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PHYTOCHEMICAL SCREENING, ANTIBACTERIAL AND FREE RADICAL SCAVENGING ACTIVITY OF THE FRUIT AND PEEL EXTRACTS OF *TRAPA BISPINOSA* (WATER CHESTNUT)

ANJALI B THAKKAR[1], SWATI K. KURTKOTI*[2] AND NANDIBATLA V.SASTRY[3]

[1]Indukaka Ipcowala Center for Interdisciplinary Studies in Science and Technology, Sardar Patel University, Vallabh Vidyanagar, Gujarat, India.
[2]Indukaka Ipcowala College of Pharmacy, New Vidyanagar-388121, Gujarat, India
[3]Head of the department, Department of Chemistry, Sardar Patel University Vallabh Vidyanagar Anand, Gujarat, India

ABSTRACT

Trapa bispinosa, commonly known as the water chestnut, is a widely used edible plant found in Asian sub-continents and parts of Africa. The entire plant is believed to have large number of health benefits and disease fighting properties. With the rising resistance to synthetic antimicrobial agents, a need for finding newer source of medicine is increasing. The aim of the present work was to investigate the antibacterial and free radical scavenging activity of various extracts of fruit and peel of the *Trapa bispinosa.* Water, methanol and acetone were chosen as the solvents to carry out the extraction. Extraction was done using cold maceration technique. Preliminary investigation of phytochemicals showed the presence of major secondary plant metabolites such as alkaloids, flavonoids, glycosides, coumarins. Based on the phytochemical screening, methanol was selected as the choice of solvent as it showed greater extraction of phytochemicals. The extracts were further tested for antimicrobial and antioxidant activity. The antimicrobial activity was done against both Gram positive and Gram negative bacteria. The methanolic extract showed highest activity, almost double, against the *Gram positive bacteria* than the acetone and aqueous extracts. However, none of these extracts were effective against *the Gram negative bacteria.* Furthermore, it was observed that the antimicrobial activity of the peel extract was higher as compared to the fruit. The antioxidant activity was carried out by the Hydrogen peroxide scavenging method and it was found that the peel has more anti-oxidant activity (27.08%) compared to the fruit which showed only 18.75% activity. From the anti-microbial study and the antioxidant study, it was thus concluded that the methanolic extract of the peel of *T. bispinosa* is more potent antimicrobial and antioxidant agent as compared to the methanolic extract of fruit of *T. bispinosa.* The presence of secondary plant metabolite in the peel makes it a powerful antimicrobial against the gram positive bacteria.

KEYWORDS: ***Trapa bispinosa, water chestnut, phytochemical screening, Antibacterial activity, Free radical scavenging activity***

SWATI K. KURTKOTI*

Indukaka Ipcowala College of Pharmacy, New Vidyanagar-388121, Gujarat, India.

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INTRODUCTION

Modern day allopathic medication antibiotics have contributed to major gain in life expectancy over the last century. However, due to the emergence of multi-drug resistant pathogens and the rapid development of resistance to drugs by micro-organisms, the clinical efficacy of the existing antibiotics is being threatened.[1] The reactive oxygen species (ROS), produced as a result of cellular metabolism are highly reactive and toxic causing many chronic health diseases. This can be prevented with the help of an antioxidant activity. The antioxidants scavenge the free radicals due to their redox hydrogen donators and singlet oxygen quencher and thereby does not allow the degeneration of lipids, proteins and nucleic acid.[2-4] Many synthetic compounds such as Butylated Hydroxyl Toluene, Butylated Hydroxyl Anisole and Tetra-butyl-hydroquinone are being used as antioxidants.[5] However, considering the high level side effects associated with these substances, need for better natural antioxidants with fewer or no side effects is desirable. The ethnomedicine is generally found to be cheaper and more effective than the modern medicine against certain ailments. These are also safer to use as they have relatively lesser side effects than the synthetic equivalents.[6,7] A large number of plants produce secondary metabolites as part of their defense mechanism and protect them from various diseases. These are identified as phytochemicals. Many researchers have identified the use of these phytochemicals for the prevention and treatment of several diseases. Curing diseases using medicinal plant is widely prevailing in many countries in the name of traditional medicines.[8] The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds. A correlation between the phytochemical and the bioactivity of that constituent becomes essential for further use in treatment of many health conditions. *Trapa bispinosa* Roxb, family Trapaceae, commonly known as water chestnut or singhara, is an aquatic plant found in freshwater wetlands, lakes ponds, and sluggish reaches to rivers in India. [9-11] The aquatic plant contains 30 different species found in tropical and subtropical and temperate zone of the world.[12] It is cultivated mainly for its edible fruit in India, China, Southeast Asia and tropical Africa. The medicinal values of the whole herb have been well recognized in folklore medicines as a cure for various diseases. The aim and objective of the present study was to screen the extracts of the fruit and the peel of water chestnut for the presence of different phytochemicals and to determine the potential of various extracts of the fruits and the peels for antibacterial efficacy and natural antioxidants in order to fully utilize the local fruit and its non-edible peel.

