Phytochemical Analysis, Antimicrobial and Anti- oxidant Activity of Guaiacum Officinale L. Stem Extract

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Abstract
Guaiacum officinale (Zygophyllaceae) commonly known as Lignum Vitae, its native to Central America, introduced to India. Resins of wood are used to treat various disorders like, chronic form of rheumatism, skin diseases, uterine problems, toothache, laxative, abortifacient and an antidote for poisoning. Plant has been enlisted in IUCN endangered category because of over exploitation. The objectives of the present study were to analyse qualitative and quantitative phytochemical constituents in G. officinale stem using standard procedures. And also, extracts were subjected to antibacterial, antifungal and antioxidant activities. The results show the presence of alkaloids, carbohydrates, proteins, tannins, saponins, flavonoids, sterols, phenols, quinones and gums in all the tested extracts. The ethyl acetate extracts show the highest total protein, total phenolic, total flavonoid, total alkaloid and hexane extract shows the highest total carbohydrate and total tannin content when compared to other extracts. The antibacterial potential was tested against E. coli and B. subtilis (Gram positive and Gram negative) bacteria by well and disc diffusion method, Ethyl acetate extract showed the highest inhibition in both the pathogens. Maximum percentage inhibition for growth was Aspergillus niger with GOEA whereas growth of Fusarium oxysporum was inhibited by GOC. The ethyl acetate extract had the highest free radical scavenging activity with IC50 of 149.77 µg/mL. Ethyl acetate and chloroform stem extract were found to possess higher reducing power with 1.45 µg/mL and 1.26 µg/mL respectively. The extracts of Guaiacum officinale exhibit good antimicrobial and anti-oxidant activity and further it may be used for the drug development and food supplements.

Keywords: Guaiacum Officinale, Secondary Metabolites, Total Phenolic Content, Antibacterial, Antifungal, Antioxidant

Introduction

Plants have been used as medicines for thousands of years ago. The relationship between man and plants has close throughout the development of civilization. Siddha, Ayurveda and Unani are the traditional systems of medicines which have been followed by India. The traditional medicines have been prepared from single part of plant or combining many more plant parts. Guaiacum officinale L. is native to Central America like Florida, Panama (1) and introduced to India (18). Plant belonging to the family Zygophyllaceae, a slow growing broad leaf, evergreen tree. Leaves are thick, composed of 2 or 3 pairs of leaflets. Flowers are bright beautiful blue in color in great profusion and almost cover the tree. Plant produces a wide range of chemical compounds which are classified into primary and secondary metabolites. A numeral

of phytochemical constituents has been reported from bark, flowers and leaves of G. officinale, mainly comprise saponins such as guaianin and guaiacin. Lignum vitae was enlisted as endangered species in IUCN 2019 for overexploitation for valuable wood and medicinal products (4).

G. officinale, one of the ancient medicinal herbs, used to treat uterine problems, angina, tonsillitis, rheumatism, fish poisoning, HIV and also used as an abortifacient (2,9,21). However, there is less findings regarding antimicrobial and antioxidant activity from stem. Therefore, the objectives of the study are to evaluate antimicrobial and antioxidant activities of stem extract and to analyze qualitative and quantitative phytochemical constituents.

