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Pharmacognostic and antibacterial activity evaluation of *Acacia caesia* (L.) *Willd*

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Abstract
Acacia caesia (L.) *Willd* has been extensively used in India as a substitute for soap and is widely acclaimed for its antibacterial properties. The aim of this study was to establish a phytochemical profile for the medicinal plant and to confirm the antibacterial property on three human pathogenic bacteria. The qualitative analysis revealed the presence of phytochemical classes such as alkaloids, Saponins, flavonoids, steroids, cardiac glycosides, alkaloids, carbohydrates, fats, amino acids, coumarins, phenols, catechins, anthraquinones, quinones, terpenoids and tri-terpenoids. Quantitative estimations of three classes of phytochemical present were carried out, which indicated the presence of 0.464859mg/ml alkaloids, 1.747573mg/ml of phenols and 0.929426mg/ml flavonoids. GC-MS analysis revealed the presence of 21 compounds present in the methanolic extract of the stem of *Acacia caesia* (L.) *WILLD* including some known antibacterial phytochemicals such as phytol and isoeugenol. The extract exhibited antibacterial activity, comparable to that of Ciprofloxacin, against the gram-positive bacteria, *Staphylococcus aureus* and gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*.

Keywords: *Acacia caesia*, antibacterial, medicinal plant, phytochemical, GC-MS

Introduction
Medicinal plant knowledge serves as an untapped reservoir of potential drug leads. In order to effectively exploit this knowledge for drug discover and development, pharmacological profiling serves the base for any further studies [1]. Phytochemical analysis helps to identify crucial chemical classes present within a plant thus giving and idea on the properties they may exhibit and Gas chromatography-Mass spectroscopy help to enlist the phytochemical constituents of a plant each of which confers certain properties to the plant and also serve as possible leads for drug identification for binding to potential targets [2].
Acacia caesia Linnaeus is a leguminous perennial climbing shrub of the family Mimosaceae, native to south-east Asia. The extract from the stem bark is a frequently used remedy among the Miza tribes of north-east India for gastrointestinal infections. The crude stem bark extract has also showed anthelmintic effects on *Raillietina echinobothrida* (a parasitic tapeworm) [3]. The stem is a potential source as a fuel wood and its pod powder is a potential substitute for soap [4]. *Acacia caesia* (L). WILLD has been found to have many medicinal properties such as anti-microbial activities, antibiotic, insecticidal, wound healing activities [5]. The leaf and bark juice mixed with salt relives stomach aches. The bark powder is sprinkled on ulcers which provide great relief [6]. The plant is widely found to be distributed along the foot hills of the Western Ghats around the altitude of 500m above MSL [7]. In this study, identification of bioactive compound present in *Acacia caesia* (L.) WILLD by quantitative and qualitative methods followed by the identification of antibacterial property of the plant against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* [3] was carried out.

Materials and Methods
Plant material
Fresh stem of *Acacia caesia* (L). WILLD was collected from Kottayam district of Kerala, India. The plant materials were taxonomically identified by Department of Botany, University of Kerala, Karyavattom, Thiruvananthapuram, Kerala where, a herbarium was desposited (Voucher Number: KUBH 10097).

Preparation of extract
The stems of *Acacia caesia* (L). WILLD was collected from wild, shade dried and ground down to powder using a mechanical grinder. Grounded *Acacia caesia* (L). WILLD stem was loaded into the thimble of a soxhlet apparatus and extracted using methanol.

The crude extract thus obtained was concentrated using a rotary evaporator and the concentrated extract was stored in a refrigerator at 4 ^{o}C until further use.

Qualitative phytochemical analysis

The preliminary phytochemical analysis was carried out by the following standard procedures [8-12].

