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COMPARATIVE ANALYSIS OF THE BACTERICIDAL AND ANTIBIOTIC MODULATORY ACTIVITIES OF THE STEM BARK EXTRACTS OF *GMELINA ARBOREA* ROXB AND *OROXYLUM INDICUM* (L.) KURZ

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Keywords:

Phytochemical analysis, Antibacterial activity, *Gmelina arborea, Oroxylum indicum, Escherichia coli, Staphylococcus aureus*

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ABSTRACT: Medicinal plants play pivotal roles in novel drug discovery or combinatorial drug surveillance against emerging infectious diseases. This study aims to explore and compare the bactericidal and antibiotic modulating activities of stem barks of *Gmelina arborea* Roxb. and *Oroxylum indicum* (L.) Kurz against two pathogenic bacterial strains, i.e., *Escherichia coli* and *Staphylococcus aureus*. Methanolic stem bark extracts of *G. arborea* and *O. indicum* were subjected to phytochemical analysis, DPPH antioxidant assay, and varieties of antibacterial assays (disc diffusion, agar well diffusion, modified agar well diffusion, and bacterial cell viability), and the results were compared. Phytochemical analysis revealed that both plants had major bioactive compounds like alkaloids, terpenoids, phenol & tannins, steroids, glycosides, etc. *G. arborea* had a total phenol content (TPC) of 27.33±8.60 mg/g of Gallic Acid Equivalent (GAE) and a total flavonoid content (TFC) of 72.33±4.90mg/g of Rutin Equivalent (RE). At the same time, *O. Indicum* was calculated to have a TPC of 48.58±1.80 mg/g of GAE and TFC of 269±0.88mg/g of RE. Though both stem barks exhibited excellent antibacterial activities against both *E. coli* and *S. aureus* strains, *O. indicum* inhibited the growth of *S. aureus* better in comparison to *E. coli* by augmenting the bactericidal activity of conventional antibiotics like ampicillin, and nitrofurantoin, while *G. arborea* supplemented the activities of streptomycin, ampicillin, and cefixime against *S. aureus*. Our results may be beneficial in recommending *G. arborea* and *O. indicum* stem barks as alternatives or combination therapy with antibiotics to treat different bacterial infections.

INTRODUCTION: Alternative medicines obtained from plants have been part of traditional healthcare for thousands of years and are also potential agents against microbial diseases [1].

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Serious side effects caused by synthetic chemicals and prevalent antibiotic resistance among the pathogens urge for safe and effective novel alternatives for different infectious diseases.

Phytochemicals isolated from various potential medicinal plant parts are used as single therapeutic agents or in combination with conventional allopathic medicines. Phytocompounds are assumed to alleviate the efficacy of synthetic drugs and diminish their side effects by lowering their required dose to a safe range, and such combination therapies are gaining popularity in both human and veterinary treatment systems [2]. Though multiple ethnomedicinal reports exist for these medicinal plants, reports on their scientific validation are limited. In this study, stem barks of two important medicinal plants, i.e., *Gmelina arborea* Roxb. (Verbenaceae) and *Oroxylum indicum* (L.) Kurz (Bignoniaceae) were selected for their phytochemical screening and antibacterial activity study. These two barks are used in the preparation of different ayurvedic formulations like *Dasamularistha*, *Dashamulaghrita*, etc., to reduce colds, coughs, asthma, chronic fever, and respiratory problems [3]. *G. arborea* is native to Asia and found all over India. It is reported to have significant antimicrobial activity to support its folkloric use against some diseases as a broad-spectrum antimicrobial agent [4].

Decoction of the root and stem bark of *G. arborea* was given orally for scorpion stings and snake bites. The bark is also used for liver disorders, stomach disorders, bone fractures, gout, and gonorrhea [5]. *G. arborea* stem bark is reported to have quercetagetin, gummadiol, glycosides of kaempferol, apigenin, luteolin and lignans such as 3,4,5- trimethoxypheol, 2,6-dimethoxy-p-benzoquinone and tyrosol, [2-(4- hydroxyphenyl) ethanol] along with other compounds like arborone and 7-oxo dihydrogmelinol, 4,8-dihydroxy-sesamine and 4 – hydroxyseasamin [6].

The stem barks of *O. indicum* are rich in different compounds like chrysin, oroxindin, sitosterol, apegenin, prunetin, ellagic acid, baicalein, biochanin-A, baicalein-7-O-glucoside, baicalein-7-O-diglucoside (Oroxylin B), antraquinone, tetuin, aloe emodin and scutellarein. These stem barks are often used for treating ulcers, diarrhea, fever, jaundice, and even cancer in Ayurvedic and folk medicine. Recently, its antiproliferative, anticancer, antioxidant, antiulcer, hepatoprotective, antiarthritic, antimicrobial, antimutagenic, photocytotoxic, anti-inflammatory, and immune-stimulant properties were reported in different *in-vivo* and *in-vitro* studies [7]. *O. indicum* fruits have significant flavonoids with excellent antibacterial and antioxidant potential. The ethanol and water extracts of seeds and fruits exhibited moderate to intermediate antibacterial properties against some clinical isolates of *Streptococcus suis* and *Staphylococcus intermedius* strains. A phytochemical study of the extracts unveiled the probable presence of some phenolics and flavonoids, including baicalein [8]. Significant antienterobacterial efficacy of *G. arborea* supplemented its traditional uses against gastroenteritis, dysentery, typhoid fever, and shigellosis [9]. Therefore, this plant could serve as a potential source of antimicrobial and chemotherapeutic agents for novel pharmaceutics development to treat diarrhea and dysentery [10]. Phytochemical screening of the barks, leaves, roots, and fruits of *G. arborea* confirmed the presence of a higher concentration of flavonoids (15.2%) and saponins (6.1%), both secondary metabolites responsible for potent antimicrobial activity [11, 12].

The water extracts were reported to contain reducing sugars, steroids, saponins, tannins, phenolics, flavonoids, and glycosides [13, 14]. It was also noted that the hexane extract comprised saponins and steroids, and the methanolic extracts had glycosides, saponins, steroids, and flavonoids [15]. *G. arborea* and *O. indicum* are well-known medicinal plants with broad applications in folklore and ayurvedic formulations. Antibacterial activities of these two plants' leaves, roots, fruits, and seed extracts have already been reported. However, limited reports were found on the bactericidal effects of the stem barks of these two plants.

Moreover, there are no records of the antibiotic modulatory activities of these two plant parts. Additionally, these therapeutic stem barks are not only used for human and veterinary health care but also, they are used in temple rites of the world-famous Jagannath temple of Puri, Odisha, India, to treat the ailing anthropogenic Lord Jagannath and his brother Balabhadra and sister Subhadra during the 14 days *Anasara*, when the lords happen to suffer from fever during July month with the advent of monsoon season. Different herbs and herbal parts are used to treat the ailing lords, and these two stem barks are among the mystic herbs (Dash and Das, unpublished data). Hence, this present study was conducted to analyze the different phytochemicals present in these two extracts along with their antioxidant, bactericidal, and antibiotic modulating potentialities. Their antibacterial activities were assessed against different standard and clinically isolated bacterial strains using multiple assay methods to compare and validate their potentiality as anti-infectious agents. Their effectiveness in combination therapy was also estimated by supplementing the extracts with different conventional antibiotics. Overall, this study includes all the parameters to validate the folkloric and divine use of these medicinal plants as anti-infectious agents.

