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## CLIMATE BASED COMPARATIVE STUDY OF ANTI-FUNGAL 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 fungal pathogens: *T. reesei*, *A. niger*, *P. funiculosus*, *C. albicans* and *F. oxysporum* by performing the agar-well diffusion method and minimum inhibitory concentration. Among selected extracts, ethanol showed better potentiality against fungi, followed by acetone. Chloroform extract of the South plant was ineffective against all the selected fungi and the only root of the North plant showed effectivity against *T. reesei* and *P. funiculosus*. Acetone extracts were the least effective against *A. niger*, *C. albicans* and *F. oxysporum*. Ethanol extracts were the least effective against *C. albicans* and *F. oxysporum*. It has been observed that both North and South plant's parts are effective on some and ineffective on some of the selected fungi.

**Keywords:** *Phyllanthus amarus*; Fungi; Agar-well diffusion; Minimum inhibitory concentration (MIC); Chloroform; Acetone; Ethanol.

### INTRODUCTION

The definition of Ayurveda is preventing the disappearance of the cultural practices of humans and provide them much simple and locally accessible medicines that can be managed by encouraging scientific research into folk medicines. Ayurveda acknowledges the best medicinal values of herbal plants and connects nature to human beings. Plants provide valuable natural antimicrobial compounds to battle against external microbes as plants are the complex storehouse of biodynamic compounds that form the primary defense line [1,2].

Preventing the disappearance of the people's cultural practices and providing 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 micro-organisms. To battle this challenge, the plant provides valuable resources of natural antimicrobial agents [1,2].

*Phyllanthus amarus* is widely grown in India as a weed in cultivated and wastelands [3]. Commonly known by the name of stone breaker, the gala of wind, carry me seed, etc, belongs to Euphorbiaceae family. It has a significant class of bioactive compounds like flavonoids, alkaloids [4], lignans [5], sterols, triterpenes, tannins and phyllanthusin D [6], volatile oils which enhance the plant's antimicrobial effect on microbes [7]. *P. amarus* is a traditional medicine from above 3000 years [8].

It is used in several diseases such as hepatitis [9], urine infection [10], diabetes [11,12,13], gastrointestinal disorders [14], cancer [15] etc. Urinary problems, jaundice, dyspepsia, anorexia, constipation, and dysentery are cured by whole plant extract [16,17]. Therefore, green medicines are safer and more dependable than highly-priced synthetic drugs with no harmful effects.

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 is commonly known by the name of carry me seed, stone breaker, the gala of wind, etc. The primary 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 a vital role in developing green medicines that are safer to use and more dependable than costly synthetic drugs with no adverse effects.

The present investigation focuses on the climate-based anti-fungal effect on selected fungi by the selected *Phyllanthus amarus* extracts collected from the North and South parts of India. Minimal research has been done on their comparison. Climate changes impact biochemicals quantity in all parts of the plant, which helps see the varying results in their antimicrobial activity.



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

### Significance Statement

Comparative study of *Phyllanthus amarus* Schum. & Thonn. is studied to know the impact of climate and habitat on the same plant from different locations through agar well diffusion method on selected solvents (chloroform, acetone and ethanol).

## MATERIAL AND METHODS

### Material Collection

The mature plants of *Phyllanthus amarus* were collected from North and South India to compare the climate effect on plant's bioactivity. Sample from North India was collected from Jaipur, Rajasthan, whereas the other sample of the same plant was picked from Salem, Tamil Nadu of South India. The reason for choosing these locations was that weather change in Rajasthan prevails every few months. During summers, the temperature rises high, while in Tamil Nadu, the weather is constant throughout the year, along with humidity. These weather changes show promising results in the plant's activity too.

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 seasons.

