Screening of Antimicrobial and Antioxidant Activity of Acetone Extracts of *Heritiera fomes* Whole Plant against Pathogens

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ABSTRACT

Background: Phytochemicals are well known to have many important pharmaceutical properties. *Heritiera fomes* grows in low saline environments. The present study was initiated to explore the biologically beneficial properties of *H. fomes*. **Methods:** The crude extracts of *H. fomes* were extracted in acetone by orbital shaker and concentrated using rota evaporator. Total flavonoid content and Total phenolic content were estimated spectrophotometrically and the *in-vitro* antioxidant capacity was estimated by DPPH (2,2-diphenyl-1-picrylhydrazyl), Ferric Reducing Antioxidant Power (FRAP) and Hydrogen peroxide (H_2O_2) Scavenging Assays. Anti-microbial activity was determined by Disc diffusion method. **Results:** Phytochemical screening of acetone extract of *H. fomes* showed the presence of major classes of phytochemicals like alkaloids, glycosides, flavonoids, saponins, carbohydrates, phenols and sterols in considerable quantity. The total amount of phenolic and flavonoid content was found to be 75.3 mg GAE/g dry weight and 61.3 mg QE/g dry weight respectively. Antioxidant activity of plant extract determined by using different assays like H_2O_2 radical scavenging, DPPH and FRAP. A positive correlation between all the pairs of antioxidant assays was observed. Further, antimicrobial activity against various pathogens was evaluated by disc diffusion method where the zone of inhibitions were found to be in range from 5± 0.35 to 12 ± 0.50 mm. **Conclusion:** The study concludes that the plant *Heritiera fomes* has its effect in scavenging free radicals and has a potential to be a power antioxidant. Several *in-vitro* studies possess significant antioxidant, antimicrobial activities. The present study plays an important tool for new drug discovery.

Key words: *Heritiera fomes*, Total phenolic content, Total Flavonoid content, H_2O_2 radical scavenging assay, DPPH assay, FRAP assay, Antioxidant activity, Antimicrobial activity, Disc diffusion method.

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INTRODUCTION

Phytochemicals are also non-nutritive chemicals that are produced from plants. Plants have wide variety of secondary metabolites such as tannins, alkaloids, phenolic compounds, flavonoids etc. that been found *in-vitro* to show the antimicrobial properties.[1] In therapeutics, the use of crude extracts from plants and their phytochemicals, known to have antimicrobial properties, are an excellent importance. *Heritiera. fomes* belongs to the family: Malvacea which grows abundantly in the Sundarbans. It is commonly known as “Sundari”. *H. fomes* is a mangrove tree which is widely spread over in the sundarbans mangrove forest, Bangladesh. By nature it is a mangrove species. It can grow up to height of 25m and its trunk can be about 50cm in diameter. This mangrove forest is named after the plant *H. fomes* and it is grown densely in this mangrove forest. It preferably grows in low saline environments. The present study was initiated to explore the biologically beneficial properties of *H. fomes*.

MATERIALS AND METHODS

Materials

Microbial culture: The bacterial strains were *Eschericia coli* (2412), *Staphylococcus aureus* (E40), *Proteus vulgaris* (2266), *Klebsiella aerogenes* (U98), *Bacillus cereus* (2128), *Staphylococcus epidermidis* (2120) were collected from MTCC Mumbai. The culture was prepared by using Nutrient Broth. Nutrient broth was added with 50µl of culture in sterile test tubes and kept for incubation for 24 hr.

Plant collection and Extraction

Plant material was collected from Sri Venkateshwara University, Tirupati. To extract the crude compounds, the plant material was diluted with acetone (1:10 w/v) and kept at room temperature for 24 hr without disturbing. After 24 hr, it was kept in orbital shaker for the next 48 hr. The diluted plant sample was filtered by using Whatman No.1 filter paper to get crude extract. Now the extract was air dried and concentrated at room temperature. The dried concentrated crude extract was weighed and stored at room temperature for further purposes.

