounds (methanol extract) Methanol: Water (50:50) as the mobile phase. The wave length of 254 nm was found to be optimal for the highest sensitivity. Presently the analysis of crude extract has revealed 3 peaks in *M.annua*. The R_f values is respective the areas (%) covered by the individual peaks are represented in **Figure 3** and **Tables** 3

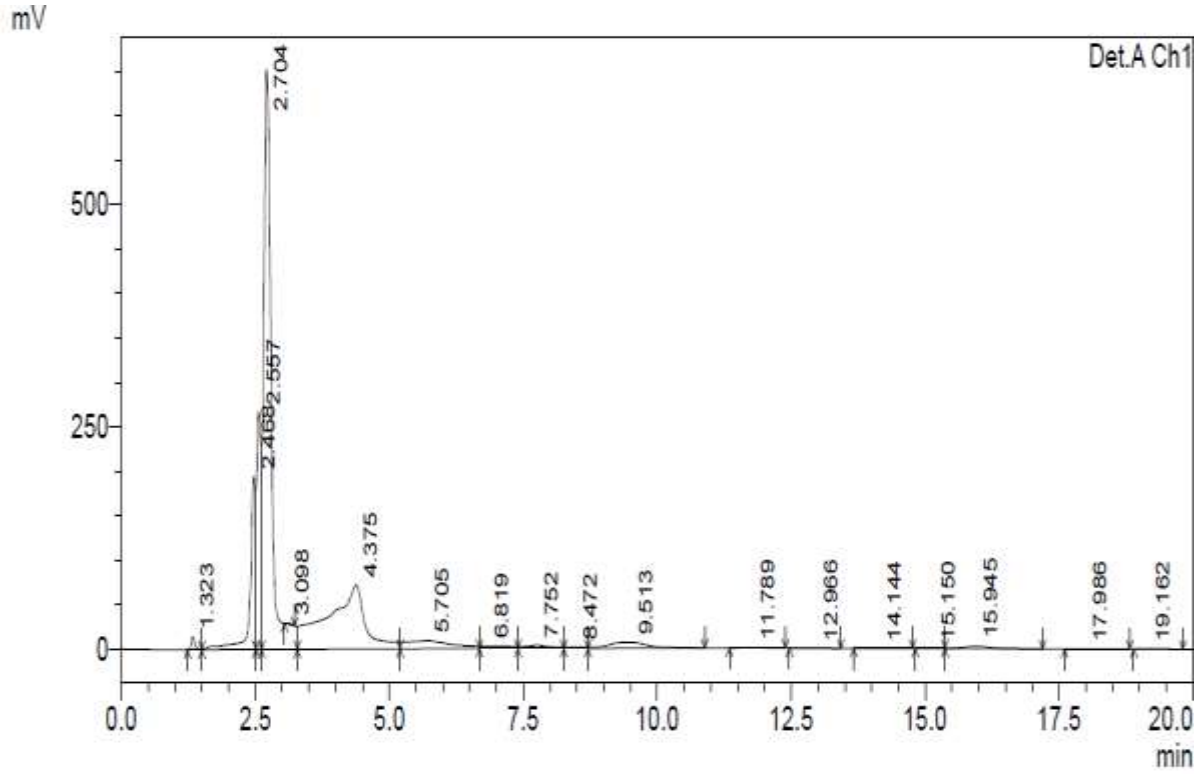


Fig.3 HPLC chromatogram of methanolic leaf extract

Table 3 The retention time, percentage peak of various bioactive compounds

PEAK#	Ret. Time	Area	Height	Area %	Height%
1	1.323	66779	14421	0.468	1.173
2	2.468	1390079	195001	9.743	15.856
3	2.557	1546439	267029	10.839	21.712
4	2.704	6444062	652326	45.165	53.041
5	3.098	8178	1344	0.057	0.109
6	4.375	3528382	72030	24.730	5.857
7	5.705	546104	8976	3.828	0.730
8	6.819	115587	2959	0.810	0.241
9	7.752	120419	3590	0.844	0.292
10	8.472	37392	1443	0.262	0.117
11	9.513	322693	6348	2.262	0.516
12	11.789	15524	544	0.109	0.044
13	12.966	9471	310	0.066	0.025
14	14.144	6669	188	0.047	0.015
15	15.150	4728	195	0.033	0.016
16	15.945	92992	2782	0.652	0.226
17	17.986	7217	210	0.051	0.017
18	19.162	5057	161	0.035	0.013
Total		14267773	1229857	100.000	100.000