### Micro-organisms Used

Root, stem and leaves of *Phyllanthus amarus* have been extracted using chloroform, acetone and ethanol for the anti-fungal activity against selected fungi. Five strains of fungi were used for anti-fungal screening. Clinical laboratory isolates of fungi were procured from the Microbiology Laboratory, SMS Medical College, Jaipur. For the present study, pure fungal strains were taken as mentioned with their activity index tested using standard (Ketoconazole) in Table 1.

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

**Table 1:** Selected fungal strains for the present study and the standard zone of inhibition for each fungus are as follows:

S.No	Fungal Strains	MTCC No.	Standard (in mm)
1	<i>Trichoderma reesie</i>	MTCC164	10.34
2	<i>Aspergillus niger</i>	MTCC282	24.02
3	<i>Penicillium funiculosum</i>	MTCC2552	16.14
4	<i>Candida albicans</i>	MTCC3958	12.04
5	<i>Fusarium oxysporum</i>	MTCC6659	19.07

### Culture and Maintenance of Micro-organisms

Pure cultures of mentioned fungal strains were used as indicator organisms. The fungus was grown in a potato dextrose agar (PDA) medium. Fungal culture was maintained on the medium for 72 h of sub-culturing, usually incubated at 37°C and stored at 4°C for future experiments. A fresh suspension of test organism in saline solution was prepared from a freshly grown agar slant before every anti-fungal assay

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



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## Preparation of Extract

From both the locations, *in vivo* parts, i.e., root, stem and leaves of *Phyllanthus amarus* were washed under tap water followed by distilled water, then shade dried. Dried parts were then coarsely powdered. Selected extracts were obtained by macerating 100 g of dried powder of different parts in selected solvents and separately kept on a rotary shaker for 24 hr. Each extract was filtered and centrifuged for 15 min at 5000 rpm, dried under reduced pressure and stored in airtight sterile bottles at 4 °C.

*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 °C in sterile, airtight bottles.

## Microbiological Screening

The antimicrobial activity was performed with the agar well diffusion method [18,19] and minimum inhibitory concentration (MIC) [20].

## Determination of Antifungal Assay

The anti-fungal activity of the experimental plant was investigated by agar well diffusion method [18,19]. The fungal strains were subcultured on Potato Dextrose Agar (PDA: Merck, Germany) medium and respectively incubated at 37°C for 24 h and 25 °C for 2 - 5 days. Suspensions of fungal spores were prepared in sterile PBS (phosphate buffered saline) and adjusted to a concentration of 10<sup>6</sup> cells mL<sup>-1</sup>. Dipping a sterile swab into the fungal suspension was rolled on the surface of the agar medium. The plates were dried at room temperature for 15 min. Wells of 6 mm in diameter and about 10 mm apart were punctured in the culture media using sterile cork borer. The test compound (40 µl) was introduced in the well. Plates were incubated at 37°C. After incubation of 72 hr, bioactivities were determined by measuring the diameter of inhibition zone (mm). The diameters of zone of inhibition produced were with those of standard ketoconazole used as standard anti-fungal agent. All the experiments were performed in triplicate and mean values were taken.

The crude extracts *in vitro* antibacterial activity 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 °C and a standardized inoculum (1.5×10<sup>8</sup> CFU/ml, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petridishes to give a solid plate. Wells (6mm and 1 cm apart) were prepared in the agar plates using a sterile cork borer. The test compound (40 µl) and standard antibiotic (60µl) were introduced in the well. The plates were incubated overnight at 37°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 ciprofloxacin. 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. The inhibition zone diameter 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 least concentration, which can inhibit the fungal growth on the media plates, is known as minimum inhibitory concentration (MIC) [21]. The most commonly employed methods are the tube dilution method and agar dilution method [22]. Less drug required for inhibiting the growth of the organism, lesser is its MIC value. Consequently, drugs that have low MIC values are more effective antimicrobial agents. The resistivity of microbes to antibiotics is seen by its MIC score and can monitor their activity.

The lowest concentration, which can inhibit visible fungal 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 means that less drug is required to inhibit 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.