MATERIALS AND METHODS:

Materials: Bacterial growth culture media obtained from Himedia, Mumbai. The solvents and chemicals were of analytic grade and obtained from SRL, Mumbai; Merck, Bangalore; and Loba Chemicals.

Plant Material Collection and Extract Preparation: Fresh stem barks of *G. arborea* and *O. indicum* were collected from Kanas, Puri, Odisha, in May and June 2023. They were identified and authenticated by taxonomist Prof. M.K. Misra and Prof. S.K. Dash, Berhampur University, and a voucher specimen was submitted to the Herbarium of Department of Botany, Berhampur University bearing voucher number (BOTBU2303, BOTBU2304). The shade-dried stem barks were ground into fine powder. The powdered plant samples were subjected to exhaustive Soxhlet extraction in 300mL methanol for 72 hours at 60-70°C. The filtered extract was concentrated by solvent evaporation in a rotary evaporator. The final crude extract was collected in specific labeled pre-weighed petri plates and left at 37°C till complete drying. The yield percentage of crude extract was calculated by using a standard formula [16]. The plates containing crude extract were sealed with a Petri seal and preserved at 4°C for future use. These extracts were dissolved in lukewarm distilled water to prepare the desired concentrations of the different extracts based on the requirement of the assay.

Thin Layer Chromatography (TLC): TLC was conducted using a standard protocol with some modifications [17]. About 2-10µl of methanolic crude extract was loaded on commercially available TLC plates with the help of capillary tubes at 1cm above the lower margins of TLC plates. The chromatograms were developed by placing the air-dried sample-loaded TLC plates in specific glass chambers containing different solvent mixtures such as Hexane: Ethyl acetate or Diethyl ether: Ethyl acetate at a ratio of 8:2, 7:3, and 6:4, respectively. The mobile phase was allowed to move through the absorbent phase up to 1cm under the top margin of the plate. After air drying, the TLC plates were developed in saturated iodine chambers. Rf values were calculated for each isolated compound, and the number of possible constituents like alkaloids, flavonoids, tannins, and phenols was noted [18].

Qualitative Phytochemical Screening: The crude methanolic extracts of *G. arborea* and *O. indicum* stem barks were investigated for the occurrence of different compounds using standard procedures [19-21]. The phytochemical tests were conducted for the presence of Alkaloids (Mayer's test, Wagner's test). Flavonoids (Shinoda test, Sodium hydroxide test). Steroids/Triterpenes (Lieberman-Burchard test and Salkowski test). Saponins (Frothing test). Tannins (Lead sub-acetate test and Ferric chloride test) in both the stem bark extracts.

Quantitative Phytochemical Screening:

Total Phenolic Content (TPC): For estimation of TPC, the folin- ciocalteu (FC) method was followed [22]. To 200 µL of crude extract (1mg/mL), 3mL of distilled water was added, then 0.2mL of FC-reagent was added and mixed gently for 8 min. Then 0.6mL of 10% Na_2CO_3 was added and left in the dark for 1hr. After that, the absorbance of the mixture was measured at 765nm. The average absorbance value of triplicate sets of tests and standards were used for calculating the TPC from the gallic acid standard curve by using the below-mentioned formula, and the values were expressed as gallic acid equivalents (GAE) per gram dry weight of different extracts.

$$C = (c \times V) / m$$

Where, C= Total phenolic content of plant extract expressed in GAE in mg/g, c = The concentration of gallic acid calculated from the standard curve in mg/Ml, V = The volume of extract in mL, m = The weight of plant extract in g.

Total Flavonoid Content (TFC): TFC was estimated by spectrophotometric method [23]. To 1mL solution of methanol extract (1mg/mL), 1mL of 2% $AlCl_3$ solubilized in methanol was added and incubated at room temperature for one hour.

Then, absorbance was noted at 415nm. Three sets of tests and standards were used to get the average absorbance value. The TFC was calibrated from the standard curve of Rutin and presented in mg of Rutin equivalent (RE) per gram dry weight of different extracts.

1 1 – Diphenyl – 2 - picrylhydrazyl (DPPH) Antioxidant Assay: The antioxidant activity of the stem bark extracts was determined by analysing their radical scavenging potential using the DPPH method [24]. DPPH free radical solution was prepared by dissolving DPPH (0.2mM) in methanol. To 20-500µl of methanolic extract, absolute methanol was added for a final volume of 1mL, then 1mL of DPPH solution was added to it. After incubating for 30 min in the dark, absorbance was measured at 517 nm. Ascorbic acid was used as positive control. IC_{50} value is the concentration of an antioxidant needed to decrease the initial concentration of DPPH by 50%. The percentage of scavenging of free radicals or 50% inhibitory concentration (IC_{50}) was calculated using the formula below.

$$\text{Inhibition (\%)} = 1 - (\text{Abs. of Sample}) / (\text{Abs. of Control}) \times 100$$

Bacterial Strains, Maintenance and Storage: The clinical isolates of *Enterococcus faecalis* (BUMCC001), *Escherichia coli* (BUMCC002), *Proteus vulgaris* (BUMCC003), *Pseudomonas aeruginosa* (BUMCC004) and *Staphylococcus aureus* (BUMCC005) were procured from MKCG Medical College and Hospital, Berhampur and standard strains of *E. coli* (ATCC25922) and *P. aeruginosa* (MTCC3541) were procured from Department of Biotechnology, Berhampur university. The identification of clinical isolates was done by 16S rDNA sequencing and blast analysis. For routine use, the cultures were maintained on Nutrient agar plates. For long-term storage, glycerol stocks were prepared and then stored at -20°C aseptically. Subcultures of these strains were maintained in Nutrient agar plates at 4°C. Before every antibacterial assay, the preserved bacterial strains were activated for one hour in Nutrient broth and then used.

Determination of Minimum Inhibitory Concentration (MIC): The MIC analysis was performed via broth microdilution techniques in a 96-well microtiter plate according to Clinical Laboratory Standards Institute (CLSI) guidelines with some modifications [25-27]. Stock extract solution (250mg/mL) and Ciprofloxacin (CIP) (250µg/mL) were prepared. Bacterial suspensions at a concentration of 10^6 cfu/mL were prepared. Duplicate wells (starting with a concentration of 12.5mg/mL of GA/OI or 12.5µg/mL of CIP) were loaded with serially diluted extract or antibiotic solutions, followed by bacterial culture. Duplicate wells with only culture media were used for background score; bacteria without extract served as control, and wells with extract without bacteria served as blank. Each well had a final volume of 200µl. The plates were sealed loosely with parafilm to prevent dehydration and incubated for 24 hours at 37°C.

After incubation, 40µl of MTT (3-(4,5-dimethyl-2thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) (0.2mg/mL) was added to each well and incubated for 30 minutes at RT. The optical densities of microplates were measured at 595nm in a Microplate reader. The development of purple coloration in the wells indicates bacterial growth. The well with the lowest coloration was noted, and its concentration was determined as the MIC value.

Antibacterial Assay: The susceptibility of different strains of *E. coli* and *S. aureus* to crude stem bark extracts was tested through different antibacterial methods such as disc diffusion [28], agar well diffusion (swab, pour plate), modified agar well diffusion method [29] and bacterial cell viability assay [30].