1. Test for Tannins - 0.5g of the dried powdered sample was boiled in 10ml of water in a water bath. The heated solution was then filtered and a few drops of 0.1% ferric chloride was added. The formation of a brownish green colour indicates the presence of tannins.
2. Test for Steroids - Liebermann-Burchard Reaction: 2ml extract was mixed with 2.5 ml chloroform. To this 1-2 ml acetic anhydride and 2 drops concentrated sulphuric acid were added. Formation of a reddish colour indicates the presence of steroids.
3. Test for Flavonoids - A portion of the aqueous filtrate of the extract was taken in a test tube to which 3ml of dilute ammonia solution followed by concentrated sulphuric acid was added. The formation of a yellow colour solution indicates the presence of flavonoids in the solution.
4. Test for Cardiac Glycosides - Kellar-Kiliani test - A small amount of the extract is dissolved in roughly 3ml of glacial acetic acid containing 2-4 drops of 5% ferric chloride solution. This mixture was then transferred to a surface containing 2ml of concentrated sulphuric acid. The formation of a reddish brown layer at the junction of the two layers followed by the formation of a bluish green upper layer upon standing indicates the presence of cardiac glycosides.
5. Test for Carbohydrates – Molisch's Test - 1ml of alcoholic alpha-naphthol solution was added to the extract and concentrated sulphuric acid was added through the sides of the test tube. A formation of Purple or reddish violet colour at the junction of the two liquids reveals the presence of carbohydrates.
6. Test for Saponins – 1ml of the extract was diluted with 5ml of water and then subjected to vigorous shaking. A persistent lather formation indicates the presence of Saponins.
7. Test for Quinones – 1 ml of concentrated sulphuric acid was added to 1 ml of the extract. Formation of a red colour indicates the presence of Quinones.
8. Test for Phenols - Ferric Chloride Test - a few drops of 10% aqueous ferric chloride was added to the extract. The appearance of blue or green colour indicates the presence of phenols.
9. Test for Alkaloids - A small amount of extract was treated with 1% HCl solution and then divided into two parts–
   Mayer's Test - To one part, few drops of Mayer's reagent was added. The formation of a cream coloured precipitate indicated the presence of Alkaloids.
   Dragendroff's Test - To second part, few drops of Dragendroff's reagent was added. The formation of an orange-red colour indicated the presence of Alkaloids.
10. Test for Oils and Fats - A small quantity of the extract was pressed between two filter papers, and the resulting stain on the filter paper was noted. The appearance of an oil stain indicates the presence of oils and fats.
11. Test for amino acids - A small amount of ninhydrin was added to the extract and kept in a water bath for 30 seconds. The formation of a characteristic intense purple colour indicates the presence of amino acids.
12. Test for Coumarins - 1ml of 10% sodium hydroxide solution was added to 1ml of extract. The formation of a yellow colour indicates the presence of coumarins.
13. Test for Phlobatannins - 2ml of aqueous solution of extract was added into dilute HCl. The formation of a red precipitate indicates the presence of Phlobatannins.
14. Test for Catechins - A matchstick was dipped in plant extract and made to dry, then moistened with concentrated hydrochloric acid and warned near a flame. The presence of a pink or red colour in the wood confirms the presence of catechins.
15. Test for Anthraquinones - 5ml of chloroform was added to a small amount of the extract and heated in a steam bath for 5 minutes. This solution was cooled and equal volume of 10% ammonia solution was added. Upon shaking, the formation of an upper bright pink coloured layer is indicative of the presence of Anthraquinones.
16. Test for Proteins – Biuret Test - 1ml of 40% sodium hydroxide solution was added to 2ml of the extract followed by the addition of 1% copper sulphate solution. The formation of a characteristic purple colour was indicative of presence of proteins.
17. Test for Terpenoids - Salkowski's test - 2ml of chloroform was added to a small amount of sample, followed by addition of some concentrated sulphuric acid along the sides of the test tube. Formation of a reddish brown colour at the interface showed the presence of terpenoids.
18. Test for Triterpenoids – Hirshonn Test - A small amount of sample was heated with trichloro acetic acid, red to purple colour was observed indicative of presence of triterpenoids.
19. Test for Phytosterols - A small amount of the extract was dissolved in few drops of dry acetic acid to which, 3ml of acetic anhydride was added followed by few drops of concentrated sulphuric acid. Formation of a blue colour indicates the presence of phytosterols.