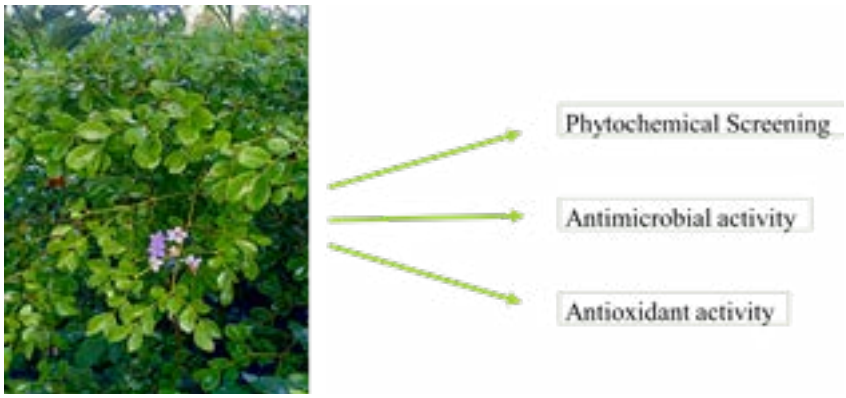

Figure 1 Graphical Representation of the Study

Materials and Methods

Preparation of the Plant Extracts

The stem of Guaiacum officianale L. were collected from Rajmandry, Andhra Pradesh, India, and one plant is located in JSS Suttur Matt, Mysore Branch (19). Plant parts were washed under running tap water and was dried in shade for complete dry. They were then ground as fine powder. 250 mL of different solvents (hexane, chloroform, ethyl acetate, ethanol, methanol and water) were added to 20 g of plant powder. The plant powder was extracted by Soxhlet extraction method at 60oC for 5 hours. The solvents of respective extracts were reduced under room temperature and stored at 4oC for further use.

Phytochemical screening of extracts

The different solvents extracts were used for the preliminary and quantitative phytochemical analysis using standard procedures (13).

Qualitative Analysis

a) Test for Alkaloids

Dragendroff's test: 2mL of plant extract was taken and 2mL of reagent was added and formation of prominent yellow precipitation indicates the presence of alkaloids.

Mayer's test: 2mL of plant extract add few drops of reagent and mixed well, formation of reddish-brown precipitate indicates the presence.

b) Test for carbohydrates

Barfoed test: few drops of reagent is added to 2mL of extract and placed in boiling water bath. Brick red precipitate indicate the presence.

Fehling's test: 2mL of plant extract was taken and equal amount of Fehling's A and B solution were added, formation of green to yellow to red precipitate indicate the presence.

c) Test for proteins and amino acid

Ninhydrin test: 3 drops of Ninhydrin added to 2mL of plant extract boiled in water bath for 10 min. Formation of purple colour shows the presence.

Biuret test: 2mL of plant extract is taken, 4% NaOH is added with few drops of 1% copper sulphate. Appearance of violet colour confirms the presence.

d) Test for phenols

Iodine test: 1mL of iodine solution is added to 2mL of extract, appearance of transient red colour indicates the presence.

Ferric Chloride test: A fraction of extract was treated with 5% ferric chloride and observed the formation of blue deep colour.

e) Test for tannins

Ferric chloride test: 1% of ferric chloride is added to 2mL of plant extract. Blackish blue colour formation indicates the presence.

Gelatine test: 2mL of extract is taken, 1mL of 1% gelatine solution with 10% sodium chloride solution is added. Formation of white precipitate indicate the presence.

f) Test for saponins

Foam test: 2mL of extract is shaken with 2mL of water, if foam persists for 10 minutes, indicates the presence.

g) Test for flavonoids

Shinoda test: Magnesium ribbons along with conc. Hydrochloric acid is added to 2mL of plant extract. Appearance of red to pink colour indicated the presence.

Ferric chloride test: few drops of ferric chloride solution is added to 2mL of extract. Blackish green colour formation indicates the presence.

h) Test for Sterols

Salkowski test: few drops of concentrated sulphuric acid was added to extract, appearance of red colour in lower layer indicates the presence.

Liebermann Burchard test: few drops of acetic anhydride added to extract, 1mL of conc. Sulphuric acid was added from the sides of test tubes, appearance of reddish-brown ring indicates the presence.

i) Test for quinones

Alcoholic KOH test: 1mL of alcoholic KOH is added to 1mL of plant extract, formation of red to blue colour indicates the presence.

Concentrated HCl test: 2mL of extract is treated with conc. HCl and formation of yellow precipitate shows the presence.

j) Test for gums

Alcohol test: 100mg of extract mixed with 100 mL of distilled water and 25mL of Alcohol results in the formation of white precipitated indicates the presence.

