INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY EDUCATIONAL RESEARCH
ISSN:2277-7881; IMPACT FACTOR :6.514(2021); IC VALUE:5.16; ISI VALUE:2.286
Peer Reviewed and Refereed Journal: VOLUME:10, ISSUE:4(6), April:2021
Online Copy Available: www.ijmer.in
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CLIMATE BASED COMPARATIVE STUDY OF ANTIBACTERIAL ACTIVITY OF SELECTED EXTRACTS OF PHYLLANTHUS AMARUS SCHUM. & THONN

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Abstract

The present study was done to compare the climate based antimicrobial activity of Phyllanthus amarus Schum. & Thonn. collected from two different locations of India i.e., Jaipur, Rajasthan and Salem, Tamil Nadu. The chloroform, acetone and ethanol solvents were used and tested against five bacterial pathogens: S. aureus, E. coli, S. griseus, B. subtilis and P. aeruginosa by performing the agar-well diffusion method and minimum inhibitory concentration. Both of the plant parts (root, stem and leaf) have shown significant activity against all bacterial strains in different extracts. North plant is more potent against S. aureus, E. coli and S. griseus when used in chloroform solvent. Acetone extracts of both plants have shown good results against most bacterias. Ethanolic extracts are known to be best against microbes but here stem part of the South plant was not effective against E. coli, S. griseus and B. subtilis. It has been observed that the North plant is more potent against bacterial infections as compared to the South plant.

Keywords: Phyllanthus Amarus; Bacteria; Agar-Well Diffusion; Minimum Inhibitory Concentration.

Introduction

Preventing the disappearance of the cultural practices of the people and provide a simple and locally accessible therapeutic alternative can be managed by promoting scientific research into traditional medicine. And this is the definition of Ayurveda in the true sense, which acknowledges the richest medicinal values of herbal plants and connects the environment to the people. Plants are the complex chemical storehouse of biodynamic compounds that serve as plant defense mechanisms against invasion by microorganisms, and to battle this challenge, the plant provides valuable resources of natural antimicrobial agents [1, 2].

In India, Phyllanthus amarus is widely distributed as a weed in cultivated and wastelands [3]. Phyllanthus amarus Schum and Thonn. herb is a traditional medicine for more than 3000 years [4]. It belongs to a family of Euphorbiaceae and commonly known by the name of carry me seed, stone breaker, gala of wind, etc. The major class of bioactive compounds like alkaloids, flavonoids, lignans, sterols, tannins, triterpenes and volatile oils enhance its antimicrobial effect on micro-organisms. Its uses are gaining momentum because of several biological activities against urine infection [5], hepatitis [6], cancer [7, 8], diabetes [9, 10, 11], gastrointestinal disorders [12], etc. The whole plant extract is also used in urinary problems, liver disease, dyspepsia, anorexia, constipation and dysentery [13, 14]. It plays an important role in the development of green medicines which are safer to use and more dependable than costly synthetic drugs with no adverse effects.

The present study is, therefore, mainly focused on the antimicrobial effect on various micro-organisms by the Phyllanthus amarus extracts collected from different locations which have distinguished climate as no research has been done in the comparative field. The impact of distinct habitats of the plant can be seen by the changes in its bioactive compounds' concentration at different parts of the plant through its antimicrobial activity.

Significance Statement

The present study is mainly focused on the comparative study of Phyllanthus amarus Schum. & Thonn. collected from different locations of selected solvents (chloroform, acetone and ethanol) against selected gram-positive and gram-negative bacteria.

Material and Methods

Material Collection

The well-grown plants of Phyllanthus amarus were collected from two different locations to compare the climate impact on the plant's bioactivity. From North India, mature plants were collected from Jaipur, Rajasthan whereas the other sample of the same species was collected from South India i.e., Salem, Tamil Nadu. Rajasthan is one of the hottest states of India and its climate is usually hot and dry whereas Tamil Nadu is hot but humidity prevails there all the seasons.

Microorganisms Used

The extracts namely Chloroform, Acetone and Ethanol were used for the determination of the antibacterial activity of all parts of Phyllanthus amarus i.e., root, stem and leaves tested selected pathogens. Five bacterial strains were selected for the antimicrobial screening. Clinical laboratory isolates of bacteria were procured from the Microbiology Laboratory, SMS Medical College, Jaipur. For the present study, pure bacterial strains were taken as mentioned in Table 1.