7.3. Compound Isolation

7.3.1. Thin Layer Chromatography (TLC)

Methanolic extract of *M. annua* was subjected to separation by Thin layer chromatography using the mobile phase Chloroform:Methanol (9:1) (**Fig. 4A**) and the clear band separation was observed under UV which is represented (**Fig 4B**). The R_f value of different

fractions were noted, the separated bands were eluted and subjected to continuous stirring for 4 hours subsequently the bands was filtered and concentrated. In semi-preparative tlc fraction was further diluted with methanol and runned in a same solvent system. Single bands were viewed under UV. The two bands were found visual in the UV light with the same R_f value (band 1 – 0.73 and band 2- 0.46). Based on the R_f values, the fractions may be flavonoids or terpenoids (Biradar et al., 2013).

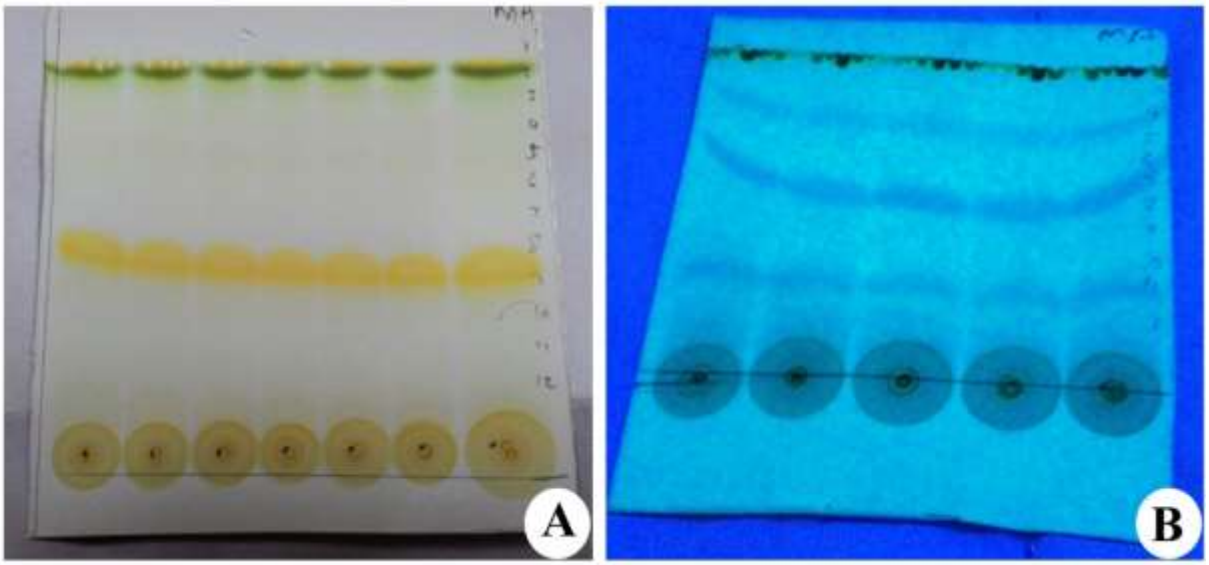


Fig.4. TLC profile of methanolic leaf extract *M.annua*

7.3.2. Antibacterial Activity (Plate and Tube Method)

Antibacterial activity of PE, MA1, MA 2 and antibiotic (Cefotaxime) was tested against 4 different bacterial strains viz., *P. aeruginosa, K. pneumoniae, E. coli and S. epidermidis* was assayed by Agar Well Diffusion Method using Muller Hinton Agar in 90 mm plate (MHA). The concentration of PE, MA1, MA 2 and antibiotic was 100 µl. The zone of inhibition in diameter was tabulated below.As a result MA2 showed higher zone of inhibition(Table 4) as compared to the PE and MA1 where the inhibition rate is higher in gram negative bacteria than the gram positive bacteria due to the difference in the cell wall of the bacteria.(Fig 5).4

