In vitro Study on the Antioxidant, Antibiotic modulatory and Bactericidal Activity of the Methanolic Extracts of *Asparagus racemosus* Willd. and *Desmodium gangeticum* (L.) DC. Root

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Abstract

There are many roots rich in different phytotherapeutic nutrients that help human beings to fight against different germs or pathogens and to overcome various deadly diseases. This study provides comparative information regarding protective role of the methanolic extract of roots of Asparagus racemosus Willd. and Desmodium gangeticum (L.) DC. against multidrug resistant (MDR) Gram-positive bacteria i.e. Staphylococcus aureus and Gram-negative bacteria i.e. Escherichia coli. Phytochemical analysis revealed that both the plants had major bioactive compounds like alkaloids, flavonoids, terpenoids, phenols, tannins, steroids, glycosides etc. A. racemosus had a total phenol content (TPC) of 41.91±7.716mg/g of Gallic Acid Equivalent (GAE) and a total flavonoid content (TFC) of 159±24.832mg/g of Rutin Equivalent (RE), while D. gangeticum was calculated to have a TPC of 48.58±1.80 mg/g of GAE and TFC of 141.47±3.96mg/g of RE. Though both roots exhibited excellent antibacterial activities against both E. coli and S. aureus strains, A. racemosus inhibited the growth of S. aureus better in comparison to E. coli by augmenting the bactericidal activity of conventional antibiotics like ampicillin and nitrofurantoin against S. aureus while D. gangeticum augmented the activities of ciprofloxacin, ampicillin and nitrofurantoin against E. coli.

Hence, it may be concluded that both extracts can be used either singly or in combination therapy with synthetic antibiotics to combat bacterial infections and infectious diseases. However, further study can confirm the mode of action of certain phytocompounds against bacterial pathogenesis and can help better in targeted alternative drug therapy.

Keywords: Phytochemical analysis, Antioxidant, Antibiotic modulatory, Antibacterial, Bactericidal, *A. racemosus, D. gangeticum, Escherichia coli, Staphylococcus aureus*.

Introduction

Worldwide, plants and plant products are always proclaimed for their great health benefits. In India, plants are always considered as the treasure of ayurvedic medicines and the knowledge of herbal medicines is mentioned in ancient literature used by people for curing various diseases. We can find the description and properties of medicinal plants in *Rig Veda, Charaka Samhita* and *Sushruta Samhita*[17]. According to World Health Organisation, 80% people utilise traditional medicines for better health and to avoid side effects of allopathy medicines[3].

Asparagus racemosus Willd. (Family: Asparagaceae), commonly known as Wild Asparagus in English and Shatavari in Sanskrit, which means “the plant with hundred roots” or "a lady possesses hundred husbands or acceptable to many" is an excellent tonic and is especially known to improve female reproductive health. It is referred as “Queen of herbs" in Ayurveda[1]. Local people used *A. racemosus* as vegetables and medicines for its gentle flavour and health benefits. *A. racemosus* is well-branched, barbed undershrub with short, tuberous roots[10]. According to ancient literature, this plant and its root have been used since pre-Vedic times as antiseptic, bitter- sweet, cooling, nervine tonic, stomachic, emollient, carminative, rejuvenating, constipating, diuretic, aphrodisiac and galactogogue. It is also effective in diarrhoea, dysentery, hyperacidity, dyspepsia, hyperdipsia, cough, bronchitis, inflammations, nervous disorders, neuropathy, hepatopathy, tumors and certain infectious diseases[37].

Fleshy roots of *A. racemosus* are rich storehouse of many vital phytocompounds ladened with potent health beneficial efficacies. The medicinal uses are stated in the British and Indian Pharmacopoeias and also in Ayurveda, Unani and Siddha for treating diseases like neurodegenerative disorders, menopause issues, lactation failure, diarrhoea etc.[35] *A. racemosus* is rich in sapogenins and saponins. The steroidal saponins such as shatavarin VI-X along with other saponin-like shatavarin I (asparoside B), schidigerasaponin D5 (asparanin A), shatavarin IV (asparinin B), shatavarin V, immunoside etc. were reported from the roots[16].

Quercetin and glycosides of quercetin (quercetin-3-O-rutinoside or rutin and quercetin 3-O-galactoside or hyperoside) were also isolated from the roots[25]. An alkaloid, asparagamine A was isolated from the ethanol extract of the roots[33]. Isoflavone 8-methoxy-5,6,4′-trihydroxyisoflavone-7-O-β-D-glucopyranoside[32] and 9,10-Dihydro-1,5-dimethoxy-8-methyl-2,7-phenanthrenediol[33] were reported from the roots of this plant.

Desmodium gangeticum (L.) DC. (Family: Fabaceae) usually known as Shalaparni (Shal leafed bush) is a vital species of the Desmodium genus. It is extensively used in Ayurveda, Unani and Siddha medicine either singly or along with some other herbs in Indian sub-continent. It is a bitter tonic, digestive, anticatarrhal, antiemitic, febrifuge and is used extensively for chest inflammation or other inflammatory or 'vata' disorders. The roots are used as expectorant and also as an antivenom in scorpion sting and snake bites[11,27]. *D. gangeticum* extract and its active principles like gangetin, desmodin and hordenine have great potentiality as antioxidant, anti-diabetic, anti-amnesic, anti-inflammatory, immunomodulator, hepatoprotective and cardio-protective agent[45].

In Indian sub-continent, this shrub is conventionally used as an astringent (cures diarrhoea, dysentery and irritable bowel syndrome), diuretic, laxative, anthelmintic, antipyretic, anticatarrhal and nervine tonic, however in China, it is often used to treat pain, fever, cough, dyspnea and neutralize toxins. Pterocarpans such as desmocarpin, desmodin, gangetin, gangetinin and gangetial were found to be present in roots[43]. Steroids like stigmastrol, β –sitosterol, 24-methylcholesta-5-en-3β –ol, 24-ethylcholesta-5,22-dien-3β-ol, lupeol and its acetate and volatile oils like 1-heptadecanol, 1-tritriacontanol, trans-5-hexadecenoic acid, aliphatic β-lactone were also detected[39].

Root of *D. gangeticum* is a major ingredient of some Ayurvedic preparations like *Dashmoolarishta, Chyavanaprasam* and *Agasthyarasayanam*, usually given to treat fever, colic pain and respiratory diseases. The polyherbal preparations of *Dasamula* and *Laghu panchamula* are used in asthma, rheumatism, pain, hysteria, renal and cardiac problems[22,23]. '*Dashmoolarishta'* and *'Dashmoolakwaath'* are suggested for postpartum care and to circumvent post-delivery complications[20]. Though there are many reports on the ethnopharmacological uses, *D. gangeticum* had negligible *in vitro*/*in vivo* scientific investigations or clinical trials.