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Table 1: Selected bacterial strains for the present study and the standard zone of inhibition for each bacteria are as follows:

S.No	Bacterial Strains	Type	MTCC No.	Standard (in mm)
1	Staphylococcus aureus	Gram-positive	MTCC3160	23.06
2	Escherichia coli	Gram-negative	MTCC1652	19.45
3	Streptomyces griseus	Gram-positive	MTCC4734	22.08
4	Bacillus subtilis	Gram-positive	MTCC441	22.77
5	Pseudomonas aeruginosa	Gram-negative	MTCC741	23.05

Culture and Maintenance of Micro-organisms

Pure cultures of the above-mentioned bacterial strains were used as indicator organisms that were grown in nutrient agar (NA) medium. Each bacterial culture was maintained on the medium for 48 h of sub-culturing usually incubated at 37 ^{o}C and stored at 4 ^{o}C for future experiments. A fresh suspension of test organisms in saline solution was prepared from a freshly grown agar slant before every antibacterial assay.

Preparation of Extract

In vivo parts of Phyllanthus amarus i.e., roots, stems and leaves collected from either location were washed with tap water and finally with distilled water, then allowed to shade dry. Dried parts were then milled to a coarse powder. Sequential extracts i.e., chloroform, acetone and ethanol were obtained by macerating 100 g of dried powder of different samples in respective solvents and kept on a rotary shaker for 24 h, separately. Each of the extracts was filtered, centrifuged at 5000 rpm for 15 min, dried under reduced pressure and stored at 4 ^{o}C in sterile airtight bottles.

Microbiological Screening

The antimicrobial activity was performed with the agar well diffusion method [15, 16] and minimum inhibitory concentration (MIC) [17].

Determination of Antibacterial Assay

In vitro antibacterial activity of the crude extracts was studied against gram-positive and gram-negative bacterial strains by the agar well diffusion method [15, 16]. Nutrient Agar No. 2 (Hi-Media, India) was used as the bacteriological medium. The extracts were diluted in 100% dimethylsulphoxide (DMSO) at the concentrations of 5 mg/ml. The Nutrient agar was melted and cooled to 48-50 ^{o}C and a standardized inoculum (1.5×108 CFU/ml, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petridishes to give a solid plate. Wells (6 mm and 1 cm apart) were prepared in the agar plates by using a sterile cork borer. The test compound (40 µl) and standard antibiotic (60 µl) was introduced in the well. The plates were incubated overnight at 37 ^{o}C. The antimicrobial spectrum of the extract was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotic ciproflaxin. For each bacterial strain, controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the diameter of the inhibition zone was measured with antibiotic zone reader to nearest mm and activity index was also calculated. The experiment was performed in triplicate to minimize the error and the mean values are presented.

Determination of Minimum Inhibitory concentration

The lowest concentration, which can inhibit any visible bacterial growth on the culture plates, is called minimum inhibitory concentration (MIC) [18]. The tube dilution method and agar dilution method are the most commonly employed methods [19]. A lower MIC value refers that less drug is required for inhibiting the growth of the organism; therefore, drugs having lower MIC scores are more effective antimicrobial agents. MIC scores confirm the resistance of microbes to antibiotics and monitor the activity of new antibiotics.

MIC is performed by a serial dilution technique of the extracts representing different concentrations. The 100, 50, 25 and 12.5 µg/ml was taken from selected extracts, namely chloroform, acetone and ethanol. Serial dilutions of the extracts with nutrient agar broth for bacterial culture were used with their respective inocula and incubated for 24 h at 37 ^{o}C. MIC is calculated with the least visible growth (on the binocular microscope) on the lowest concentration indicating 99.5% killing of the used inoculums on plates. MIC is considered 'gold standard' for determining the proneness of organisms to antibiotics, are therefore used to judge the performance of other methods used [18].

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Results and Discussion

The extracts chloroform, acetone and ethanol of the in vivo root, stem and leaves of Phyllanthus amarus showed inhibitory effects against bacterial strains. By using Agar well diffusion method and micro-dilution method, anti-bacterial activity was determined.