Quantitative analysis

Estimation of Flavonoids - 10mg of the sample was weighed accurately and made up to 1ml with DMSO. 0.5ml of the extract stock solution was taken to which 1.5ml methanol, 0.1ml aluminum chloride, 0.1ml potassium acetate and 2.8ml distilled water were added and mixed thoroughly. The blank solution was prepared in a similar manner using distilled water instead of the sample. The absorbance of both the blank and the solution was read at 415 nm using UV-VISIBLE spectrophotometer (Agilent, Cary 60). All solutions were subjected to filtration using Whatmann filter paper prior to measurement. The Quercetin standard graph was obtained using different concentrations of quercetin in methanol. 10mg of quercetin was dissolved in methanol and then diluted to 100, 200, 400, 800 and 1000µg/ml [13].

Estimation of Total Phenolic content - 1:10 ml diluted solution of Folin-Ciocalteau (2N) was prepared. 7.5% solution of sodium carbonate (anhydrous) was made with distilled water instead of the sample. 0.2ml sample was pipetted out from extract solution 5ml of Folin-Ciocalteau reagent was added. After 5 minutes, 4ml of sodium carbonate solution was added and incubated at room temperature for 2 hours. Then, absorbance was measured at 750nmusing UV-VISIBLE spectrophotometer (Agilent, Cary 60) and the

values obtained were interpreted in the standard graph of Gallic acid to get the milligram equivalents of Gallic acid. For the preparation of standard graph of Gallic acid, 10mg Gallic acid was weighed and made up to 1ml with methanol. From the stock solution (10mg/ml stock solution), solutions of concentration 100, 200, 400, 800 and 1000µg/ml were prepared. To the above solutions, 5ml of Folin-Ciocalteau reagent was added and 4ml of 7.5% sodium carbonate solution was added after 5 minutes. The resulting solution was stirred and incubated at room temperature for 2 hours. After 2 hours, absorbance of the solutions was measured at 750nm using UV-VISIBLE spectrophotometer (Agilent, Cary 60). The absorbance values were plotted against concentration and standard graph was obtained [13].

Estimation of Alkaloids - Stock solution was prepared by making up 10 mg of the sample with 1ml of DMSO. The sample to be estimated, was centrifuged for 10 minutes at 3000rpm to remove residual suspended particles and then 0.5ml extract was mixed with 1ml of HCl 0.1 N. Then 0.25ml of Dragendroff's reagent was added to the attained mixture for precipitation and the precipitate was centrifuged for 5 minutes at 3000 rpm. This precipitate was further washed with 0.25 ml of ethanol. The filtrate was discarded and the residue was then treated with 0.25ml of disodium sulphate solution (1%w/v). The brownish black precipitate formed was centrifuged for 5 minutes at 3000 rpm. This residue was dissolved in 0.2 ml of concentrated nitric acid and 0.1 ml was then pipette out and mixed with 0.5 ml of thiourea solution (3% w/v). The absorbance of this solution was measured at 435 nm using UV-VISIBLE spectrophotometer (Agilent, Cary 60) against a blank containing 0.1 ml of concentrated nitric acid and 0.25ml of thiourea solution (3% w/v)and the values obtained were interpreted using the standard graph of reserpine to get the milligram equivalents of reserpine [14].

GC-MS analysis

GC-MS analysis of the methanolic extract of *Acacia caesia* was performed using a Shimadzu GCMS - QP2010 series and analysed using the software GC-MS control software, GC-MS solution Ver. 2.6. Experimental conditions of GCMS were as follows: Rxi-5Sil MS column, dimensions: 30mtrs, ID: 0.25mm, film thickness: 0.25µm. Flow rate was set at 1.0 ml/min. in the gas chromatography part. The injector temperature was maintained at 260 ℃, the ion source temperature was maintained at 200 ℃, the oven temperature was programmed from 80 ℃ with an increase of 5℃/min to 280 ℃. For GC-MS detection, an electron ionization system was operated in electron impact mode with an ionisation energy of 0.71eV. Helium gas (99.995%) was used as the carrier gas with an injection volume of 0.50µl. Sample was dissolved in methanol and were scanned at regular intervals. The mass spectra were taken at 0.71eV and the total GC-MS run time was 51 minutes. The results were compared by using Wiley 8 and NIST 11 libraries and the relative percentage of each component was calculated by comparing its average peak area to total area.