Disc Diffusion Method: Discs of approximately 6mm diameter were cut from the Whatman no.1 filter paper. These discs were loaded with 250µg, 500µg, 750µg, and 1000µg concentrations of methanolic extracts of the aforesaid plant parts, air-dried, and then placed aseptically on nutrient agar plates swabbed with either *E. coli* or *S. aureus*, which were activated for one hour. The plates were incubated overnight at 37°C and were checked for the zone of inhibitions (ZOIs) in terms of millimeters.

Agar well Diffusion Assay by Swabbing Method: A single bacterial colony from the master culture plate was suspended in 5 mL of sterilized nutrient broth. The test tube was shaken well for proper mixing and incubated at 37°C for 1 hr activation.

The freshly activated bacterial cultures of *E. coli/S. aureus* were swabbed with sterilized cotton buds on nutrient agar plates and incubated for 15 minutes. Four wells were made in each plate and loaded with 0.5mg, 1mg, 1.5mg, and 2mg concentrations of crude extract and left at room temperature for 15 minutes to allow the extract to diffuse into the wells. The ZOIs were noted down after overnight incubation at 37°C. The zone of inhibition depends either on the bactericidal or bacteriostatic effects of the plant extract being assayed.

Agar Well Diffusion by Pour Plate Method: Around 100µl of freshly activated culture of *E. coli/S. aureus* was added to sterile plates, followed by lukewarm nutrient agar media aseptically. After proper solidification, four wells were made, and each of the methanolic crude extracts was loaded into them at four different concentrations of 0.5mg, 1mg, 1.5mg, and 2mg, separately to verify the drug-dependent ZOIs against each bacterial strain.

Modified Agar well Method: The sensitivity of 7 different bacterial strains (clinical isolates of *E. coli, E. faecalis, S. aureus, P. aeruginosa, P. vulgaris,* and standard strains of *E. coli*, and *P. aeruginosa*) were tested on a single plate using this method.

A well was made in the center of the agar plate and loaded with 1mg of crude stem bark extract of *G. arborea/ O. indicum*. All seven bacterial strains were streaked on the plate from the periphery toward the center in a zigzag manner. Then, the plates were left at RT for an hour for drug diffusion before incubating overnight at 37°C. The distance between the starting point of the bacterial growth line from the well was measured to get a comparative inhibitory result.

Bacterial Cell Viability Test by Spread Plate Method in Wild and Treated Bacteria: The bacterial cell viability was assessed by counting the colonies in wild and stem bark extract treated bacteria following the standard lab protocol [30]. Three test tubes containing 3mL of sterilized nutrient broth were inoculated with 100µl of activated culture. The first one without crude extract served as a wild/control. The second test tube was added with 100µg/mL, and the third one was added with 600µg/mL of stem bark extract and incubated for 4 hours at 37°C. Aliquots of the diluted bacterial sample were spread over sterilized agar plates using an L-shaped rod and incubated overnight at 37°C. The CFU/mL in the wild and treated bacteria was calculated by counting the colonies and multiplying it with the dilution factor.

Antibiotic Modulating Potential: The antibiotic sensitivity of the bacterial strains was tested with some research-grade antibiotic discs such as Ciprofloxacin, Nitrofurantoin, Ampicillin, Cefixime, Streptomycin, Polymyxin, Amikacin, Gentamycin, and Chloramphenicol. The zone of inhibition of each antibiotic disc was measured in millimeters against each strain. From the above list, five most and least effective antibiotics were selected for further studies.

The standard antibiotic discs of AMP 30-Ampicillin 30µg, NIT 300-Nitrofurantoin 300 µg, CIP 5-Ciprofloxacin 5µg, CFM 5-Cefixime 5µg, S10-Streptomycin 10µg were aseptically placed on *E. coli /S. aureus* swabbed nutrient agar plates. These were subjected to overnight incubation, and the inhibitory zone was measured in millimeters to determine the bacterial susceptibility.

These ZOIs were used as standards for comparing with another set of plates containing antibiotics added with crude stem bark extracts at a dose of 500µg/disc. The variations in the inhibition zones indicated how much the methanolic extracts enhanced or augmented the antibiotic discs' inhibitory capacity [30].

RESULTS:

Plant Extract: The stem barks were collected from different localities and processed as described in the methodology section; however, the detailed steps of crude extract preparation are summarized in **Fig. 1**.

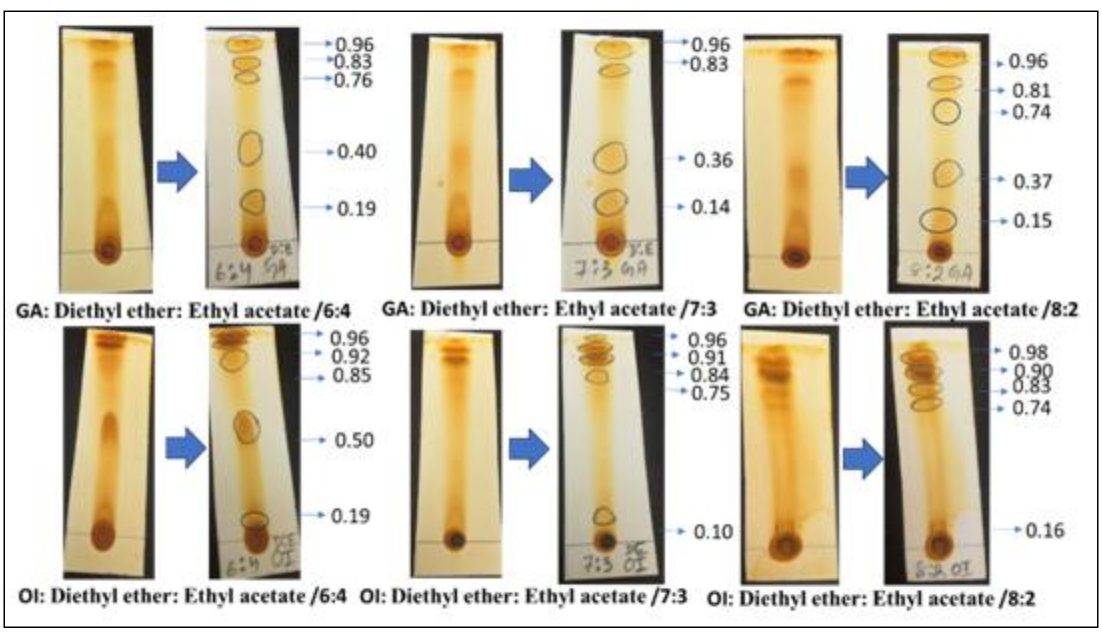
Total 56.50gm of *G. arborea* (GA) and 56.28gm of *O. indicum* (OI) dried stem bark powers were collected, from which 4.60gm and 7.25gm of crude extracts were prepared after evaporating methanol. The final dark brown semi-solid crude extracts were stored at 4°C in a dried condition. The yield percentage of crude extracts was found to be 8.14% for GA and 12.88% for OI, respectively.

FIG. 1: PREPARATION OF METHANOLIC CRUDE EXTRACT OF *GMELINA ARBOREA* AND *OROXYLUM INDICUM* STEM BARK

Thin Layer Chromatography: Thin layer chromatography gives an insight into the probable polar and nonpolar phytocompounds present in plant extracts.

Hexane: Ethyl acetate chambers and Diethyl ether: Ethyl acetate chambers at ratios of 8:2, 7:3, and 6:4 detected phytocompounds with different Rf values depending on the polarity and solubility of the compounds. In Hexane: Ethyl acetate mobile phase upto 10 compounds (Rf value 0.10 – 0.94) were detected in GA, while eight compounds (Rf value 0.16 – 0.96) were detected for OI and five compounds each (Rf value 0.15 – 0.96 for GA and 0.16 – 0.98 for OI) were detected in Diethyl ether: Ethyl acetate in both the extracts.