Table 4 Antibacterial activity on solid medium

Bacteria	Zone of inhibition Mean±SD	MA2	MA PE	Antibiotic(Cefotaxime

	(mm) MA1			
P.aeruginosa	10.33±0.50	11.00±0.00	9.00±1.00	14.33±1.5
E.coli	11.33±0.50	12.33±0.57	10.00±0.00	13.33±0.57
S. epidermidis	11.00±1.00	11.67±0.57	10.33±0.57	15.00±1.00
K. pneumoniae	12.33±0.57	15.67±0.0	10.33±0.57	23.33±1.52

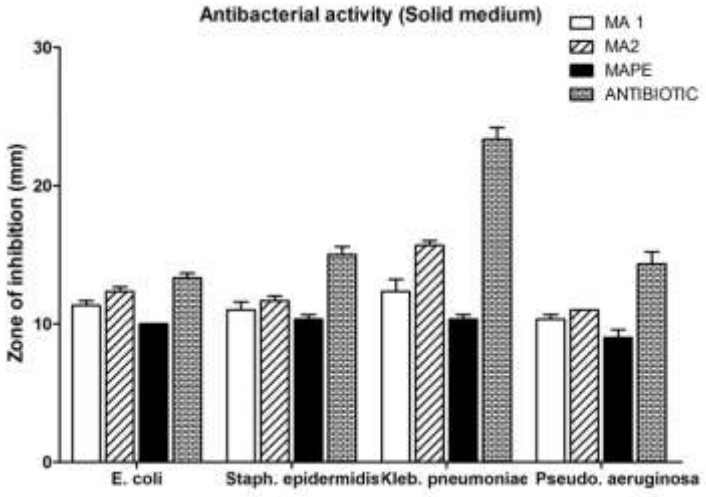


Fig 5 Graph shows efficacy of the Antibacterial activity on MA PE, MA1, MA2, and antibiotic (solid medium)

The percentage of growth inhibition in liquid medium was calculated for PE, MA1, MA2, anibiotic (Cefotaxime) against *P. aeruginosa, K. pneumoniae, E. coli and S. epidermidis* while compared with MA1 and PE, measurements of optical density (OD) were much more decreased when bacterial suspensions were treated with MA2. In addition, the percentage of growth inhibition of MA2 for all the tested bacterial strains were ranged from 32-72%.As the surface area is found to be moderate in solid media, the inhibition rate was not calculated efficiently but liquid media showed up to 72% growth inhibition for the compounds (Fig.6) .

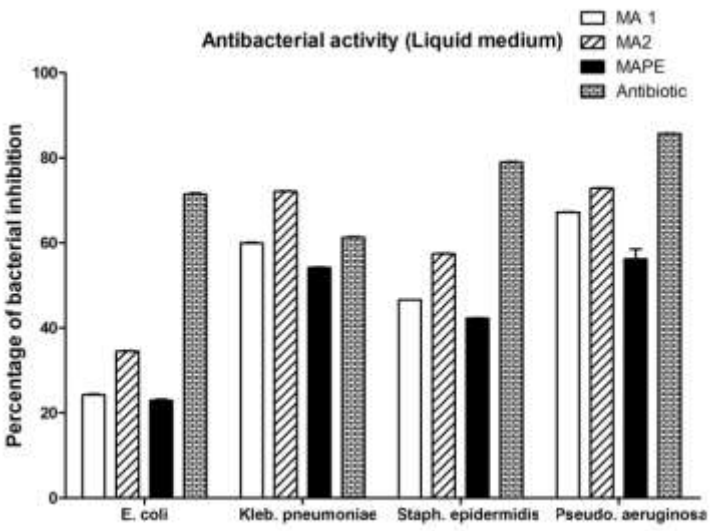


Fig. 6. Graph shows efficacy of the Antibacterial activity on MA PE, MA1, MA2, and antibiotic (liquid medium)

