

Original Article

ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF RIBES GLACIALE WALL EXTRACTS

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Received: 28 Apr 2015 Revised and Accepted: 30 May 2015

ABSTRACT

Objective: *Ribes glaciale* Wall. is used in folk medicine by the locals of North West Himalayas, Uttarakhand, India, but its pharmacological potentials are not well studied. This work evaluates antioxidant, anti-inflammatory and analgesic activity of the extract of *Ribes glaciale* Wall.

Methods: Antioxidant activity was assessed by studying free radical scavenging ability, total antioxidant capacity, ferrous ion chelation and reducing power of the extracts. The anti-inflammatory and analgesic activity was assessed by carrageenan induced rat paw edema and acetic acid induced writhing model respectively.

Results: The methanolic and ethyl acetate extracts of *Ribes glaciale* Wall. Consists considerable amount of total phenolic and flavonoid contents. The ethyl acetate extract was potent in scavenging DPPH free radical (IC₅₀ 55.7 µg/ml) while methanolic extract demonstrated higher ability to scavenge ABTS free radical (TEAC 11802.2). Methanolic extract exhibited higher ferrous ion chelation activity (61% at 400 µg/ml). The ethyl acetate extract demonstrated superior reducing power and total antioxidant capacity. The methanolic extract was evaluated for anti-inflammatory and analgesic activity. Administration of methanolic extract of *Ribes glaciale* exhibited dose dependent inhibition of paw edema induced by carrageenan and also showed significant reduction in number of acetic acid induced abdominal writhing.

Conclusion: The study revealed antioxidant potential of methanolic and ethyl acetate extract of *Ribes glaciale* Wall. The methanolic extract exhibited significant anti-inflammatory and analgesic activity and indicate the need for its further phytochemical evaluation.

Keywords: *Ribes glaciale*, Antioxidant, Anti-inflammatory, Phenolic contents, Flavonoid contents.

INTRODUCTION

Plants have provided us an enormous pool of biologically active molecules with varying structural complexity since antiquity. Extensive research in phytochemical investigation of plants with some associated traditional medicinal information is being carried out world-wide to discover novel drug molecules. *Ribes glaciale* Wall. (Family Grossulariaceae) found in North-West Himalayas, Uttarakhand, is used in Indian traditional system of medicine. The seeds are edible and the plant is used as a diuretic [1]. The secondary metabolites isolated from *Ribes* species include flavonoids, acylated flavonoids, pyranoanthocyanins, phenolic acids and nitrile group containing compounds [2, 3]. *Ribes* species are reported to be useful as diuretic, anti-inflammatory and antioxidant [4].

The traditional claim was associated with *Ribes* species and lack of scientific studies regarding anti-inflammatory and antioxidant potential of *R. glaciale* prompted us to perform this study. The present paper for the first time reports antioxidant and anti-inflammatory activity of *Ribes glaciale* Wall. extracts.

MATERIALS AND METHODS

Plant material and extraction procedure

The plant *Ribes glaciale* Wall. Was collected from North-West Himalayas, Uttarakhand, India and identified (GUH 6086) H. N. B Garhwal University (A Central University), Uttarakhand, India. Freshly collected plant material was dried under shade and the dried samples were pulverised before extraction with solvents. The air dried powdered plant material was extracted with petroleum ether, ethyl acetate and methanol in soxhlet extractor. The extracts were concentrated by rotary vacuum evaporator (40 °C) and then air dried. Ethyl acetate and methanolic extracts were used for evaluation.

Determination of total phenolic content

The total phenolic content (TPC) was determined according to the method described by Mbaebie *et al.* [5]. The reaction mixture

consisted of 0.5 ml extract, 2.5 ml of the Folin-Ciocalteu's reagent (10% v/v) and 2.0 ml of saturated sodium carbonate solution. The resulting mixture was vortexed for 15 sec and incubated (40 °C, 30 min) for color development. The absorbance of total phenolics was measured at 765 nm. Standard gallic acid solutions were used for the calibration curve and results were expressed as gallic acid equivalent per gram of extract (mg GAE/g).

