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Antimicrobial activity and phytochemical analysis of *Kaempferia rotunda L.* rhizomes

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ABSTRACT

This study was carried out to evaluate the antibacterial activity of different solvent extracts (n-hexane, methanol, ethyl acetate and water) of Ayurvedic medicinal herb Kaempferia rotunda L. against six bacterial strains, i.e. *S. aureus (MTCC 1144), H. influenza (MTCC 3826), P. aeruginosa (MTCC 2474), S. pneumonia (MTCC 655), S. pyogenes (MTCC 442), and L. acidophilus (MTCC 447) and one fungal organism i.e. Candida albicans (MTCC 227) by the agar well diffusion method. The results of antibacterial study showed that ethyl acetate extract of K. rotunda rhizomes has potential antibacterial activity against L. acidophilus (17.3±0.57mm), S. pneumonia (16.6±0.28mm) and S. pyogenes (16.6±0.28mm). While all extracts showed low antifungal activity against Candida albicans. Preliminary phytochemical analysis of K. rotunda revealed the presence of alkaloids, steroids, terpenoids, flavonoids and saponins.*

Keywords: *Kaempferia rotunda L.*, antimicrobial activity, agar well diffusion method, phytochemicals.

INTRODUCTION

Plants are the major source of flavors, fragrances and natural medicines worldwide because they contain a wide variety of biologically active chemicals like alkaloids, terpenoids, flavonoids, steroids and saponins. In the past few decades, a number of medicinal plants have been demonstrated by the researchers *in vitro* to have antimicrobial properties against human pathogens [1-4]. Antimicrobial studies of the crude extracts of plants have led to search for new antimicrobial agents *in vitro* and to isolate pure chemicals from them. Further the phytochemical screenings of plant materials provide a profile of biologically active compounds which have led to discover the natural sources of medicines [5-7]. Recently antimicrobial, antioxidant, anti-inflammatory, anti-allergic, anti-diabetics, anti-carcinogenic and wound healing have been demonstrated as the characteristic properties of phytochemicals [8-14]. The present study focused on the antimicrobial screening of different solvent extracts of *K. rotunda* rhizomes to discover the new source of antimicrobial agents. *K. rotunda* is a perennial rhizomatous herb of *Zingiberaceae* family which has been described in *Ayurveda* for its stomachic, anti-inflammatory, antitumour, antiulcer, wound healing, emetic and vulnerary actions [15]. *K. rotunda* is also known as *Bhumi-Champaka*, *Bhuu-Champaka* or *Hallakam* and widely distributed throughout India in wet and shaded regions [16].

MATERIALS AND METHODS

Plant material

Fresh rhizomes of *Kaempferia rotunda L.* were collected from Sushila Tiwari herbal garden, Rishikesh, Uttarakhand. The plant was identified by the staff of Botanical Survey of India (BSI), Dehradun, and a voucher specimen (Acc. No. 114817) was deposited at the herbarium of BSI, Dehradun. The plant picture is given in *Figure 1*.

Figure 1. *Kaempferia rotunda L.*

Glassware and chemicals

Clean, dry and good quality glassware's were used for all tests. They were properly washed with good detergent, soaked in chromic acid, and finally rinsed in acetone. Analytical grade chemicals, reagents and solvents were used in antimicrobial and phytochemical studies.

Preparation of plant material

The dried rhizomes of *K. rotunda* were grounded well using mechanical grinder into fine powder and stored into airtight glass container with proper labeling.

Extraction procedure

Crude organic extracts n-hexane, methanol (MeOH) and ethyl acetate (EtOAc) were prepared by soaking 50g of the grinded rhizomes with 200ml of each solvent separately for 72 h, whereas the water (H_2O) extract was prepared by heating 50g of grinded plant rhizomes with 200ml of distilled water in a round bottom flask at 30-40ºC for 72 h. Thereafter, the extracts were filtered through Whatmann filter paper and stored in dark colored bottles at 15°C with proper labeling. These extracts were used for further antimicrobial and phytochemical screening.

