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Comparative study of antioxidant activity and nitric oxide synthase activation property of different extracts from *Rhododendron arboreum* flower

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Abstract: The present study was conducted to evaluate the antioxidant activity and nitric oxide synthase (NOS) activation properties of the different extracts of *Rhododendron arboreum* flower (*Ra*F). Cold water, hot water and ethanolic extract of *Ra*F were evaluated for antioxidant activity against hydroxyl radical, superoxide radical and lipid peroxidation. EC_{50} values of cold water, hot water and ethanolic extracts of *Ra*F represented 260, 208 and 41.7 µg/ml respectively in case of hydroxyl radical scavenging activity; 94.7, 154.8 and 36.3 µg/ml respectively in superoxide scavenging activity and 286.3, 239 and 45.6 µg/ml respectively in case of lipid peroxidation inhibition. Ethanolic extract showed significant antioxidant activity in all the test systems. Nitric oxide (NO) production was determined spectrophotometrically by conversion of oxyhemoglobin to methemoglobin. Cold water, hot water and ethanolic extracts also increased significantly nitric oxide production (264, 472.5 and 1041.7 pmol/mg dry wt/h respectively) over the control. Results show that, ethanolic extract possesses maximum antioxidant and nitric oxide synthase (NOS) activation properties, which might be utilized as a promising source of therapeutics.
Keywords: Antioxidant activity, Hydroxyl radical, Lipid peroxidation, Reactive oxygen species, Superoxide radical.

1. Introduction

ROS or reactive oxygen species produced by sunlight, ultraviolet, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging (1). Uncontrolled production of reactive oxygen species (ROS) is responsible for several pathophysiological processes (2, 3). The cause of a majority of disease conditions like atherosclerosis, hypertension, ischemic disease, Alzheimer's disease, Parkinsonism, cancer, diabetes mellitus and inflammatory conditions are being considered to be preliminarily due to imbalance between prooxidant and antioxidant homeostasis (4). Superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. However, the damaging action of the hydroxyl radical is the strongest among free radicals. Synthetic compounds are found to be strong radical scavengers but usually they have side effects (5). Neutralization of this radical activity by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy (6).

Nitric oxide (NO) produced at the cellular level from L-arginine catalyzed by nitric oxide synthase (NOS) is a very important signaling molecule (7, 8). It is well studied in mammalian system and has been

found to have numerous roles in pathophysiology (9, 10). Cellular production of NO below physiologic level causes initiation of different diseases like hypertension, atherosclerosis, diabetes mellitus, ischemia, stroke, myocardial infarction, heart failure, hypoxia, Alzheimer's disease, fibrosis, cancer, renal failure, etc (11). Activation of NOS enzyme to elevate NO production could protect the body from these killer diseases. Thus, NOS activation by supplementation of food would find a new route of therapy.

Rhododendron arboreum, commonly known as rose tree is well known for its wide applicability as food and medicine. *R. arboreum* is an important medicinal plant which is used for treatment of various ailments in Ayurvedic system of medicine. The root, leaves and flowers of *R. arboreum* are important crude source of drugs in traditional and modern system of medicines (12-14). The flowers are sourish-sweet in taste and used in squash and cold drinks (15). The flowers are used in dysentery, fever, headache and are known to possess anti-inflammatory activity (16).

The present study was conducted to evaluate the antioxidant activity and NOS activation properties of the different extracts of *R. arboreum* flower.

2. Materials and Methods

Chemicals

Standards L-ascorbic acid and catechin were purchased from Sigma (St. Louis, MO, USA). NBT (nitro blue tetra zolium), TBA (Thiobarbituric acid), TCA (trichloroacetic acid), deoxyribose were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Sample collection and preparation

Flowers of *R. arboreum* locally known as 'Gurash' (Nepali), were collected from forests of Eastern Himalaya, West Bengal, India.

Cold water extracts were prepared from fresh flowers (100 g/100 ml of distilled water) after homogenization (in 0.1 M phosphate buffer, pH 7.4) and centrifugation at 15,000 g for 30 min at 4° C. Supernatant was lyophilized (Lyolab BII LSL Secfroid Lyophilizer) and then stored at -20° C for further use. Hot water extract was also prepared from fresh flowers (100 g/100 ml 0.1 M phosphate buffer, *p*H 7.4) and boiled in water bath for 1 h, then homogenized and centrifuged at 15,000 g for 30 min at room temperature. Supernatant was lyophilized and stored at -20° C for further use (17).

