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A Comparative Study on Secondary Metabolites Producing Microbes Isolated from Rhizospheric & Non-Rhizospheric Region of *Azadirachta Indica* and *Oscimum Tenuiflorum*

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Subject: Microbiology

Abstract

The present study is carried out by the isolation and characterization of rhizospheric and non-rhizospheric region micro flora and comparative analysis of production of secondary metabolites against three bacterial pathogens (*E. coli*, *P. aeruginosa* and *S. aureus*). *Azadirachta indica* and *Oscimum tenuiflorum* plant regions were used for isolation of microbes. The secondary metabolites will not involve in the growth of the cultures but they will be resultant of the primary metabolites. So, after isolation, the antibiogram analysis revealed the activity of isolates and further all were characterized through Bergey' manual, The isolates were *Sporosarcina*, *Streptococcus*, *Micrococcus luteus*, *Lactobacillus fermentum*, *Neisseria sicca*, *E. coli*, *Streptococcus faecalis*. It was comparatively observed that the organic solvent had increased the activity of the secondary metabolites of the all the potent isolates by many time. The best activity obtained in the non-rhizospheric region of *Oscimum tenuiflorum* plant and showed zone of inhibition of 36.5mm against *S. aureus* after treatment with the organic solvent methanol and chloroform for intracellular and extracellular secondary metabolites respectively.

Key words*: Rhizospheric, Non rhizospheric, Secondary metabolites, antibiogram*

Introduction

The plants containing medicinal substances which can be used as antibacterial, antifungal, antipyretic, anti-cancerous, etc., are termed as medicinal plants. India has one of the richest plant medical cultures in the world. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers 'all' plants as potential sources of medicinal substances. Soil microorganisms constitute world largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems [1].The potential importance of microbial activities associated with root systems in plant nutrition and coined the term "rhizosphere" to describe the zone of intense microbial activity around the root. The region of soil surrounding and including the plant root, is of crucial importance for plant health and nutrition. It has a high level of microbial activity, particularly because of nutrients secreted by plant roots in the form of soluble exudates as amino acids, organic acids and other photosynthates. It is a habitat for a vast interactive community of rhizotrophic microorganisms whose activities largely determine the physico-chemical properties of the rhizosphere soil [2]Soil as a living system inhabits assorted cluster of living organisms, both micro flora (fungi, bacteria, algae and actinomycetes) and micro-fauna (protozoa, nematodes, earthworms, moles, ants) [3]. Many bacteria are intimately associated with plants roots. Microorganisms may affect the permeability of root cells, metabolism of roots, absorption and excretion of certain compounds in root exudates [4]. Secondary metabolites are organic compounds produced from microorganisms as the resultant of their metabolism; often play an important role in plant defence against herbivore and other interspecies defences. The present study is carried out by comparative study on Secondary metabolites producing microbes, isolated from rhizospheric & non-rhizospheric region of medicinal plants *Azadirachta indica* and *Oscimum tenuiflorum* against bacterial pathogens (*Escherichia coli*, *Pseudomonas aeruginosa* & *Staphylococcus aureus).*

Material and methods

Sample collection:

Soil samples were collected from rhizospheric and non-rhizospheric region of selected medicinal plants, *Azadirachta indica* and *Oscimum tenuiflorum* from Vibhutikhand, gomtinagar Lucknow.

Isolation:
Isolation of the desired microbes was done by the serial dilution method [5,6,7].
This method is used to get the reduced number of the microbes from the sample. It is the best method to get the less populated colonies of various cultures.

Primary screening:
Primary screening was done to characterize the isolates. Spreading and streaking was done in order to get the isolated colonies, further antibiogram was performed to know the activity of isolates [5,6].

Spreading and Streaking:
The spreading method is used for the separation of microbial colonies from each other present into the diluted sample in order to get single isolated colony in a mixed culture plate. This is followed by the streaking method which is used to get the only single type of culture on to the nutrient agar plate [7].

Culture characterization:
The characterization of the cultures has been done by following the Bergey's manual of bacterial classification. This is accomplished by the various test performed according to the Bergey's manual [5,8].
Gram's staining, Endospore staining, Acid-fast staining, Catalase test, Glucose fermentation test, Mannitol fermentation test, Nitrate reduction test, Oxidase test, Lactose fermentation test, Citrate utilization test were performed according to Bergey's manual [9].

Pure culturing:
Pure culturing is done to preserve the single type of culture in the form of broth from the streak plate.