Evaluation of Antibacterial activity

Comparing zone of inhibition and antibacterial potential of the selected plant from the North and South locations with the standards activity i.e, ciproflaxin against various bacterial pathogens, effective results were observed. All the extracts were potent against all the bacteria studied. Among all three extracts, ethanol showed a high level of inhibition, followed by acetone and chloroform. The zone of inhibition of all three extracts of root, stem and leaves were compiled in the form of figures to make a clear vision of comparison.

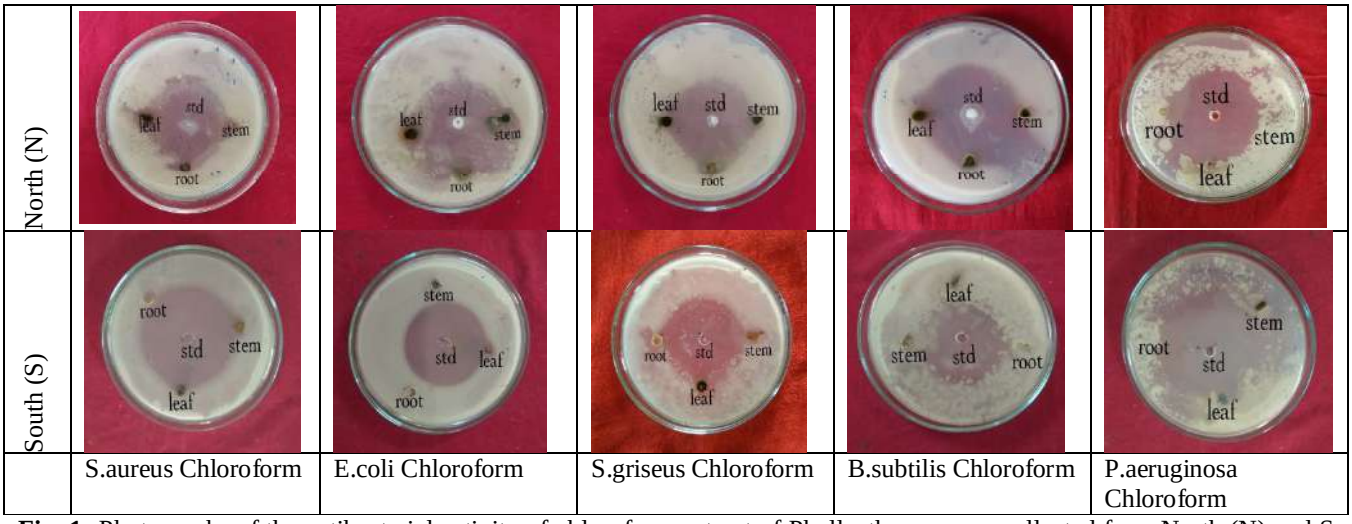

Fig. 1: Photographs of the antibacterial activity of chloroform extract of Phyllanthus amarus collected from North (N) and South (S) locations against selected bacterial strains (std – Standard).