For ethanolic extraction, fresh flowers were freeze-dried. A coarse powder (20 mesh) was obtained using a mill. A sample (10 g) was extracted by stirring at 100 rpm with 100 ml of methanol at 25° C for 24 h and filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of ethanol, as described above. Combined ethanolic extracts were then rotary evaporated at 40° C to dryness. A dark red material was obtained and stored in refrigerator (6).

Assay of hydroxyl radical

Hydroxyl radicals (OH^-) are generated from Fe^{2+}-ascorbate- EDTA- H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm (18). Reaction mixture (1 ml) contained deoxyribose (2.8 m*M*), KH_2PO_4- KOH (20 m*M*; *p*H 7.4), $FeCl_3$ (100 m*M*), EDTA (104 μ*M*), H_2O_2 (1 m*M*) and ascorbate (100 μ*M*). Reaction mixture was incubated at 37° C for 1 h and color developed as described above. EC_{50} value of deoxyribose degradation by the cold water, hot water and ethanolic extracts of *Ra*F over the control was measured. Catechin was used as a positive control.

Assay of superoxide radical scavenging activity

The method by Martinez *et al* (19) for determination of the superoxide dismutase was followed with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system (20). Each 3 ml reaction mixture contained 50 m*M* sodium phosphate buffer (*p*H 7.8), 13 m*M* methionine, 2 μ*M* riboflavin, 100 μ*M* EDTA, 75 μ*M* NBT and 500 μl sample solution of various concentrations of cold water, hot water and ethanolic extracts. Production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination by a fluorescent lamp. Identical tubes with the reaction mixture were kept in the dark and served as blank.

Assay of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+} ascorbate system in human red blood cells (RBC) and estimated as thiobarbituric acid reacting substances (TBARS) by the method of Buege and Aust (21). The reaction mixture contained RBC- packed cell (10^8 cells/ ml) in Tris- HCl buffer (20 m*M*; *p*H 7.0) with $CuCl_2$ (2 m*M*), ascorbic acid (10 m*M*) and different extracts of *Ra*F in final volume of 1 ml. The reaction mixture was incubated at 37° C for 1 h. Lipid peroxidation was measured as malondialdehyde (MDA) equivalent using trichloroacetic acid (TCA), thiobarbituric acid (TBA) and HCl (TBA-TCA reagent: 0.375 % w/v TBA; 15 % w/v TCA and 0.25 N HCl). The incubated reaction mixture was mixed with 2 ml of TBA-TCA reagent and heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 10,000 g for 5 min. Finally

malondialdehyde concentration in the supernatant fraction was determined spectrophotometrically at 535 nm. The concentrations of cold water, hot water and ethanolic extracts that would inhibit by 50%, the production of thiobarbituric acid reactive substances, i.e., EC_{50} values, were calculated. Catechin was used as control.

Determination of nitric oxide (NO) synthase activity

NO was determined according to Jia *et al* (22) by using scanning Hitachi 330 spectrophotometer. Typically, NO content was determined by conversion of oxyhemoglobin to methemoglobin. The reaction mixture containing RBC (10^8 cells) was incubated with L-arginine (10 μ*M*), hemoglobin (30 μ*M*) with different concentrations of cold water, hot water and ethanolic extracts of *Ra*F; in a total volume of 2.5 ml for different time periods at 37˚C. After each incubation period, a portion of reaction mixture was centrifuged at 8,000 g for 5 min at 37° C, and NO content of the supernatant was compared with an appropriate control set.

Statistical analysis

Results were subjected to statistical analysis using Student's *t* test. In all the cases, results are the mean ± SD of at least 3 individual experimental data, each in triplicate.

Table 1- *In vitro* hydroxyl radical and superoxide anion scavenging activity of *Rhododendron arboreum* flower extracts (EC_{50} μg/ml). Values represented as means ± SD from three independent observations. [a] = Catechin as standard. [b] = Ascorbic acid as standard.