7.4. Antibiofilm Activity

In vitro antibiofilm activity of PE, MA1 and MA2 against *S. epidermidis*, and *P. aeruginosa* was performed. The antibiofilm activity of PE, MA1 and MA2 was concentration (20, 40, 60, 80, 100 µg/ml) dependent. Results indicated that maximum reductions in cell attachment were observed in *P. aeroginosa* upto 61.79%, 71.41%, and 75.28% of PE,MA1 and MA2 respectively at 100 μg/mL concentration (Fig. 7), whereas at 20 μg/mL level the strains exhibited comparatively less biofilm activity. Similarly in *S. epidermidis* maximum reduction were observed upto 60.94%, 65.91%, and 68.96% of PE, MA1, and MA2 at 100 μg/mL (Fig. 7). As campared to the above result gram positive bacteria, gram negative shows maximum level of inhibion. MA2 shows the highest inhibition for both tested organism and are able to prevent or disturb the biofilm formation. The results showing the dose dependent response were represented in the Figure

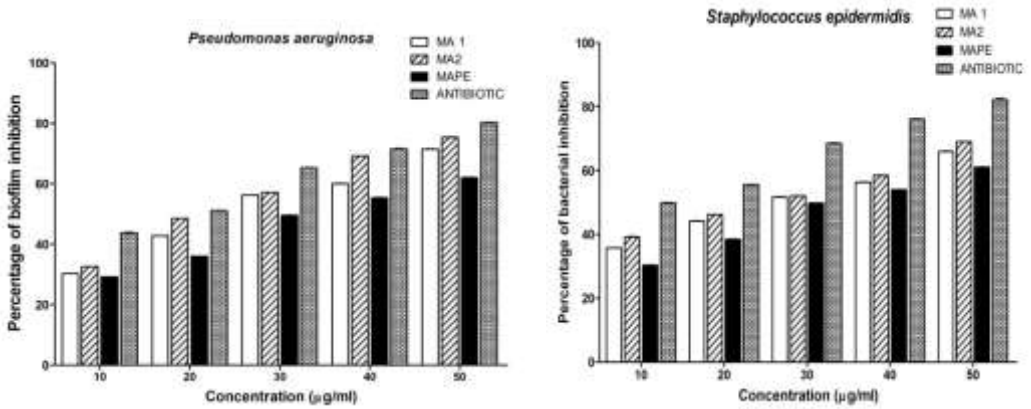


Fig.7 Graph shows efficacy of the Antibiofilm activity on MA PE, MA1, MA2, and antibiotic

7.5. Antioxidant Activity

The evaluation of the free radical scavenging potential of the crude, compound (MA1 and MA2) and Standard (Ascorbic acid) at different concentrations (20, 40, 60, 80, 100 µg/ml) were tested by the DPPH method. The dose response graph of DPPH radical scavenging activity of crude extracts and compounds of plant was observed, when compared with standard ascorbic acid and shown in (Fig. 8). Antioxidant activity in the form of IC_{50} values were calculated and shown in Table 4. Highest antioxidant activity was given by MA2 approximately at the concentration of 49.46 µg/ml among the MA PE and MA1 which is found to be more than even the ascorbic acid while activity of MA1 was found to lower to the crude. Thus it is clear that polyphenolic antioxidants in leaves of selected plants play an important role as bioactive principles and the scavenging effect can be attributed to the presence of active phytoconstituents in them

Table.5. Free radical scavenging activity of methanolic crude extracts, MA1 and MA2 of *M. annua*

Sample	**IC_{50} (µg/ml)**
Ascorbic acid(standard)	16.03
MA.PE	61.3
MA1	84.56
MA2	49.46