Antibiogram test:
Microorganisms are found in their natural habitat and are in constant exposure of undesirable chemicals, which may have antimicrobial activity against various microbes other than itself. To check the resistivity or sensitivity of a microbe against the various pathogens antibiotic sensitivity test is used to perform. This test is also termed as Antibiogram test [5,8,9]. Prepare nutrient agar plate. Spray 20 µl of selected pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*) onto the solidified nutrient agar plates. Make three wells at appropriate distance onto the plate with the help of gel puncture. Load 50 µl of the isolates' broth. Incubate it at 37ºC for overnight. Observe the plates and take measurement of the zone of inhibition, if found.

Secondary screening:
Secondary screening includes the Growth kinetics study of the culture to know about the growth rate of the culture. This is useful for the extraction of the secondary metabolites of the microbes. Microbes produce the secondary metabolites at the end of the log phase. The extraction of intracellular and extracellular secondary metabolites by the treatment of the organic solvents is also the part of the secondary screening. Finally, it is followed by the antibiogram test to check the activity of extracted intracellular and extracellular secondary metabolites after treatment with the organic solvents.

Growth kinetics:
Growth kinetics study is applied to determine the time period at which culture shows the optimum activity. The growth period of any microbe is considered under four different phases; Lag phase, Log phase, Stationary phase and death phase.

Extraction of Intracellular and Extracellular secondary metabolites:
Secondary metabolites have been extracted using solvents chloroform and methanol. Intracellular secondary metabolites extraction is carried out by dissolving it into methanol and extracellular secondary metabolites have been extracted by dissolving it into the chloroform, on the basis of their polarity and their dissolving power. The treatment of the culture with organic solvent, enhances the activity of the secondary metabolites. This is only because of their dissolving power. More the solvent dissolve the secondary metabolite, more will be the activity of the compound [10]. Take stored culture, centrifuge it at 5000rpm/5min. Take supernatant (for extracellular secondary metabolite) and add equal volume of the chloroform. Take pellet (for intracellular secondary metabolites) and add 0.5ml of the methanol (the volume of the methanol may vary according to the amount of the pellet).

Extracellular secondary metabolite extraction:
Mix it gently by inversion for about an hour. Centrifuge it at 10,000rpm/10min. Discard the top layer and take the second layer in eppendorf tubes.

Intracellular secondary metabolite extraction:
Mix it gently by inversion for about half an hour. Centrifuge it at 10,000rpm/10min. Take the supernatant into eppendorf tubes. Now allow eppendorf tubes to dry. Then add 50 µl 100mM TrisHCl (pH -8)in all the tubes. Dissolve the dried samples in Tris HCl. Collect intracellular and extracellular secondary metabolites separately. Now sample is ready for further antibiogram test.

Results

Isolation, purification and characterization of rhizospheric and non-rhizospheric bacteria of selected medicinal plants:
Soil samples of the *Azadirachta indica* and *Oscimum tenuiflorum* rhizosphere and non-rhizosphere regions were collected from the Vibhutikhand, Gomtinagar, Lucknow. The bacterial culture from the soil samples were collected by the serial dilution and spread plate technique. The total 18 culture have been isolated

from the soil samples and out of total 18 only 8 have been characterized.

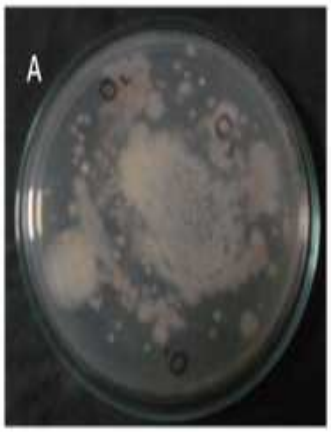


Fig 1: *Azadirachta indica* rhizospheric soil sample (A)

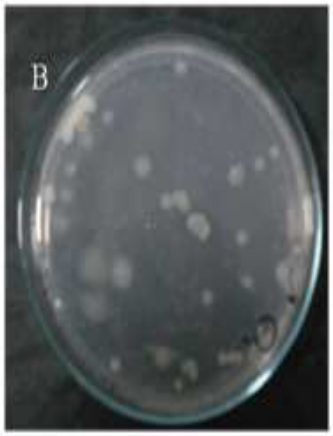


Fig 2: *Oscimum tenuiflorum* rhizospheric soil sample (B)

Spread plate A is showing the result of the *Azadirachta indica* Rhizospheric soil sample which is showing the mix culture. The active cultures isolated from this plate, are D1, S1, S2.

Spread plate B is showing the result of the *Oscimum tenuiflorum* Rhizospheric soil sample from which D1*, S2* have been isolated.

