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RESEARCH PAPER

Evaluation of Antioxidant and Antimicrobial Activity of *Rhododendron arboreum* Flowers Extract

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Abstract

Ethanolic extract of *Rhododendron arboreum* flowers was analyzed for antioxidant and antimicrobial activity. Antioxidant activity was determined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) and FRAP (Ferric reducing antioxidant power) assays. The antioxidant activity of flowers extract for DPPH assay was 134.1±2.34 mM TE/g and for FRAP assay was 140.6±2.76 mM TE/g. The antimicrobial activity was measured by agar well diffusion method against six different bacterial species out of which; three were Gram-positive and three were Gram-negative. The minimum inhibitory concentration (MIC) and the minimum bacteriocidal concentration (MBC) were also determined for each pathogen using a 96-well microtiter plate method. The results reported in mg/ml, indicated that all the bacterial strains (Gram-positive and Gram-negative) were effectively inhibited by the ethanolic extract of Rhododendron flowers. *E.coli* exhibited highest sensitivity to flowers extracts with maximum zone of inhibition (17mm) at extract concentration 50mg/ml and *Bacillus cereus* and *Bacillus subtilis* showed minimum inhibition (8mm each) at extract concentration of 25mg/ml. Highest MIC was observed against *E.coli* (1 mg/ml) and *Shigella flexneri* (1 mg/ml).

Keywords: *Rhododendron arboreum*, antioxidant, antimicrobial, minimum inhibitory concentration, minimum bacteriocidal concentration

Antioxidant defence mechanism of a cell is overcome by reactive oxygen species by oxidising biological molecules thus, inducing damage to the cell membranes, proteins, carbohydrates and DNA. Hypertension, diabetes, heart failure and several pathological situations are the cause of this oxidative stress (Gonenc *et al*., 2013). The natural antioxidants not only enhances the body's defence system, but also protect food (Chandrasekara and Shahidi, 2010, Deng *et al*., 2012; Eberhardt *et al*., 2000; Fu *et al*., 2011; Li *et al*., 2008; Li *et al*., 2007 and Stangeland *et al*., 2009). Natural antioxidants are always the first choice, because they are better than synthetic antioxidants. There is also a pressing need to develop new antimicrobial agents as pathogenic bacteria have increased the incidence of resistance causing an increase in mortality and morbidity around the world (Kraiczy and Wurzner, 2006 and Oskay *et al*., 2010). In this context, investigation has been carried out on plants source such as *Rhododendron* flower because they contain many bioactive compounds and fewer toxins for antimicrobial and antioxidant *Rhododendron arboreum*

(Srivastava, 2012). It is the state flower of Himachal Pradesh and commonly known as *Burans* (Himachal Pradesh) and *Laliguras* (Nepal). It is a native to China, Nepal, Malaysia and India. This evergreen tree grows at an elevation of 4,500 to 10,500 ft above mean sea level (Rai and Rai, 1994). Flowering season is from March to April, bearing deep red or crimson to pale pink flowers. The flowers of *Rhododendron arboreum* have been found to be rich in carbohydrates, amino acids, flavones, coumaric acid, ursolic acid and resins. Flowers and leaves of several species of *Rhododendron* contain simple phenols, flavonoids, anthocyanins and antioxidants (Takahashi *et al*., 2001; Krishnaraju *et al*., 2005; Cho *et al.,* 2008 and Chung *et al*., 1996). Flowers of *Rhododendron arboreum* also possess anti-inflammatory and cholinergic activity (Agrawal and Sharma, 1988; Tripathi *et al*., 1992).

Therefore, the main aim of this study is to evaluate antioxidant and antimicrobial activity of the flower of *Rhododendron arboreum* and the results are reported here.

MATERIALS AND METHODS

Plant material and extraction

Rhododendron arboreum flowers were collected from Mandi district, Himachal Pradesh. About 50 g sample of shade dried flower were extracted with ethanol (350 ml) using soxhlet apparatus. Extracts were concentrated to semi solid mass by evaporating the solvents and drying in a dessicator. The percentage yield was found to be 30 % w/w. Sample was kept at -20°C, till further use.