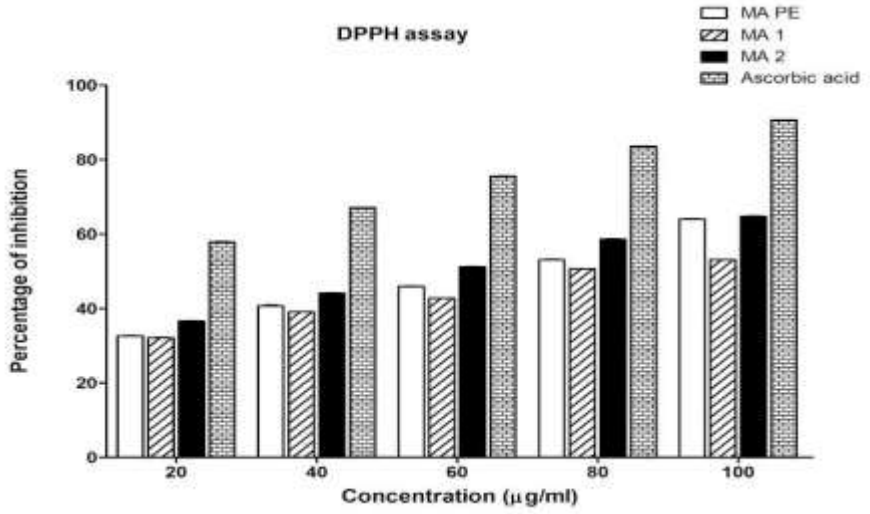


Fig.8 Graph shows efficacy of the Antioxidant activity on MA PE, MA1, MA2, and ascorbic acid (standard)

7.6. FTIR Analysis

The functional organic group vibration was given by FTIR analysis. The FTIR spectra were recorded for separated compounds MA1, and MA2 from *M. annua* methanolic of leaf extract Typical IR spectrum of MA1 showed strong peak at 3442.34cm^{-1} represent N-H (primary amines), 2922.44cm^{-1} and 2853.33cm^{-1} represent O-H stretching (carboxylic acid), 2389.41cm^{-1} represent P-H stretching (phosphours acid and ester), 1736cm^{-1} represent C=O stretching (ester, aliphatic), 1566.62 cm^{-1} represent C=O (carboxylic acid/derivatives, carboxylates (salts)), 1418.15 cm^{-1} represent N=N stretching (azo compound), 1105.16cm^{-1} C-O (esters), 797.51cm^{-1} strong C-H (aromatic),618.68cm^{-1} represent C-X (chloroalkanes), 466.84cm^{-1} represent S-S stretching (sulfur compound) group. The FTIR spectrum of MA2 represented peaks at 3438.82cm^{-1} corresponds to strong N-H (primary amines), 2852.52cm^{-1} corresponds to C-H stretching (aldehyde), 2315.30cm^{-1} phosphine P-H stretching (phosphorus compound), 1739.77cm^{-1} corresponds to C=O stretching (aliphatic, esters), 1577.96cm^{-1} C=N(pyridine),C=C stretching (various nitrogen containing compound), 1467.73cm^{-1} corresponds to C=C stretching (aromatic compound), 1109.02cm^{-1} C-H stretching (organic compound), 795.96cm^{-1} corresponds to P-O-C stretching (phosphorus compound), 723.07cm^{-1} corresponds to strong C-H (aromatic, monosubstituted benzene), 616.74cm^{-1} corresponds to =C-H bending (alkynes) 465.99cm^{-1} corresponds to P-Cl stretching (phosphorus compound).