Phytochemical Characterization

The preliminary screening of phytochemicals like alkaloids, glycosides, flavonoids, saponins, sterols, tannins, aminoacids, terpenoids, quinones, diterpenes, resins, phenols was done by taking crude extract a stock of 1mg/ml and by following standard procedures.[2]

Antioxidant Activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) method

DPPH assay of *H. fomes* extract was evaluated by using the standard method[3] with very slight modifications. Ascorbic acid of different concentrations (2.5-15µg/ml) was used as reference standard. For determining the DPPH scavenging ability, 1ml of plant extract of different concentrations (25-200µg/ml) was treated with 1ml of 0.2mM DPPH solution in ethanol. The total reaction mixture was incubated in

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dark at room temperature for 30 min. After incubation, the absorbance was measured at 517nm. The ability of plant extract to scavenge the DPPH radical was calculated as the percentage of inhibition using the following formula

$$\text{DPPH Scavenging activity (\%)} = \frac{[A_{control} - (A_{sample} - A_{sampleblank})]}{A_{control}} * 100$$

Where,

$A_{control}$=1ml of DPPH solution +1ml of ethanol.

$A_{sample\ blank}$=1ml of plant extract+1ml of methanol.

A_{sample} =absorbance of sample.

Inhibitory concentration 50% (IC_{50}) value was obtained from the linear regression line. The antioxidant activity was determined by IC_{50} value.

FRAP (Ferric Reducing Antioxidant Power Assay) method

Ferric reducing ability of *H. fomes* extract was measured by using standard protocol.[4] For this, the FRAP reagent was prepared as 10:1:1 v/v/v of 300Mm Sodium acetate buffer where pH is adjusted to 3.6, 10mM TPTZ (2,4,6-Tri-(2-pyridyl)-5-triazine) in 40Mm HCl and 20Mm Ferric chloride hexahydrate ($FeCl_3.6H_2O$). By using ferrous sulphate ($FeSO_4$) of different concentrations (0.2 - 1.0mM) standard, a calibration curve was plotted. As reference standard, Ascorbic acid (100-500µg/ml) was used. To determine the ferric ion reducing ability, 1ml of plant extract of different concentrations (200-1000µg/ml) were treated with 3ml of freshly prepared FRAP reagent and 1ml of distilled water. All these experiments were done in triplicates. The total reaction mixture was kept for incubation at 37°C for 30min. After incubation, the absorbance was measured at 593nm and is expressed as mM $FeSO_4$ equivalent/gm of plant extract.

Hydrogen peroxide scavenging assay (H_2O_2)

H_2O_2 Scavenging assay of acetone extract of *H. fomes* was evaluated by using the method[5] with slight modifications. For this, 20mM of H_2O_2 was prepared in 50mM Phosphate buffer and pH was adjusted to 7.4. In this assay, ascorbic acid of different concentrations (10-50µg/ml) were used as reference standard. For determining the scavenging ability, 1ml of plant extract of different concentrations (10-200µg/ml) was dissolved in phosphate buffer that was treated with 2ml of 20mM H_2O_2 solution. The total reaction mixture was kept for incubation at room temperature for 10 min in dark. After incubation, the absorbance was measured at 230nm using UV-Vis-Spectrophotometer. The scavenging ability of the plant extract and the standard to scavenge H_2O_2 molecules was calculated as percentage of inhibition using the formula-

$$H_2O_2 \text{ Scavenging ability (\%)} = \frac{[A_{control} - (A_{sample} - A_{sample\ blank})]}{A_{control}} * 100$$

Where,

$A_{control}$ - 2ml of H_2O_2 solution+1ml of phosphate buffer.

A_{sample} - absorbance of sample.

$A_{sampleblank}$ - 1ml of plant extract+2ml of phosphate buffer.

Phosphate buffer is used as blank solution.

The inhibitory concentration 50% of H_2O_2 molecules is obtained from linear regression line. The antioxidant activity of plant extract is evaluated from IC_{50} value.