MATERIALS AND METHOD

Plant materials

The fruit *Trapa bispinosa* (water chestnut) was purchased from the local market in Anand, Gujarat, India. The fruits were washed thoroughly under running tap water and dried at room temperature. The peel was separated manually with the help of scrapers. The fruit and peel were powdered with grinder and stored in desiccator until further use.

Preparation of extracts

Extracts were prepared by infusion extraction method with some modifications.[13] 50 g of sample was taken and separately soaked in 250 ml of Acetone, Methanol and distilled water for 24 hr. under stirring at 100 rpm using a magnetic stirrer. Extracts were then filtered using Whatman filter paper No.1 and centrifuged at 3000 rpm for 10 min to remove any solid debris. The supernatant was collected and concentrated by solvent recovering assembly (J-Sil, India), dried completely at room temperature and stored it in a refrigerator until further use.

Screening of Phytochemicals

Qualitative analysis was carried out by using standard procedures described earlier (Khandelwal, 2008 and Zhanam Z. et al., 2015)[14,15], to identify the presence of various constituents in crude extracts of fruit and peel parts prepared in Acetone, Methanol, and Distilled Water.[14,15]

Dragendroff's Test

Ethanolic extract of the plant (2 ml) was taken in a test tube and dilute hydrochloric acid (0.2 ml) was added to it. 1 ml of Dragendroff's reagent was added to the test tube and observed for the development of orange brown precipitate.

Wagner's test

The extract equivalent to 50 mg was taken in a test-tube. To it few ml od dil. HCl was added and stirred. This was then filtered. To the test-tube few drops of Wagner's reagent was added from the sides by inclining the test-tube. The test-tube was observed for formation of reddish-brown precipitates.[14,15]

Alkaline reagent test

In test tubes approximately 2ml of extract was taken and few drops of sodium chloride was added. This produced intense yellow colour. To the same test-tubes dil HCl was added and observed for disappearance of yellow colour.[14,15]

Salkowski's test

To approximately 0.5mg of extract, few ml of chloroform was added. To the chloroform containing test-tubes concentrated sulphuric acid was added slowly from the sides by slanting the test-tubes in order to form two separate layers. The test-tube was observed for formation of reddish brown colour at the interface of the two layers. [14,15]

Ferric chloride test

About 50 mg of each of the extracts were dissolved in approximately 5 ml of distilled water in separate test-tubes. To each test tube 2-3 drops of 5% ferric chloride solution were added and stirred. The test-tubes were observed for colour development. The presence of phenolic compounds would yield a bluish black colour to the test solution. [14,15]

Froth test

In a graduated cylinder about 50 mg of the extract was taken. To this distilled water was added and the voulme made up to 20 ml. The resulting suspension was shaken by shaking the graduated cylinder for 15 min. After 15 min the cylinder was observed for froth formation. A stable layer of froth indicates the the presence of saponins.[14,15]

Keller-Kiliani's test
About 50 mg of extract was taken in test-tubes. To the test-tubes 2 ml of glacial acetic acid containing one drop of 5% ferric chloride solution was added. This was followed by slow addition of 1 ml of concentrated sulphuric acid. The test-tubes were observed for formation of a brownish ring at the interface and a violet coloured ring just below the brown ring. The acetic acid layer was also observed for appearance of greenish ring.[14,15]