Antibacterial activity

Test microorganisms and growth media

Muller Hinton Agar Medium served the medium on which zone of inhibition measurement was carried out using the agar well disc diffusion method. The medium was prepared by dissolving 33.8 g of the commercially available Muller Hinton Agar Medium (MHI Agar Media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121 ℃ for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25-30ml/plate) while still molten.

Nutrient Broth (1L)

Nutrient broth was used to revive the lyophilized cultures and grow pure cultures. One litre of nutrient broth was prepared by dissolving 13 g of commercially available nutrient medium (HI Media) in 1000ml distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were chosen due to their pharmacological importance and their known susceptibilities to *Acacia caesia* (L). WILLD stem extract[3]. The bacterial cultures were incubated for 24 hours at 37 ºC on nutrient agar medium following refrigeration storage at 4 ºC. The strains were grown in Mueller-Hinton agar (MHA) plates at 37 ºC.

Antibacterial activity of plant extract by disk diffusion method

Petri plates containing 20ml Muller Hinton Agar Medium were seeded with bacterial culture of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (growth of culture adjusted according to McFarlands Standard, 0.5%) [15]. A negative methanol control was also kept to negate the possible antibacterial effects of any residual methanol. Each plate consisted of four sterile Whatmann paper discs of diameter (10mm) coated with 10ug/ml Ciprofloxacin, 10ul methanol, 25ul and 100ul extract. The zone of inhibitions of plant extract was found at two concentrations of 25ug/ml and 100ug/ml and correspondingly compared to a standard drug, Ciprofloxacin (1.5ug/ml), known to possess antibacterialbacteria against the three chosen organisms [16]. Plates were placed with sterile paper discs having respective test samples. The plates were then incubated at 37 ℃ for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the discs [15].

Results and Discussions

Qualitative phytochemical analysis

The results of the qualitative phytochemical analyses is summarized in table 1. The methanolic extract of *Acacia caesia* (L.) *WILLD* showed the presence of steroids, flavonoids, cardiac glycosides, carbohydrates, phenols, alkaloids, fats, amino acids, coumarins, catechins, anthraquinones, quinones, terpenoids and tri-terpenoids.

Table 1: Qualitative phytochemical analysis results

S. No	Compound Class	Chemical Test	Presence
	Tannins		Negative
	Steroids		Positive
	Flavanoids		Positive
	Cardiac Glycosides	Keller-Kiliani Test	Positive
	Saponins	Foam Test	Positive
	Carbohydrates	Benedicts Test	Negative

		Molisch Test	Positive
	Phenol		Positive
	Alkaloids	Mayer's Test	Negative
		Dragendroff's Test	Positive
	Fats and Oils		Positive
	Proteins	Biuret Test	Negative
	Amino Acids	Ninhydrin Test	Positive
	Coumarins `		Positive
	Phlobatannins		Negative
	Catechins		Positive
	Anthraquinones		Positive
	Quinones		Positive
	Terpenoids	Salkowski's Test	Positive
	Tri-terpernoids	Hirshonn's Test	Positive
	Phytosterols		Negative

Quantitative analysis of phytochemicals

Estimation of total alkaloids

The total alkaloid content of the stem was estimated using spectrophotometric method. The alkaloid content was found to be 0.464859mg/ml of sample stock solution expressed in reserpine equivalents. Alkaloids represents a pharmacologically and therapeutically prominent group of compounds present in natural sources [17]. Alkaloids are reported to provide antibacterial [18], anti-cancer [19], anti-analgesic [20] and vasodilator [21] properties also.

