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Shielding effect on oxidative stress and immune modulating potential of Himalayan Musk Rose (*Rosa brunonii* Lindl.)

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Rosa brunonii Lindl., a wildly growing species of rose is used widely by the folklore of the Himalayan region for various food and traditional medicinal purposes. The present study aims to evaluate the protective effects against oxidative stress markers and immunomodulation of *Rosa brunonii* Lindl. The crude extracts (chloroform, methanol, and aqueous) and subsequent fractions (chloroform, ethyl acetate, acetone, methanol, and aqueous) of both the leaves and flowers were evaluated for antioxidant potential (DPPH free radicals scavenging, chelation capacity on ferrous ions, FRAP activity, and reducing activity), DNA protection (DNA strand nicking) and immunomodulatory potential (humoral antibody titre) and cell-mediated (DTH immune responses and lymphocytic proliferation). The antioxidant study indicated that among all, methanol fraction (leaves) possessed remarkable antioxidant potential with the highest DPPH free radical scavenging (IC_{50} 32.3±1.15 µg/mL), chelation capacity on ferrous ions (66±0.22%), reducing power (EC_{50}124.4±0.17 µg/mL), FRAP (3996±2.17 µM/g dry wt.) as well as highest amounts of total phenols (250±0.54 GAE mg/g dry wt.) and flavonoids (344±1.76 QE mg/g dry wt.). Moreover, a significant reduction in plasmid DNA damage was also observed. Immunomodulatory results of methanol fraction (flowers) at 100 µg/mL showed maximum stimulation with 125% on humoral response, 166% on cell-mediated response and significant suppressive effect on mitogen-induced lymphocytic proliferation. The active constituent identified in methanol fraction (leaves) through RP-HPLC analysis was myricetin and from methanol fraction (flowers) was *p*-coumaric acid. This study concluded that the leaves of *R. brunonii* are a rich source of natural antioxidants and immune-modulating agents which can be further explored for establishing their pharmacological potential.

Keywords: Antioxidant, DNA strand nicking, Immunomodulatory, Phytochemicals, *Rosa brunonii*.

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Introduction

Free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in the biological systems of the human body. There are numerous potential sources of ROS within the cell. The important generators of intracellular oxidants are a host of enzymes such as NADPH oxidases, xanthine oxidase, cyclooxygenases, cytochrome p450 enzymes, and lipoxygenases that produce oxidants as part of their normal enzymatic function[1]. Generally, the generation of free radicals in the body is balanced by the various levels of antioxidant defence systems. An antioxidant can scavenge the free radicals like reactive oxygen/nitrogen species (ROS/RNS) to mitigate the radical chain reactions, or they inhibit the oxidants from being formed. However, exposure to adverse physiochemical, environmental or pathological agents results in the disturbance of this equilibrium resulting in oxidative stress[2].

ROS also results in the state of oxidative stress which may influence the immune system either by hyper-excitation to cause autoimmune disorders or suppress it, resulting in higher susceptibility to infections. The humoral and cell-mediated immune responses show great competence in dealing with foreign intruders like bacteria, viruses, fungi, yeasts and parasites, and prevention of cancer. Various antioxidants and immune modulators preferably of natural origin may prevent and/or correct immune dysfunction[3]. These immune modulators act by modifying the immune responses either by suppression or stimulation to fight against various diseases and infections[4-5].

Scientific studies on natural products have proved that plant-derived polyphenols prevent various

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diseases like cancer, cardiovascular, osteoporosis, neurodegenerative diseases, and diabetes[6]. Moreover, hazards associated with allopathic drugs, a continuous upsurge of serious diseases and the rising demand for natural products has warranted the search for alternative treatments using traditional medicinal plants due to easy accessibility, affordability and lesser side effects[7].

Keeping in view the therapeutic and pharmacological importance of natural products derived from medicinal plants, the present study was planned to explore *Rosa brunonii* Lindl., commonly known as Himalayan musk rose, a stout climber which is abundantly found in the North-western Himalayan region (1500-2500 m altitude). It has fragrant white flowers which blossom during April-July. This plant holds numerous applications in the Indian traditional system of medicine. It serves as an aphrodisiac and is also valuable in case of bile disorders and burning of skin[8]. Its root is commonly called '*Rajatarini*' which is used to cure eye infections[9]. Fresh flowers decoction is used for constipation, eye and skin diseases wound infection, diarrhoea, and also as heart tonic[10]. Flower paste is used against scabies. Leaves are used as fodder. However, the biological potential of this plant species has not been scientifically explored. Therefore, owing to the traditional medicinal value of this plant, this research work was designed to unearth its antioxidant and immunomodulating potential.