The results inferred that the extracts have a more significant number of nonpolar compounds than polar compounds. The TLC profiles of both extracts are presented in **Fig. 2.**



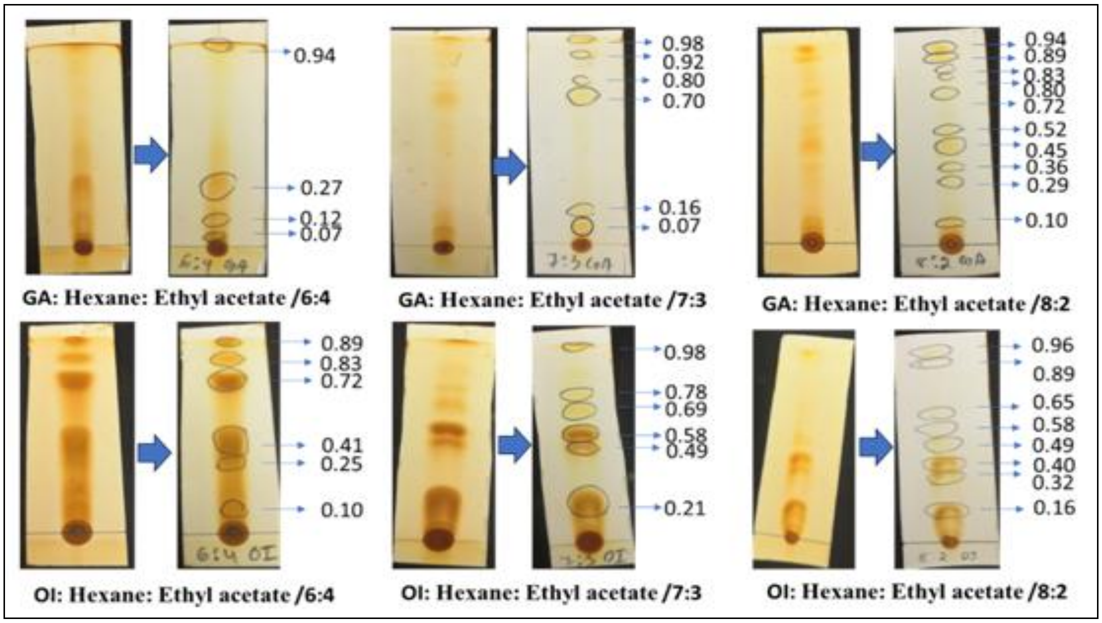


FIG. 2: THIN LAYER CHROMATOGRAPHY SEPARATION OF METHANOLIC CRUDE EXTRACT OF *O. INDICUM* AND *G. ARBOREA* STEM BARK IN DIETHYL ETHER: ETHYLE ACETATE AND HEXANE: ETHYL ACETATE VARIABLE SOLVENT MIXTURE MOBILE PHASE

Qualitative Phytochemical Analysis: The extracts of both plants were screened for the presence of biologically active phytochemicals. The methanolic extract of GA showed nine, and OI showed seven active compounds out of thirteen phytochemical tests conducted using the above standard protocol. Methanolic extracts of both plants were found to contain major bioactive compounds like alkaloids, terpenoids, phenol and tannins, steroids, glycosides, *etc*., and the results are presented in **Table 1**.

TABLE 1: PHYTOCHEMICAL SCREENING OF BIOACTIVE COMPOUNDS PRESENT IN *GMELINA ARBOREA* AND *OROXYLUM INDICUM*

Analysed phytochemicals	Name of plants	
	Gmelina arborea	***Oroxylum indicum***
Alkaloid	+	+
Terpenoid	+	+
Phenol & tannin	+	+
Reducing sugar	+	-
Saponin	+	-
Protein	+	-
Steroid	+	+
Anthocyanin	-	-
Coumarin	+	-
Leuco anthocyanins	-	-
Glycosides	+	+
Flavonoids	-	+
Phlobatanin	-	+

Note; ‘+’ sign for presence and ‘-’ for absence of phytochemicals

Quantitative Phytochemical Analysis: The total phenol content (TPC) of 27.33±8.60mg/g of Gallic Acid Equivalent (GAE) and a total flavonoid content (TFC) of 72.33±4.90mg/g of Rutin Equivalent (RE) were found in *G. arborea*. At the same time, a TPC value of 48.58±1.80 mg/g of GAE and TFC of 269±0.88mg/g of RE was calculated to be present in *O. indicum*. The phytocompounds like phenols and flavonoids were quantified by plotting standard calibration curves.

DPPH Antioxidant Assay: Extracts with potential antioxidant properties and ascorbic acid (positive reference), when mixed with DPPH solution, change the deep purple color of DPPH solution to colorless. In contrast, the purple color remained unchanged when the DPPH solution was mixed with extracts of low antioxidant activity or any negative control. Ascorbic acid had maximum scavenging activity and minimum IC_{50} value of 1.86±0.2, followed by GA extract with an IC_{50} value of 2.00±0.19, while OI had an IC_{50} value of 2.2±0.25. Since, the difference between the antioxidant properties of extracts and the standard ascorbic acid is insignificant, it can be inferred that both extracts have comparable antioxidant activities as those of the standard. The comparative analysis is plotted using MS Excel software and presented in **Fig. 3**.

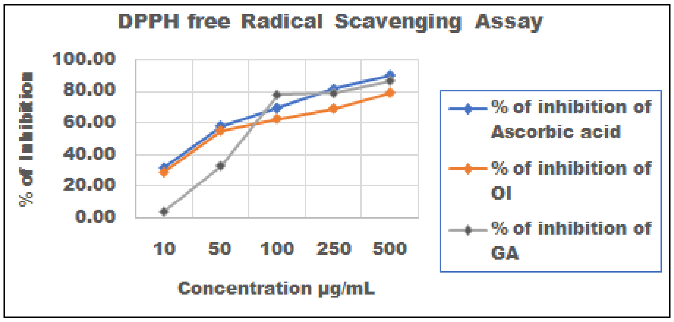


FIG. 3: *IN-VITRO* ANTIOXIDANT ACTIVITY OF *G. ARBOREA* AND *O. INDICUM*STEM BARK EXTRACTS IN DPPH FREE RADICAL SCAVENGING ASSAY

Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration (MIC) values were determined as 0.39µg/mL for standard antibiotic ciprofloxacin against the two test strains, and 3.125mg/mL against *E. coli* and 1.562 mg/mL against *S. aureus* for both OI and GA stem bark extracts.

Antibacterial Assays:

Disc Diffusion Method: Discs with different concentrations of methanolic stem bark extracts were applied against both strains to find their antibacterial effects, which were measured as a zone of inhibition (ZOI) and expressed in millimeters (mm). The results are depicted in **Table 2**.

Both plant extracts were found to have a dose-dependent effect on both test strains. *S. aureus* was found to be more sensitive to both the extracts, and GA showed more activity with a ZOI of 11.00±1.00 and 16.33±0.58 mm at a dose of 1000µg /disc in comparison to OI that exhibited 8.33±0.58 and 14.00±1.00 against *E. coli* and *S. aureus*, respectively.