Quantitative Analysis

Protein estimation: Total protein estimation of G. officinale extract was done by Biuret method described by Plummer (1988), with slight modifications. Bovine serum albumin is taken as standard. The crude extract is dissolved in Ethyl acetate solvent. 3mL of Biuret reagent is added to all test tubes. Incubated for 10 minutes art room temperature. Absorbance measured at 540nm. Carbohydrate estimation: The total sugar concentration of extract was estimated using Antrone method. Different aliquots of extract along with glucose (0-100µg) where made up to 1mL using distilled water. 5mL of antrone reagent is added. The blue green solution measured colorimetrically at 620 nm. The total sugar concentration was calculated according to standard glucose calibration curve (8).

Phenolic estimation: Total phenolic content was determined using Folin-Ciocalteu colorimetric method prescribed by Malick and Singh (1980). Gallic acid is used as standard. The crude extracts were dissolved in ethyl acetate, 1mL of Folin-Ciocalteu reagent was added and left for 5 minutes. 2mL of 10% Na2CO3 was added to the solution. Incubated at 90oC for 10 minutes. Absorbance recorded at 660 nm.

Total flavonoid estimation: Determined using aluminium chloride assay. 0.5mL of extract is taken, 2mL of distilled water was added followed by 0.1mL of sodium nitrite and allowed to stand for 6 min, 2mL of sodium hydroxide is added, volume made up to 5mL using distilled water. Incubated for 15 min. mixture turns to pink and absorbance recorded at 510nm. The total flavonoid content was expressed in mg of Quercetin equivalents per gram of extract (17).

Total Saponin estimation: Standard saponin solution is prepared by dissolving 10mg of diosgenin in 4mL of distilled water. 1mL of extract is added, 0.25mL of vanillin reagent was added and 2.5mL of sulphuric acid added slowly on the inner side of the wall. The solution was mixed and tubes were kept in 60oC water bath. Absorbance was measured at 544nm (15).

Thin Layer Chromatography

Thin layer chromatography was carried out by following procedure of Solanki et al., (2020) on TLC plates. Plates were prepared using Silica gel 10g dissolved in 20mL of distilled water, distributed over plates and allowed to dry in Hot Air oven at 110oC for 30 min. Soxhlet extracts were diluted with ethyl acetate solvents, spots were made using capillary tube and run in different solvents systems like Ethyl acetate: Methanol: Water (10:1:1); Toluene: Ethyl acetate: Formic acid (4:1:0.5); Toluene: Acetic acid (9:2); Chloroform: Water (6:4); Toluene: Ethyl Acetate (4:1); Hexane : Ethyl acetate (3:1); Hexane: Methanol and Methanol : Water (8:2). The movement of active compound was expressed by its retention factor (Rf). Rf values were calculated using the formula

"Rf= "Distance travelled by the solute" / "Distance travelled by the solvent"

Antibacterial assay: The antibacterial assay of sample was carried out by well and disc diffusion method with slight modification. Antibacterial activity was performed by Nutrient agar disc and well methods. Stock solution and agar medium were prepared and solidified. Different dilution of plant extracts, (25mg, 50mg and 100mg/mL) and standard antibiotics i.e., chloramphenicol (1mg/mL) were prepared and tested against Escherichia coli and Bacillus subtilis stains. Zone of inhibition formed around well and disc were recorded after an incubation of 24 hours. The formation of inhibition zone was considered positive for antibacterial activity of extract (6).

Antifungal assay: The antifungal activity was carried out by Potato dextrose agar well diffusion method. Different dilution of plant extracts (25, 50, and 100mg/mL) were prepared and tested against Fusarium oxysporum and Aspergillus niger. Zone of inhibition formed around the wells was recorded after an incubation of 72 hours. Nystatin (1mg/mL) is used as standard (6).

Antioxidant Activity

DPPH free radical scavenging activity: The scavenging efficacy of various concentrations (25, 50, 100, 200, 300 µg/mL of extracts) of stem extracts of G. officinale was investigated on the basis of scavenging effect against DPPH radicals (4). Ascorbic acid was used as standard. Scavenging potential of stem extracts were determined using the formula:

$$\text{Scavenging activity (\%)} = \frac{(\text{Ac-At})}{\text{Ac}} \times 100$$

Ferric Reducing Power assay: The ferric reducing efficacy of various concentrations (25, 50, 100, 200, 300 µg/mL of extracts) of stem extracts of G. officianale was investigated (4). Ascorbic acid was used as reference antioxidant. An increased in absorbance with increase of concentration indicates reducing ability of extract/ ascorbic acid.