Molisch's test
The extract were taken in different test tubes and a small amount of Molisch's reagent (α-naphthol dissolved in ethanol) was added in each of the test tubes. The samples were mixed by stirring. few ml of concentrated sulfuric acid was added from the sides of the test tube by inclining the test-tubes slowly in order to form two separate layers. Care was taken to avoid mixing of the two layers. The test tube was observed for the appearance of a purple ring at the interface between the acid and the test layers.[14,15]

Biuret test
Appromimately 50 mg of each of the extracts were taken in the test-tubes and diluted with distilled water. To each test-tube few ml of Biuret reagent was added and allowed to react and observed for development of pink colour. Pink colour indicats the presence of protein.[14,15]

Brontager's Test
The extracts were taken in separate test-tubes and 1 ml of sulphuric acid was added. The test-tubes were heated for 5 minutes and filtered while hot. The filtrate was allowed to cool. To the filtrate equal volume of chloroform was added and shaked. The lower chloroform layer was separated and collected. The chloroform layer was then shaken with half the volume of dilute ammonia. The ammoniacal layer was observed for the development of red color.[14,15]

Test for coumarin
A small amount of sample was placed in a test tube and covered with a filter paper moistened with dilute sodium hydroxide solution. The covered test tube was heated in a water bath for several minutes. The paper was then removed and exposed to ultraviolet (UV) light, and observed for green fluorescence.[14,15]

Antimicrobial Activity
Microbes
Microbial pure cultures of *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli,* and S*almonella typhi* were obtained from MTCC (Microbial Type Culture Collection, Chandigarh). The bacterial cultures were grown on nutrient agar medium (Hi-media, pH 7.4) at 37°C and maintained at 4°C. The Antimicrobial activity was carried out at B. R. Doshi School of Bioscience, Sardar Patel University.

Inoculum preparation
A fresh microbial suspension was prepared by sub culturing the bacterial colonies in the nutrient broth medium (Hi Media pH 7.4) and incubated at 37°C in order to maintain approximately uniform growth rate of each organism. The bacterial cultures from fresh media were compared with 0.5 Mc Farland turbidity standard, which is equivalent to approximately 1×10^8 bacterial cell density and was used throughout the experimentation.[16]

Cup Plate method
The antimicrobial activity of the extracts extracted from the fruit and peel tested in different solvents was screened by agar well diffusion method. The petri plates were sterilized in an autoclave at 15 lbs pressure for 15 min. Approximately 20 mL of sterilized nutrient agar medium was poured into a petri plate in the sterile condition in Laminar air flow cabinet and then allowed to solidify at room temperature. The prepared agar plates were then marked and divided into 3-4 equal parts and labeled with organism and extract name. Fresh bacterial culture inoculums of 100 µL having 1×10^8 CFU/mL were spread on agar plates with glass spreader. A well of 8 mm diameter was punched off at previously marked petri plates into agar medium with sterile cork borer and then was filled with 100 µL of each plant extract. Plates were placed in a refrigerator for 30 min. for pre diffusion of plant extract and then incubated at 37°C for 24 h for bacteria until the appearance of inhibition zone. After incubation all the plates were examined and zone of inhibition (excluding well diameter) was measured as a property of antibacterial activity. Streptomycin (25µg/mL) used as a positive control and 100% of methanol, acetone and distilled water negative control were used for bacteria. Bioassay was carried out in duplicate and repeated twice.[17,18] The results were measured and expressed in terms of zone of inhibition (ZI) of bacterial and fungal growth around each disc in millimetres as low activity (1–6 mm), moderate activity (7–10 mm), high activity (11–15 mm), very high activity (16–20 mm), no activity (-).[19]

Hydrogen peroxide scavenging assay
The ability of the *T. bispinosa* extracts to scavenge hydrogen peroxide was determined according to the method adopted by Ruch et al., 1989.[20] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (100µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM). The absorbance of hydrogen peroxide was measured after 10 mins of addition of test at 230 nm by UV-Visible spectroscopy. The phosphate buffer without hydrogen peroxide was used as a blank solution. The percentage of hydrogen peroxide scavenging of both *T. bispinosa* extracts and standard compounds were calculated:

$$\textbf{\% Scavenged } [H_2O_2] = [(AC - AS)/AC] \times 100$$

Where AC is the absorbance of the control and AS is the absorbance in the presence of the sample of *T. bispinosa* extracts or standards.[21-24]