TABLE 2: ANTIBACTERIAL ACTIVITY OF *G. ARBOREA* AND *O. INDICUM* STEM BARK EXTRACTS AGAINST *E. COLI* AND *S. AUREUS*IN DISC DIFFUSION METHOD

Extracts	Strains	ZOI measured in mm at different conc. of stem bark extracts in µg /disc			
		250	500	750	1000
GA	*E. coli*	5.67±0.58	6.33±0.58	7.33±0.58	11.00±1.00
	S. aureus	11.67±0.58	12.33±0.58	14.67±0.58	16.33±0.58
OI	*E. coli*	6.33±0.58	7.00±1.00	8.00±1.00	8.33±0.58
	S. aureus	5.67±0.58	10.33±0.58	12.00±1.00	14.00±1.00

Note: Values represent the average ± SD of triplicate sets of experiments

Agar well Diffusion by the Swabbing Method: The comparative inhibitory effects of the stem bark extracts against the test strains were verified in agar well diffusion by the swabbing method, and ZOI was expressed in mm. The results are presented in **Table 3**. Both the plant extracts exhibited dose-dependent effects against both the test strains. *S. aureus* was found to be more sensitive to both the extracts, and GA showed more activity with a ZOI of 15.33±1.15 and 28.33±2.08 mm at a dose of 2mg /well in comparison to OI that showed 14.33±3.06 and 26.67±2.08 at the highest test dose against *E. coli* and *S. aureus*, respectively.

TABLE 3: BACTERICIDAL ACTIVITY OF *G. ARBOREA* AND *O. INDICUM* STEM BARK EXTRACTS AGAINST *E. COLI* AND *S. AUREUS*IN AGAR WELL SWABBING METHOD

Extracts	Strains	ZOI measured in mm at different conc. of stem, bark extracts in mg /well			
		0.5mg	1.0mg	1.5mg	2.0mg
GA	*E. coli*	10.67±0.58	12.00±1.00	14.33±1.53	15.33±1.15
	S. aureus	18.33±1.15	26.00±1.00	26.00±3.61	28.33±2.08
OI	*E. coli*	10.67±0.58	12.33±2.52	14.00±2.00	14.33±3.06
	S. aureus	11.33±0.58	16.33±1.53	17.00±2.00	26.67±2.08

Note: Values represent the average ± SD of triplicate sets of experiments

Agar well Diffusion by Pour Plate Method: The comparative inhibitory effects of the stem bark extracts against the test strains were verified in agar well diffusion by pour plate method, and ZOI was expressed in mm. The results are presented in **Table 4**. Both plant extracts were found to have a dose-dependent effect on both test strains. *S. aureus* was found to be more sensitive to both the extracts, and GA showed more activity with a ZOI of 31.00±1.00 and 25.67±0.58mm in comparison to OI that exhibited 8.67±0.58 and 16.33±0.58 at a dose of 2mg/well against *E. coli* and *S. aureus*, respectively. In contrast to the results of disc diffusion and agar well swabbing method, *E coli* was significantly suppressed by GA stem bark in comparison to *S. aureus.*

TABLE 4: BACTERICIDAL ACTIVITY OF *G. ARBOREA* AND *O. INDICUM* STEM BARK EXTRACTS AGAINST *E. COLI* AND *S. AUREUS*IN AGAR WELL POUR PLATE METHOD

Extracts	Strains	ZOI measured in mm at different conc. of stem, bark extracts in mg /well			
		0.5mg	1.0mg	1.5mg	2.0mg
GA	*E. coli*	20.00±1.00	25.33±0.58	26.00±1.00	31.00±1.00
	S. aureus	18.67±0.58	23.67±1.15	24.33±1.53	25.67±0.58
OI	*E. coli*	7.00±1.00	7.67±0.58	8.00±1.00	8.67±0.58
	S. aureus	10.67±0.58	12.67±0.58	14.00±1.00	16.33±0.58

Note: Values represent the average ± SD of triplicate sets of experiments

Modified Agar well Diffusion Method: The inhibitory effects of the stem bark extract (1mg/well) were studied against different clinical isolates and standard strains of *E. coli, S. aureus, Pseudomonas aeruginosa, Proteus vulgaris, and Enterococcus faecalis* on a single plate using modified agar well diffusion method to find the comparative susceptibility level of different pathogens to the extract. The ZOIs were measured in mm, and the results are presented in **Table 5**. In concordance to our other results, GA was observed to inhibit the bacterial growth better than OI against all the test strains especially, *E. coli, P. aeruginosa and S. aureus.*

TABLE 5: BACTERICIDAL ACTIVITY OF *G. ARBOREA* AND *O. INDICUM* STEM BARK EXTRACTS AGAINST *E. COLI* AND *S. AUREUS*IN MODIFIED AGAR WELL METHOD

Bacterial strains	*G. arborea* (1 mg/well) ZOI in mm	*O. indicum* (1 mg/well) ZOI in mm
E. coli (ATCC 25922)	6.33±0.58	4.33±0.58
E. coli (BUMCC002)	6±1.0	3.67±1.53
E. faecalis (BUMCC001)	5.67±0.58	5.33±1.15
P. aeruginosa (MTCC3541)	5.33±1.53	4±1.00
P. aeruginosa (BUMCC004)	6.67±0.58	5.67±0.58
P. vulgaris (BUMCC003)	4±1.00	3.67±0.58
S. aureus (BUMCC005)	6.33±0.58	5±1.00

Note: Values represent the average ± SD of triplicate sets of experiments

Bacterial Cell Viability Assay by Spread Plate Method: The colony forming units per mL (CFU/mL) was determined at the log/exponential phase in the control/untreated and extract treated bacteria. The % of bacterial growth inhibition by different stem bark extracts was found to be dose-dependent. OI at a dose of 600µg/mL inhibited the CFU/mL of *S. aureus* up to 78.13% and *E. coli* up to 65.74%. Whereas, GA at 600µg/mL had almost similar % of growth inhibition of 70.78 and 70.76% against *E. coli* and *S. aureus*, respectively. The results are presented in **Table 6** and **Fig. 4**.

TABLE 6: BACTERICIDAL ACTIVITY OF *G. ARBOREA* AND *O. INDICUM* STEM BARK EXTRACTS AGAINST *E. COLI* AND *S. AUREUS*IN BACTERIAL CELL VIABILITY ASSAYBY SPREAD PLATE METHOD

Extracts	Strains	Colony forming units/mL at log phase				
		Control	Treated (100µg/mL)	% inhibition	Treated (600µg/mL)	% inhibition
GA	*E. coli*	79.4×10^7	43.6×10^7	45.08	23.2×10^7	70.78
	S. aureus	85.5×10^7	52.5×10^7	38.59	25.0×10^7	70.76
OI	*E. coli*	79.4×10^7	50.2×10^7	36.77	27.2×10^7	65.74
	S. aureus	85.5×10^7	44.6×10^7	47.83	18.7×10^7	78.13