Total flavonoid content

Total flavonoid content of acetone extract of *H. fomes* was evaluated by using Aluminum chloride colorimetric method[6] with slight modifications. Quercitin dihydrate is used as reference standard and a calibration curve was plotted by using different concentrations (20-100µg/ml). For determining total flavonoid content, 500µl plant extract of different concentrations (200-1000µg/ml) or Quercitin was mixed with 1.5 ml methanol, 0.1ml $AlCl_3$, 0.1ml potassium acetate and 2.8ml of distilled water. The total reaction mixture was incubated at room temperature in dark for 30 min. After incubation, absorbance was measured at 415nm by UV-Vis-Spectrophotometer. A blank was prepared by taking all reagents except aluminum chloride. Total flavonoid content was expressed as mg Quercitin equivalent (QE)/gm dry weight.

Total phenolic content

The total phenolic content of acetone extract of *H. fomes* was determined by using Folin-Ciocalteu method.[7] A calibration curve of gallic acid of different concentrations (20-100µg/ml) was plotted as standard. For this, 200µl plant extract of different concentrations (200-1000µg/ml) was mixed with 2.8ml of distilled water and 0.25ml of Folin-Ciocalteu reagent (1:10 v/v). After 5 min of duration, 0.75ml of Sodium carbonate (7.5% w/v) was added. The total reaction mixture was kept for incubation at room temperature in dark for 30 min. After incubation, the absorbance of standard and the sample was measured at 765nm by UV-Vis-Spectrophotometer. Distilled water was used as blank. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/gm dry weight.

Antimicrobial Activity

Disc Diffusion Method

The most widely used method for determining the anti-microbial activity is Disc diffusion method. This method is mainly used to test the effectiveness of antibiotics on organisms. Antibiotic discs were prepared by using Whatman No.1 filter paper, of size-4mm. Gram positive organisms and Gram negative organisms used in the present study were *Staphylococcus epidermidis, Bacillus cereus, Staphylococcus aureus, E.coli, Klebsiella aerogens, Proteus vulgaris* respectively.

On the surface of the agar plates, bacterial strains were inoculated and evenly spread with the help of glass rod. Discs of plant extract of concentration 8mg/ml and 16mg/ml were placed on the surface of the agar plates. These plates were closed tightly with the help of parafilm. All this work should be done in laminar air flow chamber. The anti-microbial compound starts diffusing into the surrounding agar. All these plates were kept in incubator for 24 hr at 37°C. Now, simultaneously the growth of the bacteria and the diffusion of the anti-microbial compound begins. After incubation, zone of inhibition were formed and are measured for all the bacterial strains. Simultaneously, amikacin antibiotic of different concentrations is used for comparison between plant extract of different concentrations

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

In this method, seven test tubes were taken out of which one tube is kept as blank. In all the test tubes, an equal volume (5ml) of Nutrient Broth was taken in all the test tubes. To this 500mg of plant extract was taken in the first tube. From this tube, 1ml of broth was taken and a serial dilution was done in all the tubes and 1ml of broth is discarded out from the last tube. Now, to each tube add 50µl of culture was added in all the test tubes and these tubes were incubated at 37°C for 24 hr. After incubation, the results were validated and the MIC and MBC concentrations were determined.[8,9]

RESULTS

Phytochemical screening

The quantitative phytochemicals analysis of this species exhibited the presence of alkaloids, glycosides, flavonoids, saponins, carbohydrates, phenols and sterols in considerable quantity (Table 1).

Total phenolic content and total flavonoid content

Total phenolic content and flavonoid content of *H. fomes* was determined by linear regression equation of gallic acid (y=0.0013x+0.0131, r^2=0.9956) and quercitin (y=0.0019x-0.0136, r^2=0.9904) respectively. Total phenolic content and total flavonoid content was calculated and expressed as mg GAE/g dry extract and mg QE/g dry extract.

The total amount of phenolic content present in the plant extract was 75.3 GAE/g dry weight and the total amount of flavonoid content present in the plant extract was 61.3 QE/g dry weight (Table 2).

Antioxidant activity

DPPH Scavenging Assay

DPPH is most widely used antioxidant activity. It is rapid, economic and simple activity. It contains unpaired electrons and shows maximum absorption at 517nm. It reduces the unpaired electron on DPPH by donating an hydrogen atom.[10] Therefore, a decrease in the intensity of deep violet colour is observed, which is proportional to antioxidant activity.