Determination of antimicrobial activity

Test microorganisms

Six bacterial strains, i.e. *S. aureus (MTCC 1144), H. influenza (MTCC 3826), P. aeruginosa (MTCC 2474), S. pneumonia (MTCC 655), S. pyogenes (MTCC 442), and L. acidophilus (MTCC 447)* and one fungal strain i.e. *Candida albicans (MTCC 227)* were used in vitro studies. Microorganisms were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Preparation of inoculums

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from stock cultures to test tubes of Mueller-Hinton Broth (MHB) for bacteria that were incubated without agitation for 24 h at 37°C.

Antimicrobial study

Antimicrobial activity of different extracts was tested using agar well-diffusion method [17]. *In vitro* antibacterial activity was screened by using Mueller-Hinton Agar (MHA) medium no. 173 (Hi media Pvt. Ltd., Mumbai, India). 0.1 ml of 12-16 h incubated cultures of bacterial species were mixed in molten medium and poured in pre-sterilized Petri plates. Plates were allowed to solidify for 5-10 minutes. A cork borer (6 mm diameter) used to punch wells in

medium and filled with extracts of 45 µl of 200 mg/ml final concentration of extracts. Dimethyl sulfoxide (DMSO) was used as negative control. Efficacies of extracts against bacteria were compared with broad spectrum antibiotic ofloxacin (positive control). Ofloxacin was dissolved into double distilled water. Plates were incubated at 37°C for 24 h in BOD incubator. At the end of incubation, inhibition zones formed around the well were measured with transparent ruler in millimeter. Each sample was assayed in triplicate and mean values were observed. The antimicrobial activity was interpreted from size of diameter of zone of inhibition measured to the nearest millimeter (mm) as observed from clear zones surrounding the wells.

Phytochemical analysis
The preliminary phytochemical analysis of all the extracts was performed by using following standard procedures [18, 19, 20].

Detection of proteins and amino acids
50mg of extract was dissolved in 5ml of distilled water and filtered through Whatman filter paper No.1. The filtrates of each extract were subjected to the following tests.

Xanthoproteic test
1ml of hot nitric acid was added in equal volume of extract in a test tube. The formation of yellow precipitate which is changed to a deep orange color by the addition of ammonia indicated the presence of Xanthoproteins.

Biuret test
2ml of filtrate was treated with few drops of 2% copper sulphate solution. To this, 1ml of 95% ethanol was added, followed by the excess of sodium hydroxide pellets, the development of a pink color in ethanolic layer indicated the presence of proteins.

Ninhydrin test
1ml of 0.1% alcoholic solution of Ninhydrin solution was added to 2ml of filtrate in a test tube and the contents are boiled for 1-2 min in a boiling water bath. Formation of a red-purple color indicated the presence of amino acid.

Detection of carbohydrates
50mg of extract was dissolved in 5ml of triple distilled water and filtered. The filtrate was subjected to the following test.

Fehling's test
1ml of filtrate was boiled on a water bath with 1ml each of Fehling solutions 1 and 2. The formation of a red precipitate confirmed the presence of reducing sugar.

Iodine test
To 2 ml of filtrate, 2 drops of iodine solution and 1ml of water were added in a test tube. A blue-black or reddish-purple colored precipitate indicated the presence of polysaccharides.

Detection of alkaloids
50mg of solvent free extract was dissolved in few ml of dilute hydrochloric acid and filtered. The filtrate was subjected to the following tests for the detection of alkaloids.

Mayer's test
To a few ml of filtrate, two drops of Mayer's reagent (potassium mercuric iodide solution) were added from the side of the test tube. A whitish creamy or pale yellow colored precipitate indicated the presence of alkaloids.

Wagner's test
To a few ml of filtrate, few drops of Wagner's reagent (iodine in potassium iodide) were added by the side of the test tube. A reddish-brown colored precipitate was confirmed the presence of alkaloids.

Detection of terpenoids
Salkowski's test
1ml of extract was mixed with 2ml of chloroform in a test tube. 2ml of concentrated sulphuric acid was added carefully from the side of the test tube. Formation of a reddish-brown ring immediately at the interphase of two liquids confirmed the presence of terpenoids.

Detection of flavonoids
Shinoda's test
Four pieces of magnesium ribbon were added to the extract followed by few drops of concentrated hydrochloric acid. A pink or red color indicates the presence of flavonoids. Colors varying from orange to red indicate flavones, red to crimson indicated flavonoids, crimson to magenta indicated the presence of flavonones.