Note: Values represent the average of triplicate sets of experiments

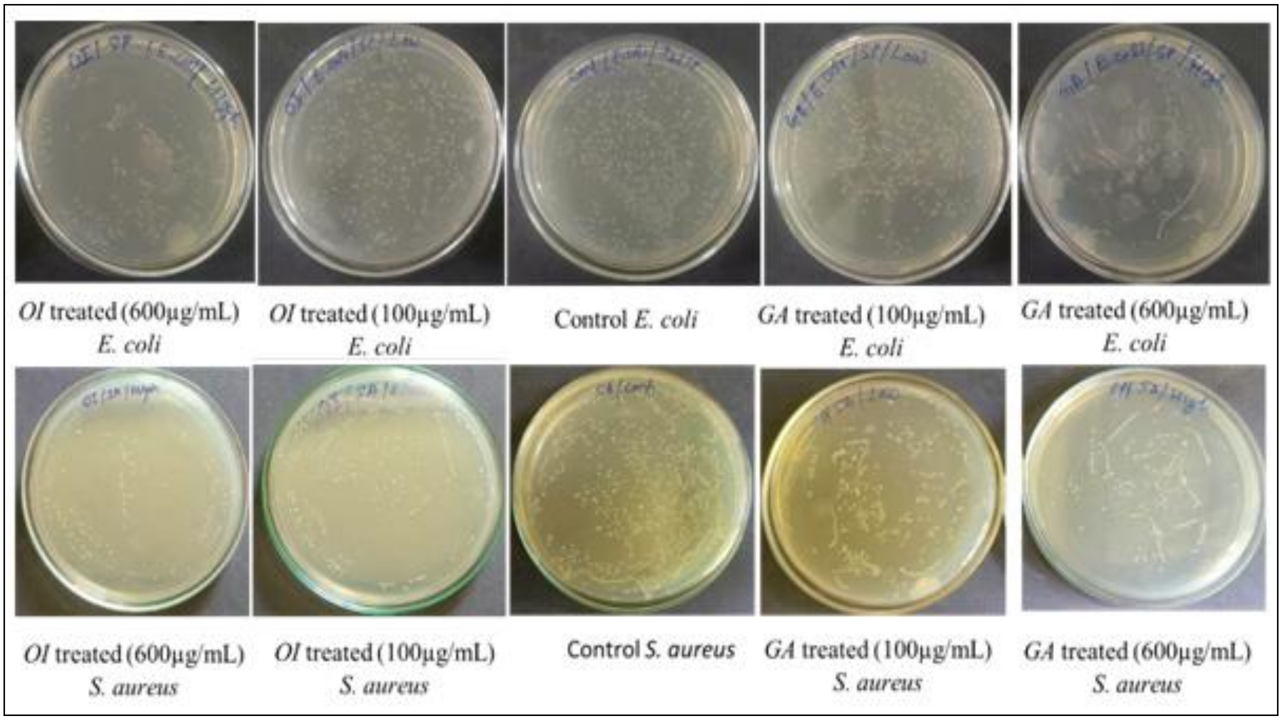


FIG. 4: *IN-VITRO* ANTIBACTERIAL STUDY OF METHANOLIC STEM BARK EXTRACTS OF *G. ARBOREA* AND *O. INDICUM* AGAINST *E. COLI* AND *S. AUREUS* BY SPREAD PLATE METHOD.

Antibiotic Modulatory Effect: The *in-vitro* antibiotic sensitivity of the bacterial strains against different conventional antibiotics like Ampicillin 10 (AMP 30), Nitrofurantoin 300 (NIT 300), Ciprofloxacin 5 (CIP 5), Cefixime 5 (CFM 5), Streptomycin 10 (S10) and their modulatory effects in the presence of the stem bark extracts of GA and OI were tested.

The increase or decrease in ZOI of antibiotic discs, against each strain was calculated by taking the difference of ZOI in mm, between antibiotic supplemented with 500µg/disc of stem bark extract and antibiotic alone. The antibiotic modulatory effects of the stem bark extracts were studied by finding the synergistic, indifferent, or antagonistic effects, and the results are presented in **Table 7**.

By supplementing GA extract to the S10 disc, the inhibition zone was increased by 2.67±0.58 mm against *E. coli* and CFM5 activity was significantly increased by 7.67±0.58 mm against *S. aureus.*

At the same time, the OI extract increased the S10, AMP10, and NIT300 activity by 1.33±0.58, 3.33±0.58, and 7.00±2.65 mm against *S. aureus*. However, the stem barks remained ineffective in changing the activity of other antibiotics against *E coli* strain.

TABLE 7: *IN-VITRO* ANTIBIOTIC MODULATING ACTIVITY OF *G. ARBOREA* AND *O. INDICUM* STEM BARK EXTRACTS AGAINST *E. COLI* AND *S. AUREUS*

Extracts	Strains	Difference in zone of inhibition (in mm) (ZD) by addition of stem bark extract (500µg /disc)to different antibiotics				
		S10	**CFM5**	**AMP10**	**CIP5**	**NIT300**
GA	*E. coli*	2.67±0.58	0.33±0.58	0.00±0.00	0.33±0.58	0.67±0.58
	S. aureus	1.33±0.58	7.67±0.58	1.67±0.58	0.67±0.58	0.67±0.58
OI	*E. coli*	0.67±1.53	0.33±1.15	0.33±0.58	0.33±1.15	0.33±1.15
	S. aureus	1.33±0.58	0.67±0.58	3.33±0.58	1.00±1.00	7.00±2.65

Note: Values represent the average ± SD of triplicate sets of experiments

All the bactericidal tests (disc diffusion, agar well, antibiotic modulatory assay) were compiled to have an overview of all the results. **Fig. 5** presents the bar diagrams/graphical results of different bactericidal assays of *G. arborea,* while **Fig. 6** represents the compiled results of *O. indicum.* All the images of assay plates are compiled and presented in **Fig. 7**.

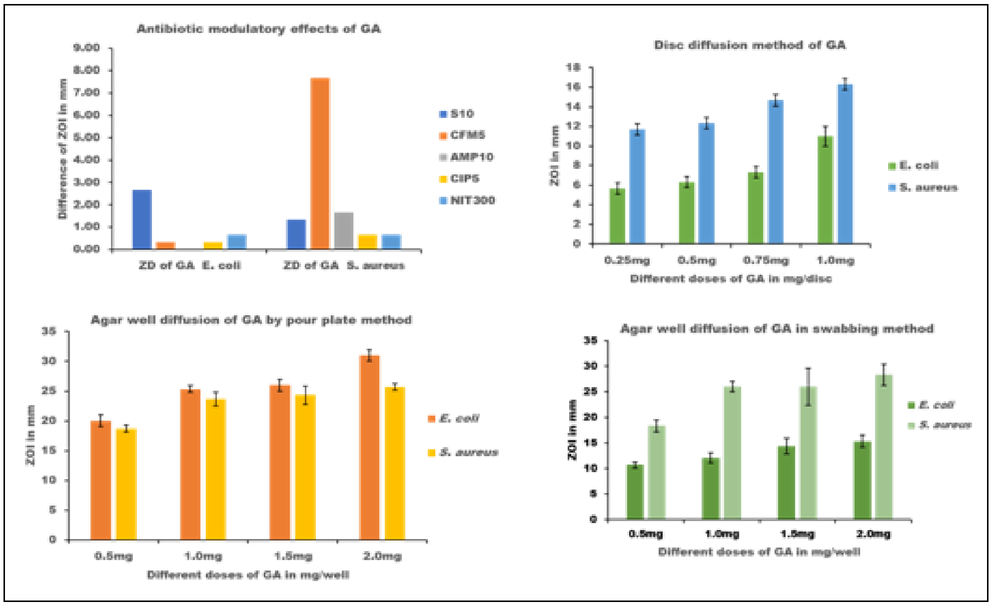


FIG. 5: COMPARATIVE GRAPHICAL PRESENTATION OF THE ANTIBACTERIAL EFFICACIES OF *G. ARBOREA* AGAINST *E. COLI* AND *S. AUREUS*