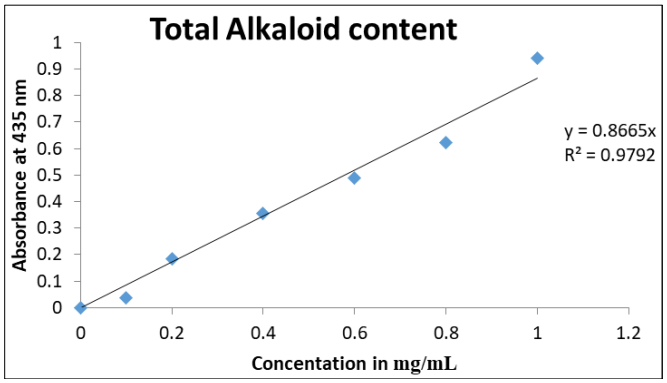

Fig 1: Standard reserpine curve

Estimation of total phenols

The total phenolic content of the stem was estimated using spectrophotometric method. The alkaloid content was found to be 1.747573mg/ml of sample stock solution expressed in Gallic acid equivalents. Alkaloids represents a vital set of biochemical involved in defence response of a plant [17]. Phenols are report to exhibit antiradical and antioxidative properties [22, 23]. Phenols have also been reported to harbour antidiabetic [24, 25] and anti-inflammatory properties [26].

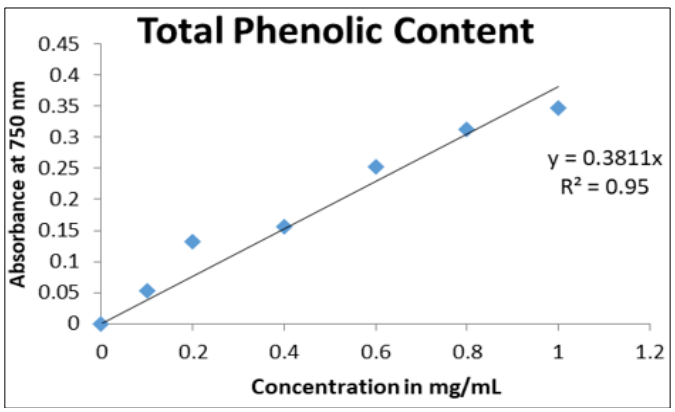

Fig 2: Standard gallic acid curve

Estimation of flavonoid content

The total flavonoid content of the stem was estimated using spectrophotometric method. The alkaloid content was found to be 0.929426 mg/ml of sample stock solution expressed in Gallic acid equivalents. Flavonoids have been reported to exhibit anti-inflammatory, anti-cholinesterase, anti-oxidative [27] and antibacterial [18] properties highlighting their therapeutic importance.