Fig 3: *Azadirachta indica* non-rhizospheric soil sample(C)

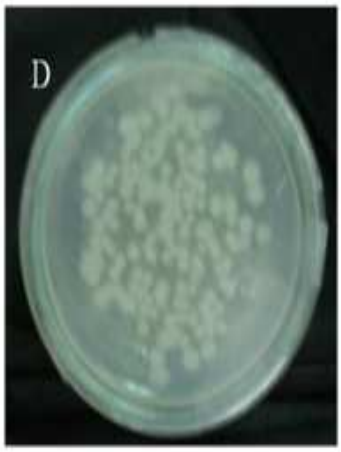


Fig 4: *Oscimum tenuiflorum* non- rhizospheric soil sample (D)

Spread plate C is showing the result of the *Azadirachta indica* non-rhizospheric soil sample. Not a single active microbe has been screened from this plate.
Spread plate D is showing the result of the *Ocimum tenuiflorum* non-rhizospheric soil sample. T1, T2 and T3 have been isolated from this plate and further screened.

Fig5: D1 isolate

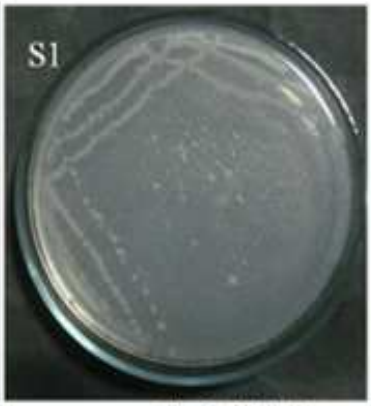


Fig 6: S1 isolate

Fig 7: S2 isolate

Fig.8: D1* isolate

Fig 9: S2* isolate

Fig 10: T1 isolate

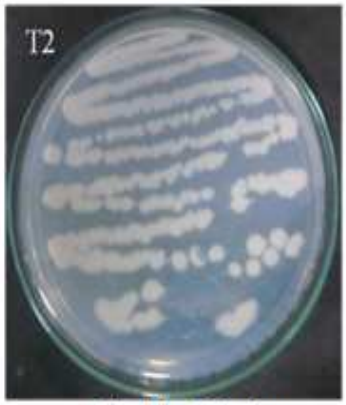


Fig 11: T2 isolate

Fig 12: T3 isolate

Figure no. 5.7 to figure no. 5.11, are showing the results of streak plates of all isolated cultures containing single type of culture.

Table 1: Showing the morphology of the isolated culture from the Azadirachta indica rhizospheric soil sample.

S. no.	Neem Rhizospheric Microbes		
1	**D1**	*Shape*	circular
		Size	0.2mm
		Colour	off white
		Texture	rough
		Elevation	flat
		Opacity	opaque
2	**D2**	*Shape*	circular
		Size	0.2mm
		Colour	off white
		Texture	smooth
		Elevation	flat
		Opacity	opaque
3	**S1**	*Shape*	circular
		Size	0.1mm
		Colour	off white (pale yellow)
		Texture	rough
		Elevation	flat
		Opacity	opaque

Table 2: Showing the morphology of the isolated culture from the *Oscimum tenuiflorum* rhizospheric soil sample.

S. no.	Tulsi Rhizospheric Microbes		
1	**D1***	*Shape*	circular
		Size	0.01mm
		Colour	off white
		Texture	smooth
		Elevation	flat
		Opacity	opaque
2	**S2***	*Shape*	circular
		Size	<0.1mm
		Colour	off white
		Texture	smooth
		Elevation	flat
		Opacity	opaque

Table 3: Showing the morphology of the isolated culture from the *Oscimum tenuiflorum* non-rhizospheric soil sample.

S. no.	Tulsi Non- Rhizospheric microbes		
1	**T1**	*Shape*	circular
		Size	<0.1mm
		Colour	off white
		Texture	hard
		Elevation	flat
		Opacity	opaque
2	**T2**	*Shape*	circular
		Size	<0.1mm
		Colour	yellow
		Texture	hard
		Elevation	flat
		Opacity	opaque
3	**T3**	*Shape*	circular
		Size	0.1mm
		Colour	off white
		Texture	smooth
		Elevation	flat
		Opacity	opaque

Table 4: Showing the gram's staining results of all isolates

S. no.	Neem Rhizoshperic Isolates	
1	**D1**	gram +ve cocci
2	**S1**	gram +ve cocci
3	**S2**	gram +ve cocci
	Tulsi Rhizospheric Isolates	
4	**D1***	gram +ve rods
5	**S2***	gram -ve cocci
	Tulsi Non- Rhizospheric Isolates	
6	**T1**	gram -ve rods
7	**T2**	gram -ve cocci
8	**T3**	gram +ve cocci

