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IN VITRO ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF ETHANOLIC LEAF EXTRACT OF *Rosemarinus officinalis* L.

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Abstract— Ethanol extract of the leaves of Rosemarinus officinalis L. was investigated for their in vitro antimicrobial properties. The plant was collected from Kolathur, Perambalur District, Tamil Nadu. The antimicrobial activities of the extract against six pathogenic strains were evaluated based on the inhibition zone using the disc diffusion assay. The ethanolic extracts of R. officinalis L. had inhibitory effects on the growth of six strains tested. The ethanolic extract of R. officinalis L. showed considerably high activity against Salmonella sp. The phytochemical screening of leaves of R. officinalis L. revealed the presence of flavonoids, tannins, alkaloids, fats and fixed oil, protein, carbohydrate, insulin and lignin.

Keywords: Rosemarinus officinalis L., ethanol extract, antimicrobial potential, phytochemical analysis.

INTRODUCTION

Since prehistoric era, people have been searching the nature in exploration of new medicinal compounds. Among which, plants are the vital source of medicinal compounds and play a major role in the human society to combat diseases (Wink *et al.*, 2005). A condensed history of the contribution of plants to medicine was given by Philipson (2001). Today a huge number of medicinal plants with therapeutic properties have been used to treat various diseases (Kalemba and Kunicka, 2003). Now about 80% of the population depends on traditional plants for primary health care worldwide. A survey of current pharmaceutical exploit revealed that, of the total prescription drugs dispensed, 25% are plant origin (Anburaj., 2009).

Plants are storehouses of a wide variety of secondary metabolites such as

tannins, terpenoids, alkaloids and flavonoids, which have demonstrated their antimicrobial properties in vitro. Worldwide medicinal plants have been used for its antibacterial, antifungal and antiviral activities (Barbour *et al.,* 2004; Yasunaka *et al.,* 2005, Annamalai et al., 2007, Anburaj, 2009; Anburaj, 2011). Researchers are increasingly focusing their attention to natural products and searching for novel medicinal plant compounds to develop better drugs against cancer, as well as viral and microbial infections (Koshy *et al*., 2009; Anburaj, 2009). India has a rich culture of medicinal herbs and spices and has a vast geographical area with high potential abilities for Ayurvedic, Unani and Siddha traditional medicines. However, only very few have been studied chemically and pharmacologically for their potential medicinal value (Gupta *et al.*, 2005; Anburaj, 2009).

R. officinalis L. (Rosemary) is a member of the mint family (Lamiaceae) and originally grows in southern Europe. Rosemary is a medicinal herb and commonly used as spice and flavouring agents in food processing (Lo *et al*., 2002). Rosemary has been widely accepted as one of the spices with the highest antioxidant activity (Peng *et al*., 2005) and it also used as an antibacterial, antifungal (Fernandez-Lopez *et al*., 2005; Kabouche *et al*., 2005) and anticancer agent (Leal *et al*., 2003). Rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram-positive and Gram-negative bacteria (Moreno *et al*., 2006). It is proved that rosemary extracts have excellent bioactive properties, but their antimicrobial activities have not been deeply studied. Thus, the objectives of the present work are to test the antibacterial activity of ethanolic leaf extract of rosemary against different bacterial pathogens using disc diffusion method and to qualitatively determine phytochemical constituents of leaf extract of rosemary.

MATERIALS AND METHODS

Collection of plant

The plant material used in this study was *R. officinalis* L. and fresh leaves of this plant were collected from Kolathur, Perambalur District, Tamil Nadu. The leaves were washed thoroughly with normal tap water followed by sterile distilled water. Then, the leaves were dried under shade conditions at room temperature, after which they were grinded to a uniform powder.

Pathogenic strains and culture preparation

Pathogenic strains of *Escherichia coli*, *Klebsiella species*, *Pseudomonas species*, *Staphylococcus species*, *Candida albicans* and *Salmonella species* were obtained from Department of Microbiology in the Dhanalakshmi Srinivasan Hospital, Siruvachur, Perambalur District, Tamil Nadu and were maintained on nutrient agar medium at 4 ºC for further experiments. The strains were inoculated in the nutrient broth and incubated at 37 ºC for 24 h.