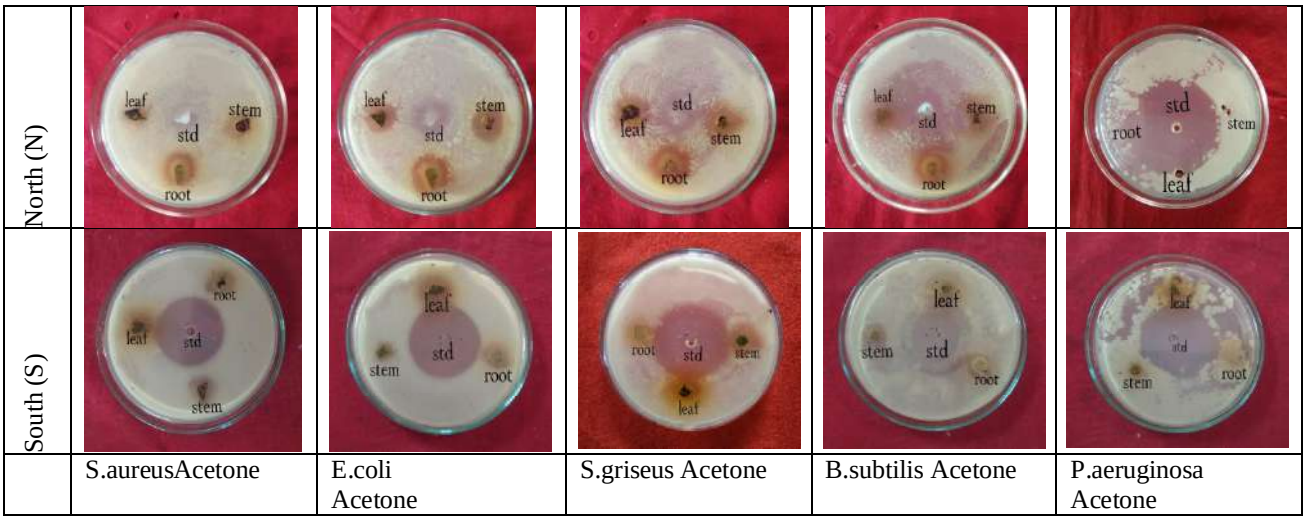

Fig. 2: Photographs of the antibacterial activity of acetone extract of Phyllanthus amarus collected from North (N) and South (S) locations against selected bacterial strains (std – Standard).

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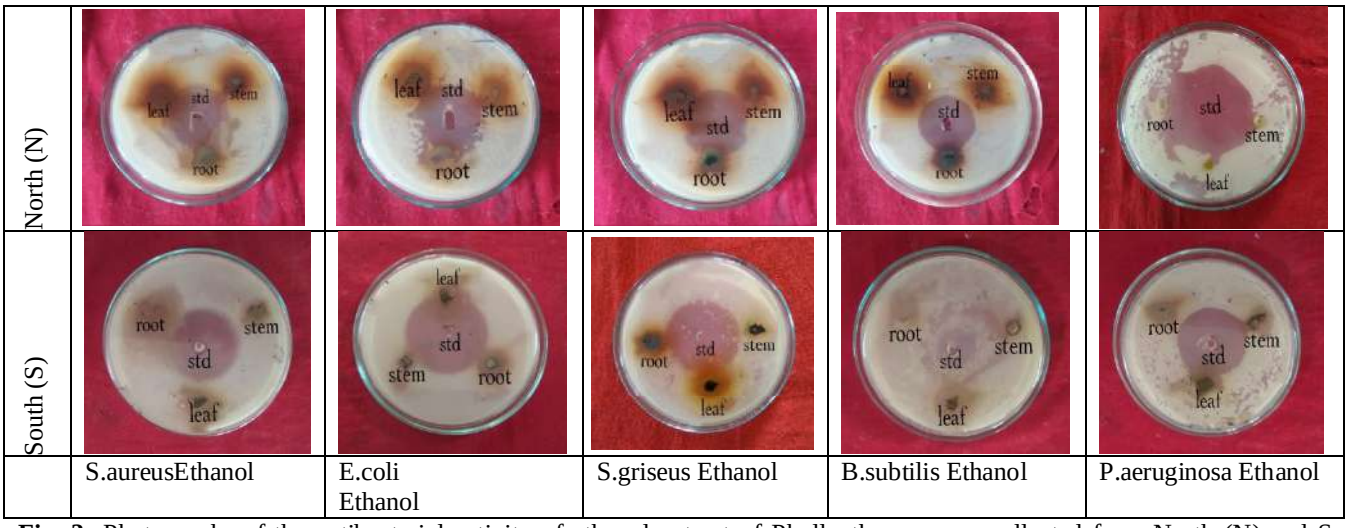

Fig. 3: Photographs of the antibacterial activity of ethanol extract of Phyllanthus amarus collected from North (N) and South (S) locations against selected bacterial strains (std – Standard).

Antibacterial Essay

Using agar-well diffusion method, the antibacterial potentiality of different extracts of different parts of the plant was measured determining the zone of inhibition (in mm) and thereby calculating its activity index. Below are the results calculated in the form of the Activity Index presented in the form of graphs. Each extract has been explained in brief to visually differentiate the potential of root, stem and leaves of North and South plants. The visible zone of inhibition was quantitatively assessed based on inhibitory activity on strains along with MIC and their Activity Index was also calculated with the help of the given formula.

