



Phytochemical, antioxidant and antibacterial activities of *Glycosmis pentaphylla* (Rutaceae) leaf extracts against selected multi-drug resistant bacteria's

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ABSTRACT

The emergence and spread of multi-drug-resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae* in associated with causing various illness in human beings. There is an urgent need for searching new drugs to treat bacterial infections. *Glycosmis pentaphylla* Retz. (Rutaceae) is a shrubby plant found all over India, used for the healing of wounds of livestock in Indian folk medicine. The crude leaf extract of *G. pentaphylla* was used to perform the screening of phytochemical, antioxidant, antibacterial activities and characterize the chemical constituents using Gas Chromatography-Mass Spectroscopy (GC-MS). The phytochemical screening and antioxidant activity was determined by free radical scavenging assay like DPPH and ABTS. The antibacterial activity was evaluated by agar well plate method. The phytochemical test results show the presence of alkaloids, saponins, tannins, phenolics except glycosides and amino acids. The antioxidant result found the ethyl acetate extract shows an excellent free radical scavenging activity of 63.4mg/ GAE and 49.10mg/g in DPPH and ABTS assays, respectively. The GC-MS spectrum reflects the presence of many biologically importance volatile constituents including, Quaterphenyl, Hexamethy and Heptatriactontadien-2-One. The better antibacterial activity was observed in *S. aureus* (17mm) followed by other organisms. MIC range was reported as 20mg/ml (the lowest concentration) against clinically isolated multi-drug resistant bacteria's. The outcome of this study suggests that use of *G. pentaphylla* plant extract is a best candidate for the treatment of infections caused by multi-drug resistant bacteria's.

Key words: *G. pentaphylla*, phyto-chemicals, antioxidants, antibacterial and GC-MS.

INTRODUCTION

Antibiotic resistance has become a serious and widespread problem in developing countries, both in hospitals and the community causing high mortality in each year [8]. Inappropriate usage of antibiotics is the most influential factor of antibiotic resistance and the global emergence of multi-drug resistant bacteria such *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae* is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure. Antibiotic resistance results in reduced efficacy of antibacterial drugs, making the treatment of patients difficult, costly, or even impossible. The impact on particularly vulnerable patients is most obvious, resulting in prolonged illness and increased mortality [52]. New therapy classes of antibiotics have become a popular choice to reduce the antibiotic resistance. However, antibiotic resistance is difficult to reduce. One strategy to avoid antibiotic resistance by using alternative therapeutic agents from plants that are effective against antibiotic resistant bacteria, safe and have low cost.

Recently the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics have reaffirmed the need to probe the antimicrobial activity of

medicinal plants [2, 12, 13 and 18]. Plants are rich source of secondary metabolites and novel therapeutic compound to enhance human health with controlled adverse effects [14]. Natural products has played vital role in pharmacological and commercial industries, produce a lot of health care and medicinal product such as antimicrobial, anti-tumour agents, antihepatotoxic, cardiotoxic, CNS stimulant, nutraceuticals, sweeteners, food additives and animal feeds [9,47]. Plants such as herbs, trees, shrubs and climbers are exploited for their various bioactive compounds for human health [5]. Moreover, plants contain important bioactive compounds such as alkaloids, flavonoids, saponins, steroids, terpenoids, polysaccharides and tannins that are largely contributing to various biological activities in traditional and modern therapeutic principles [4, 20]. Commonly used medicinal plants have promising biological activity to control various bacterial and fungal diseases [31]. The basis for separation of compound from the natural products using different polar and non-polar solvents are in important for extraction of single compound [37]. The interaction of different groups of active metabolites in the extract might have enhanced the therapeutic effect more than the single ingredient [26, 27]. Biochemical pathways or cellular mechanisms have been producing free radicals and reactive oxygen species as an end product [25]. Unstable chemical compounds are dangerous to living cells, it can cause mutation, myocardial infarction, Alzheimer disease and can be associated with other clinical disorders [1, 33]. Generally, chemical based antioxidants are used to control free radical activity and they have adverse effects on human health and the development of natural antioxidant from plant resources would be fruitful to humankind [29].