The serial dilution technique of extracts is followed when performing MIC representing different concentrations. 100, 50, 25 and 12.5 µg/ml of chloroform, acetone and ethanol extracts were used. The same agar well diffusion method was used with serial dilutions of extracts for inhibiting fungal culture with their respective inocula and incubated for 72 hr at 37°C. Now MIC is calculated based on



the least visible growth of the lowest concentration, killing 99.5% of the used inocula. For determining the susceptibility of organisms to antibiotics, MIC is considered the 'gold standard' used to judge the performance of other methods used [21].

MIC is performed by a serial dilution technique of the extracts representing different concentrations. The 100, 50, 25 and 12.5 µg/ml were 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 inoculum and incubated for 24 h at 37°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 the gold standard for determining the proneness of organisms to antibiotics, is therefore used to judge the performance of other methods used [18].

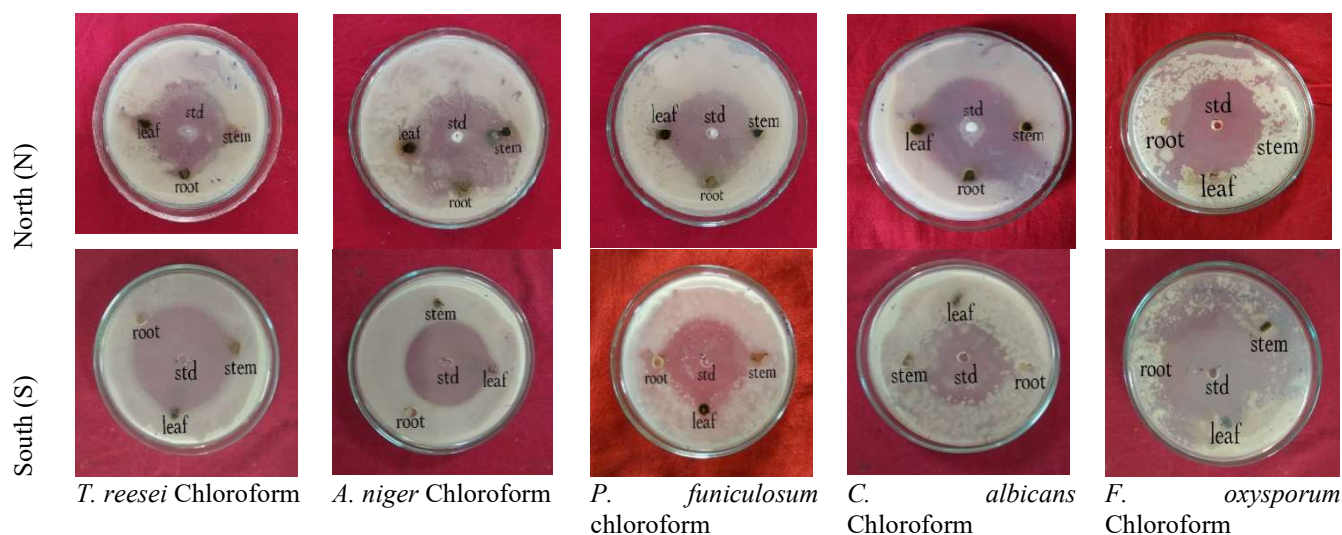
## RESULTS AND DISCUSSION

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

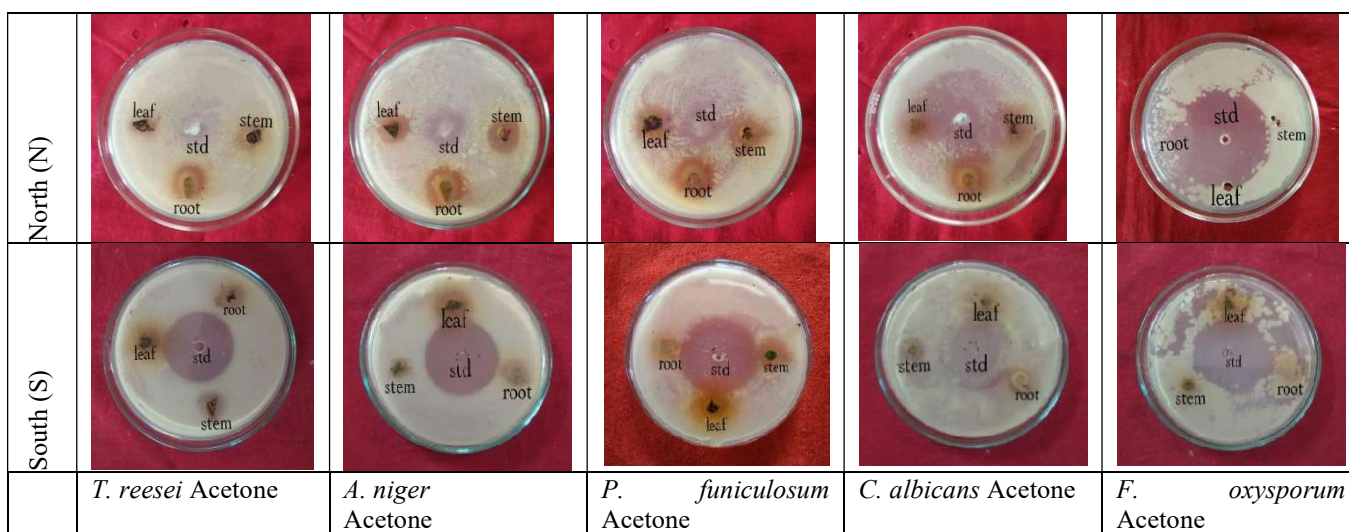