	Extracts			Standard
	Cold water	Hot water	Ethanolic	
Hydroxyl radical scavenging activity	260 ± 35	208 ± 27	42.7 ± 3.1	840 ± 25[a]
Superoxide radical scavenging activity	92.5 ± 10.5	145 ± 15	33 ± 3.5	65 ± 3.5[b]

Figure 1- Effective concentration of lipid peroxidation by *Rhododendron arboreum* flower extracts. Results are the mean ± SD of three separate experiments, each in triplicate.

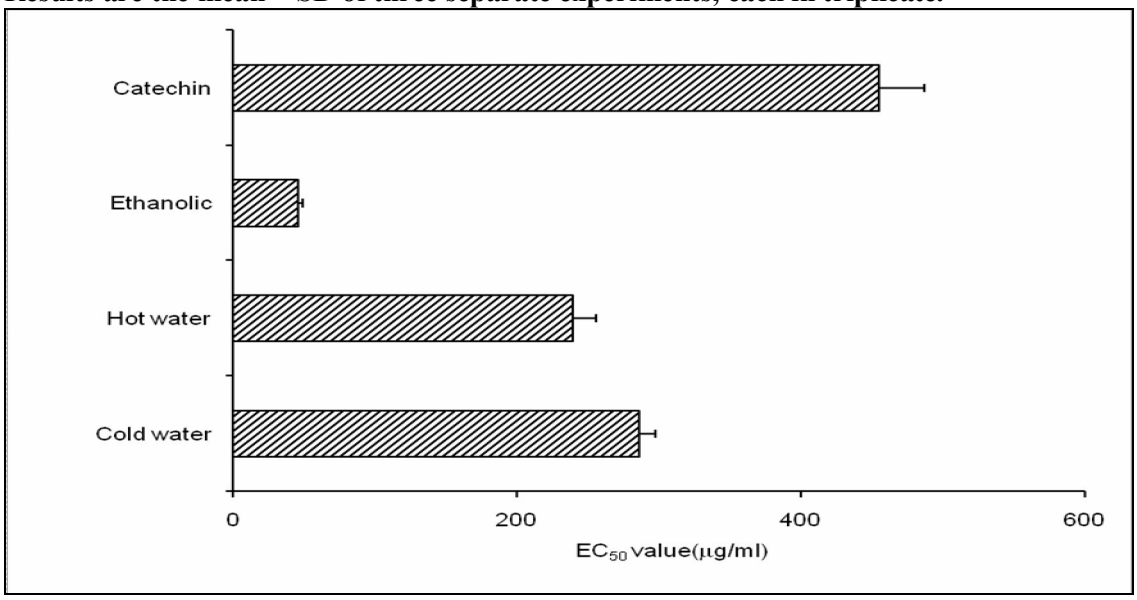
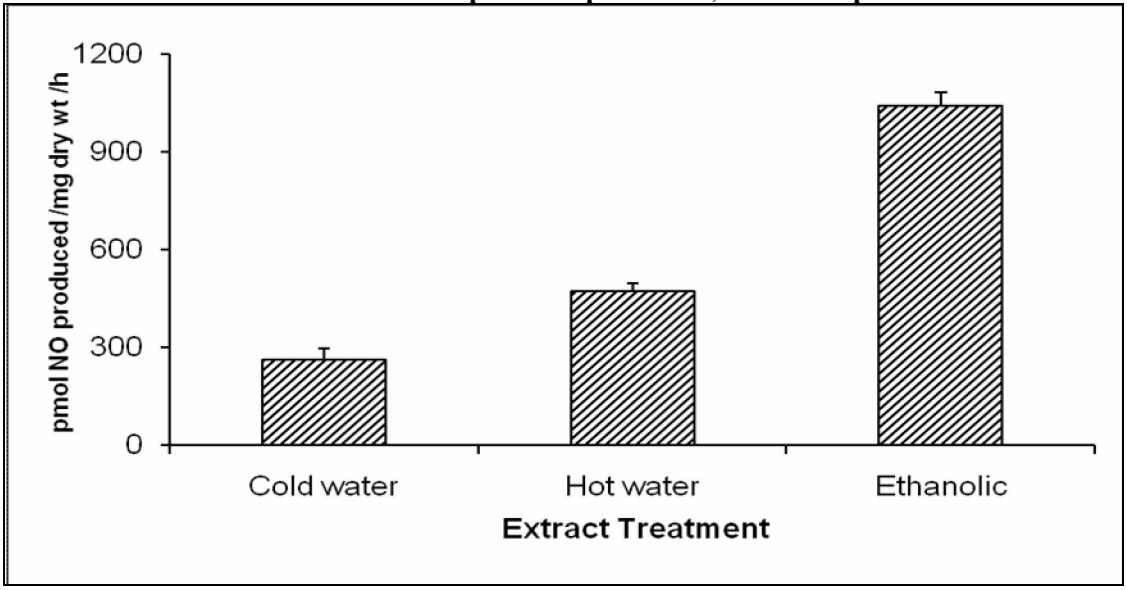