One such plant *Glycosmis pentaphylla* belongs to Rutaceae family was chosen for this study. It commonly known as Ashvashakota, Vananimbuka, Bannimbu and pannal. The plant is used in indigenous medicine for fever, cough, rheumatism, anaemia and liver disorders [38, 17]. The traditional healers in Gazipur district of Bangladesh utilize *G. pentaphylla* for prevention of all forms of cancer. Hepato- protective, anti-cancer, anti-inflammatory, anti-bacterial, anti-oxidant and anti-arthritis activities of *Glycosmis* have been already reported by different researchers [45, 26, 10, 30]. The present study was deals with to identify the chemical constituents of *G. pentaphylla* plant extracts using different solvents. Besides, the crude extracts used to check antioxidant and antibacterial potentials against MDRB's.

EXPERIMENTAL SECTION

Sample collection and identification

S. aureus, *E.coli* and *S. pneumoniae* strains were isolated from urine, blood, pus and wounds samples collected from clinical laboratory in and around Salem and Nammalkal Districts, Tamil Nadu, India. The work was approved by the institutional ethics committee (reference number PU/IEC/HR/2014/008) and approval letter dated 31/06/2014). The isolates were identified by routine microscopical, morphological characterization and biochemical tests [6] the morphological characterization was done as following National committee for clinical laboratory standard (NCCLS) protocols [28].

Antibiotics susceptibility test

The antibiotic susceptibility test of isolates (*S. aureus*, *E. coli* and *S. pneumoniae*) was performed by the Kirby-Bauer disc diffusion method as per CLSI guidelines. The following antibiotics were used in the present study namely methicillin (30 mg), penicillin (10 mg), ampicillin (10 mg), amoxicillin clavonic acid (30 mg), vancomycin (30 mg), erythromycin (15 mg), gentamicin (10 mg), streptomycin (10 mg), tetracycline (30 mg), and ciprofloxacin (10 mg) respectively. A sterile disc used as a negative control and the plates were incubated 24 h at 37°C. After the incubation period, the diameter of growth inhibition was measured.

Plant collection and identification

The fresh leaves of *G.pentaphylla* were collected from Vellimalai hills, (Latitude 11°14'46 -12°53'30'; 77°32'52'-78°53'05' East longitude) Villupuram district, Tamil Nadu, India. The taxonomic identification of plant was confirmed by Dr. D. Natarajan, Assistant Professor, Department of Biotechnology, Periyar University, Salem and the voucher specimen was deposited in a research laboratory for further reference.

Plant extraction

The plant leaves were washed with tap water, shade-dried and powdered. The powdered *G. pentaphylla* leaves (10g) were subjected to successive extraction in 250 ml of solvent with (hexane, chloroform, ethyl acetate, methanol, and acetone) using Soxhlet extractor. The extracts were dried in vacuum pump at 40°C. The dried crude extracts were stored in freezer at 0°C for further use [27].

Phytochemical studies**Preliminary phytochemical analysis**

G. pentaphylla plant crude extracts were dissolved in respective solvents used for qualitative confirmation of major phytochemical constituents such as alkaloids, flavonoids, phenolics, saponins, steroids, tannins, carbohydrates, glycoside and proteins [44,14] respectively.

Alkaloids

In a test tube containing 1ml of extract, a few drops of Dragendorff's reagent was added and colour development was noticed. Appearance of orange colour indicates the presence of alkaloids.

Flavonoids

5ml of 1% hydrochloric acids extract was shaken with sodium hydroxide, a yellow colour appeared indicate the presence of flavonoids.

Phenolics

1ml of extract was added in 2ml of distilled water and a few drops of 10% ferric chloride. Appearance of blue or green colour indicates the presence of phenols.

Proteins

4% of NaOH and few drops of 1% CuSO₄ solution were added to 3ml of the extract. Formation of violet or pink colour indicates the presence of proteins.

Saponins

One ml of the plant was boiled with 10 ml of water for a few minutes and filtered. The filtrate was vigorously shaken. The persistent froth (1cm height) was present for 1h which indicates the presence of saponins.

Steroids

The power was dissolved in 2ml of chloroform in a dry test tube. Ten drops of acetic anhydride and 2drops of concentrated sulphuric acid was added. The solution turning red followed by blue and finally bluish colour which indicates the presence of steroids.

Tannins

One drops of ferric chloride was added to 2ml of the extract, and the appearance of bluish or greenish black coloration indicates the presence of tannins.

Carbohydrate

In a test tube, 5ml of the filtrate was treated with 5ml of fehling's solutions (A&B) and heated. The appearance of a red precipitate indicates the presence of reducing sugars.

Glycosides

A total of 1ml glacial acetic acid, few drops of ferric chloride solution and conc. H₂SO₄ (slowly through the sides of the test tube) was added to the extract. Appearance of reddish brown ring at the junction of the liquids indicate the presence of de-oxy sugars.