### Evaluation of Antifungal Activity

Results were observed, comparing the zone of inhibition of the selected extracts of the North and South plant with the standard, i.e., ketoconazole against various fungal pathogens. All extracts were potent against more than two selected fungi. Among selected extracts, ethanol showed better potentiality against fungi, followed by acetone and lastly, chloroform. It has been observed that both North and South plant's parts are effective on some and ineffective on some of the selected fungi. Figures 1-3 present the inhibition zone of all three extracts of root, stem and leaves to get a clear vision of comparison.

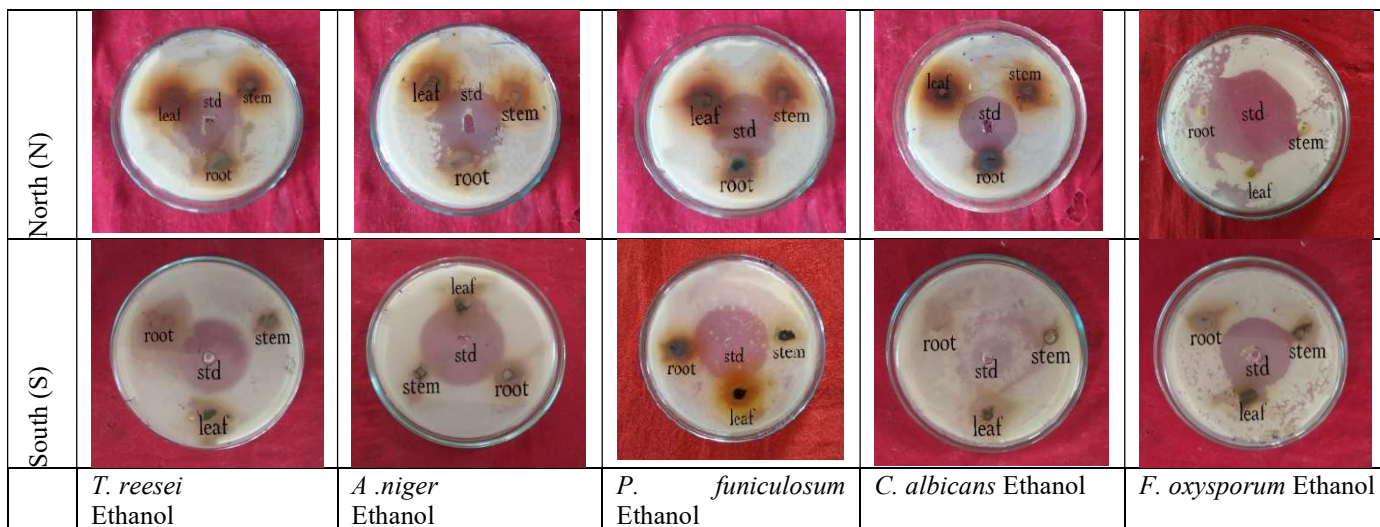
Comparing zone of inhibition and antibacterial potential of the selected plant from the North and South locations with the standards activity, i.e., ciprofloxacin against various bacterial pathogens, effective results were observed. All the extracts were potent against all the bacteria studied. Among all three extracts, ethanol showed a high level of inhibition, followed by acetone and chloroform. Figures 1-3 present the zone of inhibition of all three extracts of root, stem and leaves to make a clear comparison vision.