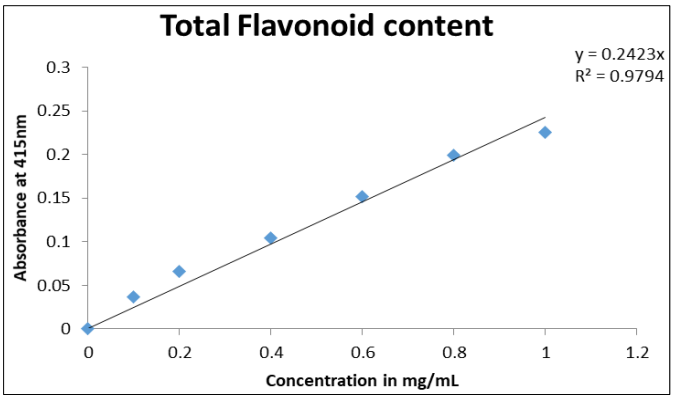

Fig 3: Standard quercetin curve

Gas Chromatography- Mass spectroscopy analysis

The Gas Chromatography - Mass spectroscopy analysis of the methanolic stem extract of *Acacia caesia* (L). WILLD showed the presence of 21 compounds as shown in table 2. The spectrum shows the presence of 21 prominent peaks between the retention times of 14.411 – 47.018 min. 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol showed the largest peak area with a retention time of 24.999min followed by 1,2-benzenedicarboxylic acid with a retention time of 38.955min. The GC-MS analysis revealed the presence of some known phytochemicals with therapeutic uses such as phytol, hexadecanoic acid, methyl palmitate and iso-eugenol. The widely publicised antimicrobial properties of the plant stem could be attributed to the interplay of the known antimicrobial compounds such as isoeugenol, methoxyeugenol, 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol, dibutyl phthalate, phytol and also other compounds present in the stem. Furthermore, the plant extract revealed the potential presence of antioxidants such as Iso-eugenol, 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol, Hexadecanoic acid, 9-octadecenoic acid (z)-, methyl ester and anti-inflammatory agents such as phytyl acetate, 4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol, Methylpalmitate, Hexadecanoic acid. These phytochemicals could serve an understanding of the successful results of the stem extract being used in traditional medicine both as an external and internal drug.