Materials and Methods

Collection of plant material and preparation of extracts

The leaves and flowers of *Rosa brunonii* were collected during September 2013 from the high altitude (2500-3500 m) region of Jammu and Kashmir State (India) and were identified by Dr. Harish Dutt (Assistant Professor, Department of Botany, University of Jammu). Spécimens bearing accession no. 8384 have been submitted in the Herbarium, Department of Botany, University of Jammu. The leaves and flowers were separately shade dried and grounded in a blender to make a fine powder. About 200 g powder of each of leaves and flowers were extracted with 500 mL of three different solvents i.e., chloroform, methanol, and water to prepare crude extracts. Methanol and chloroform extracts were obtained by continuous stirring at room temperature for 6 h whereas water extract was prepared at 60 °C overnight. This treatment was repeated thrice and the extracts were filtered and evaporated using a rotary vacuum evaporator.

Fractionation of active extracts

The active crude methanol extracts of leaves and flowers of *R. brunonii* were subjected to polarity based sequential partitioning to yield chloroform, ethyl acetate, acetone, methanol, and aqueous fractions. All the fractions obtained were evaporated to dryness using a rotary vacuum evaporator and stored at -20 °C for analysis (Fig. 1).

DPPH radical scavenging assay

In this assay, free radical scavenging activity was determined according to the method of Blois with modifications[11]. Different concentrations of the test sample (50, 100, 150, 200, and 250 µg/mL) were mixed with 1 mL from a 0.5 mM methanol solution of the DPPH radical and to this 2 mL of 0.1 M sodium acetate buffer (pH 5.5) was added. The mixtures were then allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm using a double beam UV-VIS spectrophotometer. The radical scavenging activity (RSA) was calculated as a percentage of DPPH radical discolouration from dark purple to yellow, using the equation:

$$\% \text{ RSA} = [(A_0 - A_s)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_s is the absorbance of the test compound.

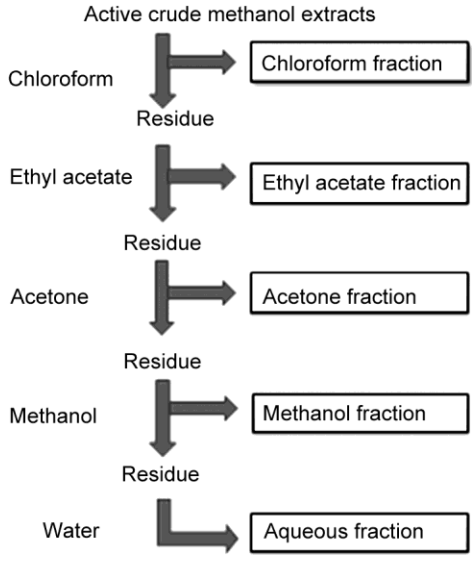

Fig. 1 — Flowchart of sequential partitioning of active crude methanol extracts of leaves and flowers of *Rosa brunonii* to obtain fractions.

Chelation power on ferrous (Fe^{2+}) ions

The chelating effect on ferrous ions of the prepared crude extracts and fractions was determined by the method of Dinis with slight modifications[12]. Briefly, 100 µL of each sample extract (10 mg/mL) was taken and raised to 3 mL with methanol. Exactly 740 µL of methanol was added to 20 µL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 µL of 5 mM ferrozine into the mixture, which was then left at room temperature for 10 min and then the absorbance of the mixture was determined at 562 nm.

FRAP Assay

Ferric ion reducing antioxidant power (FRAP) activity was measured according to the method of Benzie and Strain[13]. Briefly, acetate buffer (300 mM, pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine) 10 mM in 40 mM HCl, $FeCl_3.6H_2O$ (20 mM). The working FRAP reagent was freshly prepared by mixing the three solutions in the ratio of 10:1:1. The test sample (100 µL) was mixed with 3 mL of working FRAP reagent and absorbance was measured at 593 nm after vortexing. Methanol solutions of $FeSO_4.7H_2O$ ranging from 100 to 2000 µM were prepared and used for the preparation of the calibration curve of known Fe^{2+} concentration. The parameter equivalent concentration was defined as the concentration of antioxidants having a Ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4.7H_2O$.

Reducing power assay

The reducing power of extracts and fractions was determined by the method of Oyaizu with slight modifications[14]. Different concentrations of the test sample were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and to this 2.5 mL of 1% potassium ferricyanide ($K_3Fe[CN]_6$) was added. The mixture was incubated at 50 ◦C for 20 minutes. Exactly 2.5 mL of 10% trichloroacetic acid were added to the mixture and was then centrifuged for 10 minutes at 1036 × g. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 0.1% ferric chloride solution. The absorbance was measured at 700 nm in a double beam UV-VIS spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract/concentration.