As per earlier reports, it may be beneficial in gout, bronchitis and hypertension[44]. Though there are extensive ethnomedicinal uses and ethnopharmacological applications are available for these two mystic herbs, yet scientific reports on the antibacterial activity on the roots of *D. gangeticum* are negligible.

Though there are some reports on the antibacterial efficacies of *A. racemosus* root, yet only limited antibacterial assays like disc diffusion or agar well diffusion were conducted in those previous reports.

Hence, the present study is under taken to validate these two natural wonders and to explore their antibacterial potency against Gram positive i.e. *Staphylococcus aureus* and Gram negative i.e. *Escherichia coli* strain using multiple assay models in both solid agar and liquid broth culture media along with an antibiotic supplementary/modulatory assay to recommend these products in alternative/combinatorial therapy.

Material and Methods

Plant material collection and extract preparation: Fresh roots of *A. racemosus* (AR) and *D. gangeticum* (DG) were collected from Sakhigopal, Puri and Berhampur University campus, Odisha respectively in the month of May-June, 2022. The plants and plant parts were taxonomically identified and authenticated by taxonomist Prof. M.K. Misra and Prof. S.K. Dash, Berhampur and a voucher specimen was submitted to the Herbarium of Department of Botany, Berhampur University bearing reference numbers BOTABU2301 and BOTABU2302.

The shade dried root was ground into fine powder and subjected to Soxhlet extraction in methanol (300mL) at 60-70^{0}C for 72 hours. The filtered extract was concentrated in a rotary evaporator and left at 37^{0}C till complete drying. The crude extract yield percentage was calculated by using the yield calculation formula[2,41]. The extract was preserved at 4^{0}C for future use. These crude extracts were dissolved in lukewarm distilled water to prepare the required concentrations depending on the type of study (phytochemical or antibacterial assay).

Thin layer chromatography: The Thin layer chromatography (TLC) was conducted by following the standard method with some modifications[8]. The chromatograms were developed by placing the air-dried sample loaded TLC plate in different TLC glass chambers with different solvent mixtures like hexane: ethyl acetate or diethyl ether: ethyl acetate at a ratio of 8:2, 7:3 and 6:4. respectively. Mobile phase was allowed to move through absorbent phase up to 1cm under the top margin of the plate. After air drying, the TLC plates were developed in saturated iodin chamber. Rf values were calculated for each isolated compound[26].

Qualitative phytochemical screening: The crude methanolic extracts of *A. racemosus* and *D. gangeticum* root were investigated for the presence of various bioactive compounds using the standard procedures for qualitative phytochemical assay[15,38,40].

Quantitative phytochemical screening: The standard protocols of Folin-Ciocalteu method[36]. and the $AlCl_3$ assay[29] respectively, were used to plot the calibration curves to quantify the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of both the root extracts.

DPPH antioxidant assay: The antioxidant potential of the root extracts was deciphered by analysing the free radical scavenging method in DPPH assay[9]. Fresh solution of DPPH was prepared by dissolving 2mM DPPH in methanol. Varied concentrations (20-500µl) of methanolic root extract (1mg/ml) were added to absolute methanol to make up the

volume up to 3mL. 1mL of methanolic solution of DPPH was added to it and incubated for 30 min at room temperature. Then the absorbance was measured at 517 nm. Ascorbic acid was used as standard antioxidant. Triplicate sets were prepared for each concentration. Blank and control were included too. DPPH free radicals scavenging or inhibition % was calculated by applying the following formula:

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

The antioxidant activity was expressed as IC_{50} (the concentration of an antioxidant required to reduce the initial concentration of DPPH by 50%) values.

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* (ATCC 25922) and *P. aeruginosa* (MTCC 3541) 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 at 4°C and 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 96-well microtiter plate according to CLSI guidelines (National Committee for Clinical Laboratory Standards)[46,47] with some modifications[5,41].

Antibacterial assay: The susceptibility of different strains of *E. coli* and *S. aureus* to crude root extracts was tested through different antibacterial methods such as disc diffusion[4], agar well diffusion (swab, pour plate), modified agar well diffusion method[12] and bacterial cell viability assay[13].

Disc diffusion method: Discs of approximately 6mm diameter were cut from the Whatmann no.1 filter paper. These discs were loaded with 250µg, 500µg, 750µg, 1000µg concentrations of methanolic extracts of the aforesaid plant parts. These were air-dried and then placed aseptically on nutrient agar plates swabbed with either *E. coli/S. aureus* which were activated for one hour. The plates were incubated overnight at 37^0 C and were checked for zone of inhibitions (ZOIs) in terms of millimetre.

Agar well diffusion assay by swabbing method: A single bacterial colony from the master culture plate was suspended in 5mL of sterilized nutrient broth. The test tube was shaken well for proper mixing and incubated at 37°C for 1hr activation. The freshly activated bacterial strains *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 37^0 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 extract 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: In order to find sensitivity of different bacteria (clinical isolates of *E. faecalis, E. coli, P. vulgaris, P. aeruginosa, S. aureus* and standard strains of *E. coli* and *P. aeruginosa*), total 7 strains were tested on a single plate in this method[12]. On nutrient agar plate, a well was made in the center and loaded with 1mg of crude root extract of *A. racemosus/D. gangeticum*. All seven bacterial strains were streaked from periphery towards center in a zigzag manner on the nutrient agar plate. Then the plates were left for one hour at room temperature for drug diffusion and then left overnight in the incubator at 37^0C. The distance between the well and the bacterial growth starting point was measured to get a comparative inhibitory result.

Antibiotic Modulating Potential: The standard antibiotic discs of AMP 10- Ampicillin 10µg, NIT 300-Nitrofurantoin 300µg, CIP 5-Ciprofloxacin 5µg, CFM 5-Cefixime 5µg and S10-Streptomycin 10µg were aseptically placed on *E. coli/S. aureus* swabbed nutrient agar plates, incubated overnight and the inhibitory zone was measured in millimetres to determine the bacterial susceptibility. These ZOIs were used as references for comparing with another set of plates containing antibiotics supplemented with crude root extracts at a dose of 500µg/disc[41]. The variations in the inhibition zones indicated how much the methanolic extracts enhanced or augmented the antibiotic discs' inhibitory capacity.

Bacterial cell viability test by spread plate method in control and extract treated bacteria: The bacterial viability in untreated and extract-treated media was assessed by counting the colonies following the standard lab protocol[41].

Statistical analysis: Triplicate experimental sets were prepared for all assays. The results were expressed as mean ±standard deviation (SD) of three observations, depending

on the study. IC_{50} value was calculated by linear regression analysis in the antioxidant assay. Values and graphs were prepared using Microsoft Excel 2021 software.

Results

The roots were collected from different localities and processed; however, the detailed steps of crude extract preparation are summarized in figure 1. Total 38.23gm of AR and 49.28gm of DG dried root powders were collected from which 2.20gm and 2.50gm of crude extracts were prepared after evaporating methanol. The final dark brown semi solid crude extract was stored at 4°C in a dried condition. Yield percentage of crude extracts was calculated to be 5.75% and 5.07% for AR and DG respectively.

