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Pharmacognostic Phytochemical and Antimicrobial Activity of *Rotula aquatica* Lour

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Abstract: The present work is focused on the preliminary characterization of phytochemical constituents of *R aquatica* and analyzing for its antimicrobial and antifungal activity. Roots and stem of *R aquatica* collected from river beds was dried and the phytochemicals were extracted using various solvents. Pharmacognostic investigation of the extract was carried out by standard method. Methanolic extract showing maximum phytochemicals was analyzed for inhibitory activity against selected bacterial and fungal culture. According to the result obtained, methanolic extract showed the presence of flavonoides, tannin, saponin, *terpenoids*, phenolic compound and glycosides. The extract showed inhibitory activity against both Gram positive and Gram negative bacteria as well as *Candida* sp indicating the broad spectrum of antimicrobial activity. MIC values were between 62.5-250 mg/ml for the pathogens tested. Overall the results indicate the potency of *R aquatica* extract in inhibiting the pathogens tested for wider application as microbicidal agent.

Keywords: *R aquatica*, Antimicrobial activity, MIC, Phytochemical, Methanolic extract.

I. INTRODUCTION

The emergence of bacterial resistance to various antibiotics has increased the rate of infectious diseases worldwide, accounting for more than 41% of the global disease [1]. Emergence of multidrug-resistant organisms has forced the scientist to search for new antimicrobial substance from various sources including medicinal plants [2]. Herbal extracts are found to be more efficient, safer and better-quality drugs with improved antibacterial and antifungal activities.

From time immemorial plants are being significantly used in traditional medication to cure various diseases. Although the tradition of using plants in medicine is known from ages but people do not have the scientific insight to explain and predict the curative action these plants. Human beings have learnt the application of plants extract as remedies which are not only cost effective but also safe for health. Hence medicinal plants have maintained their popularity and are widely used in different part of the world as primary health care needs. Plants with medicinal value also generate income and livelihood improvement [3]. For example, the annual sales of herbal-based medicines ranges from 7.5-108 billion US\$ worldwide [4] and in Canada annual market sales of medicinal plants reached 400 million US\$ in 2001 [5] and are growing at a pace of 15% annually [6].

Numerous plants have been analyzed for treatment of urinary tract infections, gastrointestinal disorders, and respiratory and skin diseases [7]. The medicinal values of plant lie in their phytochemicals composition such as alkaloids, flavonoids, phenolic compounds and others like amino acid, proteins, etc. A systematic search for useful bioactivities

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from medicinal plants is now considered to be a rational approach in nutraceutical and drug research. In this regard, various studies have been conducted globally to investigate the antimicrobial activities of different plant extracts [8,9].

Rotula aquatica, belongs to the family Borogeneaceae. It is found in India along the river bed of Netravati, Assam and throughout hilly regions of central and southern India. *R aquatica* is widely used by the indigenous and tribal people. In Ayurvedic and Unani, the plants is been used in various formulations to cure various ailments. Its medicinal properties lie in their active phytoconstituents. It is reported to contain steroids, alkaloids and allantoin. In many Ayurvedic formulations *Rotula aquatica* is mainly used for kidney stone, bladder stone and other venereal ailments treatment. Patil et al. has reported antimutic activity in the root extract of *Rotula aquatica*. A root decoction of *Rotula aquatica* has been reported to exhibit diuretic activity and it is also used in the treatment of piles and in venereal disease [10,11]. In the present study, we report the phytochemical composition of *Rotula aquatica* Lour and the antimicrobial potential of various solvent extract against clinical pathogens.

II. METHODOLOGY

Plant Material Collection and Processing

The roots and stem of *Rotula aquatica* Lour were collected from Nethravati river bed, Ujjure region of Karnataka state. The identity of plant was authenticated from Dr. Harsa Hegde, Scientist, Regional Medical Research Centre (ICMR), Belgaum India. Initially, root and stem was analyzed for morphological and organoleptic characteristics. Root and stem was thoroughly washed with water to remove dust particles and was dried under shade. The coarse powder was then passed through sieve no 40 to get uniform powder. Then the uniform powder was subjected to standardization with different parameters and extraction for antimicrobial activity.