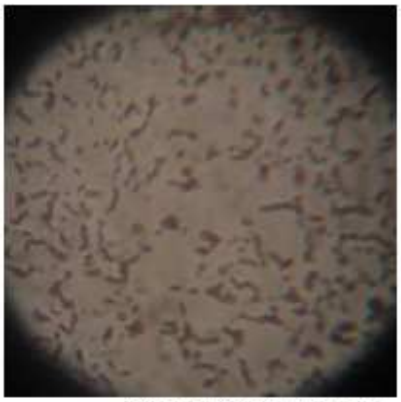


Fig 13: Gram +ve cells

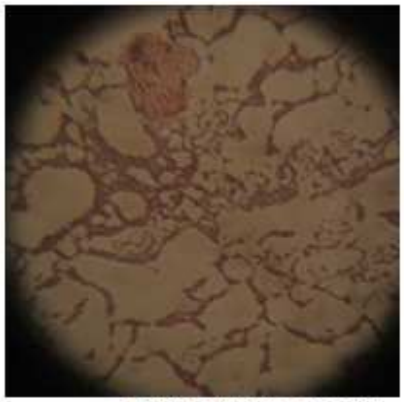


Fig 14: Gram –ve cells

Figure 12 and figure 13 are showing the results of Gram's staining. As shown in the above figure, the figure 12 is showing the purple colour of the gram +ve cells and figure 13 shows the gram –ve cells.

Fig 15: Catalase test

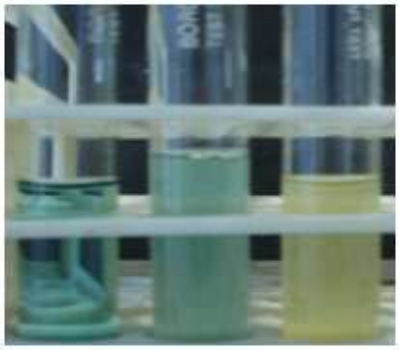


Fig 16: Glucose fermentation test

The above figure is showing the catalase positive results. Oxygen bubbles are the indicator of the positive results, as well as showing the result of the positive result of the glucose fermentation result.

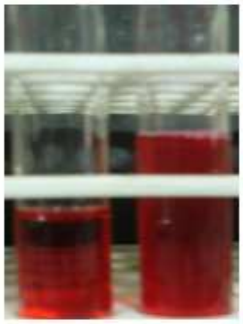


Fig 17: Mannitol fermentation test

The above picture is showing the negative result of the mannitol fermentation test by the isolate. The positive result is indicated by the colour change from red to yellow.

Table 5: Showing the group confirmed followed by Bergey's manual

S. no.	Colony	Observation	Strains assumed
1	**D1**	group V	*Sporosarcina*
2	**S1**	group VII	*Streptococcus*
3	**S2**	group VI	*Micrococcus* *Planococcus* *Staphylococcus*
4	**D1***	group III	*Lactobacillus* *Listeria* *Kurthia*
5	**S2***	group XI	*Neisseria* *Veillonella*
6	**T1**	group IX	*Neisseria* *Veillonella*
7	**T2**	group XI	*Escherichia* *Proteus* *Salmonella* *Erwinia* *Citrobacter* *Entrobacter*
8	**T3**	group VII	*Streptococcus*

Table 6: Showing the confirmed bacterial strains followed by Bergey's manual

S. No.	Culture	Bacterial Strains
1	D1	*Sporosarcina*
2	S1	*Streptococcus*
3	S2	*Micrococcus luteus*
4	D1*	*Lactobacillus fermentum*
5	S2*	*Neisseria sicca*
6	T1	*Escherichia coli*
7	T2	*Neisseria sicca*
8	T3	*Streptococcus feacalis*

Comparative analysis of the activity of secondary metabolites before and after treatment of the selected organic solvents against selected pathogens:

Table 7: Showing the results of the antibiogram before treatment of the organic solvent.