Thin layer chromatography: Thin layer chromatography gives an insight into the number of probable polar as well as nonpolar phytocompounds present in plant extracts. Hexane: ethyl acetate chambers and diethyl ether: ethyl acetate chambers at ratios of 8:2, 7:3, 6:4 detected phytocompounds with different Rf values depending on the polarity and solubility of the compounds. In diethyl ether: ethyl acetate mobile phase, up to 10 compounds (Rf value 0.10 – 0.98) were detected in AR, while 05 compounds (Rf value 0.21 – 0.98) were detected for DG and 06 compounds (Rf value 0.32 – 0.96) in AR and 07 compounds (Rf value 0.08 – 0.98) in DG were detected in hexane: ethyl acetate mobile phase.

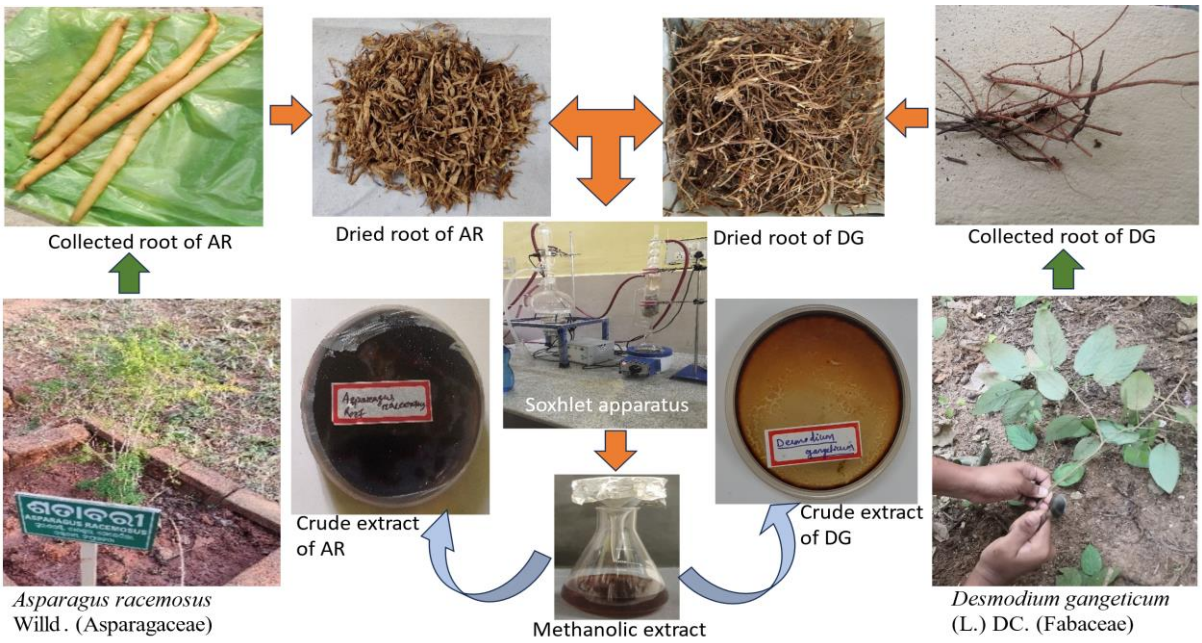

Figure 1: Preparation of methanolic crude extract of *Asparagus racemosus* (AR) and *Desmodium gangeticum* (DG) root.

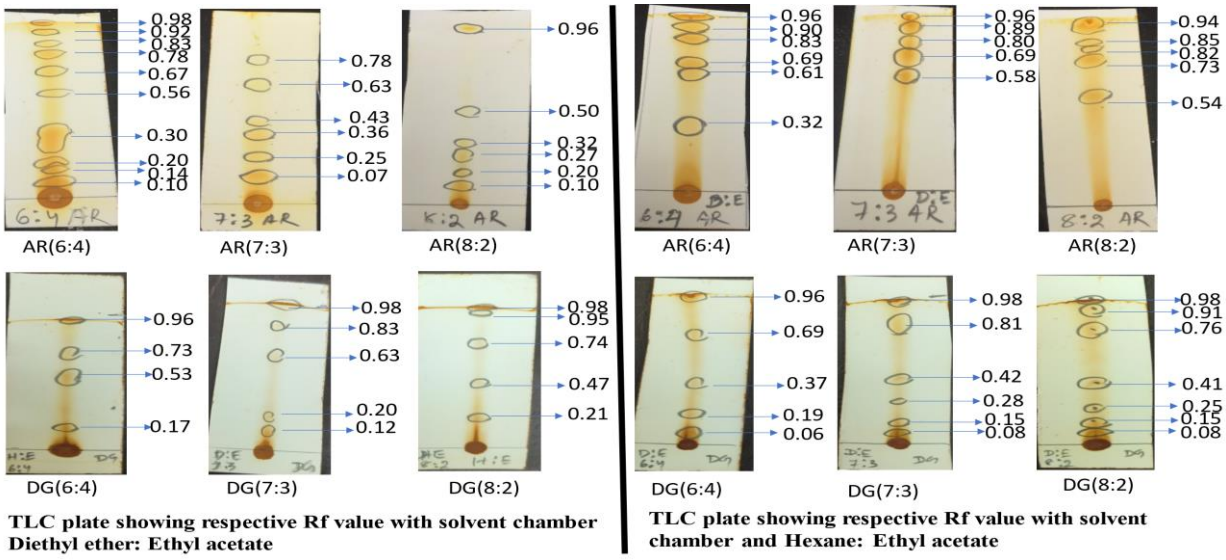

Figure 2: Thin layer chromatography profile of methanolic crude extract of *Asparagus racemosus* (AR) and *Desmodium gangeticum* (DG) root in Diethyl Ether:Ethyle Acetate and Hexane : Ethyle Acetate variable solvent mixture mobile phase

The results inferred that the extracts have a greater number of polar compounds in comparison to nonpolar compounds. The images of TLC analysis are presented in figure 2.

Qualitative phytochemical Analysis: The methanolic root extracts of both plants were screened for the presence of biologically active phytochemicals. The extract of AR showed thirteen compounds except coumarin and DG showed ten active compounds out of fourteen phytochemical tests conducted using standard protocol. Methanolic extract of both the roots had nine different major bioactive compound like alkaloids, flavonoids, terpenoids, phenol, tannin, phlobatannin, steroids, protein, glycosides etc. in common. However, the presence of anthocyanin, leuco-anthocyanin, reducing sugar and saponins was only detected in AR. The results are presented in table 1.