Pharmacognostic Analysis

Pharmacognostic analysis of *R aquatica* was carried out according to Kokate [12].

Determination of foreign matter:

Plant material (50 g) was spread into a thin layer and the foreign matter was sorted into groups either by visual inspection using magnifying lens (6X or 10X) or with help of suitable sieve. Shift the remainder of sample through a No. 250 sieve. Dust is regarded as mineral mixture. The shorted portion of foreign matter was weighed. Similarly the content in each group was weighed and expressed as per 100 g of air dried powder.

Determination of percentage of ash, acid insoluble and water soluble ash:

Accurately weighed crude powder (2-3 g) was taken in a preweighed silica dish. The dish was incinerated by gradually increasing the heat until free from carbon, cooled and then weighed. The percentage of ash was calculated with reference to the air dried drug.

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$$\text{Percentage ash} = \frac{(\text{weight of the dish after incineration} - \text{weight of the empty dish})}{\text{weight of the powder taken}} \times 100$$

Acid-insoluble ash was obtained by boiling the obtained ash for 5-10 min with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a crucible, washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated as follows:

$$\text{Percentage of acid insoluble ash} = \frac{(\text{weight of residue})}{\text{weight of the powder taken}} \times 100$$

Water soluble ash was determined by boiling the obtained ash for 5-10 min in water. The insoluble matter was collected in a crucible, washed with hot water, ignited and weighed. The percentage of water soluble ash was calculated as follows:

$$\text{Percentage of water soluble ash} = \frac{(\text{weight of residue})}{\text{weight of the powder taken}} \times 100$$

Extraction using Various Solvent

The air dried root and stem powder was extracted with 100 ml each of petroleum ether, ethyl acetate, methanol and water by cold maceration. The extraction was done for 3 days. After extraction, the extract was separated by filtration. Filtrate was preserved in a well closed container. Residue left after extraction was again subjected for extraction for 3 more days with the same amount of fresh solvent. The process was repeated once again i.e., the powder was extracted 3 times with a gap of 3 days each. The filtrates were pooled and dried under reduced pressure using rotary vacuum evaporator. The dried residue was stored in a desiccator until use.

Loss on Drying

About 2-3 g of powder is accurately weighed in a Petridish and kept in a hot air oven maintained at 110°C for 4 h. After cooling in a desiccator, the loss in weight was recorded. This procedure was repeated till constant weight was obtained.

$$\text{Loss of drying } (\%)(\text{LOD}) = \frac{\text{loss in weight}}{\text{weight of the drugs in g}} \times 100$$

Phytochemical Study of *R aquatica* Extract

Phytochemical analysis of *R aquatica* was carried out according to the methods described by Harborne [13].

Test for alkaloids:

Dragendroff's test: Few drops of dragendroff's reagent was added to 2-3 ml of extract and observed for orange brown precipitate.

Mayer's test: Few drops of Mayer's reagent was added to 2-3 ml of extract and observed for precipitation.

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Test for tannins and phenolic compound:

Lead acetate test: Few drops of lead acetate solution was added to 2-3 ml of extracts and observed for white precipitation.

Ferric chloride test: Few drops of 5% FeCl₃ was added to 2-3 ml of extract and observed for deep blue black color.

Test for terpenoids:

Chloroform (1 ml) and acetic anhydride (1 ml) were added to 2-4 ml of extract. Concentrated sulphuric acid (2 ml) was added along the sides of test tubes and observed for the formation of reddish violet color ring at the junction.

Test for carbohydrates:

Molish's test: Few drops of α -naphthol solution in alcohol were added to 2-3 ml of extract and vortex. Conc H₂SO₄ was added along the sides of the test tubes and observed for violet ring formation at the junction of two liquids.

