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IN VITRO ASSESSMENT OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF VARIOUS EXTRACTS OF *HAMELIA PATENS*

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ABSTRACT

An alarming increase in bacterial strains resistant to existing antimicrobial agents demands a renewed effort to seek agents effective against pathogenic bacteria resistant to current antimicrobials. *Hamelia patens* extracts was studied for antibacterial and antifungal activity against various clinical isolates of the bacteria and fungi, in varying concentration by Agar well diffusion method and serial dilutions. The extracts showed pronounced concentration dependent antibacterial activity against Gram positive and Gram negative bacteria and also antifungal activity. Various extracts of *Hamelia patens* inhibited the growth of *Staphylococcus aureus, Bacillus subtilis, Pseudomonas flurescens, Escherichia coli*and *Aspergillus niger, Penicillium chrysogenum, Alternaria alternata.* Six extracts of *Hamelia patens*were analyzed for their antioxidant activity by Hydrogen peroxide scavenging activity. The six extract along with the reference sample , Gallic acid were further analysed to determine toal phenolic content by Folin – Ciocalteau method. The data obtained in the in vitro models clearly establish the antioxidant potency of all extracts.

KEY WORDS: *Hamelia patens,* Antibacterial and Antifungal activity, Antibiotic resistance, Antioxidant activity

1. INTRODUCTION

Reactive oxygen species (ROS) generated from both living organisms and exogenous sources initiate reactions which damage biological molecules and also play an important causative role in disease initiation (Croft, 1999; Halliwell, 1996). Lipid oxidation, caused by free radicals, is one of the major factors for the deterioration of food products during processing and storage. Effective synthetic antioxidants such as butylated hydroxytoluene (BHT) have been used for industrial processing but these are suspected of being responsible for liver damage and carcinogenesis. Recently, there is an increasing interest in finding natural antioxidants from plant materials to replace synthetic ones. There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. The number of resistant strains of microbial pathogens is growing since penicillin resistance and multiresistance pneumococci caused a major problem in South Africa in 1977 (Maurer-Grimes, 1996; Elloff, 1998). This situation, coupled with the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections are a serious medical problem (Marchese and Shito, 2001; Poole, 2001). This has forced scientists to search for newantimicrobial substances from various sources like the medicinal plants. The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic protypes (Maurer-Grimes, 1996; Rabe and van Staden, 1997; Afolayan, 2003).

Plants contain a large variety of substances possessing antioxidant activity, such as vitamin C, vitamin E, carotenes, xanthophylls, tannins and phenolics (Chanwitheesuk, 2005). Sources of natural antioxidants are primarily plant phenolics that can be found in all parts of the plant. The plant phenolic compounds, such as flavonoids exhibit antioxidant properties due to their high redox potential (Cook & Samman, 1996). They also exhibit a wide range of biological activity, antimicrobial activity, anticarcinogenicity and antiproliferation, and many biological activities can be attributed to their antioxidant properties (Ren, 2002). Interestingly, recent research has revealed that bark, root and stem extracts of *Hamelia patens* may potentially possess antioxidant and antimicrobial properties.This study was aimed at investigating the antimicrobial and antioxidant property of *Hamelia patens.*

2. MATERIALS AND METHODS

2.1. Collection of plant materials: The fresh parts of the plant were collected; each specimen was washed under running tap water, labelled, weighed and annotated with the date of collection. Then each specimen was dried at 37^0 for 48 h, powdered and stored in air tight container.

2.2. Preparation of extracts: Exactly 100g of each powdered samples (stem, bark) and stem + bark samples (-25+25g, 35+15g, 15+35g) was soaked in the water and ethanol for 3-5 days and filter through Whatman No.1 filter paper. Further extraction of the residue was repeated 3 times until a clear colorless supernatant Extraction liquid was obtained, concentrated to dryness under reduced pressure with a vaccum evaporator, and stored at 4^0 C until the further use.

Around 100g of fresh shade dried plant maerial (bark) was powdered and wrapped in muslin cloth. It was extracted by Soxhlet apparatus with methanol and acetone. The percolation process was continued until the extraction process was completed (indicated by transparent colour). The extract was allowed to cool and then poured into a petri plate, left for drying.The dried extract was scratched and was collected in eppendorf tube and weighed , used for further phytochemical screening.