As the transfer of hydrogen atom to DPPH radical is slow, it is considered as electron transfer (ET) based assay even though there is a hydrogen atom transfer. The acetone extract of *H. fomes* has shown scavenging activity against DPPH with IC_{50} of 182.43 µg/ml (Table 3).

H_2O_2 Scvenging Assay

H_2O_2 do not contain unpaired electrons and is non-free radical. H_2O_2 is a strong oxidizing agent and undergoes reduction by accepting hydrogen from antioxidant. During this process, H_2O_2 gets neutralized to H_2O. It shows maximum absorption at 230nm. The decrease in the absorbance of H_2O_2 is proportional to the activity of antioxidant. The present study has abundant phenolic compounds which contribute to antioxidant activity with IC_{50} 71.991 µg/ml (Table 3).

Each value is the average of three analyses ± standard deviation. Means sharing the same letter are significant at P<0.01.

FRAP (Ferric reducing antioxidant power) Assay

FRAP assay is most widely used antioxidant activity. In acidic medium, the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{+3}$ was reduced to intense blue coloured ferrous complex $[Fe(II)\text{-}(TPTZ)_2)^{2+}$. Maximum absorbance was measured at 593nm wavelength and expressed as µM ferrous ion (Fe^2) equivalents. There is a clear correlation between the plant extract activity and standard, with increasing concentrations the activity increased showing the potency of the plant extract (Table 4).

Antimicrobial activity of *H. fomes* against pathogens

The Table 5 and Figures 1,2,3 depicts the antimicrobial activity of extract against the gram positive and gram negative bacteria. The acetone extract of *H. fomes*, showed maximum zone of inhibition with the diameter of 12 ± 0.50mm against the gram negative bacterium- *Proteus vulgaris*.

The acetone extract was compared with an antibiotic drug, Amikacin which showed its maximum zone of inhibition against the gram negative bacteria- *Klebsiella aerogens* with the diameter of 26mm.

Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC)

After the completion of the incubation period, the well were checked and based on the turbidity of the plates the MIC and MBC values were evaluated and plotted in the below Table 6.

Acetone extract had shown potent result inhibiting the growth of different pathogens like *Staphylococcus aureus, Escherichia coli, Staphylococcus epidermidis* and *Bacillus cereus.*

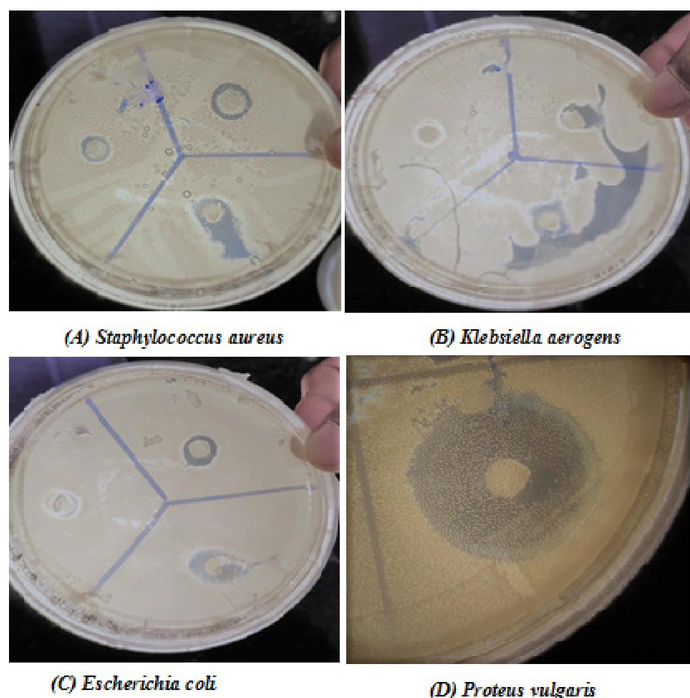

Figure 1: Antimicrobial activity of *H. fomes* of acetone plant extract.

