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Evaluation of Antimicrobial Activity of Aqueous and Ethanolic Extracts of *Glochidion Talakonense* On Selected Bacteria

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ABSTRACT

In the present study, antimicrobial activity of aqueous and ethanolic extracts of *Glochidion Talakonense* were evaluated against Staphylococcus aureus, Escherichia coli, Salmonella Enterica and Bacillus subtitles bacteria using agar disc diffusion technique. Ethanol and distilled watery were used as solvents for extraction by Soxhlet method, and extracts were analyzed by agar disc diffusion method. The antimicrobial activity was evaluated by agar disc diffusion method to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Results revealed that all extracts were effective in inhibition the growth of bacteria except aqueous extracts against Bacillus Subtilis and Staphylococcus aureus bacteria. Staphylococcus Aureus produced a significantly larger zone of inhibition for Kanamycin (23 ± 0.54 mm) than others, while Salmonella Enterica produced smaller zone of inhibition of Kanamycin (19 ± 0.65 mm). The ethanolic extract showed higher inhibition against the pathogens, especially Escherichia coli (1.56 mg/mL). Among the two solvents extracts, the ethanolic extract showed comparatively stronger antimicrobial activity than the aqueous extract, suggesting that ethanol was more effective in extracting the active phytochemicals responsible for bacterial inhibition. The extracts were particularly effective against Gram-positive bacteria (Staphylococcus aureus and Bacillus subtilis), while moderate activity was observed against the Gram-negative bacteria (Escherichia coli). However, further investigations needed to be conducted to validate the biological ingredients and test these Traditional medicinal plants' safety, efficacy, toxicity, and clinical evaluation.

Keywords: Agar disc diffusion, Staphylococcus aureus, Escherichia coli, Minimum inhibitory concentration (MIC), Minimal bactericidal concentrations (MBC).

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INTRODUCTION

Traditional medicine is a very important part of health care. Most of population in the developing countries still relies mainly on indigenous traditional medicine for satisfying their primacy health care needs. Currently, employing plant sources as an alternative to chemical additives in medicines and foods is growing [1, 2]. Antimicrobial, antioxidant, and cytotoxicity impacts of herbal extracts, Plants possess a wide variety of chemical compounds including secondary metabolites like phenols, tannins, terpenoids, alkaloids, flavonoids, etc. which are responsible for their distinctive biological properties. These secondary metabolites are produced either as intermediates or by products of primary metabolic reactions in the plant body. [3]. Herbal extracts and their components have been known to possess antimicrobial properties [4], which among them we can mention a plant known as *Glochidion talakonense* [5, 6]. *Glochidion Talakonense* a species endemic plant up to 7m high, branches spreading belongs to the family Phyllanthaceae and it is distributed in Talakona, Seshachalam biosphere reserve in Chittoor district of Andhra Pradesh, India. Glochidion genus have been used for a various biological activity in traditional medicine. All parts of the *Glochidion talakonense* such as leaves, bark, roots, seeds and fruits are used in medicinal [7]. Chemical compounds including tannins, triterpenoids, flavonoids, and saponins, glochidonol, glochidiol, glochidone have been found in *Glochidion talakonense* extracts [8]. It is a rich source of bioactive compounds, particularly triterpenoids, which possess antioxidant and antimicrobial properties. Both ingredients enhance natural elasticity and peristaltic wave observed in the intestines, without any damage to circulatory and respiratory systems [9]. Results revealed that ethanolic and aqueous extracts were effective in inhibition the growth of bacteria except aqueous extracts against Bacillus Subtilis and Staphylococcus aureus bacteria. Staphylococcus Aureus produced a significantly larger zone of inhibition for Kanamycin (23 ± 0.54 mm) than others, while Salmonella Enterica produced smaller zone of inhibition of Kanamycin (19 ± 0.65 mm) [10,11]. Since *Glochidion talakonense* has been used for centuries with no significant side effects [12], this study was aimed to evaluate of antibacterial effect of ethanolic, and aqueous extracts of *Glochidion talakonense* against Staphylococcus aureus, Escherichia coli, Salmonella enterica, and Bacillus subtitles' pathogens.