Determination of total flavonoid content

The extract (500 µl) was diluted appropriately and mixed with 1 ml NaNO₂ (5%). After standing for 6 min, 1 ml of 10% AlCl₃ and 10 ml of NaOH (1 M) were added to the mixture. The mixture was adjusted to 25 ml with 70% ethanol and allowed to rest for 15 min. The absorbance was measured at 510 nm, with 70% ethanol as a blank control [6]. Rutin was used as a reference standard and the total flavonoid content was expressed as rutin equivalents per gram of extract (mg RE/g).

Total antioxidant capacity

Sample (0.3 ml) was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 °C for 90 min. Absorbance of all the sample mixtures was measured at 695 nm [7]. Total antioxidant capacity was expressed as ascorbic acid equivalent per gram extract (mg AAE/g).

Reducing power

Different concentration of extracts (50-500 µg/ml) in 1 ml of alcohol was mixed with 2.5 ml phosphate buffers (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and 2.5 ml of 10% trichloroacetic acid was added. The reaction mixture was then centrifuged for 10 min. Further, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl₃. The absorbance was measured at 700 nm [8].

DPPH free radical scavenging activity

A 2 ml aliquot of the solution was added to 2 ml of 2×10^{-4} mol/l ethanolic DPPH solution. The mixture was shaken vigorously and the absorbance was measured at 517 nm immediately. The decrease in absorbance was determined at 15 and 30 min until the absorbance reached a steady state (after nearly 30 min). The mixture with the addition of standard antioxidants served as a positive control [9]. All the tests were performed in triplicate, and the inhibition rate was calculated according to the formula,

$$\% \text{ Inhibition} = \left[\frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \right] * 100$$

ABTS free radical scavenging activity

ABTS free radical was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 16 h at room temperature [10]. Prior to assay, the solution was diluted in ethanol and equilibrated at 30 °C to give an absorbance of 0.700 ± 0.02 at 734 nm. The stock solution of the sample extracts was diluted such that after introduction of 10 μ l aliquots into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 ml of diluted ABTS solution to 10 μ l of sample or Trolox standards in ethanol, absorbance was measured exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay and the percentage inhibition of the blank absorbance was calculated at 734 nm. Triplicate determinations were made at each dilution of the standard and percentage inhibition calculated and plotted as a function of Trolox concentration. The antioxidant activity is expressed as trolox equivalent antioxidant capacity (TEAC).

Ferrous ion chelation

The chelating activities of extracts on Fe^{2+} were estimated based on the decrease in the maximal absorbance of the iron (Fe^{2+})-ferrozine complex. 1 ml of extracts/standard was incubated with 0.5 ml of ferrous chloride (1.0 mmol). The reaction was initiated by the addition of 1 ml of ferrozine (5.0 mmol) and the total reaction volume was adjusted to 4 ml with ethanol. After the mixture had reached equilibrium (10 min), the absorbance was measured at 562 nm [11]. The negative control was prepared without the extract and EDTA was used as the positive control. The chelating activity of the extract on Fe^{2+} was calculated as follows:

$$\% \text{ Chelating activity} = \left[\left(\frac{\text{Acontrol}}{\text{Asample}} \right) - 1 \right] * 100$$

Carrageenan-induced rat paw oedema model

Paw edema was induced in the left hind paw of each rat by injecting 0.1 ml of 1% (w/v) carrageenan in physiological saline into the sub plantar tissues of the hind paw of each rat. The extract at different doses was administered orally 1 h prior to carrageenan administration [12]. The paw edema was measured before and then upto 5 h after carrageenan injection using the plethysmometer (Letica Scientific Instruments, Barcelona, Spain). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as the reference drug.

Acetic acid-induced abdominal writhing test

The method of Collier et al. as described by El-gazzar et al. Was used for this test [13, 14]. The mice were divided into five groups (n=6), three dose groups (250, 500 and 1000 mg) and one group was assigned each for negative and positive control. The mice in the negative and positive control group were orally administered distilled water (10 ml/kg) and indomethacin (10 mg/kg) respectively. After 30 min of administration of distilled water/extracts/indomethacin the mice were administered acetic acid (0.6% v/v in saline, 10 ml/kg, i. p.). The number of writhes was counted for 30 min.