STATISTICAL ANALYSIS

Two-way analysis of variance (ANOVA) was applied to the data obtained from the anti microbial study (zone of inhibition) of the fruit and peel extract of *T. bispinosa* to study the effect of different solvents on anti-microbial activity. All data are expressed as mean ± standard deviation (SD) of triplicates. A statistically significant difference was considered at $p < 0.05$. To determine whether there were any differences among the means in the determination of anti-oxidant activity of the fruit and peel extract of *T. bispinosa*, one-way analysis of variance (One–way–ANOVA) was applied to the results. All statistical analyses were carried out using Minitab 18.1.0.

RESULT AND DISCUSSION

Screening of Phytochemical

The results of Phytochemical screening of different extracts of the fruit and peel of *T. bispinosa* are shown in Table 1.

Table 1

Phytochemical analysis of fruit and peel extracts of T. bispinosa.

Phytochemicals	Methanol extract		Acetone extract		Aqueous extract	
	Fruit	Peel	Fruit	Peel	Fruit	Peel
Alkaloid	+	+	−	−	−	−
Flavanoids	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Saponins	−	−	−	−	−	−
Cardiac glycoside	−	−	−	−	−	−
Anthraquinone	+	+	+	+	+	+
Carbohydrates	+	−	−	−	+	+
Proteins	−	−	−	−	−	−
Coumarins	+	+	+	+	+	+

+ = Present − = Absent

Phenolic compounds such as flavonoids, coumarins and tannins have been reported for health benefits as they are associated with antioxidant, anti-allergic, anti-inflammatory, astringent activities. They are widely utilized in different forms of nutraceuticals in day to day life.[25] On the other hand, terpenoids have been associated with antimicrobial and antibiotic activities and are widely being studied for the same.[19-26] The phytochemical screening showed the presence of flavonoids, terpenoids, tannins, anthraquinone and coumarins in all the three different extracts. However, only methanolic extracts of the fruit and peel showed presence of alkaloids. Cardiac glycoside and proteins were absent in all the extracts. The methanolic and the aqueous extracts of the fruit showed the presence of carbohydrate whereas only the aqueous extract of the peel gave positive result for carbohydrates. Of all the extracts, methanolic extracts were found to have a rich source of phytochemicals in the fruit and the peel and hence the methanolic extract was used for all further activities.

Antimicrobial activity

The fruit and the peel extracts were tested for *in-vitro* antimicrobial efficacy. The extracts were tested against gram positive as well as gram negative bacteria. Staphylococcus aureus, bacillus subtilis, pseudomonas aeruginosa, Escherichia coli, and salmonella typhi were used respectively as gram positive and gram negative organisms. The extracts failed to show any activity against the gram negative organisms. The antibacterial activity may be attributed to the presence of phenolic compounds and the terpenoids. The methanolic extract of the fruit and the peel showed maximum activity against gram positive organism compared to the acetone and the aqueous extracts. This may be because of more quantity of phenolic compounds getting extracted in the methanolic solvent. The activities of the different extracts were prominent in *S aureus* than in *B subtilis*. The activity may be attributed to either the presence of phenolic compounds or terpenoids or a combination of both. The antimicrobial activity is shown in Table 2 and Table 3 and Figure 1. The methanolic peel extract showed the highest activity against S. aureus (ZI of 20.60 ± 3.33) followed by the acetone extract (ZI of 14.64 ± 5.11).

Statistical Analysis of Data

Statistical analysis of variance (ANOVA) applied showed that there is significant difference among the activity of the three solvent extracts tested (F- value = 25.71 > F crit 3.31 for fruit extracts and F- value = 79.34. > F crit 3.31 for peel extracts. Furthermore, there is also a significant difference among the effect of microorganisms on the inhibition zones produced by them (F-value = 19.16 > F crit 3.28 for peel extract and F-value = 40.15 > F crit 3.15 for peel extract).