MATERIALS AND METHOD

Materials.

Glochidion talakonense leaves obtained from the Talakona, chittor District and authenticated by authority of Botany department, SV University Tirupathi. and was stored in a dark place at 4°C.

Chemicals and Growth Medium.

Brain heart infusion (BHI) broth medium, blood agar medium, plate count agar medium, medium nutrient broth, Mueller Hinton broth medium, normal saline, glycerin, phosphate-buffered saline (PBS), ethanol All of the chemicals were purchased from simhapuri Scientifics, Nellore. Gentamicin was purchased from Life medicals, Nellore.

Bacterial Strains.

The used strains were (ATCC 6538) Staphylococcus aureus, Escherichia coli (ATCC 25922), Bacillus subtitles (ATCC 13048), and Salmonella enteric (ATCC 9270). All were prepared from the Pasteur Institute of India, Tamil Nadu and were stored at 4°C.

Preparation of Extracts:

Aqueous extraction:

After washing, the collected leaves were dried in a freeze dryer for 24 hr and, then, ground to fine powder by an mechanical grinder and sieved (mesh 25) [13,14]. The extraction of the *Glochidion talakonense* were carried out by known standard procedures. The 100gm of the powder is followed by 500 ml water by Soxhlet extraction method for 72 hrs separately. Solvent elimination under reduced pressure afforded the aqueous extract. The extract was dried in a vacuum desiccator to obtained constant weight. The aqueous extract of the plant parts yielded a Light Green residue (5.2 %). The extracts were then kept in sterile bottles, under refrigerated conditions, until further use. The extract was prepared by suspending the residues in hot water and used for antimicrobial studies [15].

Ethanolic extraction:

After washing, the collected leaves were dried in a freeze dryer for 24 hr and, then, ground to fine powder by an mechanical grinder and sieved (mesh 25). The extraction of the *Glochidion talakonense* were carried out by known standard procedures. The 100gm of the powder is followed by 500 ml ethanol by Soxhlet extraction method for 72 hrs separately. Solvent elimination under reduced pressure afforded the ethanol extract [16]. The extract was dried in a vacuum desiccator to obtained constant weight. The ethanolic extract of the plant parts yielded a Light Green residue (5.7 %). The extracts were then kept in sterile bottles, under refrigerated conditions, until further use [17,18]. The extract was prepared by suspending the residues in hot water and used for antimicrobial activity.

Antimicrobial testing methods:

The following three methods have been shown to consistently provide reproducible and repeatable results. They are:

1. Agar Disk diffusion method
2. Broth dilution method
3. Agar dilution method

AGAR DISK DIFFUSION METHOD:

The ethanol and aqueous extracts of *Glochidion Talakonense* were tested for antimicrobial activity by agar disc diffusion method [19].

Preparation of culture medium and inoculation:

For antibacterial activity, 35g nutrient agar and 10g agar were suspended in distilled water (1000 mL) and dissolved by boiling. Media and Petri dishes by autoclaving at pressure 15 lbs for 20 minutes. Under aseptic condition, 20 mL of media was dispended into sterilized Petri dishes to yield a uniform depth of 6mm. after solidification of the medium; the bacterial cultures were inoculated by spread plating technique [20.21]. In this study, the bacteria species such as Escherichia coli, Salmonella Enterica, Staphylococcus Aureus and Bacillus subtilis were used as the test strain.

Agar Disk Diffusion Assay.

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts [22]. The antibacterial activity of extracts was evaluated using the disk diffusion method. 20mL of suspension containing bacteria was spread on Mueller Hinton agar. After drying agar culture plates, paper discs containing 100 mg/mL of extracts were evenly distributed over the surface of the plates. Then, the wells were punched over the plates of 6.4mm diameter, and each extract was poured into separate wells. Plates soaked with Gentamicin and Penicillin (0.006 mg/mL) were used as positive control, and a plate soaked with pure solvent of aqueous solution was used as a negative control [23,24]. Then, plates were incubated at 37 ± 2°C for 24 h. After incubation, the diameter of growth inhibition zone was measured in mm and recorded. Experiments were repeated three times.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC):

Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) were measured using the microbroth dilution method [25]. Firstly, the cultures were incubated overnight and were diluted. A series of 10 test tubes were used to determine MIC (for each extract and for each studied bacterial species). 8 tubes containing Mueller Hinton Broth were employed for each one. Then, 1mL of microbial suspensions and different dilutions (1, 10, 100 mg/mL) of the extract were added to each tube. Two tubes consisting of 9mL of culture medium with 1mL of diluted extract as a positive control and 9mL of culture medium with 1mL of bacterial suspension as a negative control were prepared. All tubes were incubated at 37°C for 24 hr [26,27]. After incubation time, tubes were examined for turbidity caused by the inoculated bacteria. Experiments were repeated three times for each extract and each species. A sampling of all the tubes with no bacterial growth was performed, and the samples were cultivated using the pour plate procedure to determine MBC. So, a 1mL suspension of each tube was mixed with 20mL of Muller Hinton agar at 48°C in petri dishes. After a while, when agar solidified, plates were incubated for 24 h and tested for microbial growth. The lowest concentration of the extract at which no bacterial growth that was detected was regarded as MBC [28, 29].

RESULTS AND DISCUSSION:

Phytochemical Screening:

Aqueous extract of *Glochidion talakonense:*

Table 1: Aqueous extract of *Glochidion Talakonense*

Bioactive Constituents	Aqueous extract of *Glochidion talakonense*
Alkaloids	+
Saponins	+
Tannins and Triterpenoids	+
Flavonoids and Steroids	+
Cardiac glycosides	-
Carbohydrates and proteins	-

+ indicates present **- indicates absent**

Table 2: Ethanolic Extract of *Glochidion Talakonense*

Ethanolic extract of *Glochidion talakonense:*

Bioactive Constituents	Ethanolic extract of *Glochidion talakonense*
Alkaloids	+
Saponins	+
Tannins and Triterpenoids	+
Flavonoids and Steroids	+
Cardiac glycosides	-
Carbohydrates and proteins	+

+ indicates present **- indicates absent**

Antibacterial Activity of *Glochidion talakonense* Extracts.

Table 3: Inhibitory activity of Glochidion Talakonense leaves on Staphylococcus aureus, Escherichia coli, and Salmonella enterica and Enterobacter aerogenes in various solvents by disc diffusion method.

Solvent Extract	Concentration (mg/ml)	Inhibitory activity of *Glochidion talakonense*			
		Bacillus subtilis	Salmonella enterica	Escherichia Coli	Staphylococcus Aureus
H_2O	100	-	16 ± 0.43	16 ± 0.54	_

C_2H_5OH	100	17 ± 0.23	14 ± 0.23	18 ± 0 01	16 ± 0.34
Kanamycin	30	21 ± 0.85	19 ± 0.65	20 ± 0.65	23 ± 0.54

Result of significance test (p<0.05) for the mean of the zone of the inhibition at two solvents

Table 4: Result of significance test (p<0.05) for the mean of the zone of the inhibition at two solvents.

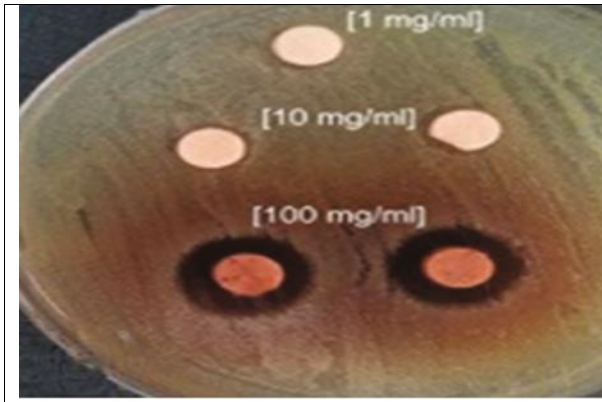
Bacteria	**t – value**	**Statistical analysis**				
		Ethanol P - value	**significant**	**t – value**	**Methanol P - value**	**Significant**
Bacillus subtilis	3.35	0.002	Yes	3.87	0.005	Yes
Salmonella Enterica	3.23	0.0065	Yes	4.54	0.0034	Yes
Escherichia Coli	4.32	0.004	Yes	3.99	0.002	Yes
Staphylococcus Aureus	2.54	0.001	Yes	3.1	0.004	Yes