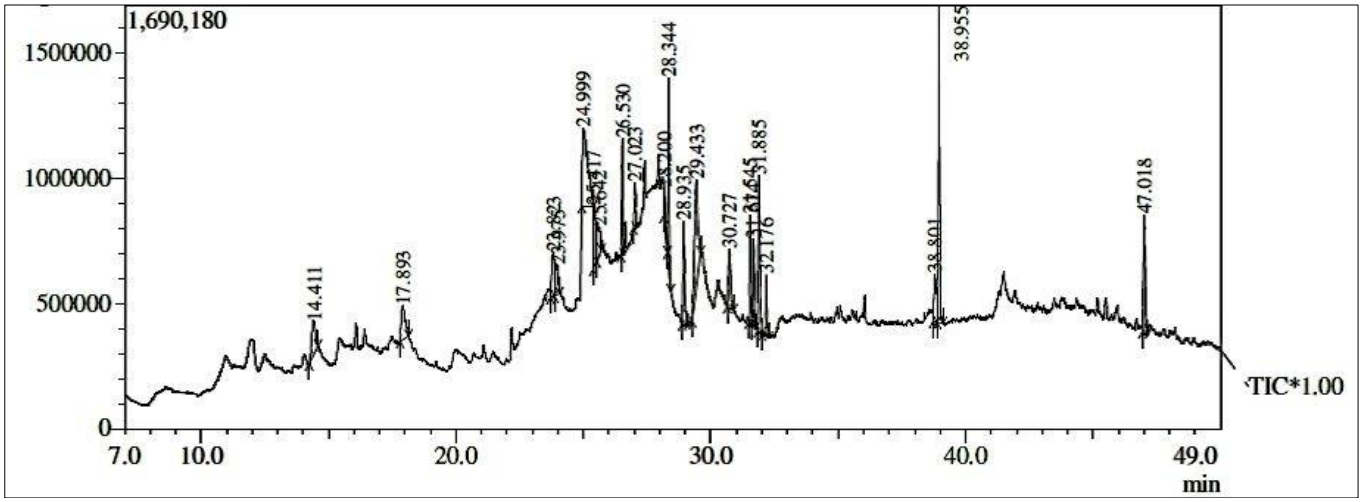

Fig 4: Gas Chromatography – Mass Spectroscopy chromatogram

Table 2: Gas Chromatography – Mass Spectroscopy Result

Peak Number	Retention time (min)	Name	Moleculuar formula	Molecular weight (g/mol)	Reported activities
1	14.411	Benzenemethanol, 2,4,5-trimethyl	$C_{10}H_{14}O$	150.22	No activity reported
2	17.893	Iso-eugenol	$C_{10}H_{12}O_2$	164.2	Antioxidant, antibacterial [28 29]
3	23.823	Methoxyeugenol	$C_{11}H_{14}O_3$	194.23	Antibacterial [30]
4	23.975	Tetradecanal	$C_{14}H_{28}O$	212.37	No activity reported
5	24.999	4-((1e)-3-hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	180.2	Antimicrobial, Antioxidant, Anti-inflammatory, analgesic [31]
6	25.417	13-methyltetradec-9-enoic acid trimethylsilyl ester	$C_{18}H_{36}O_2Si$	312.6	No activity reported
7	25.642	Aspidocarpine	$C_{22}H_{30}N_2O_3$	370.5	No activity reported
8	26.530	Phytol, acetate	$C_{22}H_{42}O_2$	338.6	Anti-inflammatory, antileishmanial, antitrypanosomal [32]
9	27.023	Phthalic acid, butyl undecyl ester	$C_{23}H_{36}O_4$	376.5	No activity reported
10	28.200	L-arabinitol	$C_5H_{12}O_5$	152.15	No activity reported
11	28.344	Methylpalmitate	$C_{17}H_{34}O_2$	270.5	Anti-inflammatory, anti-fibrotic [33]
12	28.935	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	Antimicrobial,estrogenic activity [34, 35]
13	29.433	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	Anti-inflammatory [36] Antioxidant, anticancer [37]
14	30.727	4-oxazolecarboxylic acid, 4,5-dihydro-2-phenyl-, 1-methylethyl ester	$C_{13}H_{15}NO_3$	233.26	No activity reported
15	31.545	Methyl octadeca-9,12-dienoate	$C_{19}H_{34}O_2$	294.5	No Activity reported
16	31.674	9-octadecenoic acid (z)-, methyl ester	$C_{19}H_{36}O_2$	296.5	Antioxidant, anticarcinogenic [35]
17	31.885	Phytol	$C_{20}H_{40}O$	296.5	Cytotoxicity, Anti-inflammatory, antiproliferative, Antibacterial [38, 39, 40, 41]
18	32.176	Methylstearate	$C_{19}H_{38}O_2$	298.5	No activity reported
19	38.801	Glycerol beta-palmitate	$C_{19}H_{38}O_4$	330.5	No activity reported
20	38.955	1,2-benzenedicarboxylic acid	$C_8H_6O_4$	166.13	No activity reported
21	47.018	Cholesta-4,6-dien-3-ol, benzoate, (3 beta)-	$C_{34}H_{48}O_2$	488.7	No activity reported

Antibacterial assay

Disc diffusion method was carried out to establish antibacterial activity of the *Acacia caesia* (L.) WILLD methanolic stem extract against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* compared to the drug ciprofloxacin (1.5µg/mL). The extract showed no activity at 25µl concentration against all three organism but showed significant activity at 100µl concentration comparable to that of Ciprofloxacin. The zone of inhibition for both ciprofloxacin and the methanolic plant extract for *Pseudomonas aeruginosa* was found to be 15mm whereas it was found to be 30mm and 17mm respectively for ciprofloxacin and plant extract in the case of *Escherichia coli*. For *Staphylococcus aureus* the zones of inhibition was found to be 34mm for ciprofloxacin and 11mm for 100µl of plant extract. The antibacterial assays confirm the presence of an antibacterial property for the stem extract of *Acacia caesia* (L.) WILLD against three human pathogenic bacteria. The assays Confirms the activity of the stem extract to be comparable to that of Ciprofloxacin, which suggest the drug-like potential of the extract compounds. The disc diffusion studies also shows a comparatively higher activity of the stem extract against gram negative bacteria (*E.coli* and *P.aeruginosa*) as compared to gram positive bacteria (*S.aureus*) which could prove therapeutically significant due to the inability of antibiotics to penetrate gram negative bacteria [42].