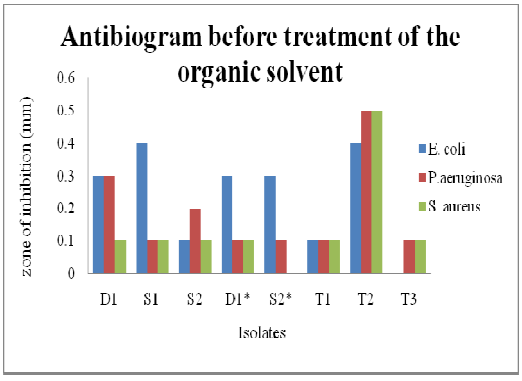
Culture	Inhibition zone(mm) against		
	E. coli	*P.aeruginosa*	*S. aureus*
D1	0.3	0.3	0.1
S1	0.4	0.1	0.1
S2	0.1	0.2	0.1
D1*	0.3	0.1	0.1
S2*	0.3	0.1	0
T1	0.1	0.1	0.1
T2	0.4	0.5	0.5
T3	0	0.1	0.1

The above table data is showing the activity of the isolates against various pathogens. The maximum inhibition zone is of 0.5mm.

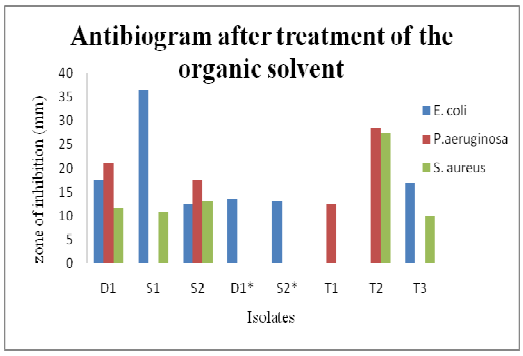
Table 8: Showing the results of the antibiogram after treatment of the organic solvent.

Culture	Inhibition zone(mm) against		
	E. coli	*P.aeruginosa*	*S. aureus*
D1	17.5	21	11.5
S1	36.5	0	11
S2	12.5	17.5	13
D1*	13.5	0	0
S2*	13	0	0
T1	0	12.5	0
T2	0	28.5	27.5
T3	17	0	10

The above data is showing the results of the antibiogram after treatment of the organic solvent. the S1 isolate has shown the many folds increased activity from 0.4mm to 36.5mm.



Graph 1: Showing the results of antibiogram of all isolates before treatment of the organic solvents against various bacterial pathogens.



Graph 2: Showing the results of the antibiogram of all isolates after treatment of the organic solvents against various bacterial pathogens.

The above graphs are showing the comparative analysis of the antibiogram results before and after treatment of the organic solvents and the drastic changes have been observed in between both the data. The activity of the isolates has increased up to 36.5mm while before treatment of the organic solvent, the activity has been observed in between the range 0mm to 0.5mm. Hence, the organic solvent treatment has shown the many fold

increment in the activity of the secondary metabolites against three selected pathogens.

Production and assessment of activity of secondary metabolites produced from rhizospheric and non rhizospheric bacteria:

The production of the secondary metabolites by the characterized culture has been done in the Nutrient broth and then is has been extracted by the treatment of the suitable organic solvents. The extracted secondary metabolites have been analyzed by the antibiogram test.

Table 9: Showing the result of the antibiogram after treatment with the organic solvent.

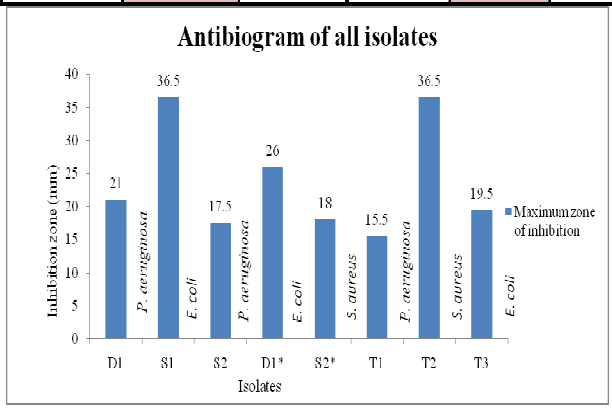
S. No.	Culture	Inhibition Zone(mm) against								
		E. coli			*P. aeruginosa*			*S. aureus*		
		E	I	E+I	E	I	E+I	E	I	E+I
1	D1	17.5	16	17.5	17.5	15.5	21	13	16	11.5
2	S1	18	17	36.5	0	0	0	11	12	11
3	S2	12.5	13.5	12.5	14	13	17.5	13	12	13
4	D1*	26	20	13.5	0	0	0	0	0	0
5	S2*	17	14	13	0	0	0	18	0	0
6	T1	0	0	0	10.5	15.5	12.5	11.5	0	0
7	T2	30	32.5	19.5	30	30	28.5	32	36.5	27.5
8	T3	11.5	19.5	17	0	10	0	0	12	10

***Note:** E= Extracellular secondary metabolite, I= Intracellular secondary metabolite, E+I= combination of extracellular and intracellular secondary metabolites.