Antioxidant assay of plant extracts**DPPH assay**

The radical scavenging activity of *G. pentaphylla* leaves extract was estimated using stable free radical of 1,1-diphenyl-2-picrylhydrazyl assay (DPPH). Free radical scavenging ability was determined as per the modified method of Siddhuraju Becker [39]. The crude extract samples were prepared at 1g/ml, the plant extract (2 ml) was taken in test tubes and 3 ml of 0.3 mM methanolic solution of DPPH was added and mixed well and allowed to incubate at 30°C for 20 mins. The absorbance value of the sample was measured at 517nm. Known antioxidant such as ascorbic acid was used as positive control. The percentage of DPPH radical scavenging activity was calculated using the following formula:

$$\% \text{ DPPH radical scavenging activity} = \frac{[\text{Reference OD} - \text{Sample OD}]}{\text{Reference OD}} \times 100$$

ABTS assay

The 2, 2-azino-bis -3-ethylbenzothiazoline -6- sulphonic acid (ABTS) radical cation was prepared adding potassium persulphate solution (2.45 mM) and an ABTS aqueous solution (7mM) and stand in the dark at room temperature for 12 h before use. After 12h, the final solution was diluted with ethanol to an absorbance at 734 nm. Briefly, the extract (50 µl) was mixed with ABTS solution (1ml) and immediately the time was taken and the absorbance was read after 1min using a Cary-50-bio varian spectrophotometer. The samples absorbance was compared with the control absorbance.

GC-MS studies

For the GC-MS analysis a 30 × 0.25 mm × 0.25 mm of a 5% diphenyl/95% dimethyl poly siloxane column; was used in Clarus 500 Perkin-Elmer gas chromatograph with a Turbo mass gold-Perkin-Elmer detector. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) used as the carrier gas at a constant flow rate of 1 mL/min. and an injection volume of 3 mL was employed (split ratio of 10:1). Injector temperature was 250°C; ion-source temperature was 280°C. The oven temperature was programmed from 110 °C (isothermal for 2 min.), with an increase of 10 °C/min to 200°C (no hold), then from 5°C/min to 280°C, ending with a 9 min. isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. Total GC running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatogram was a Turbomass version 5.2.0 [22].

Antibacterial activity

Antibacterial activity of freshly grown culture was serially diluted and 0.1 ml of diluted inoculum (106 CFU/mL) of test organism was spread on agar plates. Muller Hinton Agar (Hi media) was used for *E. coli*, *S. aureus* and *S. pneumoniae* wells (6 mm in diameter) were made in agar using a sterilized stainless steel borer. The antibacterial activity of different concentrations (50, 75, 100 µl) of plants extracts were performed by agar well diffusion methods as described by South well [40]. In the control plate, the antibiotics only added into the well. The extract was allowed to diffuse in the well for a period of hours at incubated for 24hrs. The zone of inhibition (mm) was measured.

Minimal inhibitory concentration

Minimum inhibitory concentration (MIC) of plant extract was determined by the micro dilution method [11] with minor modifications. MIC is defined as the lowest concentration of drug which controls microbial population growth. In this study, different concentrations of aqueous plant extract (100, 75, 50 and 25 mg / ml) was to use find out the effective concentration for inhibition of bacterial growth. The MIC assay was tested in 96 well plates, filled with 50µl of nutrient agar broth and 30 µl of bacterial culture, followed by 30 µl of plant extract. All the plates were incubated at 37°C for 24h. All the incubated plates were read at 560 nm in a microplate reader and the results were noted and tabulated.

RESULTS**Isolation and identification of MDRB**

A total of 86 MDRB bacterial isolates [(*S. aureus* (40), *E. coli* (44) and *S. pneumoniae* (02))] were identified according to the colony morphology and microscopic observations. *S. aureus* colonies are identified as red to yellow colour formation, (due to mannitol fermentation). *E. coli* colonies show a characteristic green metallic sheen, indicate lactose fermentation. *S. pneumoniae* colonies and characteristically produce a clear zone of alpha haemolysis. The biochemical tests were performed for all the isolated bacteria shows positive & negative reactions.