2.3. Test organisms: The test microorganisms used in the study were Bacteria: *Staphylococcus aureus* (MTCC NO. 96), *Bacillus subtilis* (MTCC NO. 2423), *Escherichia coli* (MTCC NO.1652), *Pseudomonas flurescens* (MTCC NO.2421) and fungus: *Aspergillus niger* (MTCC NO.2425), *Penicillium chrysogenum* (MTCC NO. 5108), *Alternaria alternate* (MTCC NO. 7202).The test organism were clinical isolates and obtained from the Biotech Park, Lucknow, UP, India.

2.4. Preliminary phytochemical screening: The eight extracts of *Hamelia patens* plant (bark, stem, stem + bark) were subjected to preliminary phytochemical screening for the presence and absence of various active metabolites, using standard procedures to identify the constituents as described by sofowora, Trease and Evans and Harborne.

Alkaloids: About 50 mg of Solvent free extract was stirred with 3 ml of dilute hydrochloric acid and then filtered thoroughly. The filtrate was tested carefully with various alkaloid reagents as follows:

Mayer's test: To a 1 ml of filtrate, few drops of Mayer's reagent are added by the side of the test tube. The white or creamy precipitate indicated test as positive.

Wagner's test: To a 1 ml of filtrate, few drops of Wagner's reagent are added by the side of the test tube. The color change was observed. A reddish-brown precipitates confirms the test as positive.

Dragendorff's test: To a 1 ml of filtrate, 2 ml of Dragendorff's reagent are added and the result was observed carefully. A prominent yellow precipitate confirms the test as positive.

Carbohydrates
Fehlings test: One ml of extract was boiled on water bath with 1 ml each of Fehling solutions A and B. The color change was observed. A red precipitates indicated presence of sugar.
Barfoed's test: To 1 ml of extract, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes. The color change was noted and recorded. A red precipitates indicated presence of sugar.

Benedict's test: To 0.5 ml of extract, 0.5 ml of Benedict's reagent was added. The mixture is heated on a boiling water bath for 2 minutes and the result was observed. A red precipitates indicated presence of sugar.

Glycosides
Legals test: Chloroform (3ml) and ammonia solution (10%) was added to 2ml plant extract. Formation of pink color indicated the presence of glycosides.
Proteins: The extract was dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filtrate is subjected to tests for proteins and amino acids.

Millon's test: To 2 ml of filtrate, few drops of Millon's reagent are added. The result was observed. A white precipitates indicated presences of proteins.

Biuret test: An aliquot of 2 ml of filtrate was treated with drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. The pink color in ethanol layer indicated presences of proteins.

Tannins
Ferric chloride test: The extract (5 mg) was dissolved in 5 ml of distilled water and few drops of neutral 5% ferric chloride solution were added. The formation of blue green color indicated the presence of tannins.

Flavonoids: Extract of about 0.2g was dissolved in diluted NaOH and HCl was added. A yellow solution that turns colorless indicates the presence of flavonoids.

Coumarins: 10% NaOH (1ml) was added to 1 ml of the plant extracts formation of yellow color indicated presence of coumarines.

Saponins: Distilled water 2ml was added of each plant extracts and shaken in a graduate cylinder for 15 mins lengthwise. Formation of 1cm foam indicates the presence of saponin.

Quinones: Concentrated sulphuric acid (1ml) was added to 1ml of each of the plant extract. Formation of red color indicated the presence of Quinones.

Terpenoids: Chloroform (2ml) and concentrated sulphuric acid was added carefully to 0.5 ml of extract. Formation of red brown color at the interface indicated the presence of terpenoids.

Steroids and Phytosteroids: To 0.5 ml of the plant extract equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid. Appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicated the presence of phytosteroids.

Fixed oils: A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicated the presences of fixed oil.

2.5. Antibacterial assays

2.5.1.Agar diffusion assay: The antibacterial activity of the *Hamelia patens* extract was determined in accordance with the agar well diffusion method. The bacteria was first isolated and grown in a nutrient broth for 18h before use and standardize the culture to 106cfu/ml. Mueller- Hinton agar (OXOID) was prepared and bored the wells into the agar using a sterile 4 mm diameter cork borer. 200 µl of the standardize cell culture was spread on a MH agar. Approximately 100µl of the extract were introduced into the wells, allowed to stand at room temperature for about 2 h then incubated at 37^0 C.Gentamicin at a concentration of 10 µg/ml and DMSO were used as positive and negative control, respectively.The plates were incubated at 37^0 C for 24 h and the inhibition zones were compared with that of the standard antibiotic Gentamicin. Each experiment was repeated three times (Pathan, 2012).