IZ (Inhibition zone) = in mm (Includes diameter of disc-6mm)
AI (Activity Index) = IZ of test sample/IZ of standard (as in Table 1)
***(**Values are mean of triplicate readings)
Standard: Ciproflaxin (60 µl/disc)

Chloroform Extract

As presented in Fig. 4, the Chloroformextracts of all three parts of the North plant have shown proper activity against S. aureus, E. coli and S. griseus and no effect against B. subtilis and P. aeruginosa. Only leaves of the North plant were effective against B. subtilis (0.66 AI). All parts of the South plant have shown inhibition zone against S. griseus only and near to nil effect against B. subtilis and P. aeruginosa. The maximum inhibition zone can be seen by the stem extract of the North plant against E. coli (1.03 AI) followed by stem extract of the South plant against S. griseus (0.94 AI).

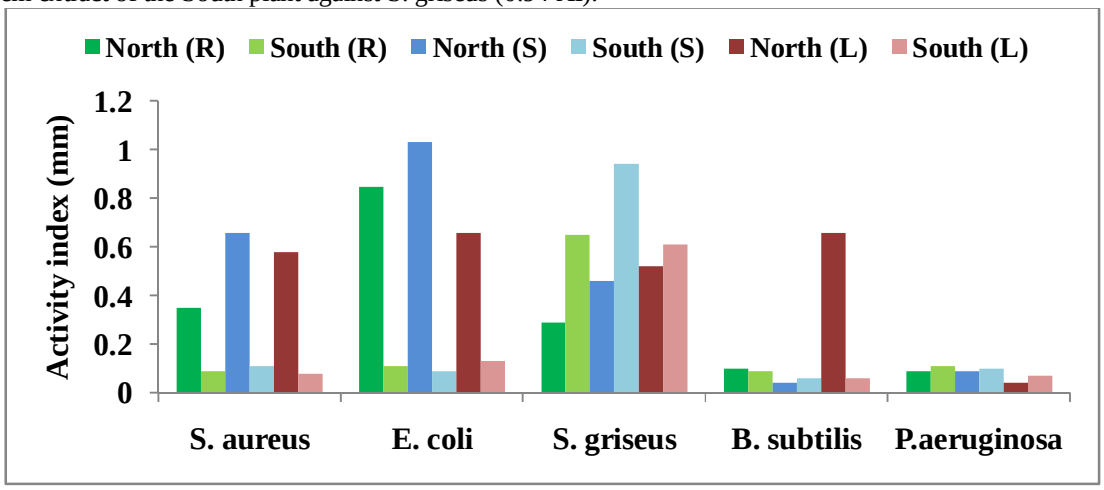

Fig. 4: Graphical representation of the comparative study of Chloroformextracts of R: roots, S: stems and L: leaves of plants collected from North (N) and South (S) locations.

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Acetone Extract

By analyzing the results (Fig. 5), acetone extracts seem to be more effective than chloroform and ethanol extracts especially for stem. Either it is North or South plant's root, stem or leaves, all are least effective against P. aeruginosa. The highest peak can be seen by the leaf extract of the South plant against S. griseus (1.08 AI) followed by leaf extract of the South plant against E. coli. Besides these, acetone extracts have shown good results against remaining bacterial strains.