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



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



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

### Anti-fungal Essay

The anti-fungal potential of different extracts of different parts of the plant was determined by measuring the zone of inhibition (in mm) and thereby calculating its activity index. Results shown (Fig 4-6) are calculated in the form of an activity index presented in graphs. Every extract has been explained in brief to visually differentiate the potential of all parts of North and South plants. The measured zone of inhibition was quantitatively assessed based on inhibitory activity against pathogens along with MIC and their activity index was also calculated by the given formula.

Using agar-well diffusion method, the anti-fungal 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 briefly to visually differentiate the potential of root,



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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. Their Activity Index was also calculated with the help of the given formula.

**IZ (Inhibition zone)** = in mm (Includes diameter of disc-6mm)

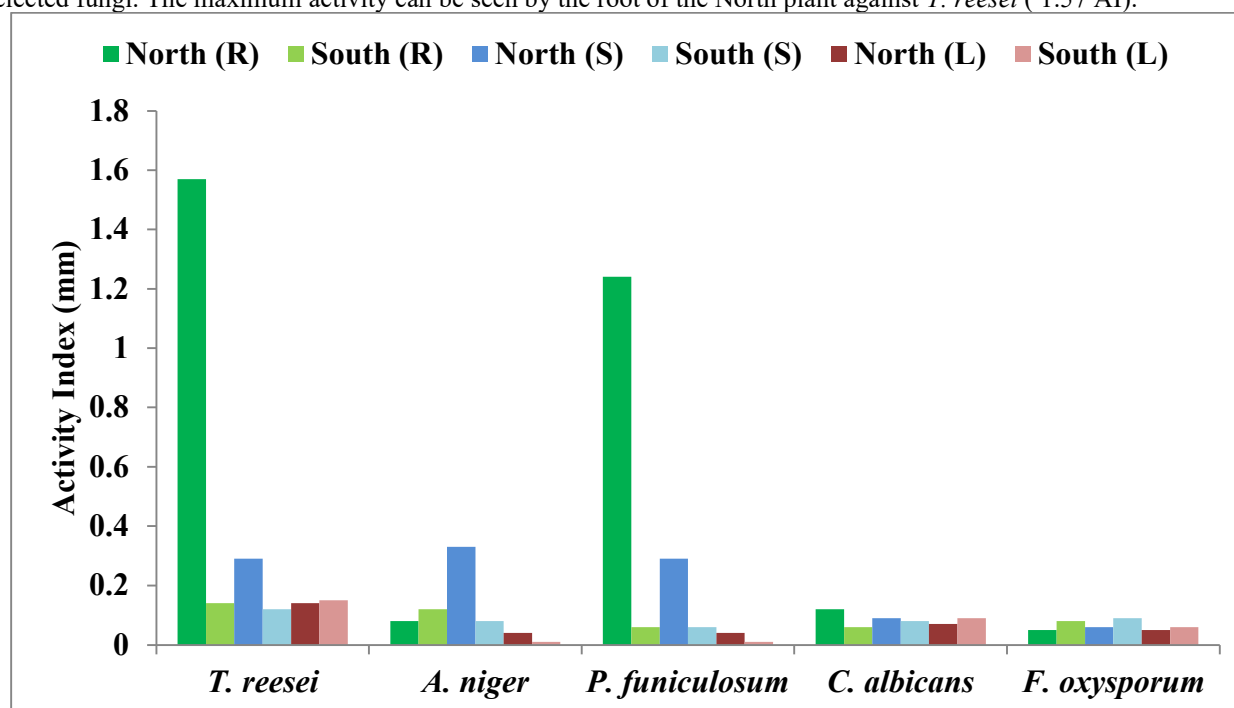
**AI (Activity Index)** = IZ of test sample/IZ of standard (as in Table 1)