Table 1
Phytochemical screening of bioactive compounds present in the methanolic extract of roots of *Asparagus racemosus* and *Desmodium gangeticum*

Analysed phytochemicals	**Type of tests**	**Name of plants**	
		Asparagus racemosus	***Desmodium gangeticum***
Alkaloid	Mayer's test Wagner's test	+	+
Terpenoid	Salkowski test	+	+
Phenol	Lead acetate test Potassium dichromate test	+	+
Tannin	$FeCl_3$ test	+	+
Reducing sugar	Benedict's test Fehling's Test Molisch Test	+	-
Saponin	Foam test Na_2CO_3 test	+	-
Protein	Biuret test Ninhydrin test Conc. HNO_3 test	+	+
Steroid	Salkowski test	+	+
Anthocyanin	Ammonia test	+	-
Coumarin	NaOH test	-	+
Leuco anthocyanins	Isoamyl alcohol test	+	-
Glycosides	Glacial acetic acid test Liebermann's test	+	+
Flavonoids	Shinoda's test Lead acetate test	+	+
Phlobatannin	Precipitate test	+	+

Note; '+' sign for presence and '-' for absence of phytochemicals

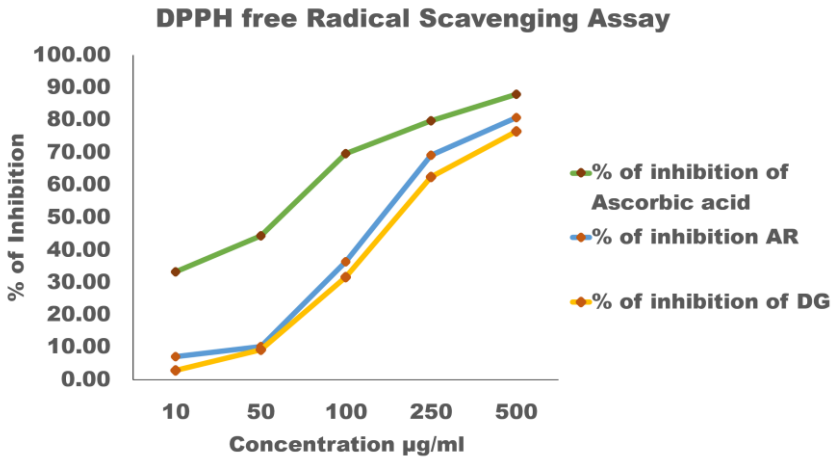

Figure 3: Graphical presentation of in vitro antioxidant activity of methanolic crude extracts of *Asparagus racemosus* (AR) and *Desmodium gangeticum* (DG) root in DPPH free radical scavenging assay

Quantitative phytochemical analysis: The total phenol content (TPC) of 41.91±7.716mg/g of Gallic Acid Equivalent (GAE) and a total flavonoid content (TFC) of 159±24.832mg/g of Rutin Equivalent (RE) were found in *A. racemosus* root extract. TPC value of 48.58±0.80 mg/g of GAE and TFC of 141.47±3.96mg/g of RE was calculated to be present in *D. gangeticum* root extract.

DPPH free radical antioxidant assay: The antioxidant activity or percentage of inhibition of DPPH free radical at the concentrations of 20 to 500 µg/ml by standard ascorbic acid, methanolic extract of the roots of *A. racemosus* and *D. gangeticum* were observed as 33.33 to 87.88%, 7.11 to 80.82% and 3.02 to 76.51% respectively. The standard ascorbic acid showed maximum scavenging activity and minimum IC_{50} value of 2.098±0.2 µg/ml followed by the IC_{50} value of 3.447±0.15 µg/ml for the methanolic extracts of AR while DG showed lesser activity with an IC_{50} value of 3.669±0.10 µg/ml. Yet, the difference between the antioxidant properties of both the extracts is not significant. It can be inferred that both the extracts have comparable antioxidant activities as that of the standard. The comparative analysis is plotted using MS excel software and is presented in figure 3.

Antibacterial assays

Minimum inhibitory concentration (MIC): Minimum inhibitory concentration (MIC) was determined as 0.62µg/ml for standard antibiotic ciprofloxacin against both *E. coli* and *S. aureus* while MIC for both AR and DG was determined as 2.5 mg/ml for *E. coli* and 1.25mg/ml for *S. aureus*.

Disc diffusion method: Discs with different concentrations of methanolic root extracts were applied against both strains to find their antibacterial effects measured as zone of inhibition (ZOI) and expressed in millimetres (mm). The results were depicted in table 2. Both the plant extracts were found to have dose dependant effect against both the test strains. *S. aureus* was found to be more sensitive to both the extracts and AR showed more activity with a ZOI of 11.00±1.00 and 13.00±1.00mm at a dose of 1000 µg /disc in comparison to DG that exhibited 8.33±0.58 and 9.33±0.58 against *E. coli* and *S. aureus* respectively.

Agar well diffusion by swabbing method: The comparative inhibitory effects of the root extracts against the test strains were verified in agar well diffusion by swabbing method and ZOI was expressed in mm. The results are presented in table 3. Both the plant extracts exhibited dose dependant effect against both the test strains. *S. aureus* was found to be more sensitive to both the extracts and AR showed more activity with a ZOI of 13.67±1.15 and 22.33±1.00mm at a dose of 2mg/well in comparison to DG that showed 12.33±0.58 and 14.33±0.58 at the highest test dose against *E. coli* and *S. aureus* respectively.

Agar well diffusion by pour plate method: The comparative inhibitory effects of the root extracts against the test strains were also verified in agar well diffusion by pour plate method and ZOI was expressed in mm. The results are presented in table 4. Both the plant extracts were found to have dose dependant effect against both the test strains. *S. aureus* was found to be more sensitive to both the extracts and AR showed more activity with a ZOI of 9.67±0.58 and 21.00±1.00mm in comparison to DG that exhibited 6.67±0.58 and 7.00±1.00 at a dose of 2mg/well against *E. coli* and *S. aureus* respectively.

Table 2

***In vitro* antibacterial activity of methanolic crude extracts of *Asparagus racemosus* and *Desmodium gangeticum* root against *E. coli* and *S. aureus* through disc diffusion method**

Extracts	Strains	ZOI measured in mm at different conc. of root extracts in µg /disc			
		250	500	750	1000
A. racemosus	*E. coli*	7.67±0.58	8.0±1.00	9.33±0.58	11.00±1.00
	S. aureus	5.67±0.58	7.33±0.58	9.33±0.58	13.00±1.00
D. gangeticum	*E. coli*	6.00±1.00	6.33±0.58	7.33±0.58	8.33±0.58
	S. aureus	6.67±1.15	7.67±1.15	8.33±0.58	9.33±0.58

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

Table 3

Bacterial growth inhibitory activity of *Asparagus racemosus* and *Desmodium gangeticum* root against *E. coli* and *S. aureus* through agar well swabbing method

Extracts	Strains	ZOI measured in mm at different conc. of root extracts in mg /well			
		0.5mg	1.0mg	1.5mg	2.0mg
A. racemosus	*E. coli*	10.67±0.58	12.00±1.00	13.00±1.00	13.67±1.15
	S. aureus	11.33±0.58	13.67±0.58	19.00±1.00	22.33±1.00
D. gangeticum	*E. coli*	6.33±0.58	10.00±1.00	11.00±1.00	12.33±0.58
	S. aureus	6.33±0.58	10.33±0.58	12.67±0.58	14.33±0.58

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

In contrast to the results of disc diffusion and agar well swabbing method, *E coli* was greatly suppressed by AR root in comparison to *S. aureus.* At lower concentration i.e. 0.5mg and 1.0mg of DG, there was no inhibition found in pour plate method but at higher doses of 1.5 and 2mg/well concentration, ZOI of 6.33±0.58 and 6.67±0.58mm and 6.67±0.58 7.00±1.00mm was observed against *E. coli* and *S. aureus* respectively which is comparatively less in comparison to the growth inhibitory effects of AR root extract.