Statistical Analysis

Experimental analyses were conducted in triplets (n=3). The results are represented as Mean± Standard Error. The data were analysed statistically by one-way analysis of variance followed by Turkey's mean range test using SPSS software ver. 14. Probability values $p<0.05$ were considered significant.

Results

Phytochemical Qualitative Analysis of Extracts

The secondary metabolites present in plants contribute significantly towards the biological activities such as antibacterial and antifungal activity. Some of the phytoconstituents possess innumerable of biological activities. The study of phytochemical screening revealed the presence of promising phytochemical compounds such as saponins, terpenoids, flavonoids, phenols, glycosides,

Table 1 Phytochemical Screening of G. Officinale Stem Extract

Sl. No.	Phytochemicals	Tests	Reagents	H	Cl	EA	Eth	Mt	AQ
1	Alkaloids	Dragendorff's test	Dragendorff's reagent	+	+	+	+	+	+
		Mayer's test	Mayer's reagent	+	-	+	-	+	+
2	Carbohydrates	Barfoed's test	Barfoed's reagent	-	+	-	-	-	-
		Fehling's test	HCl+ Fehling's solution	+ Green	+ Red	+ Bluish yellow	-	+ Green	+
3	Amino acid/ Protein	Ninhydrin test	Ninhydrin solution	-	+	+	+	+	-
		Biuret test	4% NaOH, 1% CuSO4	-	+	+	-	-	-
4	Tannins	Ferric chloride test	1% ferric chloride solution	-	+	+	-	-	+
		Gelatin test	1% gelatin +10% NaCl	-	+	-	-	-	+
5	Saponins	Foam test	Extract with water (mixed vigorously for 10 minutes)	-	+	+	+	-	+
6	Terpenoids		2mL of chloroform+ 2mL of conc. H2PO4	-	+	+	+	-	-
7	Flavonoids	Shinoda	Extract soln + Magnesium ribbon + Conc. HCl	-	+	+	+	-	-
		Ferric chloride test	1% ferric chloride solution	-	-	+	-	-	-
8	Sterols	Salkowski	Extract soln + Con. H2SO4	+	+	+	+	+	+
		Liebermann Burchard	Acetic acid+ 1 ml Conc. H2SO4	+	+	+	+	+	+

9	Phenols	Iodine test	1-2 drops of Iodine	+	+	+	+	+	+
		Ferric chloride	5% ferric chloride	-	-	-	-	-	+
10	Quinones	Alcoholic KOH	Few ml of KOH	-	-	-	-	+	-
		Conc. HCl	2 drops	-	-	-	+	+	-
11	Gums	Alcohol test	100 mg extract in 10ml dis. Water+ 25ml Alcohol, constant stirring	+	+	+	+	+	+

Note: "+" Present; "-" negative; H- Hexane; Cl- Chloroform; EA- Ethyl Acetate; Eth- Ethanol; Mt- Methanol and Aq- Aqueous.

Tannins, steroids and carbohydrates. Terpenoids were present in ethyl acetate, chloroform and ethanol extract of stem part. Flavonoids and tannins were only present in ethyl acetate and chloroform extracts. Phenolic compounds and steroids are present in all the solvent extract. Carbohydrates and alkaloids are present in all the solvent extract except ethanolic extract.

Quantitative Determination of Chemical Compounds

Total protein estimation: Total protein content of various extracts of stem of G. officinale was carried out and reading recorded in Table 2. Ethyl acetate extract of stem showed the highest total phenolic content (4.50 mg GAE/100g) compared to other solvent extracts.

Determination of Carbohydrates: Total carbohydrates content revealed highest in Hexane extract (89.3 mg/100 g), followed by methanol and ethyl acetate (87.39 and 82.80 mg/ 100 g respectively).

Total phenolic content: Total phenol content was calculated from the standard curve of gallic acid. It is found that ethyl acetate stem extracts show the highest phenolic content (9.41 µg GAE/ 1 µg).