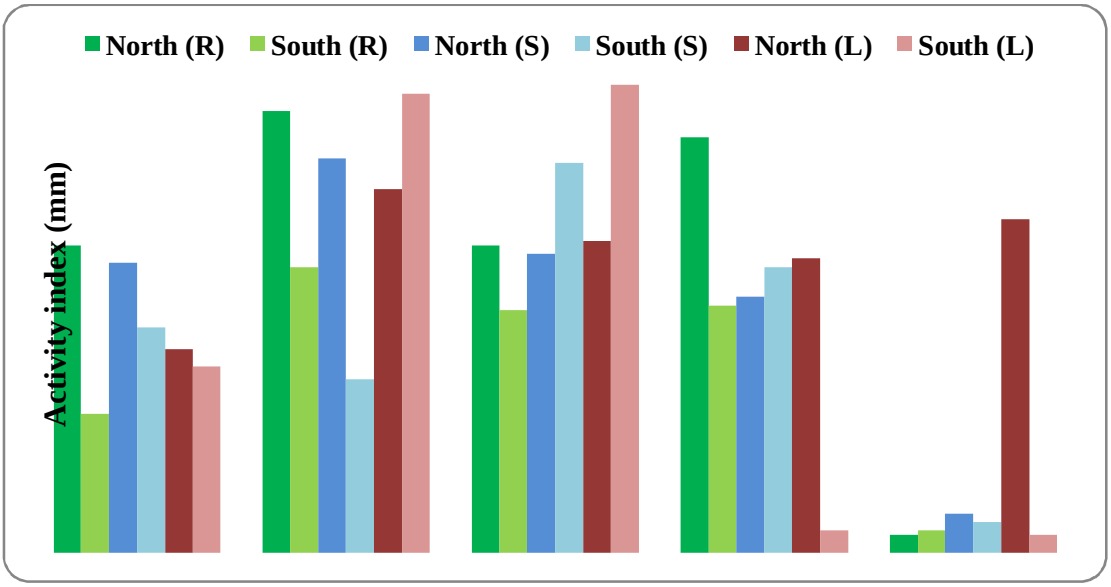

Fig. 5: Graphical representation of the comparative study of Acetoneextracts of R: roots, S: stems and L: leaves of plants collected from North (N) and South (S) locations.

Ethanol Extract

Reviewing Fig. 6, differential extracts effect on microbes, ethanol extracts are known to be more effective. Ethanol extracts of roots and leaves of both locations are implying their maximum antibacterial potentiality showing higher peaks. But ethanol extract of the South plant's stem is not as effective as that of the North except S. aureus. Like other extracts, ethanol extract is also non-effective against P. aeruginosa except the root and leaves of the North plant, which has shown considerably good potential. The highest peak is shown by ethanol extract of the North plant leaf against E. coli (1.13 AI).

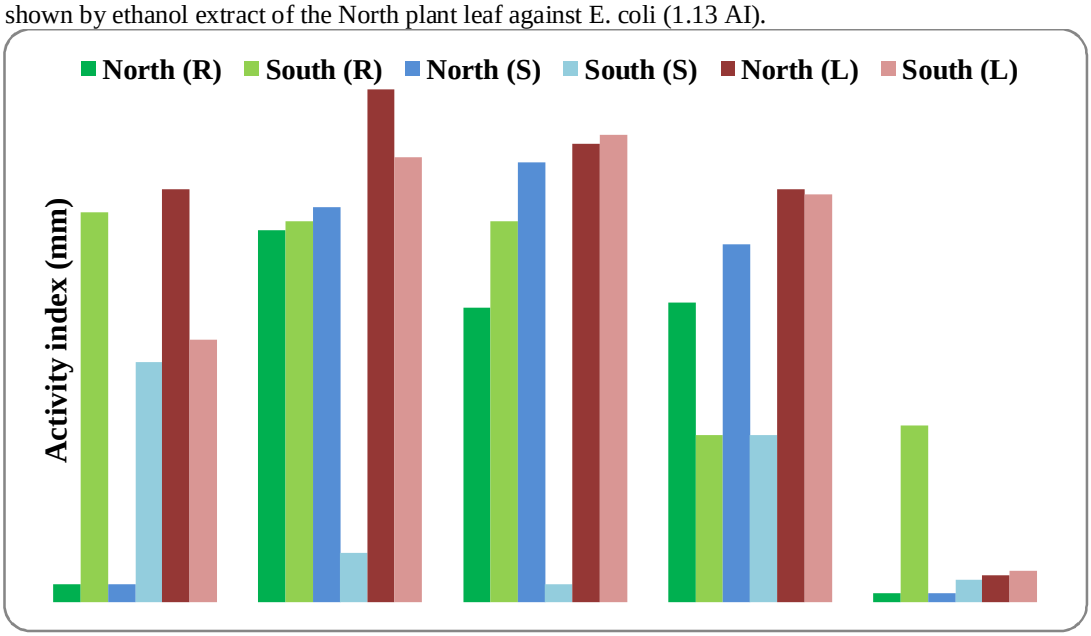

Fig. 6: Graphical representation of the comparative study of Ethanolextracts of R: roots, S: stems and L: leaves of plants collected from North (N) and South (S) locations.

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Minimum Inhibitory Concentration (MIC)

It has significant importance in diagnostic laboratories as it confirms the resistibility of the microbe against the antimicrobial solution. The highest concentration tested against bacteria was 100 µg/ml followed by 50 µg/ml and the least selected was 12.5 µg/ml.