\*(Values are mean of triplicate readings)

**Standard:** Ketoconazole (60µl/disc)

### Chloroform Extract

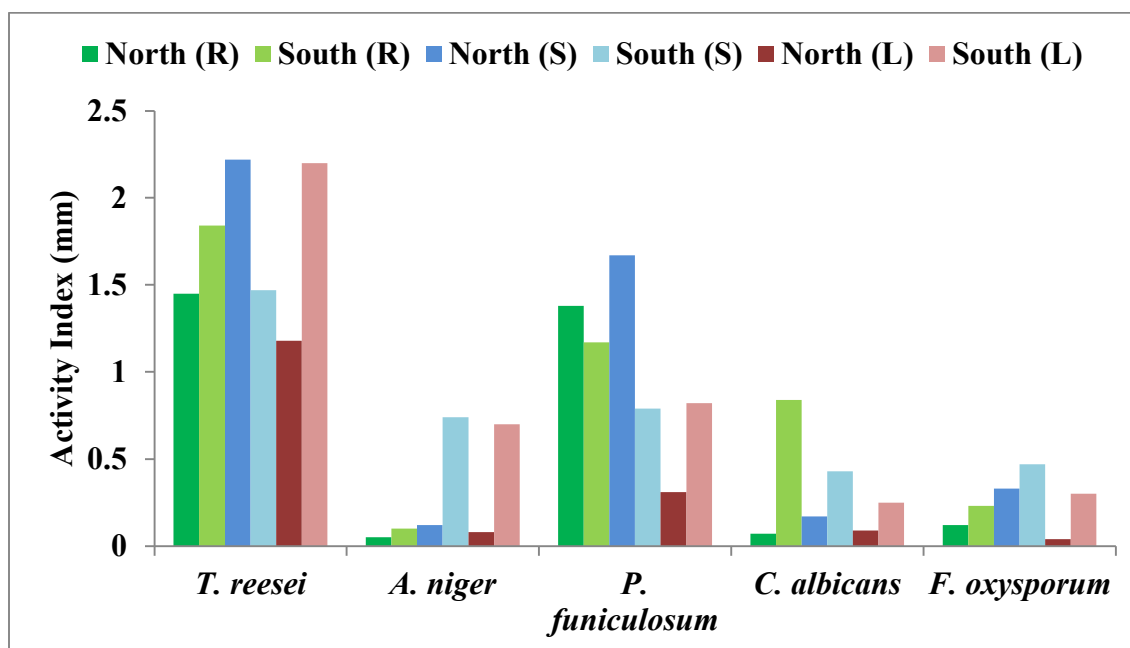
Fig. 4, clarifies the potentiality of every part of the plant of chloroform extract, either it's of North or South location. The only root of the North plant is active against *T. reesei* (1.57 AI) and *P. funiculosus* (1.24 AI). The remaining all parts are almost ineffective against all the selected fungi. The maximum activity can be seen by the root of the North plant against *T. reesei* (1.57 AI).