Test for reducing sugars:

Fehling's Test: 1 ml each of Fehling's A and Fehling's B solutions were mixed and boiled for 1 min in water bath. Equal volume of extract was added and boiled for 5-10 min and observed for the formation of yellow to brick red precipitate.

Benedict's test: Equal volume of Benedict's reagent and extract were mixed and boiled for 5 min. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test extract.

Test for amino acids:

Ninhydrin test: 3 ml of extract was mixed with 3 drops of 5% Ninhydrin solution and boiled in water bath for 10 min. Development of purple or bluish color was observed.

Test for cardiac glycosides:

Legal's test: Extract was mixed with 1 ml of pyridine and 1 ml of sodium nitroprusside and observed for the development of pink to red color.

Kellar killani test: 2 ml of extract was mixed with glacial acetic acid, one drop of 5% FeCl₃ and concentrated H₂SO₄. The reaction mixture was then observed for formation of reddish brown color at the junction of the two liquids and upper layer of bluish green.

Test for saponin glycosides:

Foam test: The extract was shaken vigorously with water and persistent foam was observed.

Test for flavonoides:

Lead acetate test: 2ml of extract was mixed with lead acetate solution and observed for yellow colored precipitate.

Alkaline test: 2ml of extract was mixed with increasing amount of sodium hydroxide until yellow coloration. Mixture was observed for decolorization after addition of acid.

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Antimicrobial Activity of *R aquatica* Extract

Bacterial cultures and growth condition:

The clinical isolates namely, *Staphylococcus aureus*, *Streptococcus* sp, *E. coli*, *Proteus* sp, *Klebsiella* sp, *Pseudomonas* sp, *Citrobacter* sp, *Enterobacter* sp, *E.coli* ATCC25922 and fungal isolates *Candida* sp and *Candida albicans* MTCC180 were used in the present study. The bacterial pathogens were grown in Muller-Hinton agar at 37°C and the fungal cultures were grown in Sabourds Dextrose (SD) media at 27°C.

Disc Diffusion Assay

Antimicrobial activity was performed by standard disc diffusion method [14]. Muller-Hinton Agar and Sabourds Dextrose (SD) Agar base plates were swabbed with the bacterial and fungal inoculum respectively. 0.5 Mc Farland concentration inoculum size 1×10^6 CFU/ml for bacteria and 1×10^7 cell/ml for *Candida* spp was taken. Petroleum ether, ethyl acetate, methanol and aqueous extract were impregnated on sterile filter paper disc (Whatman No. 1, 6 mm in diameter) at three different concentrations 1250 µg/disc (50 mg/ml) 2500 µg/disc (100 mg/ml) and 5000 µg/disc (200 mg/ml) [15,16]. Disc were left to dry for two hours in laminar air flow so as to remove residual solvent which might interfere with the determination [17]. Extract disc were then placed on the seeded agar plates. Each extract was tested in triplicate with Ciprofloxacin 30 µg per disc and fluconazole as standard for bacteria and fungi respectively. Negative control was prepared using respective solvents. The plates were kept at 4°C for 1 h for diffusion of extract and then incubated at 37°C for bacteria (24 h) and 27°C for fungi (48 h). Inhibition zone around the disc was measured and expressed as mm in diameter.

Minimum Inhibition Concentration (MIC)

MIC was performed according to NCCLS methodology and Zgoda and Porter with slight modification. Briefly, extract was dissolved in respective solvent (500 mg/ml) and then two fold serially diluted to give a range of 250 – 15.63 mg/ml [18,19]. Tests were performed in sterile U-bottom 96 wells by adding into each well 95 µl of muller Hinton broth for bacteria and SD Broth for fungi, 5 µl of inoculum (0.5 Mc Farland turbidity) was taken to this 100 ml of test extract was finally added to each well to get a final volume of 200 µl. Standard amoxicillin/ clavlanic acid was used as positive control. Flucoconozol was used as positive control for fungi. Broth and inoculum without extract was taken as negative control. Turbidity was measure at 620 nm.