In Table 2, all extracts of P. amarus from North have shown least MIC and greater antibacterial potential. The least MIC value 12.5 µg/ml was shown by chloroform extract of the leaf against S. aureus, ethanol extract of root and stem against E. coli, root and acetone extract of leaf and ethanol extract of the stem against S. griseus. Chloroform and ethanol extract of the leaf also showed the least MIC against B. subtilis. Most of the extracts showed high concentration resistance (100 µg/ml) against selected bacterias. Only a few showed resistivity of 50 µg/ml as shown in the Table 2.

Table 2: MIC (µg/ml) of selected extracts (Chloroform, Acetone and Ethanol) of Phyllanthus amarus parts (R: roots, S: stems and L: leaves) from North (N) and South (S) locations.

Bacteria		Chloroform			Acetone			Ethanol		
		R	S	L	R	S	L	R	S	L
S. aureus	N	100	100	12.5	100	100	100	100	100	100
	S	100	100	100	100	12.5	25	12.5	100	12.5
E.coli	N	100	100	25	100	50	100	12.5	12.5	100
	S	100	100	100	12.5	100	25	12.5	100	12.5
S. griseus	N	100	100	25	12.5	100	12.5	100	12.5	25
	S	50	50	50	25	12.5	12.5	100	100	12.5
B. subtilis	N	100	100	12.5	100	25	50	100	100	12.5
	S	100	100	100	12.5	12.5	100	100	100	50
P. aeruginosa	N	100	100	100	100	100	100	100	100	100
	S	100	100	100	100	100	100	100	100	100

In Table 2, observing P. amarus from south, most of the least MICs are shown by acetone and ethanol, which shows these extracts are more effective against, selected pathogens. The least MIC value tested i.e., 12.5 µg/ml was shown by acetone extract of stem & leaf and ethanol extract of root & leaf against S. aureus. Acetone extract of root and ethanol extract of root & leaf showed minimum MIC against E. coli. Acetone extract of stem and leaf and Ethanol extract of the leaf also showed its effectivity against S. griseus at 12.5 µg/ml concentrations. Against P. aeruginosa only acetone extract of root and stem showed 12.5 µg/ml MIC. The rest mostly showed minimum effectivity by 100 µg/ml antibiotic extracts.

Nature possesses a unique source of high phytochemical diversity, most of them are having potent medicinal properties [20]. This leads to much attention of researchers to extract compounds that can be prior to synthetic ones. The use of antimicrobial compounds can be widely used in therapeutic treatments for many purposes due to their efficiency against bacteria, fungi and viruses [21, 22]. Therefore, the pharmaceutical is finding its way leaning towards medicinal plants.

In the present study, Phyllanthus amarus collected from the North and South locations of India were evaluated through different extracts performing against Gram-positive and Gram-negative bacteria. Every part of the plant showed its unique capability on selected pathogens. By studying Fig. 4-6, a proper comparison can be determined and how climate can induce the same plant differently.

This investigation showed that plant parts of the North plant are more potent against selected bacteria as it is collected from the arid area of Rajasthan. It has to cope up with the harsh weather along with dry soil, therefore in comparison to the South plant, more secondary metabolites are present in it. The South plant too showed similar results in some extracts but stem and leaf part of chloroform and ethanolic extracts against S. aureus & E. coli were low as compared to the North plant. The MIC values suggest that the plant extracts were bacteriostatic at a lower concentration, but are bactericidal at higher concentrations [23, 24].

Conclusion

The present study mainly focused on the antibacterial potentiality of the different parts (i.e., roots, stems & leaves) of the Phyllanthus amarus plant obtained from the North and South part of India. Hereby, the study shows that the compounds present are of great use in the green pharmacy industry. The selected bacteria's infections can be cured by the plant extract effectively. Most of the

accumulation of antibacterial compounds could be found out in the roots, especially in the roots of the North plant which grows in an arid climate. The chloroform, acetone and ethanol extracts show varied results, consequently, ethanol extract of all parts is most effective against tested bacteria. It supports the generation of new antibacterial drugs for the wellbeing of society.

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Number of Words:	3,458 (approx.)
Number of Characters:	19,713 (approx.)