Total phenolic content

Total phenolic content was determined according to Folin–Ciocalteau method[15]. Exactly 0.5 mL of extract solution was mixed with 0.5 mL of 1N Folin–Ciocalteau reagent. The mixture was kept for 5 minutes, followed by the addition of 1 mL of 20% Na_2CO_3. After 10 minutes of incubation at room temperature, the absorbance was measured at 750 nm using a spectrophotometer. The concentration of phenolic compounds was calculated according to the regression equation:

$$y = 0.0375 x + 0.0369$$

Total flavonoid content

Flavonoid content in the extract/fractions was determined by a colourimetric method[16]. Plant extracts were diluted with distilled water to a volume of 3.5 mL and 150 µL of a 5% $NaNO_2$ solution. After 5 minutes, 300 µL of 10% $AlCl_3 \cdot H_2O$ solution were added. After 6 minutes, 300 µL of 1 M NaOH and 550 µL of distilled water were added to prepare the mixture. The solution was mixed well and the absorbance was observed at 510 nm using a UV-VIS spectrophotometer. The concentration of flavonoid compounds was calculated according to the regression equation:

$$y = 0.001 x + 0.0312$$

DNA protective effect

The fractions of leaves and flowers were also tested for the protective effect on oxidative damage of DNA. The method of hydroxyl radical-induced DNA breakage in plasmid pUC18 was modified[17]. Briefly, 2 µL of test compounds (1 mg/mL) was mixed with 2 µL of EDTA-Na_2 (10.09 g/l), KH_2PO_4 buffer (0.05 mol/l, pH 7.4), H_2O_2(1.02 g/l), $FeSO_4$ (2.42 g/l), and 0.1µg/mL of pUC18 plasmid DNA in a 500 µL micro-centrifuge tube. The molar ratio of $FeSO_4$/EDTA was kept at 0.53. The final volume of the reaction mixture was brought to 12 µL with de-ionized distilled water and incubated for 1 h at 37 °C. Following incubation, 3 µL of autoclaved distilled water and 3 µL of 6×DNA loading dye (Fermentas) was loaded onto a 1.2 g/100 mL of agarose gel. Electrophoresis was conducted at 80 V in a Tris-acetate-EDTA.Na_2 (TAE) buffer (7.25 g/L of tris acetate and 0.29 g/L of EDTA, pH 7.4). DNA bands were visualized under the illumination of UV light and photographed with a Gel Doc system.

Immunomodulatory studies

Mitogen activity test by MTT assay

The mitogen activity test was based on the method described by Mosmann[18]. All the *in vivo* experimental

protocols were approved by the Institutional Animal Ethics Committee, CPCSEA of Indian Institute of Intergrative Medicine, Jammu (IAEC No.51/02/17). Animals (Balb/c mice, 18-22 g) were sacrificed; their spleens were removed in sterile conditions. A single-cell suspension was prepared in 5 mL of incomplete Roswell Park Memorial Institute (RPMI) Medium. The cell suspension was centrifuged at 1200 rpm for 10 minutes and the supernatant was discarded. RBCs were lysed by Tris-ammonium chloride treatment. The cells were centrifuged twice at 1200 rpm for 10 minutes, the supernatant was discarded and resuspended in complete RPMI. The viability of cells was checked with trypan blue. A $1x10^6$ cells/mL suspension was prepared and 100 µL of it was poured into each well of 96 well microtitre plate. An aliquot of 50 µL of standard mitogens i.e., Concanavalin A (Con A) ¼ 10 mg/mL and Lipopolysaccharide (LPS) ¼ 10 mg/mL and test materials were added. The extracts in different concentrations (10^{-4}, 10^{-5} and 10^{-6} M) were dissolved in DMSO and added to each well of flat bottom microtitre 96 well plate. Plates were placed on a shaker for 5 min. The plates were incubated for 48 hours in a CO_2 incubator (37 °C, 5% CO_2 and 90% relative humidity). After 48 hours of incubation, plates were taken out from the CO_2 incubator and reading was taken on an ELISA plate reader at 540 nm. Thereafter, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well. The contents were placed on a shaker for 5 minutes and plates were incubated for 4-6 hours in a CO_2 incubator (37 °C, 5% CO_2 and 90% relative humidity) for MTT to be metabolized. Then, the plates were centrifuged at 2000 rpm for 10 minutes and the medium was removed (dry plate on paper towels to remove residue if necessary). The formazan crystals (MTT byproduct) were resuspended in 100 mL DMSO and reading was measured at a wavelength of 570 nm.