Preparation of plant extraction

Leaves were extracted using a conventional solvent extraction procedure. Homogenized dry leaves were preliminary extracted with water and different alcoholic solvent such as ethanol, methanol, acetone and hexane. Among different solvents tested, ethanol enhanced complete extraction of leaves and thus ethanol was selected for further investigation. For ethanol extraction, different grams of dried leaves such as 5, 10 and 15 was soaked into 100 mL of 90% ethanol for 24 h at room temperature. After extraction, samples were filtered with Whatman No. 1 filter paper and the filtrate was concentrated on a rotary evaporator at 45 ºC for ethanol elimination (Anburaj 2007; Anburaj et al., 2012; Demetrio et al., 2015).

Antimicrobial assay

The disk diffusion test was performed using the standard procedure (NCCLS). The inoculum suspension of each bacterial strain was swabbed on the entire surface of nutrient agar. Sterile 9-mm filter paper discs were aseptically placed on nutrient agar surfaces and crude ethanol extracts were immediately added to discs in volumes of 50 µL. The plates were left at ambient temperature for 15 min to allow excess pre diffusion of extracts prior to incubation at 37°C for 24 h. Diameters of inhibition zones were measured. A control test was performed with some antibiotic discs tetracycline. There were used as experimental positive control and ethanol as negative control. The tests were performed in duplicate for the evaluated microorganism (Anburaj 2011; Anburaj et al., 2012; Demetrio et al., 2015; Anburaj et al., 2017)

Phytochemical Screening

Phytochemical screening was performed using standard procedures (Trease *et al.*, 1989; Anburaj 2009).

Test for Flavonoids (Alkaline reagent test)

2 mL of extracts was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow colour, which

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becomes colourless on addition of dilute hydrochloric acid, indicates the presence of flavonoids (Kathiresan et al., 2011; Kathiresan et al., 2013; Kathiresan et al., 2016).

Test for Saponins (Foam test)

2 mL of extract was added with 6 mL of water in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins (Anburaj, 2009; Asmathunisha et al., 2010; Anburaj 2011; Anburaj et al., 2012; Anburaj et al., 2020).

Test for Tannins (Braymer's test)

2 mL of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish colour solution (Asmathunisha et al., 2013; Kathiresan et al., 2016; Anburaj et al., 2020).

Test for Alkaloids (Wagner's reagent)

A fraction of extract was treated with 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 mL of water) and observed for the formation of reddish brown precipitate (or colouration) (Kathiresan et al., 2014).

Test for Cardiac glycosides (Keller Kelliani's test)

5 mL of each extract was treated with 2 mL of glacial acetic acid in a test tube and a drop of ferric chloride solution was added to it. This was carefully underlayed with 1ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form.

Test for Phlobatannins (Precipitate test)

Deposition of a red precipitate when 2 mL of extract was boiled with 1 mL of 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

Test for fixed oil and fats

Petroleum ether extracts were pressed between two filter papers. Oil stains on the papers indicated the presence of fixed oils. To the petroleum ether extracts, few drops of 0.5N alcoholic potassium hydroxide and a drop of phenolphthalein were added and heated on a water bath for 1-2 hours. Formation of soap and/or partial neutralization of alkali indicated the presence of fixed oils and fats.

Test for Amino acids and Proteins

2mL of filtrate was treated with 2-5 drops of ninhydrin solution (1% ninhydrin solution in

acetone) placed in a boiling water bath for 1-2 minutes and observed for the formation of purple colour.

Test for Carbohydrates (Molisch's test)

Few drops of Molisch's reagent were added to 2 mL portion of the various extracts. This was followed by addition of 2 mL of conc. H_2SO_4 down the side of the test tube. The mixture was then allowed to stand for two-three minutes. Formation of a red or dull violet colour at the interphase of the two layers was a positive test.

Test for glycosides (Liebermann's test)

2 ml of the organic extract was dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added in it. The solution was cooled well in ice. Sulphuric acid was then added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus.

Statistical analysis

Data were presented as means +SD. Statistical analysis was conducted using the SPSS 11.5 statistical package.