2.5.2. Minimum inhibitory concentrations (MICs): The minimum inhibitory concentration (MICs) of the extract was estimated for each of the test organisms in triplicates. 2 ml of varying concentration of the extracts (100, 50, 25, 12.5, 6.25, 3.125 and 1000, 500, 250, 125, 62.5, 31.25 µg/ml), 2ml of MHB added and then a loopful of the test organism previously diluted to 0.5 McFarland turbiity standard (for bacterial isolates) was introduced to the tubes. The procedure was repeated on the test organisms using the standard antibiotics (Gentamicin). A tube containing MHB only was seeded with the test organisms as described above to serve as control. Tubes containing bacterial cultures were then incubated at 37^0 C for 24h. After incubation the tubes were then examined for microbial growth by observing for turbidity.

2.5.3 Minimum bactericidal concentrations (MBC): To determine the MBC, for each set of test tubes in the MIC determination, a loopfull of broth was collected from those tubes which did not show any growth and inoculated on sterile MHA by streaking. MHA only was streaked with the test organisms to serve as control. Plates inoculated with bacteria were then incubated at 37^0 C for 24 h. After incubation the concentration at which no visible growth was seen noted as the minimum bactericidal concentration (Doughari, 2006)

2.6 Antifungal activity: Agar well diffusion method was modified. PDA (Potato dextrose agar) was used for fungal cultures. The culture media was inoculated with the fungal strains (*Aspergillus niger, Penicilium chrysogenum, Alternaria alternate*). 100 µl of standardized fungal spores suspension was spread on the PDA using a glass spreader. Sterile 4mm diameter of cork borer was used to bored wells into the PDA. Approximately 100µl of the different concentration of the extract were introduced into the wells and allowed to stand (1h) for proper diffusion of the extract into the media. The plates were observed for zone of inhibition after 72 h at 25^0 C and compared with the Amphotericin (Pathan, 2012)

2.7 Antioxidant activity

2.7.1 Scavenging of hydrogen peroxide: The ability of the *Hamelia patens* to scavenge hydrogen peroxide was determined according to the method of Ruch et al (1989). A solution of Hydrogen peroxide (20 mM) was

prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230nm in a spectrophotometer. Extracts (100-500 µg) in distilled water were added to a hydrogen peroxide solution. Absorbance of hydrogen peroxide at 230 nm was determined after 10 minute against a blank solution containing in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of various extracts of *Hamelia patens* and standard compounds was calculated using the following equation:

$$\% \text{ Scavenged } H_2O_2 = \{(A_0 - A_1)/A_0 \} \times 100$$

Where, A_0= Absorbance of control

A_1= Absorbance of sample/standard

2.7.2 Determination of total phenolic contents: 50 mg/ml (0.5 ml) of the extracts, was mixed with Folin-Ciocalteau's reagent (0.25ml) and distilled water and mixed for 1min. Sodium carbonate 0.2 ml was added to the mix. The solution had its volume adjusted to 10 ml with distilled water. After 2 h, absorbance was measured at 760 nm. A standard curve was prepared using Gallic acid with a concentration range from 10-50 µg/ml. Total phenolic content was expressed as mg gallic acid equivalents (GAE) /g of sample, calculated from the formula:

$$T = C \times V/M$$

Where, T = Total phenolic content in mg/g plant extract, in GAE

C = Concentration (mg/ml) of Gallic acid obtained from the calibration curve

V = Volume of extract (ml)

M = Weight (g) of plant extract

3. RESULTS AND DISCUSSIONS

Various parts of the *Hamelia patens* plant (stem, bark) were used to estimate the presence of active constituents. Preliminary phytochemical screening of various extracts of *Hamelia patens* plant was shown in Table 1.

Table.1.Chemical constituents present in various extracts of *Hamelia patens*

Phytochemicals	Bark			Stem		S+B	S+B	S+B
						25+25 grams	15+35 grams	35+15 grams
	AE	ACE	ME	AE	EE	AE	AE	AE
Alkaloids	+	+	+	+	+	+	+	+
Carbohydrate	-	-	-	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-	-	-
Saponins	+	+	+	+	+	+	+	+
Quinones	-	-	-	-	-	-	-	-
Terpenoids	-	+	-	-	-	-	-	-
Steroids	-	+	+	-	+	-	-	-
Fixed Oils	-	-	-	-	-	-	-	-
Proteins	+	+	+	+	+	+	+	+

(AE: Aqueous extract, ACE: Acetone extract, ME:Methanolic extract, EE:Ethanolic extract, S+B: Stem + Bark)