The above table is showing the result of the activity of the secondary metabolites after treatment with the organic solvents. The best activity has been observed by the two isolates S2 (*Micrococcus luteus*) and T2 (*Neisseria sicca*). The S2 culture has shown the zone of inhibition of 36.5mm against *E. coli* containing both intracellular and extracellular secondary metabolites. And the T2 culture has shown the zone of inhibition of 36.5mm against *S. aureus* containing only the intracellular secondary metabolites.

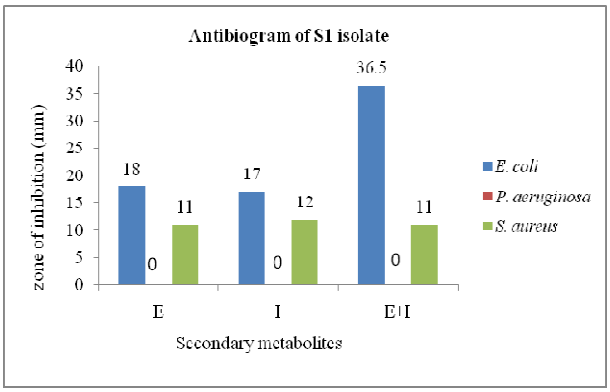
Comparative analysis of the secondary metabolites of the most potent isolate of rhizospheric and non-rhizospheric soil sample:

Maximum zone of inhibition shown by two isolates against three selected pathogens, have been shown in the following graph. According to the following graph, the best activity has been observed by S1 and T2 cultures.



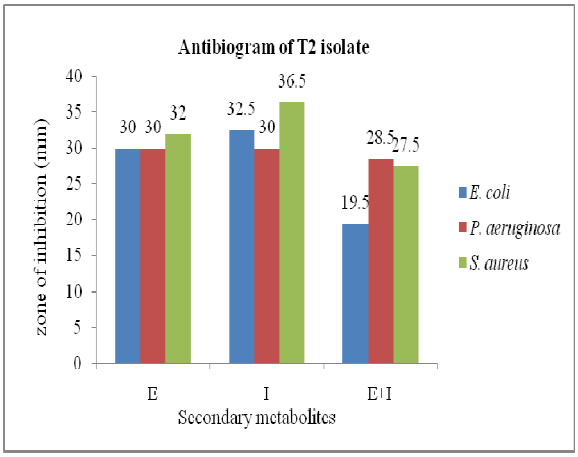
Graph 3: Showing the antibiogram result of all isolates

This graph is showing the result of antibiogram of all characterized isolates.



Graph 4: Showing the antibiogram of the S1 isolate

This graph is showing the activity E, I and E+I of S1 isolate against selected pathogens. The S1 isolate has not shown the activity against *P. aeruginosa* and the combination of E+I has shown the best activity against *E. coli*.



Graph 5: showing the result of antibiogram of T2 isolate

This graph is showing the activity E, I and E+I of T2 isolate against selected pathogens. The T2 isolate has shown the best activity with I against *S. aureus*. Graph 2 and graph 3 are showing the antibiogram result of the most potent isolates, S1 and T2 respectively. These graphs are plotted to show the best activities of E, I and E+I. Hence, according to the above graphs the most potent microbe among all eight isolates, is T2 which has shown the best activity against *E. coli*, *P. aeruginosa* and *S. aureus.*

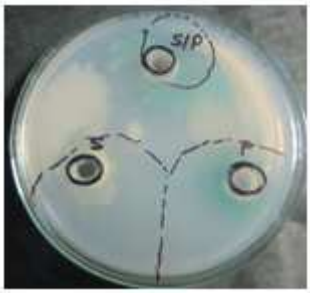


Fig 18: Antibiogram of T2 with *E. Coli*

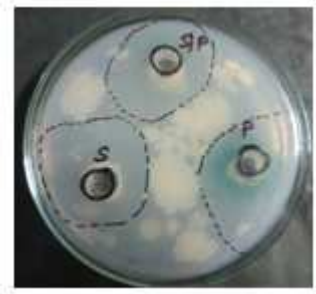
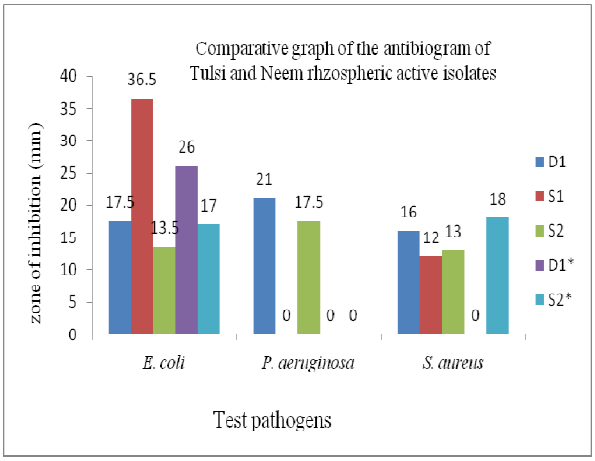