Total flavonoids content: The total flavonoid content was high in ethyl acetate stem extract (5.63 mg RE/100 g) followed by ethanol extract (3.58 mg RE/ 100 g).

Estimation of saponin content: The total saponin content showed highest in ethyl acetate stem extract (7.05 mg DE/100 g).

Table 2 Quantitative Analysis of Different Extracts of G. Officinale Stem

	Hexane	Chloroform	Ethyl Acetate	Ethanol	Methanol
Protein	1.71±.02	2.60±.014	4.50±.008*	3.20±.005	2.61±.018
Carbohydrates	89.3±0.005*	7.85±0.017	82.80±0.003	80.43±0.07	87.39±0.44
Phenols	4.25±0.02	7.94±0.02	9.41±0.01*	9.2±0.01	6.67±0.03
Flavonoids	1.97±0.002	3.74±0.002	5.63±0.012*	3.58±0.006	2.92±0.007
Saponins	0.64±0.00	2.51±0.005	7.05±0.012*	5.79±0.002	3.05±0.001

Thin Layer Chromatography

The results of TLC profiling are summarized in Table 3. Ehthyl acetate extract showed the presence of alkaloid (0.95); flavonoid (0.88); steroid (0.875); tannins (0.875) and phenols (0.84). The Rf values of each phytoconstituents present in various extracts in different mobile phases is given in the following table 3 and Fig. 2.

Thin layer chromatography is usually done for a better identification of the bioactive compounds. In the present study TLC profiling of stem extract revealed the presence of alkaloids, flavonoids, phenols, steroids and tannins. It was observed that among all the solvents (hexane, chloroform, ethyl acetate, ethanol, methanol), ethyl acetate was found more effective in extracting maximum

number of secondary metabolites. Rf values serves as a polarity indicator, guiding the choice of optimal solvent systems for isolating and purifying individual compounds from complex plant extracts (5). Compounds exhibiting high Rf values in less polar systems tend to be non-polar, whereas low Rf values suggest polar compounds (24).

Table 3 TLC of G. Officinale Stem Ethyl Acetate Extract

Sl. No.	Phytochemical	Solvent system	Fluorescence spot at 224nm	Rf value
1	Alkaloids	Ethyl acetate: Methanol: Water (10:1.35:1)	Pink Orange Yellow Green Green	0.825 0.625 0.55 0.375 0.3
		Toluene: Ethyl Acetate (4:1)	Pink Pale pink Pale pink Yellow	0.95 0.75 0.575 0.55
		Toluene: Ethyl Acetate: Formic acid (4:1:0.5)	Pale Yellow Yellow Orange	0.84 0.75 0.5 0.12
2	Flavonoid	Toluene: Acetic acid (9:2)	Brown Baby pink Pink Orange Yellow	0.88 0.66 0.44 0.4 0.26
3	Steroids	Hexane: Ethyl acetate (3:1)	Orange Brown Pink Yellow Light orange Crimson Pink	0.875 0.7 0.625 0.5 0.425 0.35 0.2
4	Tannin	Chloroform: Water (6:4)	Pink Yellow Baby pink Pink	0.875 0.75 0.5 0.375
5	Phenol	Methanol: Water (6:3)	Pink Yellow	0.75 0.3
		Methanol: Water (8:2)	Pink Pale Yellow	0.84 0.78 0.5

Figure 2 TLC Plates of Ethyl Acetate Extract on Different Solvent Mixture

Antibacterial Activity

Several studies show the crude solvent extracts from plants exhibits antibacterial activity even against resistant strains of pathogenic bacteria. In the present study, stem extract exhibit inhibition against tested strains namely E. coli and B. subtilis. E. coli is one of the common bacteria which is used to cure gastroenteritis, urinary tract infection and B. subtilis is biopesticide bacteria which is used for diarrhoea. Bothe well and disc method were followed for antibacterial activity of ethyl acetate stem extracts shown in Fig. 3. Phenolic exudates have been observed in E. coli and showed highest antibacterial activity compared to B. subtilis in well method. In disc method, the inhibition zone was less compared to well method ad highest inhibition observed in E. coli than B. subtilis. Hayet et al. (2010) reported methanolic extract of P. harmala leaves showed strong antibacterial property against Gram positive than Gram negative bacteria. In the present study the Gram-negative bacteria E. coli is showing highest inhibitory activity than Gram positive bacteria B. subtilis.