RESULTS AND DISCUSSION

Total phenolic contents

The presence of phenolic contents in the extracts was determined by

the Folin-Ciocalteu method which is based on oxidation of phenol by molybdothiophosphoric reagent to yield a colored product that can be estimated by measuring absorbance at 765 nm [15]. Gallic acid was used as the reference standard and the phenolic content of the extracts was expressed in mg Gallic acid equivalents per gram of extract (table 1). Total phenolic content per gram extract of ethyl acetate extract (367 ± 22.6 mg GAE/g) of *Ribes glaciale* was higher than that of methanolic (234.6 ± 15.7 mg GAE/g).

Total flavonoid contents

Flavonoids are responsible for antioxidant activity owing to the presence of hydroxyl groups in their chemical structures and as a group possess a wide range of bioactive capacities including antioxidant activity [16]. The determination of total flavonoid content using aluminium chloride is based on the formation of the stable complex between aluminium chloride and keto and hydroxyl groups of flavonoids. The total flavonoid content of the extracts of *Ribes glaciale* is expressed as rutin equivalents in mg/g extract (table 1). The ethyl acetate extract showed the presence of higher flavonoid contents. The high amount of flavonoids in the ethyl acetate and methanolic extracts suggested the possible antioxidant potential of the *Ribes glaciale* extracts.

Total antioxidant capacity

Total antioxidant capacity determination by phosphomolybdenum method is based on the reduction of molybdenum VI (Mo^{6+}) to form a green phosphate/ Mo^{5+} complex at acidic pH which can be estimated by measuring absorbance at 695 nm. High absorbance values indicate potential antioxidant capacity of the sample. Ascorbic acid (AA) was used as a standard. The calibration curve of ascorbic acid was used to determine the total antioxidant capacity of *Ribes glaciale* extracts and results are expressed as an equivalent of ascorbic acid (AAEmg/g extract). The antioxidant capacity expressed as an equivalent of ascorbic acid of ethyl acetate and methanolic extracts are 27.3 and 21.2 respectively (table 1).

Reducing power

Reducing power assay is based on the ability of extracts/reference compounds to reduce a yellow color Fe^{3+} /Ferric cyanide complex to form Fe^{2+} -ferrous complex. The amount of Fe^{2+} was monitored by measuring the formation of the blue color at 700 nm. A higher value of absorbance implies higher concentration of Fe^{2+} -complex and indicates higher reducing power of the extracts/reference compounds. The ethyl acetate extract demonstrated significantly reducing power which was comparable to ascorbic acid in the concentration range of 50-500 $\mu\text{g/ml}$ (fig. 1). The correlation between the polyphenolic constituents and reducing power activity has been reported for several plant extracts. Phenolic contents in the extract are reported to function as an electron and hydrogen atom donors and terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products [17]. In our study a positive correlation between total phenolic contents and reducing power of the extracts was observed. The results also indicate that ethyl acetate extract of *Ribes glaciale* consists of chemical structures with fair ability to donate electrons and convert reactive free radicals into stable products.

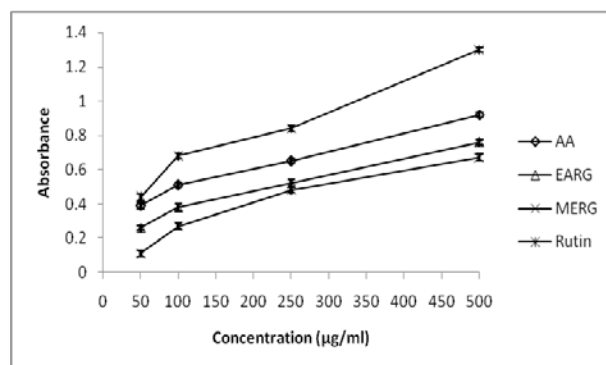


Fig. 1: Reducing power of *Ribes glaciale* Wall. Extracts

DPPH free radical scavenging ability

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical changes its color from purple to yellow in the presence of an antioxidant and is one of the most widely used to evaluate the antioxidant potential of extracts. The method is based on the hydrogen donating capability of the extract which scavenges DPPH free radical. Scavenging of the DPPH radical is also linked to the inhibition of lipid peroxidation. The results of the assay demonstrated antioxidant activity of *Ribes glaciale* extracts suggesting that the extracts are capable of donating hydrogen and acting as natural antioxidants. The ethyl acetate extract was potent in scavenging DPPH radical in comparison to methanolic extract (table 1). The radical scavenging ability was significantly low when compared to synthetic antioxidants like BHA and ascorbic acid. The scavenging ability of the ethyl acetate extract was however comparable to rutin. The potential to scavenge DPPH radical was measured by determining IC₅₀ value which indicates the concentration required to inhibit 50% of DPPH free radicals. IC₅₀ value of the ethyl acetate extract (55.7 µg/ml) was much lower than that of methanolic (126.2 µg/ml) extract of *Ribes glaciale* (table 1). IC₅₀ value of ethyl acetate extract was comparable to rutin (45