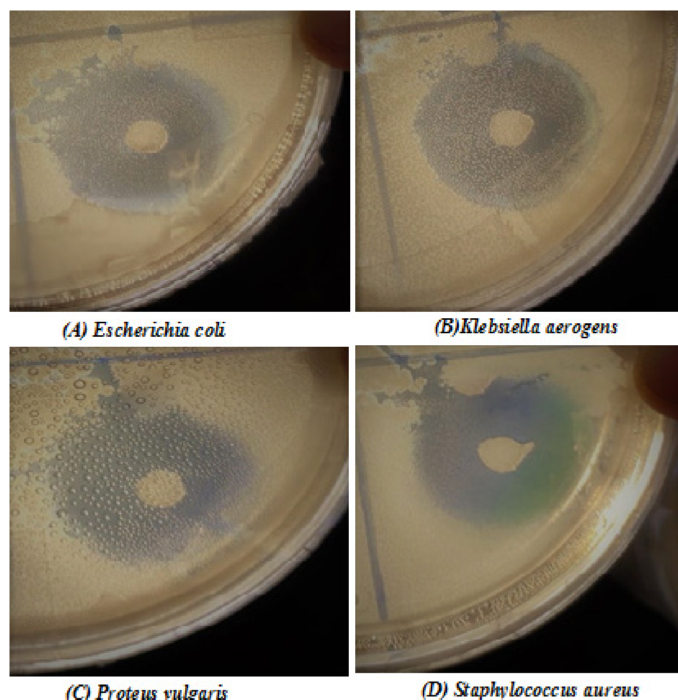

Figure 2: Antimicrobial activity of an Antibiotc (Amikacin).

DISCUSSION

Heritiera fomes is a very predominant mangrove species. As per literature available on this species regarding its biological activities, it has enoromous potential. *H. fomes* contains 0.09% chlorophyll b, 0.25% chlorophyll A, 0.11% carotenoids, 21.12% tannins, 39.45% polyphenols.[11] The presence of reducing sugars, alkaloids, saponins, glycosides, steroids, tannins, flavonoids and gums was demonstrated by phytochemical screening of leaf extract.[12] Leaf contains 29.22% protein.[13] The bark of *H. fomes* possess high amount of proanthocyanidins[14] and 7– 36% tannin.[15] The bark of stem contains high content of procyanidins, which are found to be trimeric, pentameric and hexameric.

Leaf extract of *Heritiera fomes* was evaluated for both qualitative and quantitative antioxidant activity. Quantitative assay technique was carried out by DPPH assay (hydrogen donation assay) and qualitative assay was carried out by thin layer chromatographic technique followed by DPPH spray. Leaves extract exhibited noteworthy antioxidant activity with the IC_{50} 26.30 µg/mL.[12] Bark extracts of *H. fomes* exhibited potent antioxidant activity with IC_{50} 22 µg/mL.[2]

Heritiera fomes leaf extract at dosage of 250 µg/disc and 500 µg/disc exhibited effective antimicrobial activity with the zones of inhibition against tested gram-negative and gram-positive pathogens at a range of 7.86 to 13.45 mm and 3.92 to 7.63 mm respectively.[12] The bark extracts of *H. fomes* has shown promising antibacterial activities against *P.*

Table 1: Results of phytochemical constituents.

Test performed	Phytochemical constituent	Acetone extract
Wagner's test	Alkaloids	+
Keller-kellani's test	Glycosides	+
Alkaline reagent test	Flavonoids	+
Foam test	Saponins	+
Molisch's test	Carbohydrates	+
Ferric chloride test	Phenols	+
Liebermann-Burchard test	Sterols	+
Braymers test	Tannins	-
Ninhydrin test	Amino acids and Proteins	-
Salkowki's test	Terpenoids	-
Concentrated.HCL test	Quinones	-
Copper acetate test	Diterpenes	-
Turbidity test	Resins	-

Table 2: The total phenolic and flavonoid content of *H. fomes*.