Sodium hydroxide test
To a few ml of extract, 10% aqueous sodium hydroxide was added in a test tube. The development of yellow color which becomes colorless on addition of dilute hydrochloric acid indicated the presence of flavonoids.

Detection of steroids
Salkowaski's test
2ml of concentrated sulphuric acid was carefully added to a 2ml chloroform solution of extract, the chloroform layer shows a reddish-brown color and the acid layer shows a green fluorescence.

Keller-Kilani test
2ml of crude extract was mixed with 2ml of glacial acetic acid containing 1 drop of 5% $FeCl_3$ solution. The mixture was then poured into another test tube containing 2ml of concentrated sulphuric acid. The formation of a brown ring at the interphase indicated the presence of steroids.

Detection of saponins
Foam test
2ml of extract was diluted with 6ml of distilled water in a test tube. The mixture was shaken vigorously. The formation of persistent foam indicated the presence of saponins.

Froth test
5ml of extract was diluted with 20ml distilled water in a graduated cylinder and shaken vigorously then was left to stand for 10 minutes. The persistent of about 1.5 cm layer of froth confirmed the presence of saponins.

Detection of phenolic compounds and tannins
100mg of crude extract was dissolved in 10ml of distilled water in a test tube and filtered. The filtrate was subjected to the following tests for the detection of phenolic compounds.

Ferric chloride test
To 5ml of this filtrate, few drops of neutral 5% ferric chloride solution were added. The appearance of a dark green color indicated the presence of phenolic compounds.

Gelatin test
To 5ml of this filtrate, 2ml of 1% solution of gelatin containing 10% sodium chloride was added to it. Formation of a white precipitate indicated the presence of phenolic compounds.

Detection of anthraquinone glycosides
Born trager's test
1ml of extract and 1ml of dilute sulphuric acid was boiled in a boiling tube for 5 minutes. The content was filtered while hot. The filtrate was cooled and shakes with equal volume of dichloromethane or chloroform. The organic layer of dichloromethane or chloroform was separated and again shakes with half of its volume of ammonia. A rose pink or cherry red color is immediate produced in the ammoniacal layer indicates the presences of anthraquinone glycosides.

RESULTS AND DISCUSSION

Antimicrobial activity

The results of antimicrobial studies of different solvent extracts showed that *K. rotunda* possess significant antimicrobial activity against respiratory tract pathogens (*Table 1*). Among the four solvent extracts tested, ethyl acetate extract showed maximum zone of inhibition against the *L. acidophilus (MTCC 447)* (17.3±0.57mm), *S. pneumoniae* (16.6±0.28mm), *S. pyogenes* (16.6±0.28 mm) and *P. aeruginosa*, (15.3±0.28 mm). The methanol extract exhibited moderate activity against *P. aeruginosa*, (15.6±0.28 mm), *S. pneumoniae* (15.3±0.57 mm) and S. *pyogenes* (15.3±0.28mm). In addition, methanolic extract of *K. rotunda* reportedly showed potential antiplatelet aggregation (Jantan *et al*., 2008), antihyperglycemic and antinociceptive properties (Sultana *et al.*, 2012).

Table 1. The inhibition zone diameter of various extracts of *K. rotunda L.* against respiratory tract pathogens

S.No.	Pathogens	Diameters of inhibition zone (mm)				Positive Control (Ofloxacin)
		Hexane	MeOH	EtOAc	H_2O	
1.	*S. aureus* (MTCC 1144)	8.6±0.28	10.3±0.57	14.3±0.28	13.3±0.57	33.0±0.50
2.	*H. influenzae* (MTCC 3826)	9.0±0.50	12.0±0.50	13.6±0.57	13.3±0.57	35.3±0.28
3.	*P. aeruginosa* (MTCC 2474)	10.6±0.28	12.3±0.28	15.3±0.28	15.6±0.28	34.3±0.57
4.	*S. pneumoniae* (MTCC 655)	11.6±0.28	13.3±0.57	16.6±0.28	15.3±0.28	32.6±0.57
5.	*S. pyogenes* (MTCC 442)	9.3±0.28	12.6±0.28	16.6±0.28	15.5±0.57	32.6±0.57
6.	*L. acidophilus (MTCC 447)*	10.3±0.28	12.6±0.28	17.3±0.57	14.3±0.57	34.3±0.57
7.	*Candia albicans* (MTCC 227)	7.3±0.28	11.0±0.50	11.6±0.57	11.3±0.76	27.0±0.50