Antifungal Activity

The antimicrobial activity of ethyl acetate stem extract was carried out in the present study against two strains of fungi; F. oxysporum and A. niger using well method. A. niger showed the maximum inhibition compared to the F. oxysporum shown in Fig. 4.

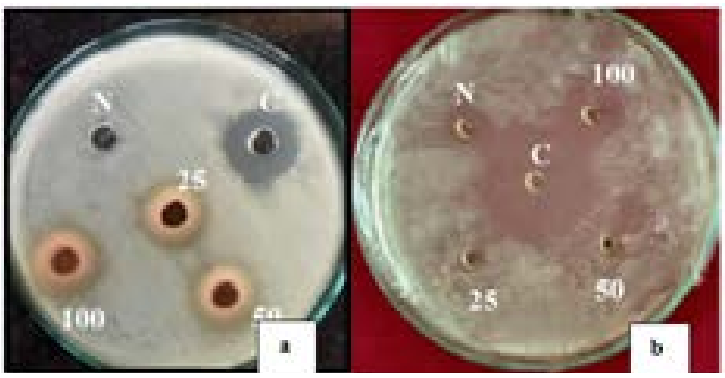

Figure 3 Antibacterial Activity of Ethyl Acetate Extracts of G. Officinale Stem, a & b well Method E. Coli & B. Subtilis Rep.; c & d Disk Method E. coli & B. subtilis resp

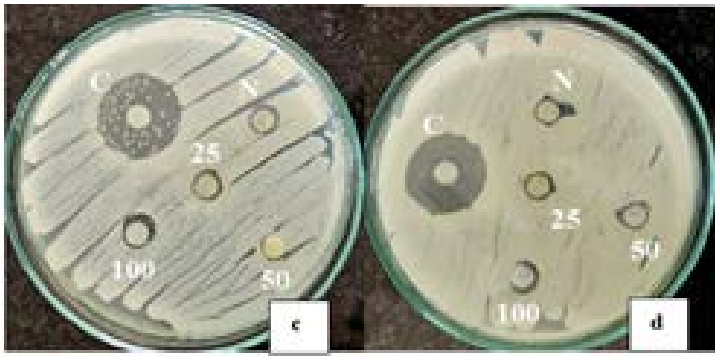

Figure 4 Antifungal Well Method F. Oxysporum & A. Niger Rep

Determination of Antioxidant Activities

Antioxidant activity of G. officinale stem extracts showed promising antioxidant activity in DPPH and ferric reducing power assay.

Determination of DPPH Radical Scavenging Activity

The DPPH of stem extracts revealed significant results of inhibition ($p \geq 0.05$). the decrease in absorbance indicates an increase in free radical scavenging activity regarding the colour changes from deep purple to colourless solution. The result showed the highest percentage of activity in ethyl acetate stem extract with IC50 of 15.41 µg/ml followed by hexane extract with IC50 of 16.18 µg/ml. With 149.77 µg/ml radical scavenging activity in ethyl acetate extract followed by chloroform extract with 104.2 µg/ml (Fig. 5).

Determination of Ferric Reducing Antioxidant Power Assay

The reducing power assay of G. officinale stem was measured at 700nm. An increase in absorbance indicates an increasing reducing activity with the colour changes to light blue to dark blue colour. The ethyl acetate stem extract shows the highest reducing power compared to other extracts. Ethyl acetate extract shows 1.45 µg/ml, followed by chloroform extract 1.25 µg/ml (Fig. 6).