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III. RESULTS AND DISCUSSION

The search for potential antimicrobial compounds from plants is a thrust area of research. Herbal extracts are capable of modulating cell systems including inhibition of cell membrane synthesis, cell wall disruption, nucleotide inhibition or may affect protein synthesis. These activities suggest for possible antimicrobial and antifungal activity.

Plants are well known reservoirs of valuable phytochemicals. Many plants have been investigated worldwide for their phytochemical constituents and their activity against various pathogens. The present study has been carried out to evaluate the phytochemical composition of *R aquatica* and antimicrobial activities of methanol, petroleum ether, ethyl acetate and water extracts of roots and stem.

Phytochemical Analysis of *R aquatica* Extract

Initially, dried powder of *R aquatica* root and stem was investigated for morphological characteristics. The course powder was pale yellow colored with characteristic odor, bitter taste and rough or fibrous texture. Table 1 represents the preliminary characteristics of *R aquatica* powder.

Table 1. Preliminary characteristics of *R aquatica* powder

SI No	Tests	Parameter	Observation
1	Physical	Nature	Course powder
		Color	Pale yellow
		Odor	Characteristic
		taste	Slight bitter
2	Extraction value (%w/w)	Petroleum ether	10
		Ethyl acetate	12
		Methanol	24
		Aqueous	16
3	Loss on drying (%w/w)		2
4	Ash values (%w/v)	Total ash	6.66
		Acid insoluble ash	0.33
		Water soluble ash	0.66
5	Fluorescence analysis		No fluorescence
6	Foreign matters		Nil

Phytochemical analysis updates the knowledge on medicinal value of plants. Further, based on pharmacognostic studies including anatomical characters and chemical composition plants have been differentiated for traditional medicine. Hence the chemical components of *R aquatica* powder were extracted with various solvent to unveil its composition (Table 2). Methanol extract showed maximum yield (6.37%) followed by aqueous extract (5.62%).

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Table 2. Percentage yield of the extract

SI No	Extract	Nature	Weight	Yield (%)
1	Petroleum ether	Semi solid	7.4	1.48
2	Ethyl acetate	Semi solid	16.2	3.2
3	Methanol	Solid	31.8	6.37
4	Aqueous	Solid	28.1	5.62

Phytochemical analysis of solvent extracts of *R aquatica* demonstrated the presence of various chemical compositions which are depicted in Table 3. The data show the presence of tannins, flavanoids, *terpenoids*, anthraquinones, saponins and cardiac glycosides in methanolic extracts of *R aquatica*, whereas in aqueous extract only tannins, saponin and *terpenoids* were identified. However, the petroleum ether and ethyl acetate extract showed negative results for all the tests conducted. Methanolic extract also showed positive results for reducing sugars, saponin, terpenoids, phenolic compound and tannin tests.

Table 3. Phytochemical composition of *R aquatica* extract

SI No	Parameter	Test	Extract			
			Petroleum ether	Ethyl acetate	Methanol	Aqueous
1	Carbohydrates	Molish's test	-	-	+	-
2	Reducing sugars	Fehling's test	-	-	+	-
		Benedict's test	-	-	+	+
3	Amino acids	Ninhydrin test	-	-	+	-
4	Glycosides					
	Cardiac glycosides	Legal's test	-	-	+	-
		Killer killanis test	-	-	+	-
	Antherquinine glycosides	Borntrager's test	-	-	+	-
Saponin glycosides	Foam test	-	-	+	+	
5	Flavonoides	Alkaline reagent test	-	-	+	-
		Lead acetate test	-	-	+	-
6	Tannins and phenolic compound	FeCl ₃ test	-	-	+	+
		Lead acetate test	-	-	+	+
7	Terpenoides	Liebermann burchard test	-	-	+	+

Qualitative phytochemical analysis of *R aquatica* powder showed that only the methanolic extracts contain maximum number of phytoconstituents compared to other solvent system. This observation may be due to higher solubility property of methanol for different phytoconstituents present in the plant species [20-22]. It is clear from the present study that the isolation of antimicrobial compound present in the plant material is largely dependent on the type of solvent used in the extraction procedure.