Effect on humoral and cellular response in immune-suppressed mice

Swiss albino mice (*Mus musculatus*) 10-12 weeks old, 20-25 g body weight and male Charles Foster rats (*Ratus norvegicus*) 10-12 weeks, 100-150 g body weight in groups of six were employed for the study. In every experiment, one group of animals was used as a vehicle control while another received a standard drug Azathioprine (Aza) at a dose of 3 mg/kg orally. The test sample was freshly prepared as a homogenised suspension in 1% w/v acacia gum administered daily (orally) once a day for the duration of the experiment.

Antigen (SRBC)

Fresh sheep red blood cells (SRBC) collected aseptically from the jugular vein of sheep was stored in cold sterile Alsever's solution. It was washed three times with pyrogen-free sterile normal saline (0.9% NaCl w/v) and adjusted to a concentration of $5x10^9$ cells/mL for immunization and challenge at the required schedule.

Humoral antibody response (Hab)

Groups of six mice each were immunized by injecting 0.2 mL of $5x10^9$ SRBC/mL intraperitoneally (i.p.) on day 0 and challenged 7 days later by injecting (i.p.) an equal volume of SRBC. Blood samples were collected on day +7 (before challenge) for primary antibody titre. Hemagglutination antibody titres were determined following the microtitration technique described by Nelson and Midenhall[19]. The value of the highest serum dilution causing haemagglutination was taken as a titre. BSA saline alone served as a control.

Delayed type hypersensitivity response (DTH)

The method of Doherty was followed to assess SRBC induced DTH response in mice[20]. Mice were immunized by injecting 20 µL of $5x10^9$ SRBC/mL subcutaneously into the right hind footpad. Seven days later the thickness of the left hind foot was measured with a spheromicrometer (0.01 mm pitch) and was considered as a control. These mice were then challenged by injecting the same amount of SRBC intradermally into the left hind footpad. The foot thickness was measured again at 0, 4, and 24 hours after the challenge.

Acute toxicity study

Acute toxicity study of active methanol fractions of leaves and flowers was conducted as per OECD guidelines 420 (Fixed-dose procedure) using Swiss mice. Each animal was administered test samples at a dose of 2000 mg/kg by oral route. The animals were observed for any changes continuously for the first 4 hours and up to 24 hours for any mortality. The animals were then kept for 14 days to observe daily cage-side observations and mortality[21].

Observations

Physiological and behavioural changes were observed at least once daily. Feed and water consumption were recorded for all treated and control

groups every day. The weekly body weight of all animals was recorded for both control and treated groups. Any animal that died during the study period was autopsied and those that survived were sacrificed to study pathology on termination of the study.

Biochemical analysis

At the end of the treatment schedule whole blood was collected in a separate tube without anticoagulant and allowed to coagulate, centrifuged at 1000 rpm for 10 minutes to obtain the serum. The serum was assayed for the determination of glucose, creatinine, uric acid (UA), bilirubin, urea, cholesterol, triglycerides (TG), SGOT, SGPT, and total protein by commercial kits procured from Bayer Diagnostics, Baroda, India using a clinical biochemistry analyzer Chem-7 (Erba, Manheim, Germany) in the process.

Haematology

Haematology parameters were measured in whole blood collected in tubes containing 5% EDTA as an anticoagulant from retro-orbital plexus of all surviving animals. The haematological analysis was carried out for RBC, WBC, platelet (Pt) and differential leukocyte count, haemoglobin (Hb), hematocrit (Ht), clotting time (CT), and bleeding time (BT) using an automated haematology analyzer (Humacount: Human, Weisbaden, Germany).

Column chromatography of the active fractions

The active methanol fractions prepared from the sequential fractionation of crude methanol extracts of *R. brunonii* leaves and flowers were further separately subjected to column chromatography with different combinations of MeOH/Dichloromethane (DCM) as solvent systems. The column (2.5 cm x 60 cm) was packed with silica gel and saturated with DCM. 20 g of methanol fraction was added to silica gel and dissolved in methanol to form a slurry. The slurry was dried to fine powder and loaded on top of the column. The column was eluted with a gradient of methanol and DCM in various proportions, starting with 1:10 ratio in the order of increasing polarity to obtain 230 sub-fractions (10 mL each). The sub-fractions were collected and analysed by TLC. Again, they were pooled based on similar TLC patterns to obtain pooled sub-fractions. These pooled sub-fractions were then analysed for their potential to identify the most potent fraction. The active sub-fractions were dried and recrystallised to isolate the active principles.