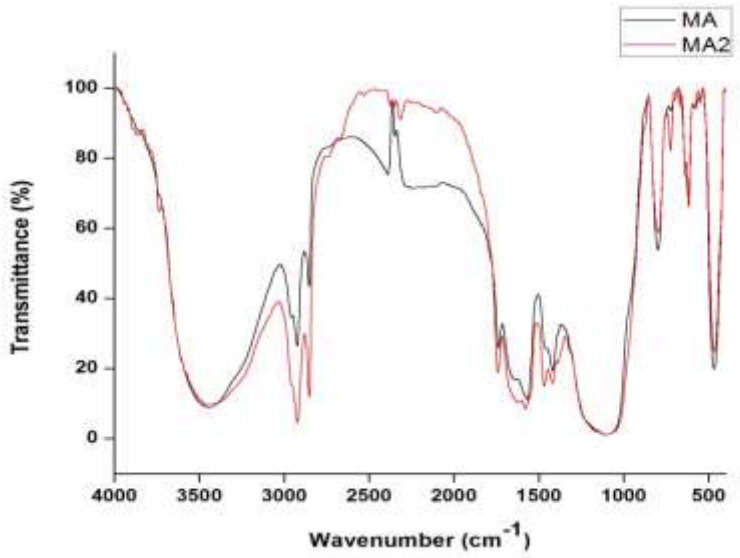


Fig.9 FTIR analysis for fractions MA1 and MA2

7.7 GC-MS analysis for separated fractions

GC-MS analysis for separated and isolated fraction of MA1 and MA2 were subjected into the Gc-Ms analysis using THERMO GC - TRACE ULTRA VER: 5.0,THERMO MS DSQ II. Gc-Ms analysis of fraction MA1 showed three major peaks(Fig.10)of 2-tert-Butyl-4isopropyl-5-methylphenol (12.93%), 3,6-bis(t-Butyl)fluorenone (21.70%), 13-Docosenamide, (Z) (35.31%). Similarly MA2 showed two major peaks (Fig.11) of Hexadecanoic acid, methyl ester (21.6%) 13-Docosenamide, (Z) (35.30%).Structure and nature of compound were depicted in the table (Table 6,7) Hence the fraction of MA1 and MA2 possess the above compounds. These compounds may be responsible for the antibacterial, antibiofilm and antioxidant activity similar compounds and its activity were reported earlier (Harada et. al., 2002)

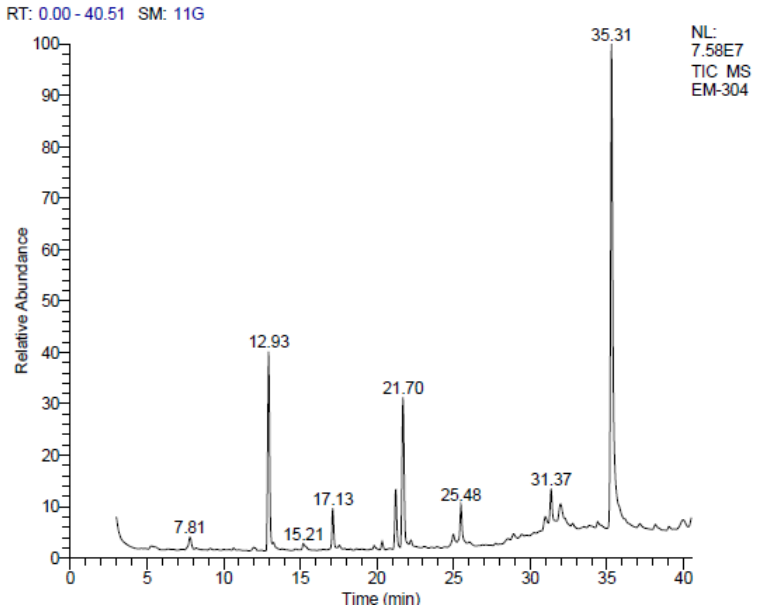
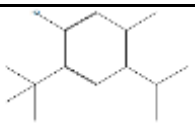
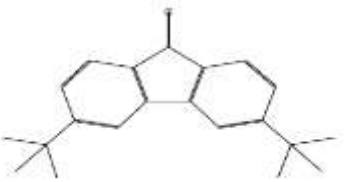
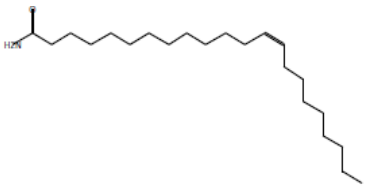