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Antimicrobial Activity of Methanolic Extract of *R aquatica*

As the maximum phytoconstituents was observed in methanolic extract, the same was used to check the inhibitory activity against various pathogens. According to the results obtained, the antimicrobial activity against the test bacteria showed varied levels of inhibition. The results of zone of inhibitory activity of methanolic extract at three different concentration (50, 100 and 200 mg/ml) against the pathogenic bacteria are shown in Fig. 1 and Table 4. The results of antimicrobial activity of the methanol extract showed broad spectrum of activity against tested organisms. Ciprofloxacin 30 µg used as positive control depicted higher antimicrobial activity. However, inhibitory activity of methanolic extract was found to be dose dependent showing increase in the zone of inhibition with increase in the concentration. Similarly, the methanolic extract showed maximum inhibition zone against *Candida* sp at 200 mg/ml concentration as compared to other extracts (Table 5). Flucanazole, used as positive control showed prominent inhibition zone (24.67 mm) at a concentration of 10 µg/ml.

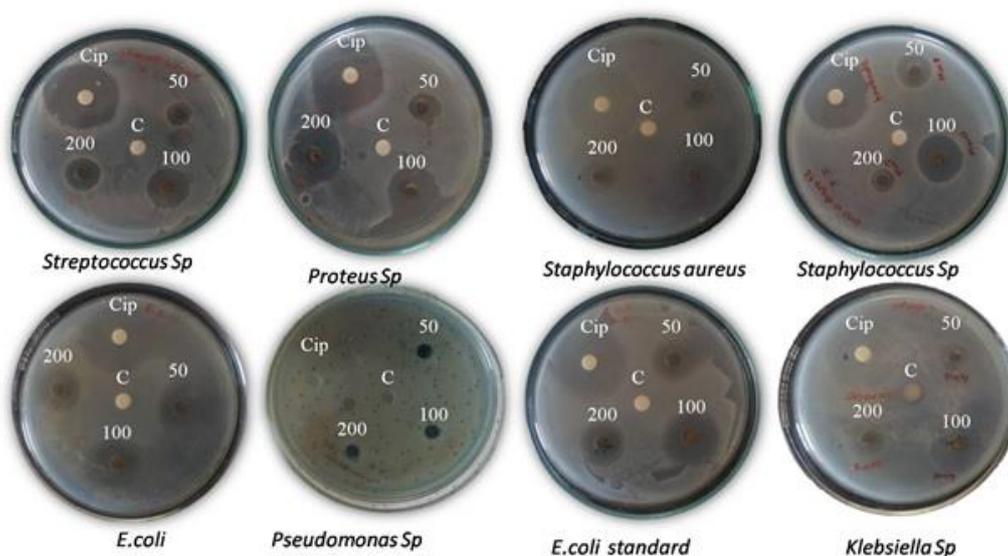


Fig. 1. Antibacterial activity of methanolic extract of *R aquatica* at different concentration methanolic extract (50, 100 and 200 mg/ml), Cip: Ciprofloxacin 30 µg, C: control methanol

Table 4. Antibacterial activity of methanolic extract of *R aquatic*

Test Pathogen	Methanolic extract			Ciprofloxacin (30 µg)
	50 mg	100 mg	200 mg	
<i>Staphylococcus aureus</i>	2.00 ± 0.00	6.17 ± 0.14	10.05 ± 0.06	27.00 ± 0.00
<i>Streptococcus sp</i>	2.00 ± 0.00	7.33 ± 0.21	11.75 ± 0.06	25.00 ± 0.00
<i>E.coli</i>	4.50 ± 0.39	5.20 ± 0.31	11.80 ± 0.46	20.00 ± 0.00
<i>Proteus sp</i>	3.33 ± 0.52	6.20 ± 0.09	11.60 ± 0.35	21.00 ± 0.00