S. No	Assay	Content
1	Total Phenolic content	75.3 GAE/g dry weight
2	Total flavonoid content	61.3 QE/g dry weight

Table 3: IC_{50} of *H. fomes*. and reference standard.

S. No	Antioxidant assay	IC_{50} values (µg/ml)
1	DPPH Assay	182.43[a]
2	H_2O_2 Scvenging Assay	71.991[a]
3	Standard (Ascorbic acid)	14.78[a]

Table 4: Ferric Reducing antioxidant power of *H. fomes*.

	Ascorbic acid (Standard) Concentration (µg/ml)				
	100	200	300	400	500
$mMFeSO_4$ per gram dry weight of ascorbic acid	0.169	0.598	0.953	1.111	1.364
	Acetone plant extract Concentration (µg/ml)				
	200	400	600	800	1000
$mMFeSO_4$ per gram dry weight of extract	0.099	0.485	0.766	0.780	0.800

Table 5: Zone of inhibition evaluated with the acetone extract of *H. fomes* and Antibiotic (Amikacin).

S.No	Pathogens	Concentration (µg/ml)	Zone of inhibition in mm: Acetone extract (µg/ml)	Antibiotic (Amikacin) (50 µg/ml)
1	*Escherichia coli*	50	5 ± 0.35	25 ± 0.35
		100	7 ± 0.45	
		150	9 ± 0.25	
2	*Klebshella aeurogenes*	50	5 ± 0.50	26 ± 0.50
		100	6 ± 0.25	
		150	9 ± 0.35	
3	*Proteus vulgaris*	50	6 ± 0.25	24 ± 0.25
		100	8 ± 0.35	
		150	12 ± 0.50	
4	*Staphylococcus aureus*	50	6 ± 0.15	25 ± 0.75
		100	8 ± 0.25	
		150	10 ± 0.25	

Table 6: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of plant extract against susceptible test organisms.

Pathogen	MIC (mg/ml)	MBC (mg/ml)
Staphylococcus aureus	0.8	4
Escherichia coli	0.8	4
Staphylococcus epidermidis	10	50
Bacillus cereus	4	20

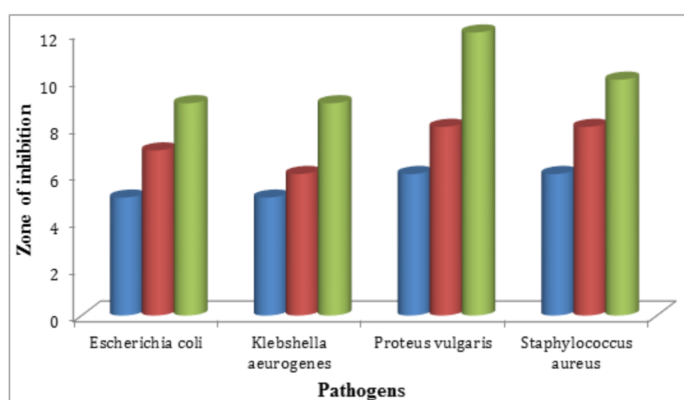

Figure 3: Zone of inhibition of various pathogens of *H. fomes* acetone extract. Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC).

aeruginosa, K. rhizophila, S. aureus and *B. subtilis*.[2] *In vitro* antibacterial study between the pneumatophores of *H. fomes* and *Xylocarpus moluccensis* demonstrated comparable antibacterial profiles in the most cases with the zone of inhibitions >10mm. Pneumatophores extract of *H. fomes* presented a potent antimicrobial activity against *Enterobacter aerogenes* with the diameter of zones between 19 and 21mm. The MIC (minimum inhibitory concentration) of *H. fomes* extract was evaluated by broth dilution method and showed significant minimum inhibitory concentration (MIC = 400 and 500 μg/mL) against *Shigella boydii* and *Shigella sonnei*, respectively.[16]

CONCLUSION

The study concludes that the plant *Heritiera fomes* has its effect in scavenging free radicals and has a potential to be a power antioxidant. Several *in-vitro* studies possess significant antioxidant, antimicrobial activities. The present study plays an important tool for new drug discovery.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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