Identification of the active constituents by Reverse-phase -HPLC

Analytical chromatographic separation of crude extracts, fractions and active phytoconstituents was carried out on a chromolith performance RP-18e column (100x4.6 mm, Merck, Germany) protected by a chromolith guard column of the same company. The column temperature was maintained at 30 °C. The flow rate was optimized to 0.6 mL/min and the sample injection volume was 10 µL. The mobile phase consisted of solvent A (water and 0.1% formic acid) and solvent B (acetonitrile). Gradient elution was programmed as follows: 0–10 min, 10-35% B; 10–15 min, 35–50% B; 15–17 min, 50-10% B and 17-19 min, 10% B. Identification of active constituents was based on retention times compared with the standard phenolic compounds. Stock solutions of standards i.e., caffeic acid, chlorogenic acid, syringic acid, *p*-coumaric acid, rutin, myricetin, and ellagic acid were prepared in volumetric flasks separately in the mobile phase. Standard working solutions were then obtained by mixing and making appropriate dilutions of stock solutions using the mobile phase. All standard solutions were filtered through a 0.2 µm membrane filter (Millipore) and injected directly.

Isolation and characterization of bioactive constituents from active methanol *R. brunonii* fraction of leaves

Bioactivity-guided fractionation was followed to isolate the active constituents from *R. brunonii* leaves. The active methanol fraction (RBL1-M) was subjected to column chromatography with different combinations of methanol (MeOH) - dichloromethane (DCM). The column (2.5 cm x 60 cm) was packed with silica gel and saturated with MeOH. Exactly 20 g of methanol fraction was added to silica gel and dissolved in methanol to form a slurry. The slurry was dried to a fine powder and eluted with a gradient of MeOH and DCM in various proportions to isolate the active principle which was analysed by HPLC and then characterized by ^{1}H NMR, Infrared Spectral analysis and ESI/MS to elucidate the molecular weight and chemical structure.

Statistical analysis

All experiments were carried out in triplicate. Data values are expressed as mean±standard deviation using ANOVA statistical analysis.

Results

DPPH radical scavenging assay

The crude extracts of leaves and flowers of *R. brunonii* were analysed for *in vitro* antioxidant

potential in terms of DPPH free radical scavenging activity. The results showed that the crude methanol extract of leaves possessed remarkable scavenging activity with an IC_{50} value of 62±1.13 µg/mL followed by that of aqueous extract (IC_{50} value 89.3±0.27 µg/mL). In the case of flowers, the methanol extract exhibited significant scavenging potential with an IC_{50} value of 71.3±0.67 µg/mL) followed by aqueous extract (IC_{50} value 85.4± 1.13 µg/mL) (Table 1). Chloroform extracts of both leaves and flowers showed negligible scavenging potential. Since the crude methanol extracts of each of leaves and flowers showed good radical scavenging potential. Therefore, they were further sequentially fractionated with solvents based on polarity and the fractions obtained were again tested for DPPH radical scavenging activity. The results as depicted in Table 1 & Fig. 2 showed that the methanol fraction of crude methanol extract of leaves possessed maximum radical scavenging potential in comparison to all the other fractions. The order of scavenging potential of fraction of leaves was in the decreasing order of: methanol fraction (IC_{50} 32.2±1.15 µg/mL)> acetone fraction (50±1.09 µg/mL)> aqueous fraction (71.3±0.34 µg/mL)> ethyl acetate fraction (93.5±0.03 µg/mL). In case of flowers, the order of activity was found be as: methanol fraction (IC_{50} 49.8±0.03 µg/mL)> aqueous fraction (61.7±0.65 µg/mL)> acetone fraction (69±0.12 µg/mL)> ethyl acetate fraction (95.2±0.78 µg/mL). In both leaves and flowers, the methanol fractions were found to possess noteworthy free radical scavenging potential (Fig. 2a).

Chelation power on ferrous (Fe^{2+}) ions

The chelation capacity of ferrous ions was determined for all the crude extracts and fractions of *R. brunonii* leaves and flowers (Table 1). It was observed that the crude methanol extract of leaves exhibited the highest chelation capacity of 71.2±0.44% at 100 µg/mL followed by that of crude aqueous extract (66±0.15%) at the same concentration. The crude methanol extract of flowers (64±1.37% at 100 µg/mL) showed good chelation capacity followed by that of aqueous extract (48±0.07% at 100 µg/mL) (Fig. 3). The fractions of leaves and flowers when tested for chelation power on ferrous ions showed that the methanol fraction of leaves demonstrated maximum chelation capacity as compared to all other fractions. The order of chelation activity of fractions of leaves at 100 µg/mL was found to be: methanol fraction (66±0.22%) > ethyl acetate fraction (39.5±0.97%) > aqueous fraction (28±0.49%). Chloroform and acetone fractions showed negligible chelation of ferrous ions. In the case of flowers, the methanol fraction of flowers also showed considerable chelating capacity with 52.6±0.51% chelation at 100 µg/mL followed by an aqueous fraction (30.6±0.53%) (Table 1, Fig. 2b).