Fig 19: Antibiogram of T2 with *P. Aeruginosa*

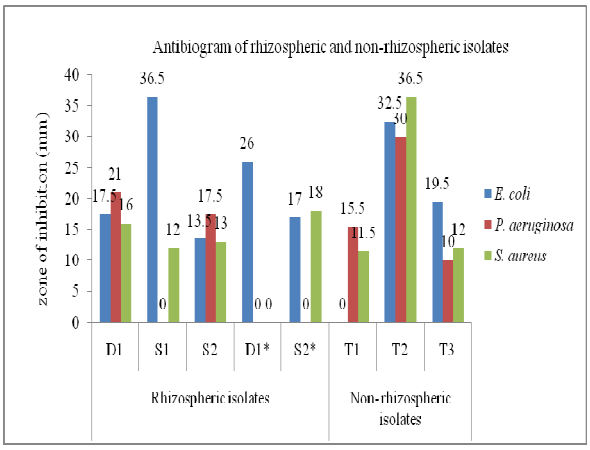
Fig 20: Antibiogram of T2 with *S. Aureus*

***Note:** In figure5.18 to figure5.20, **S**= Supernatant; which has been used for extracellular secondary metabolite and **P**= Pellet; which has been used for intracellular secondary metabolites. The above figure 17, figure 18 and figure 19 are showing the results of the T2 isolate's activity against selected pathogens.



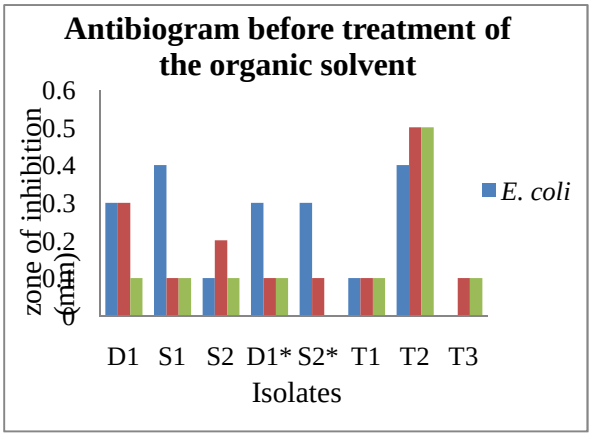
Graph 6: showing the comparative antibiogram results *Azadirachta indica* (D1, S1, and S2) and *Oscimum tenuiflorum* (D1* and S2*) rhizospheric soil microbes.

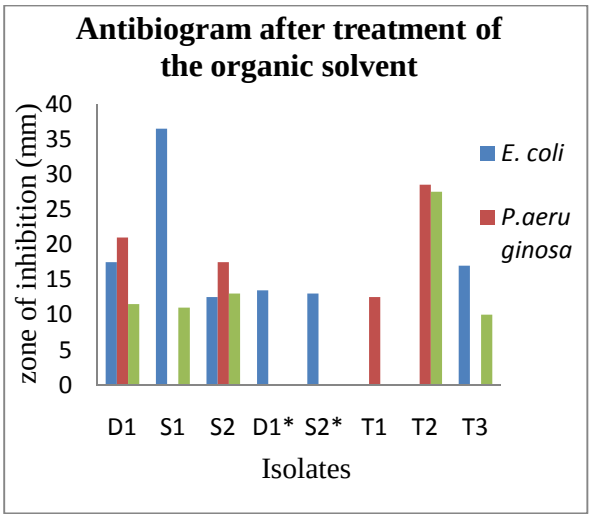
According to the above comparative graph, it is observed that the most potent microbes among all isolates are found in the *Azadirachta indica* rhizospheric soil sample against all pathogens.



Graph 7: Showing the comparative graph of activity of rhizospheric (D1, S1, S2, D1* and S2*) and non-rhizospheric (T1, T2, T3) isolates.

The above graph is showing the comparative analysis of antibiogram of rhizospheric and non-rhizospheric isolates, which shows that non-rhizospheric microbes are the most potent in comparison to the rhizospheric isolates.