Table 5: Antibacterial activity inhibition zone diameter (IZD)

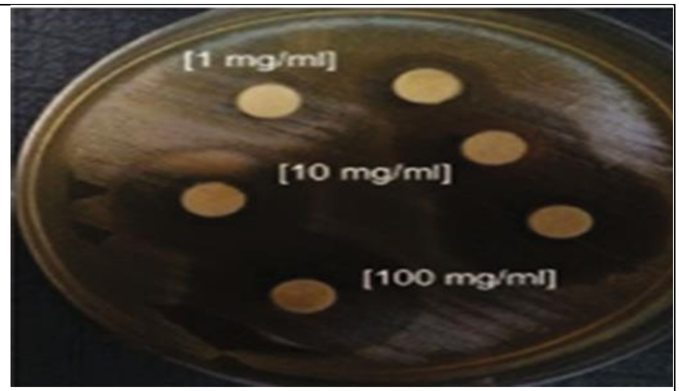
Escherichia Coli			**Staphylococcus Aureus**			**Salmonella Enterica**			**Bacillus subtilis**		
	Conc. mg/ml	IZD mm		Conc. mg/ml	IZD mm		Conc. mg/ml	IZD mm		Conc. mg/ml	IZD mm
H2O A	100 10 1	14±0.23 7±0.34 -	H2O C	100 10 1	11±0.45 - -	H2O E	100 10 1	16±0.65 11±0.63 8±0.34	H2O G	100 10 1	09±0.65 10±0.24 10±0.12
	Conc. Mg/ml	IZD mm		Conc. mg/ml	IZD mm		Conc. mg/ml	IZD mm		Conc. mg/ml	IZD mm
EtOH B	100 10 1	20±0.34 18±0.23 12±0.98	EtOH D	100 10 1	16±0.43 10±0.42 2±0.65	EtOH F	100 10 1	18±0.12 13±0.34 12±0.54	EtOH H	100 10 1	10±0.11 14±0.23 10±0.12

Antibacterial activity inhibition zone diameter (IZD):

Table 5: Antibacterial activity inhibition zone diameter (IZD), mm of Glochidion Talakonese extracts on Staphylococcus aureus, Escherichia coli, Salmonella enterica, and Enterobacter aerogenes in various solvents by disc diffusion method.



(a)



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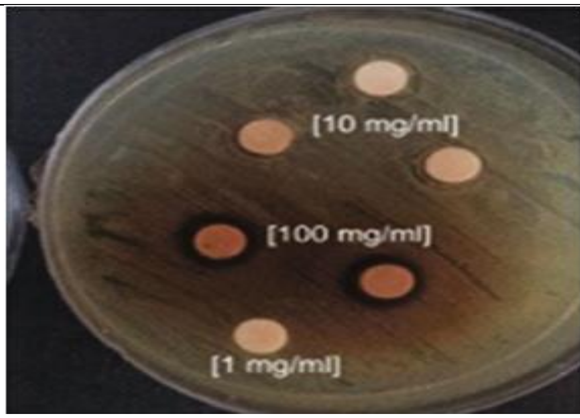

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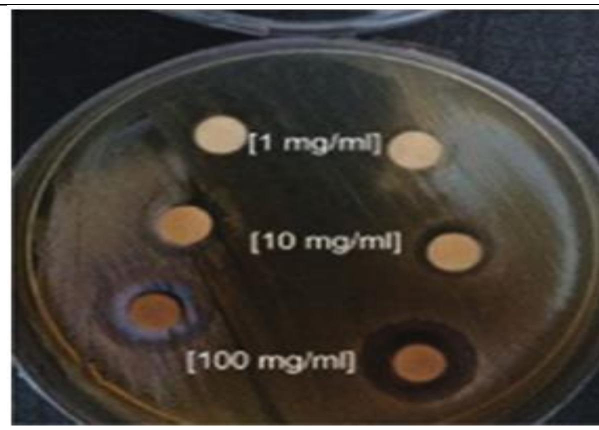