Figure 2- Production of nitric oxide by different extracts of *Rhododendron arboreum* flower over the control. Results are the mean ± SD of three separate experiments, each in triplicate.



3. Results and discussion

Hydroxyl radical scavenging activity

Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at *p*H 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low *p*H (23). When the test extracts were added to the reaction mixture, they removed hydroxyl radical from the sugar and prevented their degradation. All the extracts, i.e., cold water, hot water and ethanolic extracts of the *Ra*F showed potent hydroxyl radical scavenging activity. With regard to the scavenging ability of hydroxyl radicals, various extracts were effective in order of their EC_{50} value: ethanolic > hot water > cold water extracts, which was higher than catechin (840 µg/ml), a synthetic antioxidant (Table 1).

Assay of superoxide radical scavenging activity

The superoxide radical scavenging activity of cold water, hot water and ethanolic extracts of *Ra*F was expressed as EC_{50} value (Table 1). The ethanolic extract showed a lower EC_{50} value (36.3 µg/ml), i.e., higher scavenging activity than cold water extract (94.7 µg/ml) and hot water extract (154.8 µg/ml) and the EC_{50} value of ascorbic acid was 65 µg/ml.

Assay of lipid peroxidation

A free radical prefers to steal electrons from the lipid membrane of the cell, initiating a free radical attack on the cell induced lipid peroxidation in polyunsaturated lipid rich areas like brain and liver (24). The results presented in Figure 1 showed that all the extracts of *Ra*F inhibit Fe^{2+}- ascorbate induced lipid peroxidation much better than standard catechin. The effective concentration value of ethanolic extract (45.6 µg/ml) of *Ra*F seems to be approximately one tenth when compared to standard (EC_{50} = 455 µg/ml for catechin).

Determination of nitric oxide synthase activity

Nitric oxide is recognized to be an inter- and intra- cellular mediator of several cell functions. It acts as a signal molecule in immune, nervous and vascular systems (25). Further study was made to evaluate the nitric oxide synthase activation properties of cold water, hot water and ethanolic extracts of *Ra*F. All the three extracts showed significant increase in nitric oxide production over control (Figure 2). These were 264, 472.5 and 1041.7 pmol/mg dry wt/h, respectively. Use of 10 µ*M* N^G methyl - L - arginine acetate ester (NAME), a competitive inhibitor of nitric oxide synthase (NOS) (26), in the reaction mixture showed complete inhibition of NO production in all cases, indicating the increased production of NO was due to the activation of NOS. Ethanolic extract showed considerable NOS activation properties when compared to the other extracts.

Conclusion

Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence, antioxidants have significant importance in human health. Peroxide radicals in biological system are regarded to be associated with a number of pathological complications. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive species (2). One risk of the

superoxide generation is related to its interaction with nitric oxide to form peroxinitrite (27) which is a potent oxidant that causes nitrosative stress in the organ systems. Lipid peroxidation *in vivo* destroys biological membranes leading to change in fluidity and permeability (28). The beneficial role of NO in different pathophysiologic condition is well documented. From the above investigation, it could be concluded that the ethanolic extract of *Ra*F possessed significant antioxidant activity and NOS activation properties, thus suggesting the therapeutic value of this mushroom, which might be used as medicine for several killer diseases. These results should encourage further *in vivo* studies which could ultimately lead to an inclusion of this medicinal plant in different pharmaceutical formulations.

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