Fig.10 GC MS analysis for isolated fraction MA1

Table 6 GC-MS analysis of isolated fraction MA1

RT	**Molecular weight**	**Compound name**	**Formula**	**Nature of the compound**	**Structure**
12.93	206	2-tert-Butyl-4isopropyl-5-methylphenol	$C_{14}H_{22}O$	alcohol	
21.70	292	3,6-bis(t-Butyl)fluorenone	$C_{21}H_{24}O$	Aromatic organic compound	
35.31	337	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	Fatty acid	

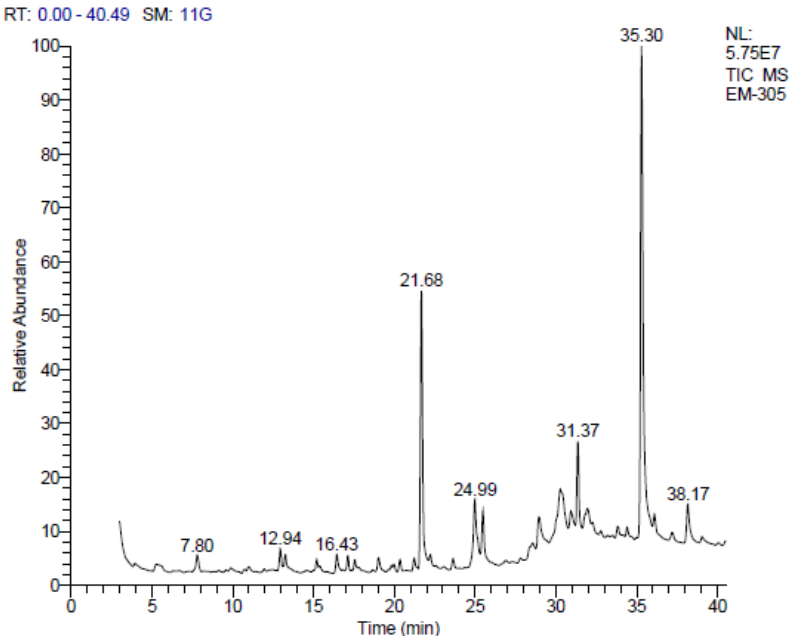


Fig.11 GC-MS analysis of isolated fraction MA1

Table 7 GC-MS analysis of isolated fraction MA2

RT	**Molecular weight**	**Compound name**	**Formula**	**Nature of the compound**	**Structure**
21.68	270	Hexadecanoic acid, methy ester	$C_{17}H_{34}O_2$,	Palmitic acid	
35.30	337	13-Docosenamide, (Z)-	$C_{22}H_{43}NO$	Amide o docosenoic acid	

8. Conclusion

Medicines derived from plants have made immense contribution towards the betterment of human health and act as a source of motivation for novel drug compounds. *M. annua* has numerous phytochemicals like tannins, alkaloids, phenols, carbohydrates that have potential to be used in the area of pharmacology. Thin layer chromatography was performed to separate the active compounds present in the methanolic extracts of *M. annua*. Plant extract, MA1 and MA2 were tested for antibacterial, antibiofilm and antioxidant activity. In the antibacterial activity of both liquid and solid medium, MA2 showed higher inhibition rate in the gram negative bacteria. In the antioxidant activity, MA2 showed maximum inhibitory concentration at 49.46µg/ml while compared with crude and MA1. A spectrum of GC-MS analysis revealed that compounds from methanolic extract of plant showed strong antibacterial, antioxidant, and anti inflammatory activities. Moreover, the separated fractions from methanolic leaf extract of *M. annua* were also characterized by GC-MS and FTIR analysis. GC-MS analysis of MA1 and MA2 showed different compounds which has antibacterial and antioxidant activity as reported earlier and results of FT-IR also confirmed the presence of functional groups. Due to the presence of these bioactive compounds in the isolated fraction of MA1 and MA2 may be responsible for the antioxidant, antibiofilm, and antioxidant activity. As the complex of compound has been present in fractions, further purification and structure of the fraction will be elucidated for the potential compound in future.

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