Reducing power

The reduction of ferric ions to ferrous ions also determines the level of antioxidant potential. Therefore, a reducing power assay was performed for all the extracts and fractions (Table 2, Fig. 2d). The crude methanol extract of *R. brunonii* leaves showed the best reduction power with an EC_{50} value of 199±1.15 µg/mL as compared to other crude extracts. Further, among the fractions of leaves and flowers, the methanol fraction of leaves showed the highest reducing behaviour with EC_{50} value 124.4±0.17 µg/mL followed by that of acetone fraction (EC_{50} value 134±0.75 µg/mL) and aqueous fraction (EC_{50} value 170±0.23 µg/mL) (Fig. 2d). Chloroform and ethyl acetate fractions did not show any noteworthy

Table 1 — Solvent recovery, DPPH radical scavenging activity and chelation capacity of crude extracts and fractions of *R. brunonii*

Extracts/ Fractions	Yield of extracts (%)		DPPH radical scavenging activity (IC_{50} µg/mL)		Chelation capacity (% at 100 µg/mL)	
	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
(Crude extracts)						
Chloroform	3.88	2.28	269±0.12	351±0.56	23±1.06	21±0.65
Methanol	22.57	12.78	**62±1.13**	**71.3±0.67**	**71.2±0.44**	**64±1.37**
Aqueous	5.10	4.00	89.3±0.27	85.5±1.13	66±0.15	48±0.07
(Fractions)						
Chloroform	26	22	na	na	29.2±1.32	15.7±1.16
Ethyl acetate	5	6.6	93.5±0.03	95.2± 0.78	39.5±0.97	14±1.87
Acetone	11	5.7	50±1.09	69±0.12	28±1.17	9.4±0.42
Methanol	40	26.6	**32±1.15**	**49.8±0.03**	**66±0.22**	**52.6±0.51**
Aqueous	12	4.3	71.3±0.34	61.7±0.65	38±0.49	30.6±0.53

*Values are expressed as mean ± standard deviation. 'na' signifies negligible activity