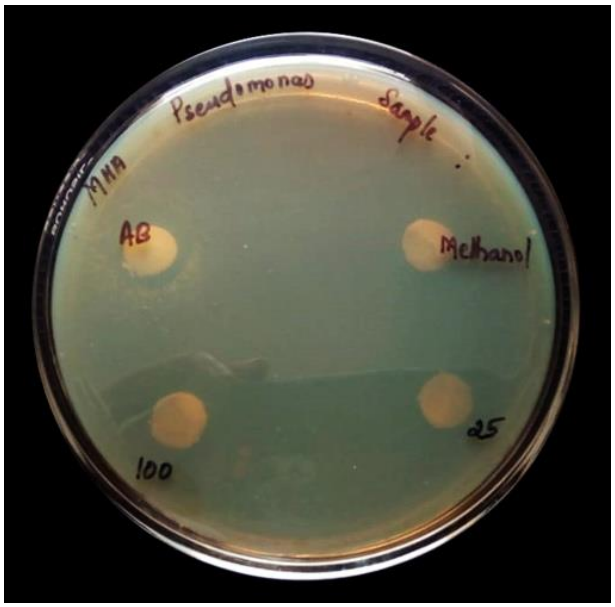

Fig 5: Disc diffusion plate of *Pseudomonas aeruginosa*

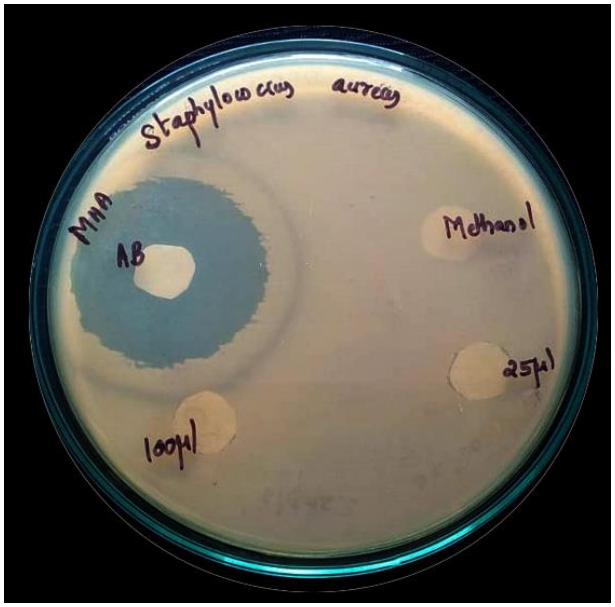

Fig 6: Disc diffusion plate of *Staphylococcus aureus*

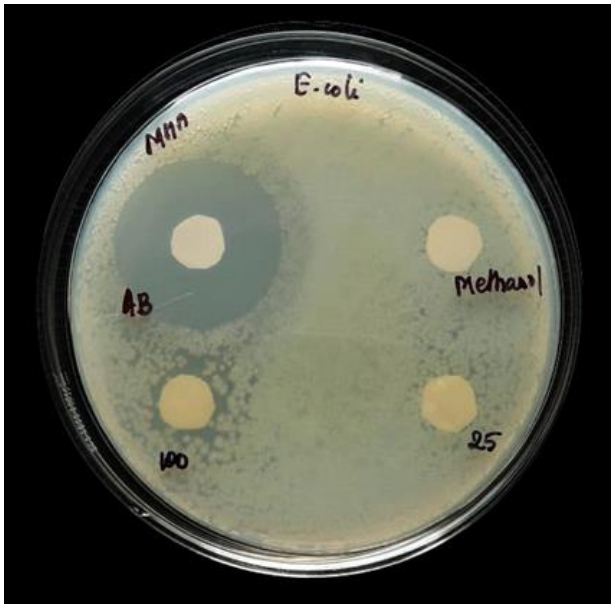

Fig 7: Disc diffusion plate of *Escherichia coli*

Conclusion

The qualitative and quantitative phytochemical analysis further helped in understanding the diverse phytochemical classes present in the stem of *Acacia caesia* (L.) *WILLD* and thereby sheds light on their success in traditional medicine. The GC-MS analysis gave a rich profile of the potential phytochemicals present in the stem with the presence of known therapeutic agents and those with unknown functions thus opening a scope for further research into the understanding of these phytochemicals. The antibacterial studies proved that potent antibacterial activity of the stem extract against three known human pathogens – namely, the gram-positive bacteria, *Staphylococcus aureus* and gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*. These studies show the immense potential of usage of the stem as an antibacterial agent and also further aids to promote phytochemical investigations in order test the drug potential of these phytochemicals.

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