**Values are means of three replicates, Cork borer diameter: 6 mm*

Phytochemical analysis

The phytochemical study of *K. rotunda* revealed the presence of alkaloids, steroids, terpenoids, flavonoids and saponins which are important pharmacologically active compounds. Alkaloids and flavonoids were found to be present in all solvent extracts while proteins, amino acids, carbohydrates and phenolic compounds were found to be absent in all the extracts. Terpenoids and steroids were found in bulk in hexane and ethyl acetate extracts whereas they were found absent in methanolic and water extracts. Flavonoids were found in bulk in hexane, methanolic and water extracts whereas saponins were more prominent in water extract. Anthraquinone glycosides were found absent in hexane and methanolic extracts, while they were present in ethyl acetate and water extracts. Stevenson *et al.* (2007) reported seven compounds including six polyoxygenated cyclohexane derivatives i.e. (-)-6-acetylzeylenol (1), four acylated derivatives of 1-benzoyloxymethyl-1,6-epoxycyclohexan-2,3,4,5-tetrol (3-6), a Diels-Alder adduct of 3-benzoyl-1-benzoyloxymethylcyclohexa-4,6-dien-2,3-diol (7), and a triacylated derivative of salicin (9), and a cyclohexane diepoxide, crotepoxide (8), in methanolic extract *K. rotunda* rhizomes. Recently three flavanones namely 5-hydroxy-7-methoxyflavanone, 7-hydroxy-5-methoxyflavanone, and 5, 7-dihydroxyflavanone have also been isolated from the methanolic extract of *K. rotunda* by Atun *et al*., 2013, which showed significant antimutagenic activity.

Table 2. Results of phytochemical studies of rhizomes extracts of *K. rotunda L.*

Phytoconstituent		Hexane	MeOH	EtOAc	H_2O
Proteins and amino acids	Xanthoproteic test	–	–	–	–
	Biuret test	–	–	–	–
	Ninhydrin test	–	–	–	–
Carbohydrates	Fehling's test	–	–	–	–
	Iodine test	–	–	–	–
Alkaloids	Mayer's test	+	+	+	+
	Wagner's test	+	+	+	+
Terpenoids	Salkowaski's test	++	+	++	–
Flavonoids	Shinoda's test	++	++	+	++
	Sodium hydroxide test	++	++	+	++
Steroids	Salkowaski's test	++	+	++	–
	Keller-Kilani test	++	+	++	–
Saponin's	Foam test	–	+	–	++
	Forth test	–	+	–	++
Phenolic compounds	Ferric chloride test	–	–	–	–
	Gelatin test	–	–	–	–
Anthraquinone glycosides	Born Trager's test	–	–	+	+

[++]= more prominent, [+] = present, [-] = absent

Phytochemicals variation present in different solvents as shown in *Table 2* might be expected due to the different polarities of the solvents. Many evidences gathered in earlier studies which confirmed that these phytochemicals exhibiting potential antimicrobial properties [21-24]. Saponins were found in methanolic and water extracts which demonstrated detergent like property [25] and antimicrobial activity [26]. Phytochemicals reportedly showed several medicinal and biological properties like alkaloids exhibit analgesic [27] and anti-inflammatory properties [28], terpenoids exhibit antifungal [29] and antitumor properties [30], whereas flavonoids exhibit antioxidant, antiviral [31], anti-inflammatory and anticancer actions [32]. Therefore, the antimicrobial activities of extracts might be assigned to a number of small terpenoids and phenolic compounds which in pure form also demonstrated high antibacterial activity.

CONCLUSION

The results of antibacterial screening suggest that the ethyl acetate and water extracts have significant antibacterial activity against to *L. acidophilus*, *S. pyogenes* and *S. pneumonia*, suggesting that the *K. rotunda* rhizomes can be significant to cure respiratory infections, pneumonia, skin and mouth diseases. The phytochemical study of the different solvent extracts of *K. rotunda* rhizomes revealed the presence several bioactive phytochemicals including alkaloids, steroids, terpenoids, flavonoids and saponins having significant antimicrobial potential against respiratory tract pathogens. Further, the isolation, purification and identification of these bioactive constituents from the extracts would be highly recommended to get the effective antimicrobial agents.

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