µg/ml); however it was much higher than synthetic antioxidant BHA (10 µg/ml) and ascorbic acid (21 µg/ml).

ABTS radical cation scavenging assay

ABTS is a stable radical cation with blue color and characteristic absorption at 734 nm. ABTS radical cations are more reactive than DPPH radicals and involve an electron transfer process. In this method, an antioxidant is added to preformed ABTS radical cation and after a fixed time period the remaining ABTS is quantified spectrophotometrically [18]. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) a water soluble analog of vitamin E is used as standard to represent the antioxidant strength of the sample. In ABTS radical cation scavenging assay, the activity of the tested sample extracts was expressed as Trolox equivalent antioxidant capacity (TEAC) defined as micromolar trolox solution having an antioxidant capacity equivalent to 1 g extract. The extracts exhibited good ABTS radical scavenging ability and were capable of decolorizing the ABTS radical. Trolox equivalent antioxidant capacity (table 1) for methanolic extract and ethyl acetate are 11802.2 and 6372.5 respectively.

Table 1: Antioxidant potential of *Ribes glaciale* extracts

Extract/Standard	TPC ^a (mg GAE/g)	TFC ^b (mg RE/g)	TAOC ^c (mg AAE/g)	DPPH IC ₅₀ (µg/ml)	ABTS ^d (TEAC)
EARG	367 (15.6)	157.5 (13.4)	27.3	55.7	6372.5
MERG	234.6 (21.7)	91.2 (8.7)	21.2	126.2	11802.2
Rutin	-	-	-	45	-
BHA	-	-	-	10	-
Ascorbic acid	-	-	-	21	-

Note: EARG and MERG are ethyl acetate and methanolic extracts of *Ribes glaciale* Wall. Respectively. BHA is butylated hydroxy anisole; ^atotal phenolic contents (TPC) are expressed as gallic acid equivalent; ^btotal flavonoid contents (TFC) are expressed as rutin equivalent; ^ctotal antioxidant activity (TAOC) is expressed as ascorbic acid equivalent; ^dTEAC is trolox equivalent antioxidant capacity defined as micromolar trolox solution having antioxidant activity equal to 1g extract; values in parenthesis indicate SD (n=3)

Ferrous ion chelation

Elemental species such as ferrous iron (Fe²⁺) can help in production of reactive oxygen species (ROS) within the body and accordingly the ability of substances to chelate iron can be a measure of its antioxidant capacity [19]. Transition metals acts as catalyst in Fenton type reactions and catalysis of hydroperoxide. Chelation of these ions by antioxidants prevents the catalytic action of metal ions [20]. Chelating agents also reduce the redox potential and thus stabilize the oxidized form of the metal ions and serve as antioxidants. The *Ribes glaciale* extracts were also evaluated for iron (II) chelating ability (fig. 2). At 400 µg/ml, the methanolic and ethyl acetate extract inhibited 61% and 47% of ferrous ion respectively. The chelating activity of the extracts was higher than rutin and significantly lower than the standard EDTA. It has also been reported that phenolic compounds with a properly oriented functional groups can chelate with metal ions and therefore the phenolic compounds in the methanolic extract with properly oriented functional groups may be held responsible for its higher chelating activity.