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<i>Klebsiella</i> sp	4.00 ± 0.00	7.43 ± 0.26	12.25 ± 0.06	24.00 ± 0.00
<i>Pseudomonas</i> sp	3.37 ± 0.29	5.53 ± 0.29	10.65 ± 0.06	27.00 ± 0.00
<i>Citrobacter</i> sp	3.27 ± 0.27	5.83 ± 0.05	13.50 ± 0.12	28.00 ± 0.00
<i>Enterobacter</i> sp	4.00 ± 0.00	7.67 ± 0.52	12.00 ± 0.00	28.00 ± 0.00
<i>E.coli</i> 25922	3.33 ± 1.03	8.00 ± 0.00	10.00 ± 0.00	27.00 ± 0.00

Table 5. Antibacterial activity of methanolic extract of *R aquatica*

Test Pathogen	Extraction solvent	50 mg	100 mg	200 mg	Flucanzole
					(10 µg/ml)
<i>Candida</i> sp	Petroleum ether	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	24.67 ± 1.03
	Ethyl acetate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	24.67 ± 1.03
	Aqueous extract	1.00 ± 0.01	4.00 ± 0.10	6.00 ± 0.02	24.67 ± 1.03
	Methanol extract	5.00 ± 0.11	9.00 ± 0.09	16.00 ± 0.09	24.67 ± 1.03
<i>Candida albicans</i> -MTCC 183	Petroleum ether	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	24.67 ± 1.03
	Ethyl acetate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	24.67 ± 1.03
	Aqueous extract	1.00 ± 0.11	4.00 ± 0.09	6.00 ± 0.02	24.67 ± 1.03
	Methanol extract	5.00 ± 0.03	9.00 ± 0.06	16.00 ± 0.70	24.67 ± 1.03

Currently, the determination of antimicrobial activity of various plant extracts is of great interest due to the worldwide issue of increasing microorganisms's resistance against antibiotics. In this view, the study was undertaken to demonstrate the antimicrobial activity of methanolic extract of *R aquatica*. Accordingly, the data represents a dose dependent increase in the inhibitory spectrum against bacterial cultures and fungal pathogen tested. Similarly, Prashanthi et al. studied antimicrobial activity of aqueous root extract of *R aquatica* against gram positive as well as negative pathogens and found dose dependent activity against *Bacillus cereus*, *Salmonella abony* and *Klebsiella pneumonia* [23]. Aswathanarayan and Vittal studied antimicrobial activity of methanolic extract of medicinal herbs *R aquatica* and *Ancistrocladus hyneanus* [24]. The extracts showed marked antibacterial activity against various food borne bacteria including *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumonia*, *Shigella flexneri* and *Salmonella typhi*. Raj and Joseph have also studied antibacterial activity of various extract of medicinal herbs against urinary tract infection causing bacteria and found *Eupatorium odoratum* and *Rotula aquatica* is the most effective extract against *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [25].

Previous studies have shown that most of the plant extracts are active against Gram positive bacteria than Gram negative bacteria [26-28]. However, methanolic extract of *R aquatica* showed inhibitory activity against both gram positive and gram negative bacteria and also against fungal species tested. The broad spectrum inhibitory activity of

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methanolic extracts might be due to the involvement of more than one active ingredient which has biological significance, which is in agreement with Ming et al. [29].

Minimum inhibitory concentration of methanolic extract against clinical pathogens was carried out with concentrations ranging from 15.63-250 mg/mL (Fig. 2). The MIC values against each pathogen is presented in Fig. 3. A concentration of 62.5 mg/ml of methanolic extract was efficient in inhibiting *Citrobacter* spp, whereas a concentration 250 mg/ml was required to inhibit *Staphylococcus aureus*, *E. coli*, *Proteus* sp, *Klebsiella* sp, *Enterobacter* and *E. coli* 25922.