Discussion

In the present study, two medicinal plants have been selected, *Azadirachta indica* and *Oscimum tenuiflorum*. The rhizospheric and non-rhizospheric region have been targeted to take the soil sample. The rhizosphere is the region adjacent to the plant root. The root exudates and the secondary metabolites secreted by the micro flora of the soil may affect each other and also to the plant health. There are total 18 cultures were isolated from these soil samples in which only 8 cultures have been screened. These 8 isolates are active against the selected pathogens, *E. coli*, *P. aeruginosa* and *S. aureus*. The characterized 8 cultures were *Sporosarcina*, *Streptococcus*, *Micrococcus luteus*, *Lactobacillus fermentum*, *Neisseria sicca*, *Escherichia coli and Streptococcus faecalis*. To characterize these cultures, Bergey's manual have been followed. According to this, gram's staining, catalase test, endospore test, acid fast staining, glucose fermentation test, mannitol fermentation test, lactose fermentation test, citrate utilization test, oxidase test, glucose oxidation test, and nitrate reduction test have been performed. The comparative analysis of antibiogram of the entire characterized active isolates has been observed before and after treatment of the organic solvents against selected pathogens. This has shown the drastic change in the activity of the isolates. The organic solvents are able to dissolve the secondary metabolites on the basis of their polarity and hence the secondary metabolites have shown the many fold increment in the activity of the isolates. Further, the isolates have been tested for the activity to inhibit the growth of the selected pathogens by antibiogram test. This is followed by the growth kinetics study to know when the culture is reaching to their optimum growth condition. Then, the intracellular and extracellular secondary metabolites have been extracted by treating the culture to the appropriate organic solvents to enhance the activity of the secondary metabolites.

The extracellular and intracellular secondary metabolites are again tested against the selected pathogens to check the activity and affect the organic solvent. The activity of the extracellular and intracellular secondary metabolites and the combined action of the extracellular and intracellular secondary metabolites, have been tested against the test organism. And it has been observed that mainly the intracellular secondary metabolite has given the best inhibition in comparison to the extracellular and combination of the extracellular and intracellular secondary metabolites. The comparative analysis of antibiogram of all isolates, have been observed. There are two potent isolates have been found, which has shown the best activity against selected pathogens. Those isolates are S1 (*Streptococcus*) and T2 (*Neisseria sicca*). S1 has shown the inhibition zone of 36.5mm for *E. coli* and T2 has shown the inhibition zone of 36.5mm for *S. aureus*. The combination E+I of the S1 isolate has shown the best activity against *E. coli* and the secondary metabolites of S1 isolate is not able to inhibit the growth of the *P. aeruginosa*. The T2 is the much more potent culture in comparison to other one. The intracellular secondary metabolite of this culture has shown the best result against *S. aureus* in contrast to other pathogens.

Between both the potent isolates, the T2 culture has the maximum activity against all the selected pathogens in contrast to the S1 with all extracellular, intracellular and the combination of the extracellular and intracellular secondary metabolites .Among all the potent isolates of *Azadirachta indica* and *Oscimum tenuiflorum* rhizospheric soil micro flora,

Azadirachta indica rhizospheric microbes has shown the better activity against all the selected pathogens in comparison to *Oscimum tenuiflorum* rhizospheric microbes. This has been observed by comparing all the isolates activity of *Azadirachta indica* and *Oscimum tenuiflorum* rhizospheric isolates together.

According to the result basis the non-rhizospheric isolates have shown the best result against all three pathogens, while the rhizospheric isolates have shown the best result only against the *E. coli*.

Conclusion

The 18 cultures were isolated from the rhizospheric and non rhizospheric region of the *Azadirachta indica* and *Oscimum tenuiflorum.* Out of 18, only 8 were the potent isolates. The characterized 8 cultures were *Sporosarcina*, *Streptococcus*, *Micrococcus luteus*, *Lactobacillus fermentum*, *Neisseria sicca*, *Escherichia coli and Streptococcus faecalis*. Further it was comparatively analysed that the organic solvent treatment had the positive effects over the activity of the secondary metabolites against various pathogens. It means, organic solvent had increased the activity of the secondary metabolites on the basis of their polarity and its ability to dissolve the secondary metabolites into it. Furthermore, the activity of the intracellular and extracellular secondary metabolites was observed and after various comparisons, it was concluded that the best activity have been shown by the *Oscimum tenuiflorum* non-rhizospheric isolate which is of *Neisseria sicca* against all three pathogens (*S.aureus, P.aeruginosa* and *E.coli*). The activity of the rhizospheric isolates were showing best result against *S. aureus*. The activity of the rhizospheric soil isolates may be affected by the plant root exudates.

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