All the extract demonstrated antimicrobial activity against both the test bacteria and fungi with the acetone extracts demonstrating the highest activity (17mm). The water extract of the bark had activity against *E.coli* (15mm zone of inhibition), Pseudomonas flurescens (16 mm zone of inhibition), *Bacillus subtilis* (14 mm zone of inhibition), *S.aureus* (13mm zone of inhibition). Methanolic extract of the bark had no activity against *E.coli.* The ethanolic and aqueous extract of the bark had no activity against *E.coli, P.flurescens, B.subtilis, S.aureus* but the ethanolic extract of the stem demonstrated antifungal activity against *Penicillium chrysogenum.* The aqueous extract of the stem + bark (25 + 25g) had activity against *E.coli, Pseudomonas flurescens* and no activity against *B.subtilis and Staphylococcus* aureus but the aqueous extract of the stem + bark (15 + 35g) had good activity against *S.aureus, P.flurescens*. The aqueous extract of the stem + bark demonstrated activity against *E.coli, S.flurescens, and B.subtilis* but no activity against *S.aureus*. (Table 2 and 3)

Table.2.Antibacterial activity of the various extract of the *Hamelia patens*

Extract	Concentration (µg/ml)	Zone of inhibition (mm)			
		E.coli	*P.flurescens*	*B.subtilis*	*E.coli*
Aqueous extract of bark	400	9	-	-	06
	500	11	04	-	07
	600	13	07	-	07
	700	15	9	10	
	800	-	12	-	12
	900	-	12	9	13
	1000	-	16	14	13
Acetone extract of bark	400	06	10	06	-
	500	07	17	07	-
	600	07	-	07	-
	700	10	-	07	07
	800	12	-	09	08
	900	13	-	13	11
	1000	16	-	19	-

Table.2.Antibacterial activity of the various extract of the *Hamelia patens* continution..

Extract	Concentration (µg/ml)	Zone of inhibition (mm)			
		E.coli	*P.flurescens*	*B.subtilis*	*S.aureus*
Methanolic extract of bark	50	-	-	-	06
	75	-	-	-	08
	100	-	-	-	09
	400	-	-	-	-
	500	-	04	-	-
	600	-	07	-	-
	700	-	09	06	-
	800	-	12	08	-
	900	-	12	10	-
	1000	-	16	11	-
Ethanolic extract of stem	50-1000	NA	NA	NA	NA
Aqueous extract of S+B (25 + 25g)	500	-	08	-	-
	600	05	10	-	-
	700	06	11	-	-
	800	09	13	-	-
	900	09	13	-	-
	1000	11	16	-	-
Aqueous extract of S+B (15 + 35g)	800	-	-	-	07
	900	-	-	-	09
	1000	-	-	-	11
	1.5mg/ml	-	0.5	-	16
	2mg/ml	09	-	19	-
Aqueous extract of S+B (35+15g)	600	-	06	-	-
	800	05	06	08	-
	900	07	10	10	-
	1000	09	10	11	-
	1.5mg/ml	09	-	13	-
	2mg/m	11	-	-	-

Table.3.Antifungal activity of various extract of *Hamelia patens*

	Zone of Inhibition (in mm)			
	Amphotericin	Acetone Extract (bark) 1000µg/ml	Ethanolic Extract (Stem)	
			900µg/ml	1000µg/ml
Aspergillus niger	25	-	-	-
Penicillium chrysogenum	20	10	06	11
Alternaria alternata	20	-	-	-

The MIC of the aqueous extract of the bark against *E.coli* is 50µg/ml, *B.subtilis* 500µg/ml, *P.flurescens* 62.5 µg/ml, *S.aureus* 62.5 µg/ml and the MBC for the aqueous extract of the bark against *E.coli* and *B.subtilis* is 1000µg/ml. The MIC of the acetone extract of the bark for *E.coli* 6.25 µg/ml, *Bacillus subtilis* 12.5µg/ml,

P.flurescens 12.5 µg/ml, *S.aureus* 12.5 µg/ml and the MBC of the acetone extract is 1000 µg/ml for *E.coli*, 250 µg/ml for *B.subtilis*, 1000µg/ml for P.flurescens, and 250 µg/ml for S.aureus. The MIC of the methanolic extract of the bark against E.coli 6.25 µg/ml, B.subtilis 25µg/ml, P.flurescens 6.25 µg/ml, S.aureus 12.5 µg/ml and MBC for *E.coli* 1000µg/ml, *B.subtilis* 500µg/ml, P.flurescens 1000µg/ml, S.aureus 250 µg/ml.Most of the MIC values were lower than the MBC values indicated that the extracts could be bactericidal in action. Low MIC and MBC values are an indication of high efficacy (Table.4).