Modified agar well diffusion method: The inhibitory effects of the root extracts (1mg/well) were studied against different clinical isolates and standard strains of *E. coli, S. aureus, P. aeruginosa, P. vulgaris* and *E. faecalis* on a single plate using modified agar well diffusion method to find the comparative susceptibility level of different pathogens to the root extracts. The ZOIs were measured in mm and the results are presented in table 5. AR had better inhibitory activity against all the test strains in comparison to DG root extract especially against *E. coil, P. aeruginosa* and *S. aureus* bacterial strains.

Bacterial cell viability test by spread plate method: The colony forming units per mL after 4 hours or log phase was determined in three conditions: untreated (control), low (100µg/ml) and high (600µg/ml) dose of different root extracts treated bacteria through spread plate method. The cfu/ml was calculated by multiplying the bacterial colonies with their dilution factor and then compared. The % inhibition of different extracts was found to be dose dependent. In concurrence to our earlier bactericidal tests on solid agar media, AR root extract had better growth inhibitory activity against both the test strains i.e. 62.72% and 84.91% against *E. coli* and *S. aureus* respectively. However, DG root extract inhibited *E. coli* up to 50.48% in comparison to a growth inhibition of 41.67% against *S. aureus.* The results are presented in table 6 and figure 4.

Table 4
Bacterial growth inhibitory activity of *Asparagus racemosus* and *Desmodium gangeticum* root against *E. coli* and *S. aureus* through agar well pour plate method

Extracts	Strains	ZOI measured in mm at different conc. of root extracts in mg /well			
		0.5mg	1.0mg	1.5mg	2.0mg
A. racemosus	*E. coli*	6.33±0.58	7.00±1.00	8.67±0.58	9.67±0.58
	S. aureus	10.67±0.58	15.00±1.00	16.67±0.58	21.00±1.00
D. gangeticum	*E. coli*	0	0	6.33±0.58	6.67±0.58
	S. aureus	0	0	6.67±0.58	7.00±1.00

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

Table 5
Bactericidal activity of *Asparagus racemosus* and *Desmodium gangeticum* root extracts against different bacterial strains through modified agar well method

Bacterial Strains	AR (1 mg/well) ZOI in mm	DG (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 (MTCC 3541)	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

Table 6
Bacterial cell viability test of methanolic crude extracts of *Asparagus racemosus* and *Desmodium gangeticum* root against *E. coli* and *S. aureus* by spread plate method

Extracts	Strains	Colony forming units/mL at log phase				
		Control	Extract treated 100µg/ml	% inhibition	Extract treated 600µg/ml	% inhibition
A. recemosus	*E. coli*	79.4×10^7	53.6×10^7	32.49	29.6×10^7	62.72
	S. aureus	85.5×10^7	40.9×10^7	52.16	12.9×10^7	84.91
D. gangeticum	*E. coli*	82.4×10^7	58.3×10^7	29.24	40.8×10^7	50.48
	S. aureus	83.5×10^7	75.6×10^7	9.46	48.7×10^7	41.67

Note: Values represent the average of triplicate sets of experiments

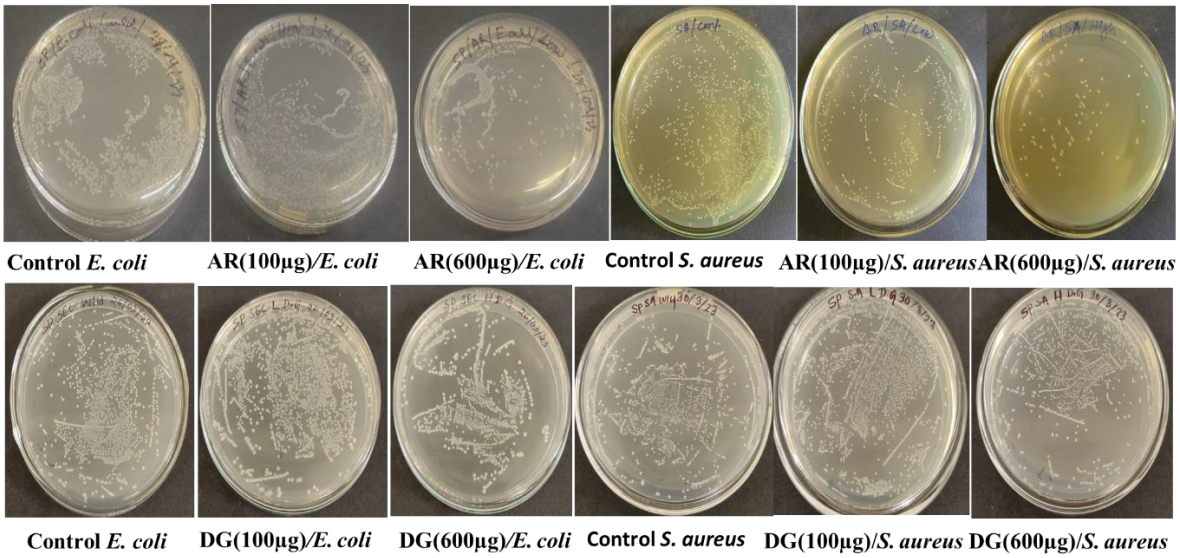

Figure 4: Plates of *in vitro* antibacterial study of methanolic extract of *Asparagus racemosus* and *Desmodium gangeticum* root treated at 100 µg/ml (low) and 600µg/ml (high) dose against *E. coli* and *S. aureus* by spread plate method.