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

### Acetone Extract

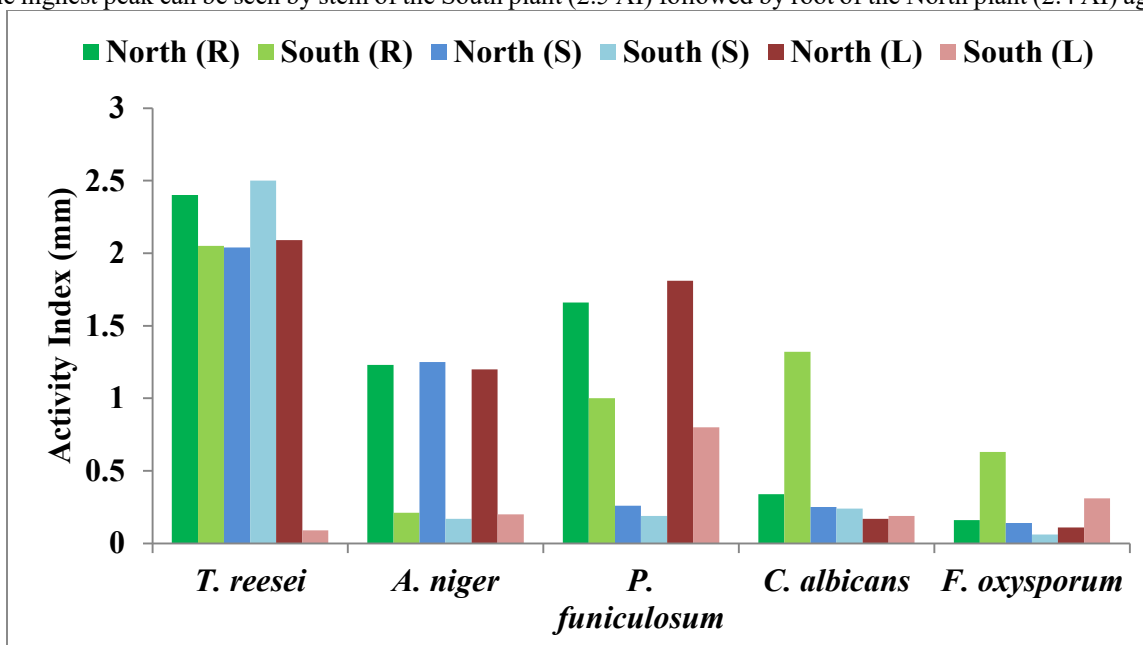
By investigating the results of Fig. 5, Acetone extracts of both locations are well active against *T. reesei* and the next against *P. funiculosus*. Acetone extracts are least reactive against *A. niger*, *C. albicans* and *F. oxysporum*. The highest activity index can be seen by the stem of North plant (2.22 AI) followed by leaves of South plant (2.20 AI) against *T. reesei*. The stem of the North plant shows the highest activity index among other parts against *P. funiculosus* (1.67 AI).



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

#### Ethanol Extract

Ethanol extracts are best known among other differential extracts. In Fig. 6, activity is seen more comparing to chloroform and acetone extracts. Against *T. reesei*, activity index is at their peak except by leaves of South plant. Against *A. niger*, only North plant parts are potent. Roots and leaves are better against *P. funiculosum*. The only root of the South plant is potential against *C. albicans* (1.32 AI). The highest peak can be seen by stem of the South plant (2.5 AI) followed by root of the North plant (2.4 AI) against *T. reesei*.



**Fig. 6:** Graphical representation of the comparative study of Ethanol extracts 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 fungi was 100 µg/ml followed by 50 µg/ml and the least selected was 12.5 µg/ml.