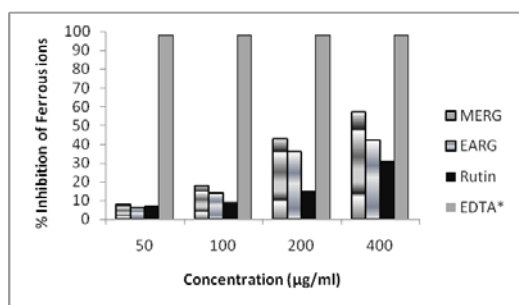


Fig. 2: Ferrous ion chelation by extracts of *Ribes glaciale* Wall
Note: *EDTA concentration (10 µg/ml)

Carrageenan induced rat paw edema

Administration of methanolic extract at the dose of 500, 1000 and 1500 mg/kg significantly reduced the volume of carrageenan induced paw edema (fig. 3). The inhibiting effect of the methanolic extract at the dose of 1000 mg/kg ($p < 0.05$) and 1500 mg/kg ($p < 0.01$) was significant when compared to control group. The reduction in paw volume edema 3 h after carrageenan injection was 33% and 50% respectively by indomethacin (10 mg/kg) and methanolic extract (1500 mg/kg). Similarly, the reduction in paw volume edema 5 h after carrageenan injection was 48% and 47% respectively by indomethacin and methanolic extract.

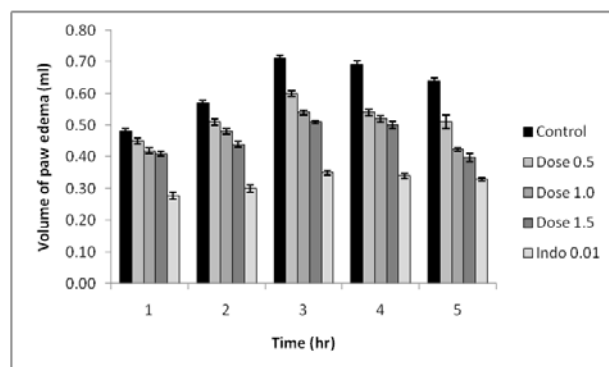


Fig. 3: Inhibition of Carrageenan induced rat paw edema by *Ribes glaciale* Wall. methanolic extract
Note: Dose in gm/kg body weight; n=6

Acetic acid induced writhing

The methanolic extract significantly ($p < 0.05$) inhibited the number

of writhes induced by acetic acid in comparison to control (fig. 4) at the dose of 500 mg/kg and 1000 mg/kg. The extract at the dose of 250, 500 and 1000 mg/kg inhibited 21%, 36% and 51% of the writhes induced by acetic acid respectively. Indomethacin (10 mg/kg) inhibited 65% of writhes in comparison to control ($p < 0.001$).

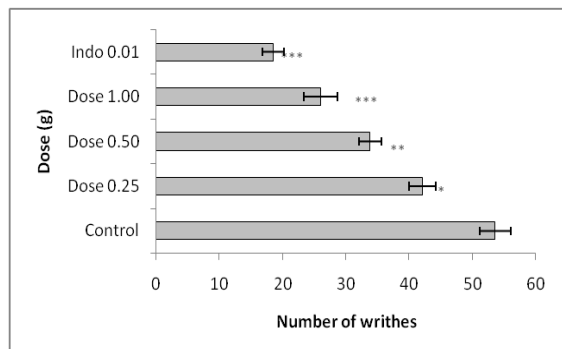


Fig. 4: Inhibition of acetic acid induced writhing by *Ribes glaciale* Wall. methanolic extract

Note: Dose in gm/kg of body weight; * = $p < 0.05$ compared to control; ** = $p < 0.01$ compared to control; * = $p < 0.001$ compared to control; n = 6**

CONCLUSION

Ribes glaciale Wall. Extracts were evaluated for the presence of total phenolic and flavonoid contents. The evaluation revealed high amount of phenolic and flavonoid contents in methanolic and ethyl acetate extracts. The extracts exhibited fair ability to scavenge DPPH and ABTS free radicals. The ethyl acetate extract of *R. glaciale* demonstrated higher reducing power. The methanolic extract exhibited higher chelating activity. The methanolic extract was further evaluated for anti-inflammatory and analgesic activity. The extract demonstrated significant anti-inflammatory and analgesic activity in comparison to control. The results clearly indicate antioxidant ability of the polar extract of *Ribes glaciale* and potential anti-inflammatory and analgesic activity of its methanolic extract. The results of the study also indicate the need for further phytochemical investigation of methanolic extract of *Ribes glaciale* Wall.

ACKNOWLEDGEMENT

This research work was funded by an University Grants Commission (UGC), New Delhi, India under Major Research Project Grant [File no. 41-221/2012(SR)]

CONFLICT OF INTERESTS

Declared None

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