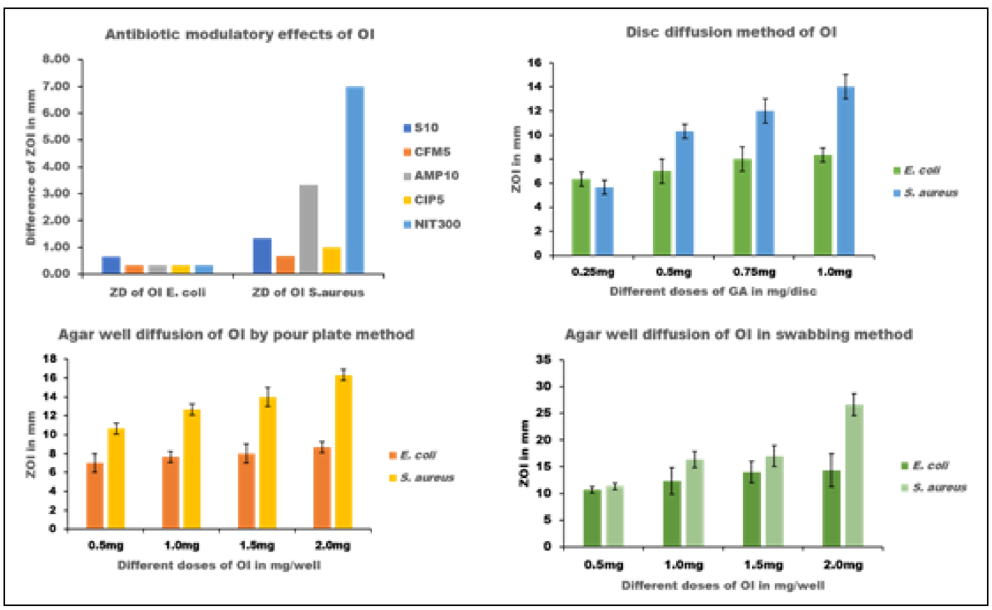


FIG. 6: COMPARATIVE GRAPHICAL PRESENTATION OF THE ANTIBACTERIAL EFFICACIES OF *O. INDICUM* AGAINST *E. COLI* AND *S. AUREUS*

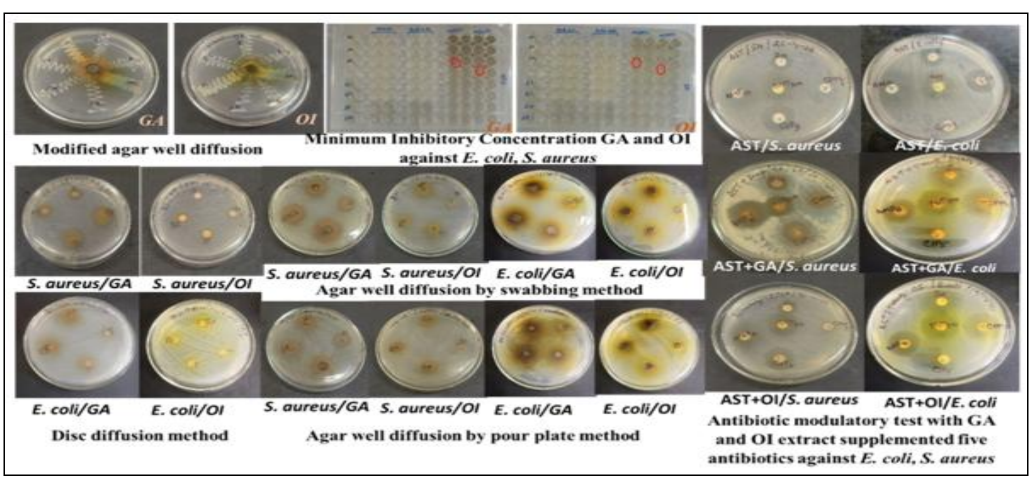


FIG. 7: COMPILED RESULTS OF THE BACTERICIDAL ACTIVITIES OF *G. ARBOREA* AND *O. INDICUM* AGAINST *E. COLI* AND *S. AUREUS*

DISCUSSION: Earlier reports confirmed the presence of phytochemicals like tannins, flavonoids, alkaloids, saponins, etc, in leaves of *O. indicum* [31]. *O. indicum* is rich in various flavonoids that have significant antioxidant, antiviral, anticancer, anti-inflammatory, and antiallergic properties and also play a significant role in cell signaling pathways [32]. The extracts of *O. indicum* (root, stem, bark) are reported to have antimicrobial activity [33]. Mature fruits are sweet, acrid, stomachic, and anthelmintic in nature. The leaf decoction cures cough and bronchitis and heals ulcers, splenomegaly, and rheumatic pain [34]. Leaves are rich in various phytoconstituents like baicalein, scutellarein, flavones, anthraquinones, aloe emodin and glycosides. In contrast, fruits are reported to have chrysin, oroxylin, aloe emodin, and ursolic acid [35, 36]. Significant antibacterial activity against *S. aureus* was also recorded for the stem extracts of *O. indicum* [37].

All the plant parts of *O. indicum* were rich in major flavonoids (chrysin, baicalin, and baicalein) responsible for appreciable antioxidant and antibacterial properties against *β-E. coli*, *S. suis*, *S. intermedius* and *P. aeruginosa* [38]. The alcoholic leaf extracts having alkaloids, terpenoids, flavonoids, glycosides, carbohydrates, and reducing sugars along with phenols, phlobatannins and tannins exhibited antibacterial activity against *B. subtilis* and *P. aeruginosa* [31]. *O. indicum,* either singly or in combination with other herbs, treated different diseases successfully [39]. The hexane extract had a better bactericidal effect than other extracts. It showed equal potency as the standard antibiotic Streptomycin (2mg/mL) against *S. aureus* and *E. coli* [40].

The methanol extract of *G. arborea* leaf showed positive results for the presence of flavonoids, phenolic compounds, triterpenes, saponins, steroids, and alkaloids. The ethyl acetate fraction had a maximum ZOI of 29mm against some test strains compared to other fractions. For the ethyl acetate fraction, MBC was noted to be 12.5mg/mL, and MIC was calculated to be between 6.25 and 12.5mg/mL [41]. The antibacterial potency of mature and ripe, immature and green fruit sap of *G. arborea* was studied against some pathogenic bacteria, i.e., *E. coli*, *Shigella dysentariae*, *P. aeruginosa*, *Salmonella typhi*, *Streptococcus pneumoniae, S. aureus,* and *Bacillus cereus*. A comparable ZOI was observed, similar to the standard antibiotic Ampicillin. Steroids, glycosides, and saponin, the probable phytochemicals responsible for bacterial inhibition, were present in all fruit saps. The MBC values of all fruit saps ranged between 25 – 250 mg/mL while MIC ranged between 12.5 – 50 mg/mL [42].

G. arborea is reported to have tannins, alkaloids, saponins, carbohydrates, cardiac glycosides, and anthraquinones, which were associated with their bioactivities. The leaf and stem bark crude extracts repressed the growth of *E. coli, S. dysentery, S. typhi, Proteus mirabilis,* and *Klebsiella pneumonia.* However, the bactericidal effects of the extracts depended on the test organism and the solvent used for extraction. Tetracycline showed better potency than the extract [10]. Plants enriched with diverse metabolites like alkaloids, flavonoids, terpenoids, tannins, etc., have been reported to have antimicrobial activities [43]. The phenolic antioxidants are primarily used as nutraceuticals to combat various human ailments [44]. The MIC values of *G. arborea* extract ranged between 6.25-12.5 while MBC of 25-50mg/mL in accordance with the findings of El-Mahmoud *et al.,* 2010 against a few members of Enterobacteriaceae [43].