**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
<i>T. reesei</i>	N	12.5	100	100	100	100	12.5	12.5	12.5	100
	S	100	100	100	12.5	12.5	12.5	12.5	12.5	12.5
<i>A. niger</i>	N	100	100	100	100	100	100	100	100	100
	S	100	100	100	100	12.5	12.5	100	100	100
<i>P. funiculosum</i>	N	100	100	100	50	100	100	12.5	100	50
	S	100	100	100	25	100	100	12.5	100	12.5
<i>C. albicans</i>	N	100	100	100	100	100	100	100	100	100
	S	100	100	100	12.5	100	100	25	100	100
<i>F. oxysporum</i>	N	100	100	100	100	100	100	100	100	100
	S	100	100	100	100	100	100	12.5	100	100

In Table 2, Chloroform extract showed the least MIC, i.e., 12.5 µg/ml only by root extract of North plant against *T. reesei* among all parts against all fungi (100 µg/ml). Acetone extracts showed all the selected MICs against different fungi. All parts of the South plant and only leaves of the North plant of acetone extracts showed 12.5 µg/ml against *T. reesei*. Only root of the North plant showed 50 µg/ml against *P. funiculosum* in acetone. In Ethanol extract too, all parts of the South plant and root & stem of the North plant showed the least MIC. Here leaves of the North plant showed 50 µg/ml against *P. funiculosum*. Against *C. albicans* root of South plant showed 25 µg/ml. The rest mainly showed MIC of 100 µg/ml.

Antimicrobial compounds can be vastly used in therapeutic treatments because of their wide application and efficiency against microbes [23,24]. Nature is a unique source of phytochemical diversity, having potent medicinal properties [25]. Most of the attention of researchers is to extract compounds from plants rather than synthetic ones. Therefore, pharmaceuticals are leaning towards medicinal plants.

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.

The present study shows that *Phyllanthus amarus* collected from India's locations were determined by studying different extracts performing against fungal isolates. Against fungi, results are not as good as against bacteria. By studying Fig. 4-6, a comparative study between different extracts of selected parts of the plant can be done and observe how climate induces the same plant differently.

In the present study, *Phyllanthus amarus* collected from India's North and South locations were evaluated through different extracts performing against Fungal isolates. 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.

It has been observed that both North and South plant's parts are effective on some and ineffective on some of the selected fungi. Chloroform extract of the South plant was ineffective against all the selected fungi and the only root of the North plant showed effectivity against *T. reesei* and *P. funiculosum*. Acetone extracts were the least effective against *A. niger*, *C. albicans* and *F. oxysporum*. Ethanol extracts were the least effective against *C. albicans* and *F. oxysporum*. Hot and harsh weather of Rajasthan or the hot and humid climate of Tamil Nadu does not show much difference against fungi against bacteria; the North plant was more potent than the South plant. The MIC values suggest that the plant extracts are fungistatic at lower concentrations and fungicidal at a higher concentration of 100 [26,27]. Consequently, MIC might not necessarily respond in vivo to the anti-fungal agent [28,29].



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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 with the harsh weather and dry soil; therefore, more secondary metabolites are present in it compared to the South plant. The South plant, too, showed similar results in some extracts, but the stem and leaf parts 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 investigation focused on the anti-fungal potentiality of the root, stem, and leaves of *Phyllanthus amarus* from India's North and South locations. The study shows that biochemical compounds obtained from plants are of great use to green pharmaceuticals. Among the selected fungi, plant extracts were most effective against *T. reesei* and *P. funiculosus*. Roots were better anti-fungal reagents than other parts as most of the compound accumulation is seen in either roots or leaves, which consequently shows better results. Therefore, these herbal extracts are the next generation for medicines without adverse effects.

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 India's North and South parts. As a result of this, 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 anti-fungal 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 well-being of society.

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