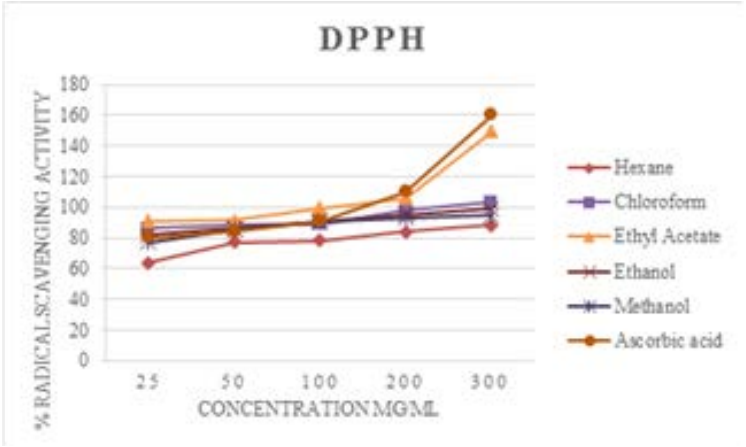

Figure 5 DPPH Radical Scavenging Activity of Various Solvent Extracts of G. Officinale Stem

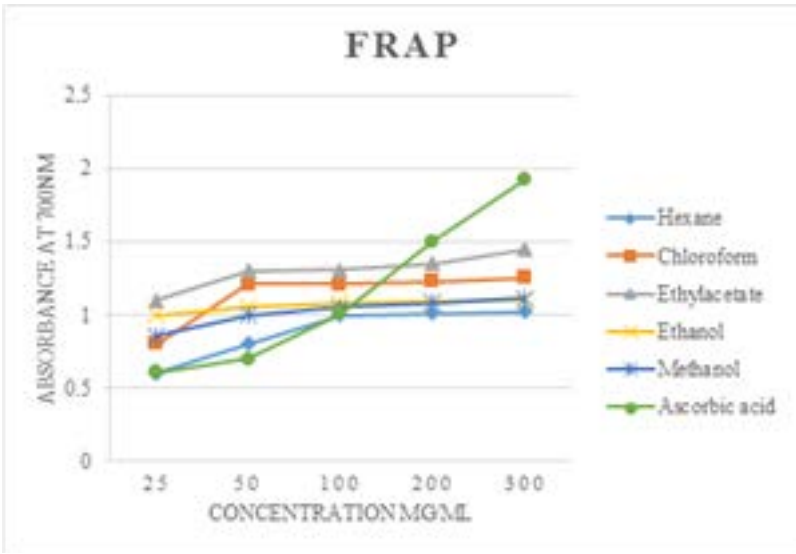

Figure 6 Ferric Reducing Antioxidant Power Assay of Various Solvent Extracts of G. Officinale Stem

Discussion

Preliminary phytochemical and quantitative analysis done for the different solvent extracts of stem of G. officinale, shows the presence of all secondary constituents like saponins, tannins, flavonoids, phenols, alkaloids, steroids and carbohydrates. Ahmad et al., (1984) confirm the presence of saponin as a major bioactive compound in G. officinale fruits.

According to some other findings, Mabhiza et al., (2016), Hanada et al., (1980), the presence of alkaloids and polyphenols shows the highest bacterial inhibition activity which is similar to the present study where phenolic exudates were observed during E. coli well diffusion method. The data reflects that all ethyl acetate extract shows significant zonation against both Gram positive and Gram negative (22). There is much finding for antibacterial activity of G. officinale, some reports were on Guaiacum coulteri which exhibits anti-tuberculosis and anti-helicobacter activity (20 and 25).

The DPPH assay was applied to access antioxidant potential of G. officinale. Mabhiza et al., (2016) findings show the presence of antioxidant potential. Ethanolic twig extract of G. officinale showed the highest scavenging activity and compared with Vitamin C plant, which is used to cure cardio-vascular dieses and anti-cancerous agent (16). Gan et al., (2017) revealed the presence of phytoconstituents such as alkaloids and polyphenols which are responsible for strong antioxidant activity. This finding is similar to the present work.

Conclusion

From the present study we can conclude that plant can be excellent source of Phytoconstituents, in particular ethyl acetate extract possess highest phenolic content, antimicrobial activity against bacteria and fungi and exceptional antioxidant capability. This natural antioxidant holds great promise as a therapeutic agent. Future work includes the purification and manufacture of new drug.

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