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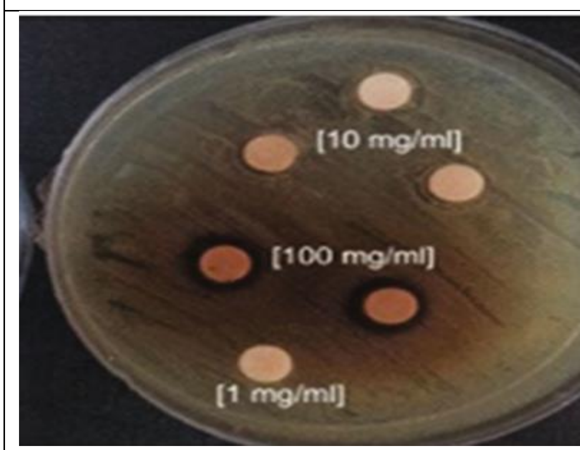

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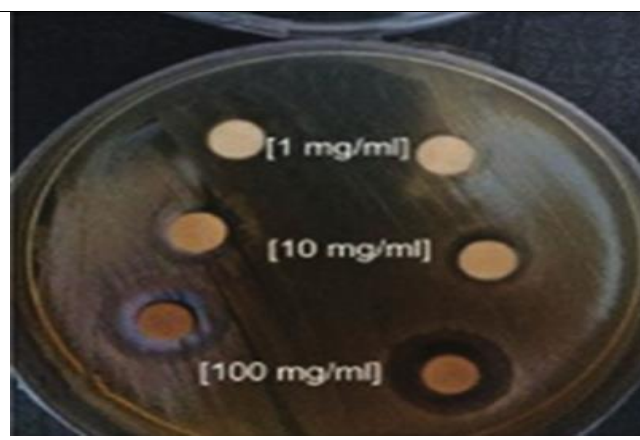

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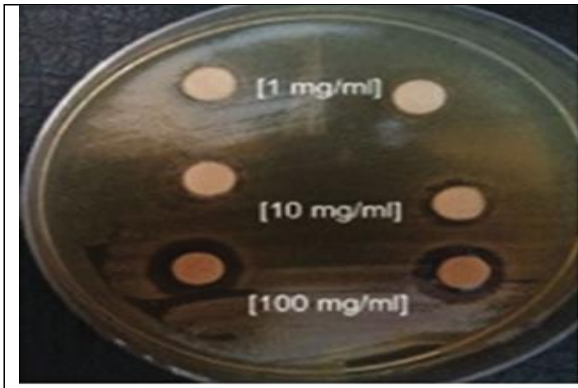	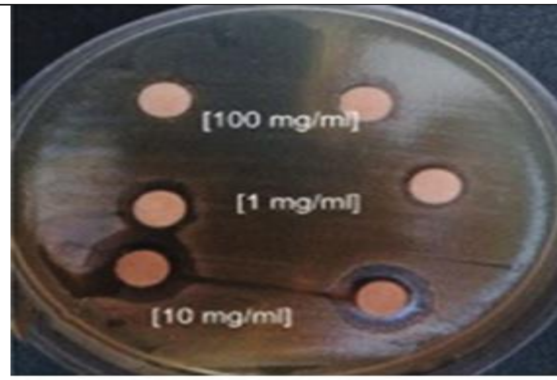
(g)	**(h)**

Figure 1: Inhibitory activity of Glochidion Talakonense Leaf Extracts on Staphylococcus aureus, Escherichia coli, Salmonella Enterica , and Bacillus Subtilis in various solvents by disc diffusion method, Escherichia coli (a) aqueous, (b) ethanolic. Staphylococcus aureus (c) aqueous, (d) ethanolic; Salmonella enterica (e) aqueous, (f) ethanolic,; and Bacillus Subtilis (g) aqueous, (h) ethanolic.