Table 7
In vitro* antibiotic modulating activity of crude extracts of *Asparagus racemosus* and *Desmodium gangeticum* root against *E. coli* and *S. aureus

Extracts	Strains	Difference of Zone of inhibition (in mm) (ZD) by addition of root extract (500µg /disc) to different antibiotics				
		S10	CFM5	AMP10	CIP5	NIT300
AR	*E. coli*	0.33±0.58	0.67±0.58	1.00±1.73	0.33±1.53	0.33±0.58
	S. aureus	0.67±0.58	0.67±0.58	3.00±1.73	0.67±0.58	4.67±0.58
DG	*E. coli*	1.33±0.58	0.33±0.58	1.67±0.58	3.00±1.00	4.00±1.00
	S. aureus	0.67±0.58	0.33±0.58	4.00±1.00	0.33±0.58	1.33±0.58

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

Antibiotic modulatory effect: The *in vitro* antibiotic sensitivity of the bacterial strains against different conventional antibiotics was tested and their zone of inhibitions were measured in millimetres. Five most and least effective antibiotics were selected to study their modulatory effects in presence of the root extracts of AR and DG. The increase or decrease in ZOI of antibiotic discs like ampicillin 10 (AMP 10), nitrofurantoin 300 (NIT 300), ciprofloxacin 5 (CIP 5), cefixime 5 (CFM 5), streptomycin 10 (S 10), against each strain was calculated by taking the difference between antibiotic added with 500µg/disc of root extract and antibiotic alone. The antibiotic modulatory effects of the root extracts were studied by finding the synergistic, indifferent or antagonistic effects and the results are presented in table 7.

By supplementing AR extract to NIT300 disc, the inhibition zone was increased by 4.67±0.58 mm against *S. aureus* and similarly the inhibition zone was increased by 4.00±1.00 mm against *E. coli* with supplementing DG extract to NIT300. Combination of AR/DG with AMP10 significantly increased activity by 3.00±1.73 mm and 4.00±1.00 against *S. aureus* respectively. From the results, it is evident that the different phytochemicals present in AR and DG modulate differentially the action of different antibiotics against the test strains.

From the experimental outcomes, it is evident that root extracts of *A. racemosus* and *D. gangeticum* had significant bactericidal and antibiotic modulatory activity against the test Gram positive and Gram-negative strain. For an easy understanding and comparative analysis, all the graphical result presentations are compiled and presented in figure 5 and 6 for AR and DG root extracts respectively. Figure 7 presents the overall compilation of all the culture plates of different assays conducted during the entire study.

Discussion

A. racemosus is a well-known medicinal plant widely used to treat different ailments such as anxiety, dysmenorrhea, leucorrhoea, angina, hyperlipidemia, hypertension, urinary tract infections and benign prostatic hyperplasia. It contains mainly steroidal saponins along with different secondary metabolites like alkaloids, steroids, flavonoids, furan compounds, derivatives of dihydrophenanthrene and essential oils that contribute to its varied bioactivities[14]. The presence of primary as well as secondary metabolites like

steroids, alkaloids, saponins, carbohydrates, flavonoids, amino acids except tannins was confirmed.

High performance thin layer chromatography (HPTLC) revealed the presence of seven polyvalent phytochemical compounds with variable Rf values and concentrations. These compounds possibly contributed for the significant antibacterial activity against the pyogenic bacteria i.e. *E. coli, P. aeruginosa, Streptococcus pneumoniae, Streptococcus pyogenes, S. aureus and Staphylococcus epidermidis*[18]. A crude methanol extract of *A. racemosus* root at a dose of 150 µg/ml inhibited growth of *E. coli, Salmonella typhi, Salmonella typhimurium, Vibrio cholerae, Shigella flexneri, Shigella dysenteriae, Shigella sonnei, Pseudomonas putida, S. aureus* and *Bacillus subtilis*[24]. The chloroform and ethanolic root extracts exhibited predominant bactericidal activity against *P. aeruginosa, E. coli, V. cholerae, S. dysenteriae, S. flexneri, B. subtilis, S. aureus and Micrococcus luteus*[37].

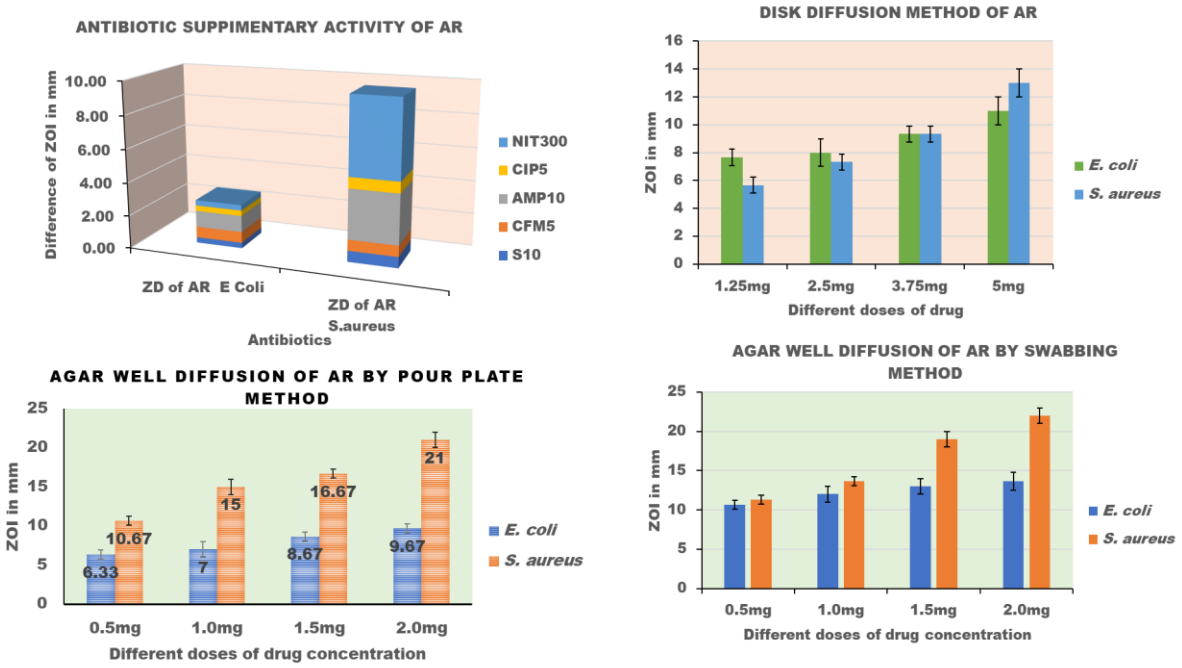

Figure 5: Comparative graphical presentation of the antibiotic modulatory and bactericidal efficacies of *Asparagus racemosus* root extract against *E. coli* and *S. aureus*