Table 2
Anti-microbial activity of the fruit and the peel extracts of T. bispinosa

Micro-organism	Methanol extract		Acetone extract		Aqueous extract	
	Fruit	Peel	Fruit	Peel	Fruit	Peel
S. aureus	+	+	+	+	−	+
B. subtilis	+	+	+	+	−	−
P. aeruginosa	−	−	−	−	−	−
E. coli	−	−	−	−	−	−
S. typhii	−	−	−	−	−	−

+ = Present − = Absent

Table 3
Antimicrobial activity of different extracts of T. bispinosa at different concentrations

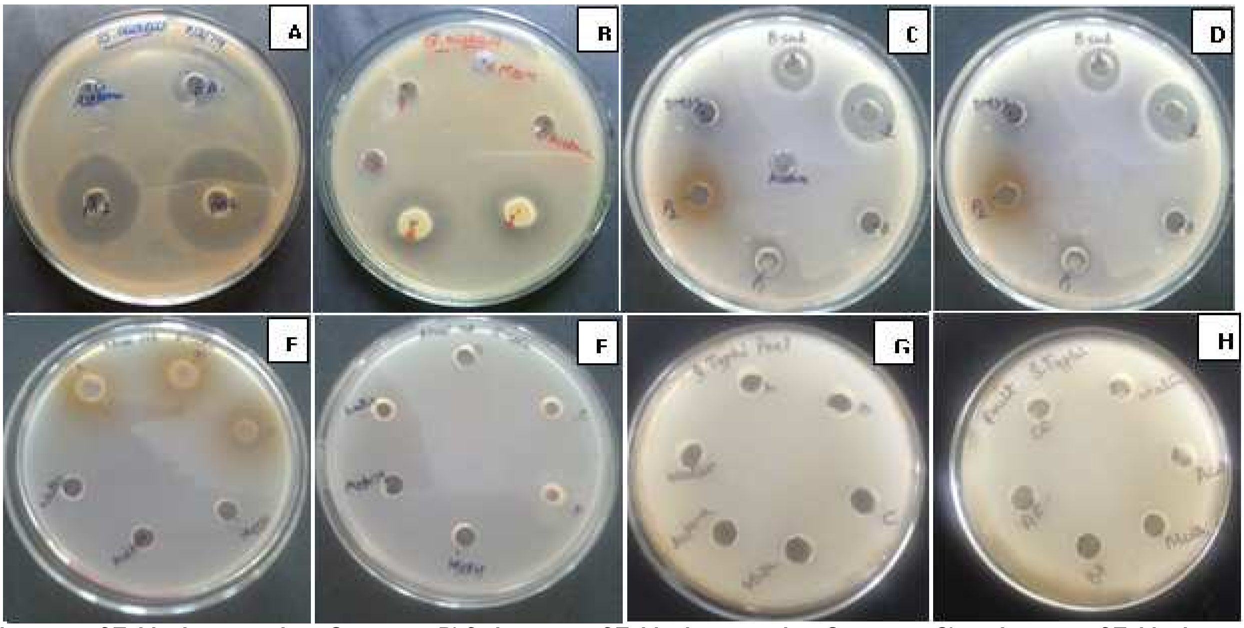
Micro-organism	Conc µg/ml	Zone of inhibition (mm) , (mm ± SD)					
		Methanol extract		Acetone extract		Aqueous extract	
		Fruit	Peel	Fruit	Peel	Fruit	Peel
S. aureus	100	8.53±1.06	20.83±1.60	7.02±1.27	15.18±1.22	−	10.36±1.09
	80	7.20±3.21	18.23±1.89	3.23±0.11	13.09±1.67	−	7.89±2.34
	60	5.43±1.23	15.34±5.32	−	8.34±1.77	−	4.32±1.55
	40	2.01±0.98	9.67±3.98	−	4.61±3.28	−	1.68±0.61
	20	−	5.22±3.22	−	2.21±1.35	−	−
B. subtilis	100	3.89±1.63	8.95±0.80	1.8±1.24	2.50±1.15	−	−
	80	1.08±0.18	5.23±2.11	−	−	−	−
	60	−	1.39±0.89	−	−	−	−
	40	−	−	−	−	−	−
	20	−	−	−	−	−	−
P. aeruginosa	100	−	−	−	−	−	−
	80	−	−	−	−	−	−
	60	−	−	−	−	−	−
	40	−	−	−	−	−	−
	20	−	−	−	−	−	−
E. coli	100	−	−	−	−	−	−
	80	−	−	−	−	−	−
	60	−	−	−	−	−	−
	40	−	−	−	−	−	−
	20	−	−	−	−	−	−
S. typhii	100	−	−	−	−	−	−
	80	−	−	−	−	−	−
	60	−	−	−	−	−	−
	40	−	−	−	−	−	−
	20	−	−	−	−	−	−