Antibacterial Activity of Glochidion *Talakonense*:

The test of antibacterial activity is carried out by measuring the clear zone developed in petri dish. The results showed that all extracts were effective in inhibiting the growth of bacteria except aqueous extract against Bacillus Subtilis and Staphylococcus aureus bacteria (Table 3). The diameter of the bacterial growth inhibition zone in control samples (Kanamycin) was larger than the inhibition zone of the extracts. Staphylococcus Aureus produced a significantly larger zone of inhibition for Kanamycin (23±0. 54mm) than others, while Salmonella Enterica produced a smaller zone of inhibition for Kanamycin (19±0 .65 mm). Produced inhibition zone by Escherichia coli and Bacillus Subtilis for kanamycin were 20 ±0:65 and 21±0.85 mm. Salmonella Enterica were significantly inhibited by ethanolic Extract (14±0:23) extract, while there is no inhibition zone for aqueous extract [30,31].

Furthermore, our findings disclosed that the extracts were more effective against Bacillus Subtilis and Staphylococcus Aureus as gram Positive bacteria and Escherichia Coli as gram Negative bacterium. However, extracts showed a good zone of inhibition against the growth of gram-positive bacterium. due to the presence of outer membrane of gram-negative bacteria and the enzymes of the periplasmic spaces which act as a barrier against numerous antibiotic molecules and can breakdown foreign molecules, respectively. According to results, *Glochidion Talakonense* is rich in tannins, terpenoids, alkaloids, and flavonoids, and Triterpenoids (glochidonol, Glochidiol , glochidone) in is effective *Glochidion Talakonense* to inhibit gram-positive bacteria. The statistical results of disc diffusion analysis presented the significance for all pathogens (Table 4). The extracts showed a good zone of inhibition ($p < 0:05$) against all of pathogens.

Results of MIC and MBC

To evaluate the effectiveness of the extracts to inhibit the growth of Staphylococcus aureus, Escherichia coli, Salmonella enterica, and Bacillus subtilis, MIC assay was employed. Low-MIC and high-MIC values indicate high and low activity of extracts against pathogens, respectively. As a result, the ethanolic extract had a considerable antibacterial activity with MIC value against Staphylococcus Aureus [32,33]. According to, the MIC value of the extracts agreed with their corresponding antibacterial activities[55]. The results of MIC revealed that Aqueous and ethanolic extracts have antibacterial activities against all pathogens, while aqueous extracts have a lower antibacterial effect due to the existence of nonpolar molecules in the extracts. the ethanolic extract showed higher inhibition against the pathogens, especially Escherichia coli (1.56 mg/mL). Also, ethanolic *Glochidion Talakonense* leaf extract displayed the lowest MBC against Staphylococcus aureus and Bacillus Subtilis (3.12 mg/mL). Moreover, reported to that using disk diffusion approach, aqueous extract of *Golchidion Talakonense* at the concentration of 100 mg/mL inhibited bacterial growth of Escherichia coli, Salmonella enterica, and Staphylococcus aureus with similar efficiency (similar inhibition zone sizes) [34,35].,

CONCLUSION

Aqueous and Ethanolic extracts of *Glochidion talakonense* showed an antibacterial effect on both gram-positive and gram-negative bacteria. Among the two solvents extracts, the ethanolic extract showed comparatively stronger antimicrobial activity than the aqueous extract, suggesting that ethanol was more effective in extracting the active phytochemicals responsible for bacterial inhibition. The extracts were particularly effective against Gram-positive bacteria (Staphylococcus aureus and Bacillus subtilis), while moderate activity was observed against the Gram-negative bacterium (Escherichia coli). In addition, results disclosed that the extracts were more effective against Escherichia coli as a gram-negative bacterium when compared with other pathogens. For Escherichia coli, a minimum inhibitory concentration (MIC) of 1.56 mg/mL was established. However, further investigations needed to be conducted to validate the biological ingredients and test these traditional medicinal plants' safety, efficacy, toxicity, and clinical evaluation.

Data Availability

Data is available on request.

Conflicts of Interest

All the authors declare that there are no conflicts of interest.

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