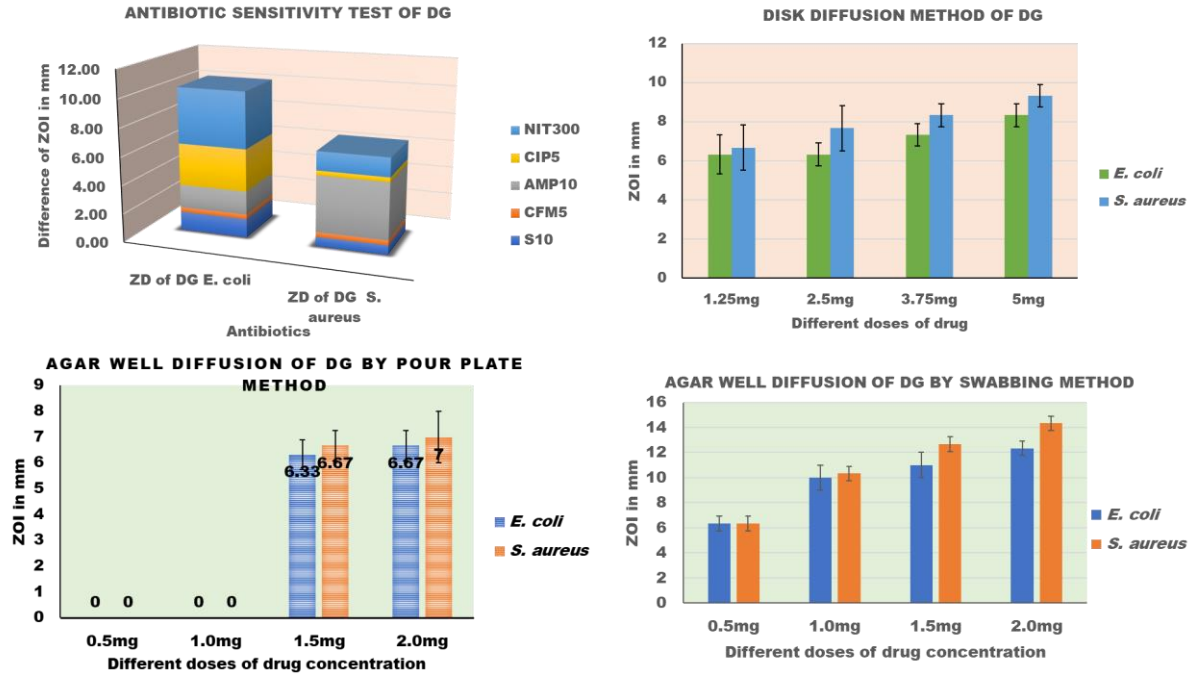

Figure 6: Comparative graphical presentation of the antibiotic modulatory and bactericidal efficacies of *Desmodium gangeticum* root extract against *E. coli* and *S. aureus*

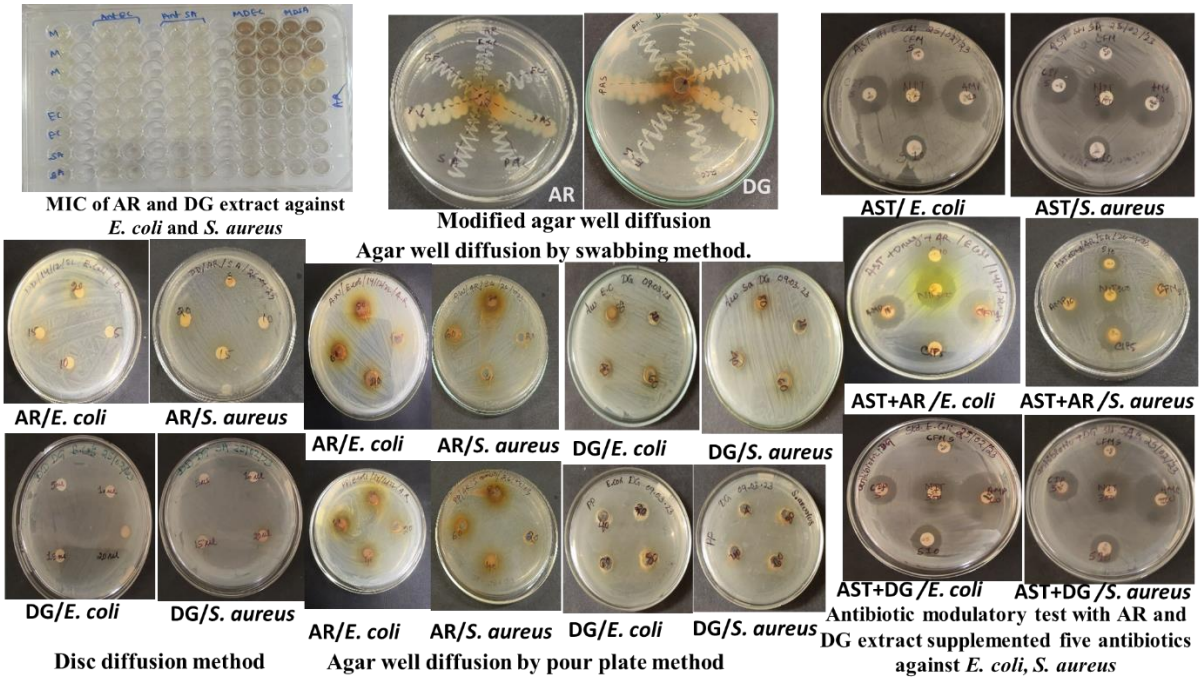

Figure 7: Compiled images of the plates of all antibacterial assays of *Asparagus racemosus* and *Desmodium gangeticum* root extracts against *E. coli* and *S. aureus*

Ethanol and acetone extract of *A. racemosus* root inhibited growth of *E. coli, S. typhi, S. aureus, B. subtilis* and *Klebsiella pneumonia* in an agar well diffusion assay[34]. The methanolic plant extracts of both *A. racemosus* and *W. somnifera* showed effective antibacterial activity against uropathogens such as *E. coli, Klebsiella pneumoniae, P. aeruginosa, Proteus mirabilis* and *S. aureus* in a disc diffusion assay[7]. In contrast to these reports, the ethanolic extract of *A. racemosus* at 100, 200 and 300 µg did not show any antibacterial activity in comparison to the standard drug ciprofloxacin at 30 µg in a disc diffusion assay[19]. This could be due to the insufficient concentration of the antimicrobials to exhibit any significant bacterial growth inhibition.

The different solvents (petroleum ether, methanol, chloroform, acetone, ethyl acetate and water) extracts of *A. racemosus* root at concentration of 25, 50 and 100µg/ml had comparable antibacterial activity to streptomycin at 5µg/ml against Gram positive (*B. subtilis*, *S. aureus*) and Gram negative (*E coli, Pseudomonas)* bacteria as well as potential antifungal activity against *Candida utilis* equivalent to standard drug fluconozole (5µg/ml)[28]. Root extract of *A. racemosus* exhibited significant antibacterial activity at higher concentrations including 10000 µg/ml and 1000 µg/ml against *S. aureus*. The maximum antibacterial activity at 100 µg/ml was assessed for *S. aureus* (14.5±0.707)[42].