Values are mean ± SD; (n = 3), * p value < 0.05
ZI were expressed as mean ± standard deviation of three replicates. Low activity (1–6 mm), moderate activity (7–10 mm), high activity (11–15 mm), very high activity (16–20 mm), no activity (–).

Table 4
Antioxidant activity of different solvent extracts of T. bispinosa.

Sample		% Hydrogen peroxide scavenging activity (mean ± SD)
Standard	Ascorbic Acid	85.67±0.34
Fruit	Methanolic Extract	54.23±1.20
	Acetone Extract	27.08±0.87
	Aqueous Extract	15.67±1.11
Peel	Methanolic Extract	62.98±0.22
	Acetone Extract	56.43±0.65
	Aqueous Extract	18.75±1.23

Values are mean ± SD; (n = 3)



A) Peel extract of T. bispinosa against S. aureus, B) fruit extract of T. bispinosa against S. aureus, C) peel extract of T. bispinosa against B. subtilis, D) fruit extract of T. bispinosa against B. subtilis, E) peel extract T. bispinosa against E. coli, F) fruit extract of T. bispinosa against E. coli, G) peel extract of T. bispinosa against S. typhi, and H) fruit extract of T. bispinosa against S. typhi

Figure 1
The Antimicrobial activity (zone of inhibition, mm)

Hydrogen peroxide scavenging assay

The hydroxyl free radicals are produced during aerobic respiration.[27,28] These free radicals cause damage to living organisms by reacting with proteins, DNA and lipids.[29] Free radicals generally lead to decreasing membrane fluidity, loss of enzyme receptor activity and damage to membrane protein. [30] The free radicals lead to different disorders like ageing, cancer, cardiovascular disease, diabetes, rheumatoid arthritis, epilepsy and degradation of essential fatty acid.[27,28,31] These disorders can be treated with the help of antioxidants. Antioxidant activity of the different extracts of *T. bispinosa* fruit and peel is shown in Table 4. The activity of the peel extract was found to be higher than that of the fruit extract. This may be due to the presence of flavanoids and the phenolic compounds present in the extract. The Antioxidant activity was measured using the Hydrogen Peroxide Scavenging Activity. The one way ANOVA showed that there is significant difference between the antioxidant activity of the three solvent extracts (F-value = 215.89 > F crit 5.14).

CONCLUSION

In the present work the fruit and the peel of *Trapa bispinosa* was studied for its potential anti-microbial and antioxidant activity. The preliminary study was done to determine the types of phytochemicals present in both the fruit and the peel. It was found that methanol when used as a solvent for extraction gave maximum extractive value as compared to the other solvents. Furthermore, it was also observed that the peel extract showed higher number of beneficial phytochemicals as compared to the fruit extract. Both the peel and fruit extracts were tested against Gram positive and Gram negative bacteria for potential antimicrobial activity. It was observed that both the extracts were effective against the gram positive bacteria but no efficacy was seen against the gram negative bacteria. Furthermore, the anti-microbial activity of the peel extract was more profound then the fruit extract. Similar results were also observed for antioxidant activity, where the peel extract showed higher efficiency then the fruit extract proving that the waste material obtained from the edible plant has beneficial use in therapeutics. However, for the use of these extracts in therapy further studies needs to be done. Study on other pathogens, in-vivo activity and identification of the principle components can offer scope for further research.

AUTHORS CONTRIBUTION STATEMENT

Anjali B. Thakkar and Swati K. Kurtkoti designed and performed the experiments. Swati K. Kurtkoti derived the models and analysed the data in consultation with Nandibatla V. Sastry Anjali B. Thakkar performed the the antimicrobial and antioxidant activities in assistance with Swati K. Kurtkoti. Swati K. Kurtkoti wrote the manuscript in consultation with Anjali B. Thakkar and Nandibatla V. Sastry.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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