Table 1 Preliminary phytochemical constituents of *G. pentaphylla* plant extract

S. no	Secondary metabolites	Hexane	Ethyl acetate	Acetone	Methanol
1	Alkaloids	+	+	+	+
2	Amino acid	-	-	-	-
3	Carbohydrate	+	-	+	+
4	Flavonoids	-	+	-	+
5	Glycosides	-	-	-	-
6	Phenols	-	+	-	+
7	Proteins	-	-	-	-
8	Saponins	-	+	+	+
9	Tannins	-	+	+	+
10	Quinine	+	-	-	-

+ Present – absent.

Table 2. Major phyto- continents identified from ethyl acetate extract *G. pentaphylla*

S. no	Peak area	Molecular weight	Compound name	Molecular formula	Biological activity	Reference
1	26.99	430	Vitamin E	C ₂₉ H ₅₀ O ₂	Analgesic, Antidiabetic, Antitumor, Antispasmodic, Antidiarrhetic, Antileukemic, Anticancer, Hepatoprotective,	15
2	25.85	306	1,1':3,1"':3",1"''-Quaterphenyl	C ₂₄ H ₁₈	Not known	
3	24.69	410	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-Hexamethyl-, (All-E)-	C ₃₀ H ₅₀	Antibacterial, Antioxidant, Antitumor, Cancer preventive	24
4	21.30	223	Thioanthranilic Acid, N-Methyl-, S-Butyl Ester	C ₁₂ H ₁₇ ONS	Not known	
5	28.31	436	Henriacontane	C ₃₁ H ₆₄	Antifungal against fungal spores germination, Antioxidant, Antitumor activity and Antibacterial	21
6	11.69	204	Caryophyllene	C ₁₅ H ₂₄	Antimicrobial and Anticancer	37
7	31.30	516	Z,Z-6,27-Hexatriactontadien-2-One	C ₃₆ H ₆₈ O	Vasodilator	23
8	12.83	222	1-Methylene-2b-Hydroxymethyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-Cyclohexane	C ₁₅ H ₂₆ O	Antimicrobial, Antihyperlipidemic	26
9	19.58	530	Z,Z-6,28-Heptatriactontadien-2-One	C ₃₇ H ₇₀ O		

Table 3 Antioxidant activity of *G. pentaphylla* plant extracts

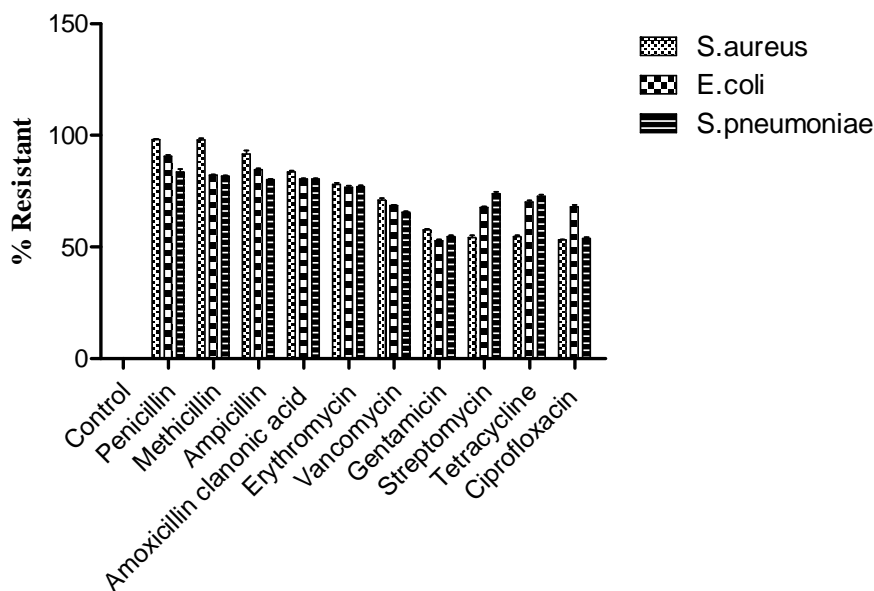
S. No	Antioxidant assays	Ethyl acetate	Hexane	Methanol	Standard
1.	DPPH assay	31.63±0.04	22.79± 0.02	33.29±0.05	67.29±0.04
2.	ABTS assay	30.14±0.03	29.46±0,04	34.80±0.05	69.23±0.05

A Mean values (n=3) with significant difference at P<0.05.

B Percentage of inhibition due to extract concentration of 100 µg/ml.