Antioxidant activity

DPPH scavenging activity

The measurement of the DPPH radical scavenging activity was performed according to (Niciforovic *et al*., 2010) with some modifications. Stock solution of DPPH (24 mg DPPH with 100 ml methanol) was prepared and kept at -20°C, until needed. Working solution was prepared by mixing 10 ml stock solution with 45 ml ethanol to obtain an absorbance of 1.1 ± 0.02 units at 515 nm. The reaction mixture consisting of 150 µl extract and 2,850 µl DPPH solution was allowed to stand for 24 h in the dark. Absorbance was measured at 515 nm. Trolox was taken as a standard for preparation of standard curve and results were expressed in mM Trolox equivalents/g fresh mass.

FRAP radical scavenging assay

The FRAP radical scavenging assay was done according to Benzie and Strain (1996). 150 µl of flower extract in methanol was mixed with 2,850 µl FRAP reagent and allowed to stand for 30 minutes in dark. Absorbance was taken at 593 nm. Trolox was taken as a standard for preparation of standard curve and results were expressed in mM Trolox equivalents/g fresh mass. The assay was performed in triplicates.

Determination of antimicrobial activity

Test microorganisms

The antimicrobial activity of flower extract was assessed against six bacteria species: *Bacillus subtilus* (ATCC 6051), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923) (Gram-positive) and *Escherichia coli (*ATCC 25922*), Salmonella enteric (ATCC* 14028*), Shigella flexineri (ATCC* 12022*) (Gram-negative). Cultures were incubated overnight at* 37°C.

Antimicrobial screening

Antibacterial activity assay was carried out using agar well diffusion method (Gutierrez *et al*., 2008). 100 µl of bacterial cell suspension was spread over the nutrient agar plate and 6 mm wells were punched into the agar with a sterile cock borer. 50 µl of flower extract of different concentration (25 and 50 mg/ml) were added to each well. Agar plates were now incubated at 37°C, overnight. Triplicate plates of each organism were prepared. 10 µg/ml of gentamicin was used as positive controls of inhibition.

Broth micro-dilution assay for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of ethanolic extract of flower was determined by well microtiter plate method (Gutierrez

et al., 2008). MIC is the minimum concentration at which microbial growth is inhibited. The first row of each plate contained 100 µl of nutrient broth and 100 µl of each fraction from extract re-dissolved in methanol at 16 mg/ml. From second row onwards 100 µl broth was added in each well and two fold dilution of extract was done present in first row. Dilution was done till eleventh column. Eleven final concentrations (16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625 mg/ml) were tested. Twelfth column was taken as a positive control and row as negative control. In positive control, growth media along with microorganism was investigated, whereas in negative control each fraction of extract along with different dilution along with media added.

Lastly, 10 µl of microbial culture at 10^6 CFU/ml was added in each well excluding negative control. Plates were inoculated at 37°C for 24 hr. After incubation 20 µl of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) dye is added into each well and again re-incubated to develop colour from yellow to pink. MIC was determined by lowest concentration at which no colour development occurs i.e. well remained as yellow. The minimum bactericidal concentration (MBC) was determined by sub-culturing samples from the wells with concentrations above the MIC on new plates. The MBC was considered as the lowest concentration of the extract associated with no bacterial culture. The results were expressed in mg/ml.

RESULTS AND DISCUSSION

DPPH scavenging assay

Rhododendron arboreum ethanolic extract scavenged DPPH radical in such a manner that it shows higher activity than Trolox as standard control. Flowers extract has good scavenging activity (134.1±2.34 mM TE/g) as shown in Fig. 1, conferring the extract as rich in maximum composition of phenolics providing them the potential of scavenging free radicals.

FRAP Assay

Fig. 1 showed higher value of antioxidant activity in FRAP assay as (140.6±2.76 mM TE/g). It is due to polyphenols present in *Rhododendron* flower extract.

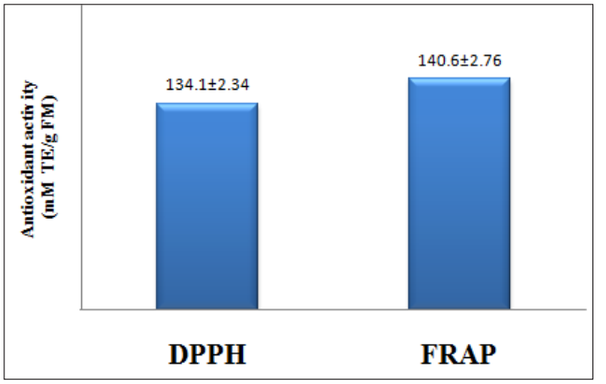

Fig. 1: Antioxidant activity of *Rhododendron arboreum* flower extract determined by the DPPH and FRAP assays

From the above results, apparent that *Rhododendron arboreum* flowers showed good antioxidant activity. Antioxidant activity is based on free radical scavenging activity. DPPH scavenging assay is based on ability of reducing the violet colour as sign of antioxidant activity. DPPH is the stable free radical that contains a decentralized electron. When DPPH is mixed with flower extract it gives hydrogen atom to reduce the free radical and changes colour from deep violet to yellow. Greater change in colour indicates increase in antioxidant activity (Naik *et al*., 1993).