The minimum inhibitory concentration (MIC) of the DG extract was found to be 10 mg/ml which exhibited inhibitory effects against *E. coli*. The results of agar disc diffusion assay showed that the DG extract was effective in inhibiting the growth of *E. coli*, suggesting its potential as an antimicrobial agent[31]. Out of the different solvents (methanol, ethanol, chloroform and aqueous) extracts of *D. gangeticum* plant against various bacterial pathogens i.e. *K. pneumoniae, E. coli, S. typhi, P. aeruginosa* and *Streptococcus mutants*, methanolic extract presented maximum zone of inhibition (ZOI) of 24±2.3mm against *S. mutants* and minimum ZOI of (7±0.08) was observed with aqueous extract against *P. aeruginosa*[21].

In this present investigation, two widely used ethnomedicinal roots of *Asparagus racemosus* and *Desmodium gangeticum* were chosen to investigate their extensive bactericidal and antibiotic modulatory activity. Both the roots had a low yield of 5.75% and 5.07% respectively in methanol extraction. Both the extracts had MIC of 2.5mg/ml and 1.25mg/ml against *E. coli* and *S. aureus,* while standard antibiotic ciprofloxacin had a MIC of 0.62µg/ml against both strains. In disc diffusion, agar well diffusion, modified agar well diffusion and the bacterial cell viability assays, we had a common observation i.e. AR had better activity than DG and *S. aureus* is better inhibited by both the root extracts in comparison to *E. coli*. It may be possible that the Gram-negative bacteria *E. coli* had a complex cell wall, which might be prohibiting the entry of bactericidal phytochemicals into it.

In disc diffusion assay, at a dose of 1mg/disc both AR and DG inhibited *S. aureus* greatly presenting a zone of inhibition (ZOI) up to 13.00±1.00mm and 9.33±0.58mm and *E. coli* up to 11.00±1.00mm and 8.33±0.58mm (Table 2). In agar well diffusion assay, swabbing method was more effective than the pour plate method and AR and DG inhibited *S. aureus* up to 22.33±1.00mm and 14.33±0.58mm and *E. coli* up to 13.67±1.15mm and 12.33±0.58mm at a dose of 2mg/well in swabbing method (Table 3). The low concentration doses of DG in pour plate method did not

show any effect. However higher doses of 1.5 and 2mg/well presented antibacterial effect. AR root extract at a dose of 2mg/well had a ZOI of 21.00±1.00mm against *S. aureus* in pour plate method (Table 4). Both extracts inhibited the bacteria in a dose-dependent manner.

Seven different standard and clinical isolate strains of *E. coli, E. faecalis, P. vulgaris, P. aeruginosa* and *S. aureus* were streaked on a plate with one centrally placed well carrying phytoextract to understand the effect of phytochemicals on the growth of different bacteria on same plate and in this current study, AR was found to be more effective than DG against *E. coli, P. aeruginosa* and *S. aureus* while DG inhibited the growth of *E. faecalis, P. vulgaris, P. aeruginosa* and *S. aureus* effectively (Table 5). In the bacterial cell viability assay, in broth culture media, AR inhibited the cell viability of *S. aureus* up to 84.91% and *E. coli* up to 62.72% at a dose of 600µg/ml (Table 6).

In the antibiotic modulatory assay, AR augmented the activity of different antibiotics by presenting a difference in zone of inhibition (ZD) of 3.00±1.73mm with AMP10 and 4.67±0.58mm with NIT300 against *S. aureus*. DG root extract supplemented the activity of CIP5 with 3.00±1.00mm, NIT300 with 4.00±1.00mm and AMP10 with a ZD of 1.67±0.58mm against *E. coli* (Table 7). All these varied outcomes proved the differential activity of different phytochemicals present in both the extracts in inhibiting bacterial growth differentially. Further, this is also confirmed that both Gram positive and Gram-negative bacteria respond to the phytochemicals differently.

The superior bactericidal potential of AR root extract was due to the number of antimicrobial phytochemicals present in them as evident from its TLC profile, which showed a maximum of ten constituents in diethyl ether : ethyl acetate mobile phase at a ratio of 6:4 in comparison to maximum seven constituents observed in DG extract in hexane : ethyl acetate mobile phase at a ratio of 8:2. Out of the fourteen phytochemical tests conducted, AR showed presence of almost all the phytochemicals except coumarins. AR also revealed presence of anthocyanins, leuco-anthocyanins, saponins and reducing sugars which were absent in DG root extract (Table 1). Chloroform and ethanolic extract of AR showed the presence of carbohydrates, steroids, tannins, alkaloids and phenolic compounds[37].

In the current study, methanolic extract of AR root showed the presence of alkaloid, flavonoid, terpenoid, phenol and tannin, reducing sugar, saponin, protein, steroid, anthocyanin, leuco anthocyanins, glycosides and phlobatanin. The superior bactericidal activity of AR root in comparison to DG root is possible due to the presence of more number of effective antimicrobial phytocompounds present in AR root.

Ethanol fraction of *A. racemosus* roots had significant effect against Gram negative bacteria. It exhibited excellent free radical scavenging activity in DPPH assay at par with standard ascorbic acid. The TLC profile showed four major spots with the Rf values 0.20, 0.22, 0.24 and 0.58. Total phenol and flavonoid content were reported to be 165.22mg/GAE and 188.3 mg/g quercetin equivalent respectively[30]. The methanolic root extract had a yield of 29.2%. The TPC and TFC were determined as 12.90 ± 0.002 mg/g of GAE and 0.80±0.001 mg/g of RE respectively with a total antioxidant activity value of 132.53±0.12 mg of ascorbic acid equivalents per gram[6].

In this present study, TPC and TFC of 41.91±7.716mg/g of GAE and 159±24.832mg/g of RE were noted in *A. racemosus* root extract while 48.58±0.80 mg/g of GAE and of 141.47±3.96mg/g of RE were found to be present in *D. gangeticum* root extract. In DPPH free radical scavenging assay, the IC_{50} values were determined as 2.098±0.2, 3.447±0.15 and 3.669±0.10µg/ml in standard ascorbic acid, *A. racemosus* and *D. gangeticum* root extract respectively.

These findings support the bactericidal and antibiotic modulatory results that the roots of *A. racemosus* had better activity than the roots of *D. gangeticum*. The presence or absence of different phytochemicals and the amount of total phenols or flavonoids or the level of antioxidant activity in the roots of AR or DG was reported earlier. The finding presented in this study can vary, which might be due to the differences in season, location and other biological or physiological parameters. However, all the assays presented significant bactericidal activities for both the extracts.

Conclusion

From the findings of this investigation, it could be inferred that *Asparagus racemosus* has better bactericidal and growth inhibitory activity against the test Gram positive (*S. aureus*) and negative bacteria (*E. coli*) in comparison to *Desmodium gangeticum* root extract. It has also better antibiotic modulatory activity against the MDR *S. aureus* strain. The mechanism behind the antibiotic synergistic effects of the phytocomponents can be identified which will be beneficial.

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