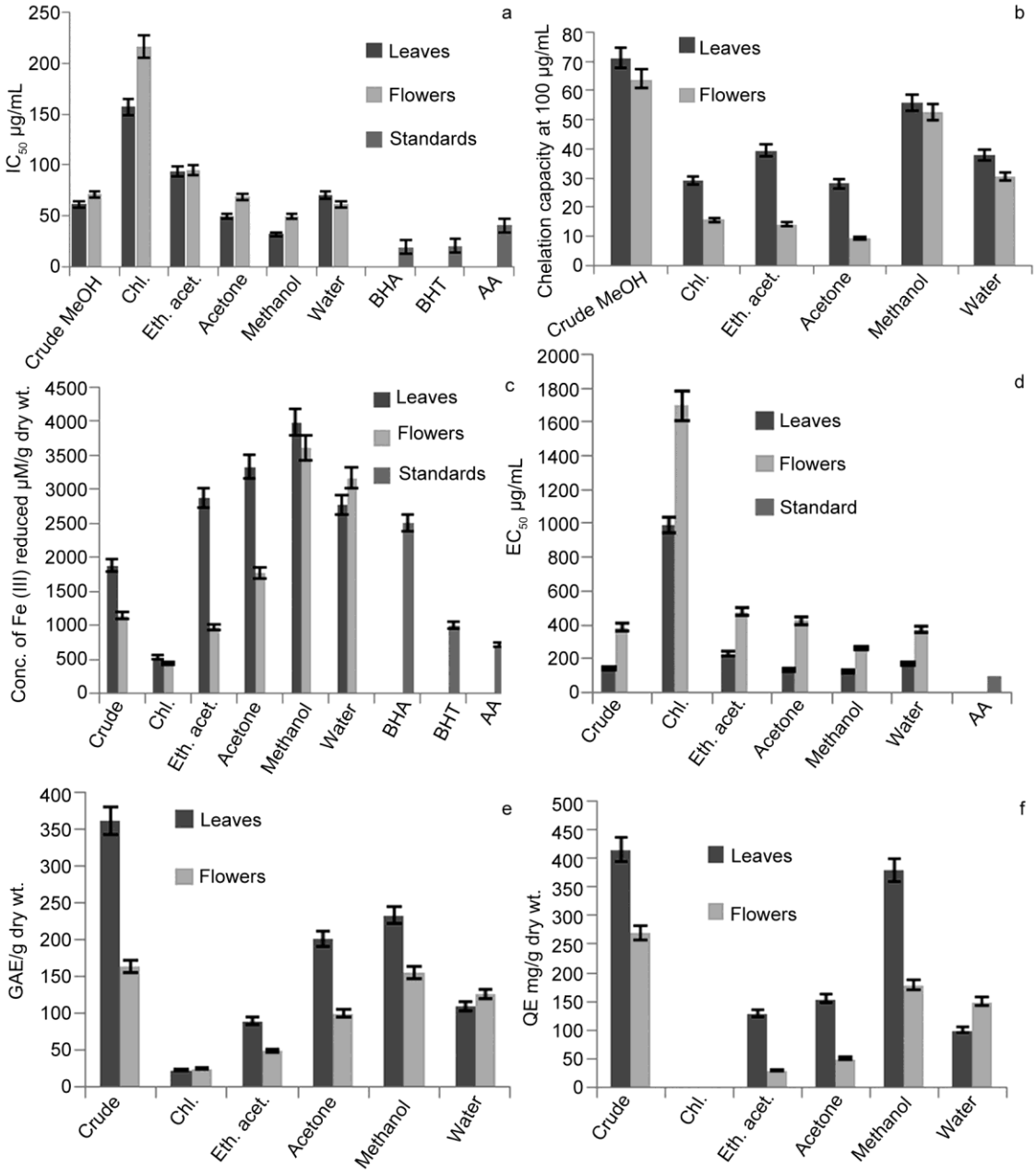

Fig. 2 — Results of a) DPPH scavenging activity of fractions of leaves and flowers of *R. brunonii*, b) Chelation power on ferrous ions of leaves and flowers of *R. brunonii*, c) FRAP activity of fractions of leaves and flowers of *R. brunonii*, d) Reducing power of fractions of leaves and flower of *R. brunonii*, e) Total phenolic content of fractions of leaves and flowers of *R. brunonii*, f) Total flavonoid content of fractions of leaves and flowers of *R. brunonii*.

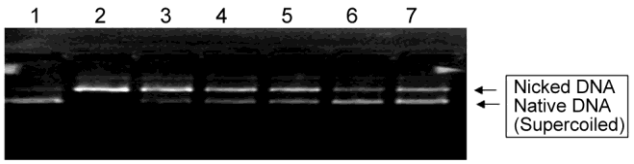

Fig. 3 — Protective effect of fractions of *R. brunonii* leaveson DNA nicking caused by hydroxyl radicals1: DNA, 2: DNA+FR, 3: DNA+RBL1-C+FR, 4: DNA+RBL1-E, 5: DNA+RBL1-A+FR, 6:DNA+RBL1-M+FR, 7: DNA+RBL1-Aq.+FR.

reducing potential. In the case of flowers, again methanol fraction showed the highest reducing potential with EC_{50} value 265±0.73 µg/mL in comparison to other fractions of flowers.

FRAP Assay

In this assay, reduction of ferric tripyridyltriazine (Fe^{3+}- TPTZ) complex to ferrous form which has an intense blue colour can be monitored by measuring

Table 2 — Reducing power and ferric reducing antioxidant power (FRAP) of crude extracts and fractions of *Rosa brunonii*

Extracts/ Fractions	Reducing power (EC_{50} µg/mL)		FRAP (µmoL/g dry wt.)	
	Leaves	Flowers	Leaves	Flowers
(Crude extracts)				
Chloroform	na	na	na	na
Methanol	**199±1.15**	**351±1.91**	**1890±1.22**	**1256±0.32**
Aqueous	421.4±0.76	490.6±0.18	1152±0.67	1022±0.21
(Fractions)				
Chloroform	995.8±0.23	1691±1.56	535±0.94	446±0.25
Ethyl acetate	289.3±0.43	480±1.24	2876±1.16	976±0.34
Acetone	134±0.75	427.5±0.65	3336± 2.18	1776±1.17
Methanol	**124.4±0.17**	**265±0.73**	**3996±2.17**	**3616±1.83**
Aqueous	170±0.23	376±0.11	2776±1.85	3176±1.42