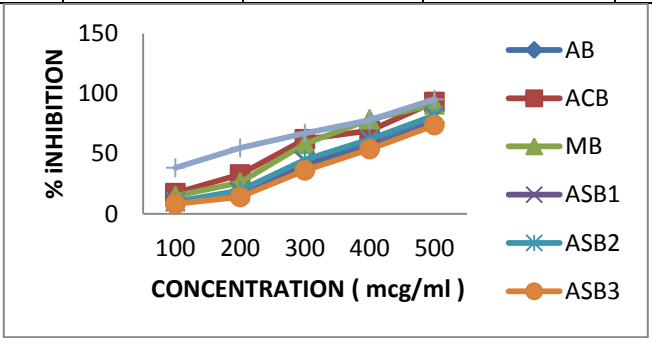
Table.4.Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of various extracts of *Hamelia patens* plant against bacterial strains (MIC and MBC in µg/ml)

Bacterial pathogens	Bark					
	MIC			MBC		
	AE	ACE	ME	AE	ACE	ME
Escherichia coli	50	6.25	6.25	1000	1000	1000
Bacillus subtilis	500	12.5	25	1000	250	500
Pseudomonas flurescens	62.5	12.5	6.25	-	1000	1000
Stephylococcus aureus	62.5	12.5	12.5	-	250	250

Scavenging of H_2O_2 by extracts may be attributes to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. The ability of the extracts to effectively scavenge hydrogen peroxide, determined according to the method of Ruch. The extracts were capable of scavenging hydrogen peroxide in concentration-dependent manner. Hydrogen peroxide itself is not very reactive; it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cells. Thus, removing H_2O_2 is very important throughout food systems (Table.5).

Table.5.Observation of antioxidant activity in terms of hydrogen peroxide method

Extracts	% SCAVENGING (MEAN ± SEM)				
	100µg/ml	200µg/ml	300µg/ml	400µg/ml	500µg/ml
Aqueous extract of Bark	9.45±0.01	15.06±0.11	39.22±0.18	56.07±0.01	76.11±0.01
Acetone extract of Bark	17.23±0.01	32.76±0.17	62.08±0.06	69.29±0.12	93.07±0.06
Methanolic extract of Bark	15.02±0.11	26.25±0.13	58.35±0.12	78.67±0.08	91.09±0.12
Aqueous extract of S+B (25+25g)	10.06±0.13	18.15±0.08	43.55±0.04	61.23±0.15	81.09±0.12
Aqueous extract of S+B (35+15g)	10.24±0.17	19.35±0.12	45.05±0.21	62.12±0.19	82.13±0.20
Aqueous extract of S+B (15+35g)	8.17±0.15	14.04±0.01	36.18±0.16	53.89±0.03	73.88±0.01
STD (Ascorbic acid)	38.37±1.39	54.95±0.69	67.11±0.32	78.15±0.27	95.25±0.77



Graph showing hydrogen peroxide scavenging activity

(AB:Aqueous extract of bark, ACB:Acetone extract of bark, MB:Methanolic extract of bark,ASB1:Aqueous extract of S+B(25+25g), ASB2:Aqueous extract of S+B (15+35g), ASB3:Aqueous extract of S+B (35+15g), STD: Ascorbic acid)

Total phenol contents, as determined by Folin-Ciocalteau method, as reported as Gallic acid equivalents by reference to standard curve (y = 0.0063x, r^2= 0.987).It was noted that various extract of *Hamelia patens* had significant higher total phenol contents. The high amount of phenols in extracts may explain their high antioxidant activities (Table.6).

Table.6.Total phenol contents of various extract of *Hamelia patens* plant

Sample	Total phenol contents (mg/g)
Aqueous extract of bark	413.8
Acetone extract of bark	303.6
Methanolic extract of bark	310.8
Aqueous extract of S+B (25+25g)	354.3
Aqueous extract of S+B (15+35g)	437.5
Aqueous extracts of S+B (35+15 g)	296.2

4. CONCLUSION

This study has demonstrated the antioxidant and antimicrobial activities of various extracts from *Hamelia patens* plant. Acetone was better solvent for extraction of antioxidant and antibacterial substances compared to other solvents but providing high extraction yields and also strong antimicrobial and antioxidant activities. The activities determined in the extracts could be attributed to the phenolic components. Thus, *Hamelia patens* plant can be considered as an easily accessible source of natural antimicrobials and antioxidants agents.

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