Consequently, MIC of methanolic extract against *Candida* spp was found to be 125 mg/ml (Fig. 4). However Fluconazole, the standard antifungal drug exhibited MIC of 8 µg/ml (Fig. 4).

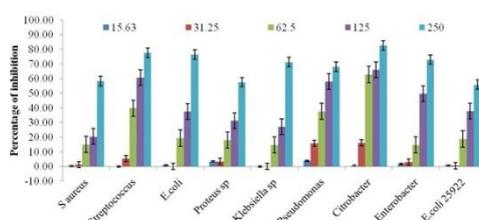


Fig. 2. Percentage inhibition of clinical bacterial pathogens by various concentration of methanolic extract (15.63 -250 mg/ml). Values are mean ± SD of triplicate values

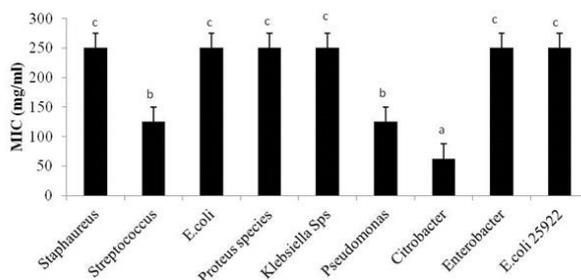


Fig. 3. Minimum inhibitory concentration of methanolic extract to inhibit the tested clinical bacterial pathogens. Values are mean ± SD (n=3). Mean values with different lowercase are significantly different ($p < 0.05$) according to Duncan multiple range test

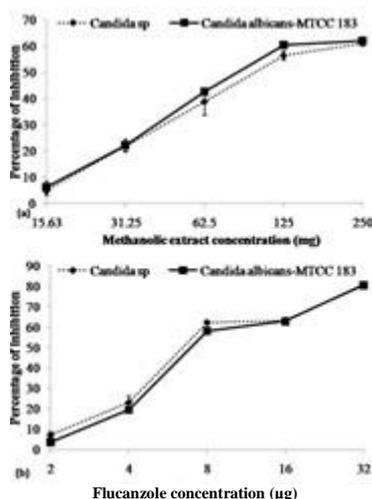


Fig. 4. Percentage of inhibition of *Candida* spp. at different concentration of (a) methanolic extract (50, 100 and 200 mg/ml) (b) Standard antifungal agent fluconazole (2-32 μ g/ml)

The present study reveals the antimicrobial activity of methanolic extracts of *R aquatica* against the tested pathogens. This antimicrobial activity of might be due to the presence of various phytochemicals analyzed including alkaloids, tannins, phenols, flavonoids and glycosides. Similarly, earlier studies have also reported the role of phytochemicals in the inhibitory activity against various pathogens [30,31]. MIC in the range of 100-1000 mg/mL is considered and classified as antimicrobials [32]. In the present study, the MIC value was between 62.5 to 250 mg/ml which indicate the efficiency of the methanolic extract in attenuating the pathogens tested.

IV. CONCLUSION

The excessive use of antibiotics provide a way for secondary ailments and resistance among different bacterial species, hence there is an urgent requirement for replacement of chemical antibiotics with natural remedies because of their non toxicity and lesser side effects and better cure. In this regard, the inhibitory spectrum of methanolic extract against tested pathogens indicates the great potential of *R aquatica* in treating infectious diseases. The present study confirms that fractions of *R aquatica* have significant antibacterial and antifungal activity along with valuable phytochemicals. These results recommend the application of *R aquatica* leaf component extracts in human protection against the test pathogenic bacteria. However, further research is required for the isolation and purification of the bioactive substances and for the determination of their respective antibacterial potencies with the view to formulating novel microbicidal agents.

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VI. CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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