RESULTS AND DISCUSSION

Antimicrobial activity

The antimicrobial activity is varying with the bioactive compounds of different species and different pathogenic strains (Kumar *et al*., 2006). In the present study, initially leaves of Rosemary were extracted with different alcoholic solvents and among which ethanol enhanced complete extraction and was selected for further studies. The antimicrobial activity of ethanolic leaf extraction of Rosemary against six different clinical microbial strains was qualitatively (disc diffusion method) screened by the presence or absence inhibition zones. Table 1 summarizes the microbial growth inhibition by ethanolic extracts of Rosemary. Rosemary ethanol extract produced a zone of inhibition against all the pathogen tested. However, the aqueous extracts of the leaves of Rosemary have exhibited differential antimicrobial activities on both fungal and bacterial species. Among which, *Salmonella* sp. showed maximum zone of inhibition and this was followed by *Staphylococcus* sp.

The agar diffusion bioassay showed that leaf extracts have the highest activity against all gram-positive and they also showed good activity against gram-negative bacteria. This is in agreement with observations by other researchers (Ali *et al*., 2001; Chattopadhyay *at al*., 2002; Anburaj et al., 2020). The least zone of inhibition was observed in *Klebsiella* sp. The reason for this variation between fungal and

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bacterial species could be ascribed to the morphological differences between these microorganisms. Diluting extracts usually weakens their antimicrobial activity. In the present study, a maximum zone of inhibition was observed in higher concentration of extract. At lower concentration, it shows micro biostatic or loss of antimicrobial activity. Low doses may inhibit growth; whereas high doses may arrest the microbial growth (Benkendorff, 2001; Anburaj, 2007; Anburaj, 2009). Moreno *et al.* (2006) reported that carnosic acid was the major bioactive compounds of the rosemary extract exhibiting antimicrobial activity.

Phytochemical analysis

According to Mallikharjuna *et al.* (2007) preliminary phytochemical tests are useful in the detection of bioactive principles and subsequently may lead to drug discovery and development. The results of the present study revealed the presences of flavonoids, tannins, alkaloids, fats and fixed oil, protein, carbohydrate, insulin and lignin (Table 2). Whereas, saponins,cardiac glycosides, phlobatannins and glycosides were absent. Ahmad *et al.* (2006) reported that flavonoids and tannins have been linked to antibacterial activity and antidiarrheal activity. In another study, Lui (2003) reported that flavonoids, tannins and alkaloids have anti-inflammatory effects. The presence of flavonoids and phenolic compounds in plants have been reported to use several biological effects which includes antioxidant, free radical scavenging abilities, anti-inflammatory and carcinogenic etc (Lalitha and Jayanthi, 2012; Anburaj et al., 2012; Anburaj et al., 2015; Anburaj et al.,2020).

Table 1. Antimicrobial activities of ethanolic leaf extract of *R. officinalis* L. against some microbial pathogens

Clinical Pathogen	Zone of inhibition (mm)		
	5g\50 µL disc	10g\50 µL disc	15g\50 µL disc
Bacterial strain			
Gram positive			
Staphylococcus sp.	3.5±0.90	4.6±0.55	5.1±0.72
Gram negative			
Escherichia coli	2.0±0.00	2.7±0.07	3.0±0.31
Klebsiella sp.	1.0±0.02	1.8±0.48	2.6±0.00
Pseudomonas sp.	2.8±0.14	3.6±0.17	4.2±0.42
Salmonella sp.	4.6±0.87	5.6±0.36	6.2±0.70
Fungal strain			
Candida albicans	2.6±0.04	2.8±0.19	3.2±0.21

Table 2. Phytochemical analysis of Ethanolic leaf extract of *R. officinalis* L.

S.No	Name of the test	Ethanol extract
1	Flavonoids	+
2	Saponins	-
3	Tannins	+

4	Alkaloids	+
5	Cardiac glycosides	-
6	Phlobatannins	-
7	Fats and fixed oil	+
8	Proteins	+
9	Carbohohydrates	+
10	Glycosides	-

+ Positive - Negative

Conclusion

As a conclusion, the present results may suggest that ethanolic extract of Rosemary possess compounds with antibacterial and anticandial properties which can be used as antimicrobial agents in new drugs. The results of this study may also consider sufficient to further studies for the isolation and identification of the active compounds.

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