Table 4 MIC values of ethyl acetate extract of *G. pentaphylla* against multi drug resistant bacteria's

S. No	MDR target strains	Ethyl acetate MIC	Gentamicin (control)
1.	<i>S. aureus</i>	0.85	1.10
2.	<i>E. coli</i>	3.55	1.70
3.	<i>S. pneumoniae</i>	2.52	0.99



Antibiotics

Fig 1 Antibiotics resistant pattern of MDRB's

Antibiotics resistant pattern of MDRB

Analysis of the antibiotics susceptibility of isolates resulted out of 86 isolates, 50 showed difference in their multi drug resistance target bacteria's (*S. aureus*, *E. coli* and *S. pneumonia*) against different antibiotics like penicillin

(99, 90 & 87 %), methicillin (98, 86 & 85%), ampicillin (97, 87 & 80%), amoxicillin clavonic acid (85,80 & 80%) and erythromycin (77,70 & 71%) respectively (Fig. 1)

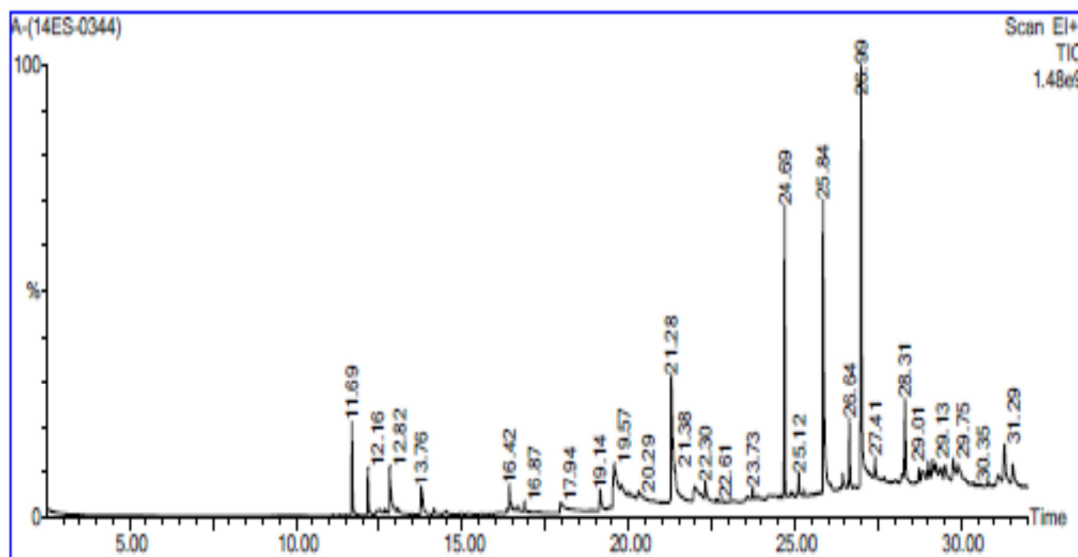


Fig 2 GC-MS chromatogram ethyl acetate extract of *G. pentaphylla*

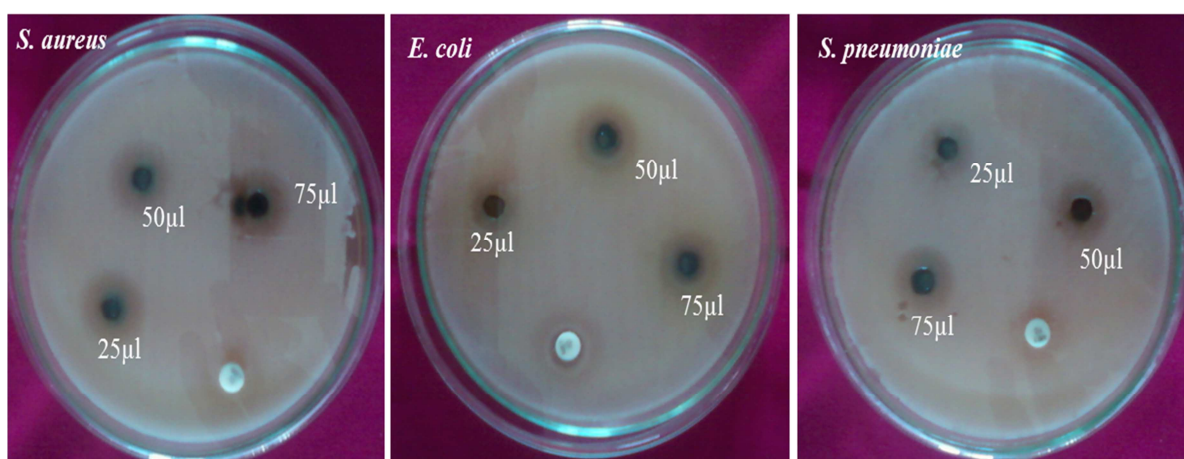


Fig 3 Antibacterial activity of *G. pentaphylla* ethyl acetate extract against MDRB

Phyto-chemical analysis of *Glycosmis pentaphylla* extracts

Preliminary phytochemical analysis of plant extract show positive result for the presence of saponins, tannins, alkaloids and phenolics in all extracts (Table 1).