Antibacterial activity of *G. arborea* leaves, fruits, and stem bark extracts was reported earlier [13, 45]. The antibacterial activity of *G. arborea* fruit extracts (hexane and methanol) was explored against different strains of *E. coli, P. aeruginosa, S. typhi, S. pyogenes, S. aureus,* and *Proteus morganis.* The hexane extract had the highest MIC of 100μg, while the methanol extracts had the lowest MIC of 0.001μg, significant bactericidal activity, and ZOI was highly dose-dependent [46]. The crude extract of *G. arborea* leaves displayed the presence of steroids, flavonoids, alkaloids, saponins, tannins, cyanogenic glycosides, carbohydrates, and carbonyl compounds. Saponins, carbohydrates, and carbonyl compounds were found in higher concentrations than other compounds. The crude leave extract presented maximum activity against *E. coli*, *S. aureus*, and *Streptococcus spp,* with ZOI of 20 mm at 1.0 mg/mL and MIC of 0.5 mg/mL and a ZOI of 15 mm and MIC of 0.5 mg/mL was reported against *Salmonella spp* [47].

O. indicum is reported to treat stomach-ache [48]. Flavonoids are prevalent in different parts of *O. indicum* and Baicalin inhibited *β*-hemolytic *E. coli* while some unidentified flavones exhibited significant activity against *P. aeruginosa, S. aureus, S. intermedius,* and *S. suis* [49, 50]. The ethanol, methanol and water extracts of *O. indicum* displayed significant chemopreventive and cytotoxic effect in HeLa cell lines with IC_{50} values of 119, 89.43 and 114.1 µg/mL, respectively while the standard doxorubicin presented an IC_{50} value of 3.895 µg/mL [51]. As per a molecular docking study, the key molecules from this plant, scutellarein 7-rutinoside, scutellarin, and 6-hydroxyluteolin, baicalein and 5,7-Dihydroxy-2-phenyl-6-[3,4,5-trihydroxy – 6 - (hydroxymethyl) oxan-2-yl] oxychromen-4-one effectively targeted the proteins of Epstein-Barr virus (EBV) and reduced the tumor size and other consequences of nasopharyngeal carcinoma patients. The molecular dynamics simulations also showed stable binding between these molecules [52].

The ethanol extract of *G. arborea* leaves was effective against pathogenic human and veterinary strains and methanol extract had potent antimicrobial activity against *Xanthomonas oryzae, Pseudomonas fluorescens* and *Aspergillus flavous* [54]. The methanol and ethyl-acetate extracts of root bark presented effective antibacterial activities against *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 6571 [55]. Nephrotoxicity is a major limitation of adriamycin (ADR) chemotherapy. The combined therapy of GA and fosinopril decreased the raise of serum creatinine, cystatin C, $β_2$-microglobulin blood urea nitrogen, and loss of total protein in urine in nephrotoxic rats. The aqueous extract of GA stem bark exerted a dose-dependent protection against ADR-induced nephrotoxicity in vivo. Hence, it is proposed to be a promising adjunct in ADR chemotherapy [56].

There are multiple reports on the phytochemical screening and antibacterial activities of leaves, roots, and fruits, and also few reports on the bioactivities of *O. indicum* and *G. arborea* stem bark extracts using limited methods of disc diffusion, agar well diffusion, and MIC/MBC determination. Since, both are essential ethnomedicinal plants being extensively used in different ayurvedic medicines either singly or in combination with other herbs, this present study is designed to explore and compare the antibacterial efficacies of the methanolic extracts of *O. indicum* and *G. arborea* stem barks against *E. coli* and *S. aureus* using multiple *in-vitro* antibacterial assays. Plant extracts are also reported to modify the efficacies of certain antibiotics. Hence, the antibiotic modulatory assay was also conducted for both the extracts against both strains. Among the two test strains, *S. aureus* was found to be an antibiotic-resistant strain compared to *E. coli*. In most of our investigations, *S. aureus* was found to be better inhibited by both the extracts, including the antibiotic modulatory assay. *G. arborea* augmented the activity of antibiotics, i.e., AMP10 and NIT300, appreciably against *S. aureus,* whereas *O. indicum* increased the inhibitory activity of CFM5 against *S. aureus* substantially **Table 7**.

In the disc diffusion assay, both extracts greatly inhibited the growth of *S. aureus.* However, *G. arborea* was more effective with a ZOI of 11.00±1.00 and 16.33±0.58 mm in comparison to *O. indicum*, which showed a ZOI of 8.33±0.58 and 14.00±1.00 at a dose of 1000µg/disc against *E. coli* and *S. aureus,* respectively **Table 2**. The agar well diffusion assay results revealed that both extracts had a dose-dependent bactericidal effect on both test strains. *S. aureus* was found to be more sensitive to both the extracts, and GA showed maximum activity with a ZOI of 31.00±1.00 and 25.67±0.58mm in comparison to OI that exhibited 8.67±0.58 and 16.33±0.58 at a dose of 2mg/well against *E. coli* and *S. aureus,* respectively in agar well pour plate method. GA had maximum inhibition against *E. coli* in this assay, which might be due to the better phytoextract diffusion and inhibition in the pour plate method compared to the swabbing method, where bacteria are present on the surface of the plate **Table 3, 4**.

In a modified agar well diffusion assay, multiple strains were streaked on a single plate to judge the efficacy of phytoextracts. Both extracts were effective against all the test strains **Table 5**. Both extracts were equally competent in reducing bacterial growth in the bacterial cell viability assay. Bacterial growth inhibition studies conducted on solid plate media and broth culture media expose the test strains differently since bacterial exposure to bactericidal phytocompounds depends on the solubility and diffusibility of the compounds. However, in broth culture media, the bacterial strains get uniform exposure to bactericidal phytocompounds, which could be the reason for the acquired results. In the qualitative phytochemical screening assay, around nine compounds were detected in *G. arborea* compared to seven compounds in *O. indicum*. The phenolic and flavonoid contents were higher in *O. indicum* than in *G. arborea*. TLC separation detected more phytoconstituents in *G. arborea* than in the *O. indicum* in the Hexane: Ethyl acetate chamber. However, equal number of compounds were present in the Diethyl Ether: Ethyl Acetate chamber created by mixing at a variable ratio.

CONCLUSION: From the findings of this investigation, it could be inferred that *G. arborea* has better inhibitory activity against the test gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*) compared to *O. indicum* stem bark extract. It also has better antibiotic modulatory activity against the MDR *S. aureus* strain. However, further study is needed to confirm the results and decipher the mode of action of any specific phytocompound responsible for the exhibited effect. Further, the mechanism behind the antibiotic synergistic effects of the phytocomponents can be identified, which might be effective in treating bacterial infections while reducing the unwarranted complicacies of post-antibiotic therapies since these alternative therapies are safe and age-tested. The test strains under investigation are known to cause multiple human ailments, and antibiotics often fail when an outbreak or epidemic results from any MDR strain. In such scenarios, herbal phytoproducts like *G. arborea* and *O. indicum* can be used as an alternative to antibiotics or in combination therapy to prevent or treat variable infectious diseases.

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