Free radical scavenging activity determined by FRAP assay is based on reduction of ferric tripyridyltriazine [Fe (III)-TPTZ] complex to the ferrous tripyridyltriazine [Fe (II)-TPTZ] at low pH. Phenols and flavonoids are known for free radical scavenging activity. *Rhododendron arboreum* flowers are found to have good amount of phenolic compounds which are known as hydrophilic antioxidants that might be responsible for the high antioxidant activity (Rajan and Muthukrishnana, 2013; Kar, 2007).

Antimicrobial assay

Table 1 showed that flower extract have antimicrobial activity against both Gram-positive and Gram-negative bacteria. Activity was directly proportional to concentration. *Rhododendron arboreum* showed very

good antimicrobial activity against *E.coli* (17 mm zone at 50 mg/ml conc.)

The extracts of the flower of *Rhododendron arboreum* containing multiple organic components including flavonoids, saponins, alkaloids all of which are known to have antibacterial affects (Ani *et al*., 2011). As the extract is rich in phenolic compounds, hence showing high antimicrobial properties. The main antimicrobial mechanism of phenolics is thought to be focussed on the presence of cytoplasmtic membrane and the outer lipid membrane of the Gram negative bacteria which provides additional protection to these bacteria from antimicrobial compounds. This explains the lesser antimicrobial activity of extract against *Salmonella enterica* (Burdulis *et al.*, 2009). This inhibition could also be caused other compounds present in the extract like weak organic acids or phenolic compounds through other mechanisms that are not membrane based (Vattem *et al*., 2004).

Bacteriocidal effect

The flower extract was tested with micro-dilution method using INT dye to determine MBC and MIC values of bacteria. Six different Gram positive and Gram negative bacteria are taken to determine the MIC. Results showed that flower extract have good bactericidal property against Gram negative bacteria in Table 2. Ethanolic extract of flowers showed MIC ranging from 1 mg/ml to 8 mg/ml. Best MIC values were against *E.coli* (1mg/ml) and *Shigella flexneri* (1 mg/ml). Flower extract also showed good results against *Bacillus subtilis* (2 mg/ml).

The micro dilution method with INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) dye is used to determine the MIC and MBC of bacteria against most of the Gram-positive bacteria which showed high bacteriocidal activity at low inhibitory concentration as compared to Gram negative bacteria. This could be due to outer lipid membrane, which provides additional protection to Gram negative bacteria (Burdulis *et al.*, 2009). It has been observed that, extract showed higher antimicrobial activity with inhibitory potential at lower concentrations.

Table 1: Antimicrobial activity of *Rhododendron arboreum*

Sample	Conc. of extract (mg/ml)	Zone of inhibition against Gram-positive bacteria (mm)			Zone of inhibition against Gram-negative bacteria (mm)		
		Bacillus subtilus	*Bacillus cereus*	*Staphylococcus aureus*	*E.coli*	*Salmonella enterica*	*Shigella flexneri*
R.arboreum	25	8	8	9	12	9	11
	50	10	11	11	17	11	14

Table 2: Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ethanolic extracts of *Rhododendron arboretum* flowers

Bacterial isolates	Concentration (mg/ml)	
	MIC	MBC
Bacillus subtillus	2	>4
Bacillus cereus	8	16
Staphylococcus aureus	8	>16
Escherichia coli	1	2
Salmonella enterica	8	>16
Shigella flexneri	1	2

CONCLUSION

In the present study, ethanolic extract of flowers of *Rhododendron arboreum* showed very good antioxidant and antimicrobial activity. As the study showed good results of MIC and MBC against potent disease causing microorganisms, this can be used as bacteriostatic and bacteriocidal agent in the food industry for the preservation of food products. Based on the results, flowers of *Rhododendron arboreum* has the potential for development of nutraceuticals, functional foods as well as therapeutic agents.

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