GC-MS characterization of *G. pentaphylla* ethyl acetate crude extract

The result pertaining to GC-MS analysis leads to the identification of twenty one major and minor compounds present in the (GC) fraction of ethyl acetate extract of *G. pentaphylla* leaves (Fig. 2). These compounds were identified through mass spectrometry attached with GC. The nine major compounds were identified from GC-MS analysis and their biological activities are presented in table 2.

Free radical scavenging activity on *G. pentaphylla* plant extract

The antioxidant activity ethyl acetate extract of *G. pentaphylla* showed an excellent DPPH radical scavenging activity (43.4%) compared with gallic acid standard. Other extracts have moderate scavenging properties of 31.63% and 32.29% respectively. In addition, hexane extract has lowest radical scavenging activity at 22.79%. ABTS free radical scavenging activity was analysed using BHT as standard. Aqueous plant extracts revealed significant ABTS radical scavenging activity (46.64%), followed by hexane (34.8%) and methanol extracts (30.14%) respectively (Table -3).

Antibacterial activity of *G. pentaphylla* extracts

Antibacterial activity of ethyl acetate extracts of *G. pentaphylla* (Fig -3) show the ethyl acetate extract exhibited more effective antibacterial activity against target multi-drug resistant bacteria's. Higher inhibitory effect was noticed against *S. aureus* (17 mm) followed by *E. coli* and *S. pneumonia* (11mm and 10mm). The MIC range of test extract was reported as 20mg/ml in lowest concentration of ethyl acetate extract having better result against *S. aureus* (0.85) followed other bacteria's (Table -4).

DISCUSSION

Infections caused by multi drug resistant bacteria have increased over the past decade. The ability of *S. aureus* to cause a disease may be due to the production of large number of enzymes, toxins and other substances, some of which may play an important role in their capacity to cause conjunctivitis. The increasing occurrence of *S. aureus* resistant not only to methicillin but to a wide range of antimicrobial agents including vancomycin, penicillin and cefixime has made therapy more difficult [16]. The present investigation, *S. aureus*, *S. pneumoniae* and *E. coli* of clinical origin were found to be multi-drug resistant and the resistant character of these microbes compared to standard antibiotics.

The traditional medicinal plants has been the source of innumerable therapeutic agents, are of great importance to the health of individuals and communities [7]. Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities [36]. Several studies have been reported the antioxidant properties of various parts of medicinal plants, which are rich in phenolic compounds [49, 50]. *G. pentaphylla* is a widespread medicinal plant used in the pharmacological system of medicine to care for various degenerative diseases [32]. In this study, preliminary phytochemical analysis result revealed the presence of saponins, tannins, alkaloids and phenolics. Natural antioxidants mainly derived from plants in the form of phenolic compounds, such as flavonoids, phenolic acids, tocopherols etc [48]. From this study, the ethyl acetate extracts of *G. pentaphylla* provide an excellent free radical scavenging property and also contains rich amount of flavonoid components. Flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and they have been found to be antimicrobial substance against wide array of microorganism *in vitro*. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall [34]. More than 2000 flavonoids have been reported among woody and non-woody plants [35]. Activity of ethyl acetate extract of *G. pentaphylla* was comparable to the reference standard drug chloramphenicol disc. The *G. pentaphylla* extract exhibited the broad spectrum of antibacterial activity. The ethyl acetate extracts show maximum growth inhibition zone against *S. aureus* followed by others.

CONCLUSION

Glycosmis pentaphylla consists of many useful compounds, such as of saponins, tannins and alkaloids and phenolics. The antioxidant activity of the plant extracts are high due to flavonoids content. The broad spectrum of anti-bacterial properties of *G. pentaphylla* was observed against the selected bacteria. Totally, nine major compounds were identified from GC-MS analysis. Hence, the result supports the *G. pentaphylla* is a promising source of natural useful therapeutic agents. This work is strongly recommends for further work is required to the isolation, purification and characterization of the active constituents responsible for the bioactivity.

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