*Values are expressed as mean±standard deviation. 'na' signifies negligible activity

the change in absorption at 593 nm. Upon FRAP analysis of all the crude extracts and fractions, it was observed that the crude methanol extract of both leaves showed the best reduction with a FRAP value of 1890±1.12 µmol/g dry wt. followed by crude aqueous extract (1152±0.67 µmol/g dry wt.). Also, the crude methanol extract of flowers showed significant FRAP activity (1256±1.13 µmol/g dry wt.) followed by that of an aqueous extract with a FRAP value of 1022±0.21 µmol/g dry wt. The chloroform extracts showed negligible FRAP activity. In the case of fractions of leaves as depicted in Table 2 & Fig. 2c, the order of FRAP activity was observed as methanol fraction (3996±2.17 µmol/g dry wt.) > acetone fraction (3336±2.18 µmol/g dry wt.) > ethyl acetate fraction (2876±1.16 µmol/g dry wt.) > aqueous fraction (2776±1.85 µmol/g dry wt.). Among the fractions of flowers, the methanol fraction depicted significant FRAP activity with a FRAP value of 3616±1.83 µmol/g dry wt. followed by that of aqueous fraction (3176±1.43 µmol/g dry wt.) and acetone fraction (1776±1.17 µmol/g dry wt.) (Table 2, Fig. 2c).

Total phenol content

Many studies have revealed that the phenolic content in plants is associated with their antioxidant activities probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. The measure of phenolic contents was made by the Folin-Ciocalteau method and it was found that the methanol fractions of both leaves and flowers possessed a considerable amount of phenolic. Among the fractions of leaves, its subsequent methanol fraction showed the highest phenolic content, (250±0.54 mg GAE/g dry wt.) followed by acetone fraction (188±1.26 mg GAE/g dry wt.). The other fractions did not show any noteworthy presence of phenolic compounds. Among the fractions of flowers, the methanol fraction exhibited the highest phenolic content (344±1.76 mg GAE/g dry wt.) followed by an aqueous fraction (204±0.79 mg GAE/g dry wt.) (Table 3 & Fig. 2e).

Table 3 — Total phenolic and flavonoid content of crude extracts and fractions of *Rosa brunonii*

Plant part	Fractions	TPC (mg GAE/g dry wt.)	TFC (mg QE/g dry wt.)
Leaves	Chloroform	31±0.22	-
	Ethyl acetate	103±0.21	94±0.16
	Acetone	204±1.12	244±1.56
	Methanol	**250±0.54**	**344±1.76**
	Water	116±0.43	154±0.78
Flowers	Chloroform	36±0.56	-
	Ethyl acetate	59±0.24	-
	Acetone	108±0.35	44±0.38
	Methanol	**188±1.26**	**204±0.79**
	Water	13±0.98	99±0.32

Total flavonoid content

Flavonoids are a group of benzo-γ-pyran derivatives found in plants which harbour biological potentials like antioxidant activity, protective effect against DNA damage etc. The crude extracts and fractions were also tested for the presence of flavonoid compounds. As depicted in Table 3 & Fig. 2f, the highest flavonoid content was detected in the crude methanol extracts of leaves (416±1.21 mg QE/g dry wt.) and flowers (206±0.72mg QE/g dry wt.). Among the fractions, the methanol fractions of leaves and flowers harboured the highest flavonoid content 344±1.76 and 204±0.79 mg QE/g dry wt. respectively. The rest of the fractions did not show any significant presence of flavonoids.

DNA protective effect

The hydroxyl free radicals generated during oxidative stress also induce DNA by creating nicks.

Antioxidants have been proved to protect DNA against such damage by quenching such free radicals. In the present study, the fractions of both leaves and flowers were analysed for their protective effect on oxidation induced DNA damage. The Fenton reaction mixture is used in the assay to generate hydroxyl free radicals. The DNA strand nicking assay predicted the protective effects of fractions of *R. brunonii* leaves (Fig. 3). It was observed that the methanol (RBL1-M) lane 6 and water (RBL1-W) lane 7 fractions had maximum shielding effects on the OH radical-induced DNA damage. These fractions prevented the formation of nicks in native DNA (Supercoiled).

The fractions of *R. brunonii* flowers when analysed for protective effects on DNA damage, it was observed that the acetone (RBF1-A) lane 5, methanol (RBF1-M) lane 6 and aqueous (RBF1-W) lane 7 fractions showed a maximum protective effect on DNA damage followed by moderate effects by chloroform (RBF1-C) lane 3 and ethyl acetate (RBF1-E) fractions lane 4 (Fig. 4). The maximum shielding effect on DNA damage by the methanol fractions may be attributed to the presence of high content of phenols and flavonoids in comparison to other fractions.

Immunomodulatory studies

Humoral antibody response

The fractions of *R. brunonii* leaves and flowers were also analysed for immune-modulating potential and noteworthy results were obtained. As depicted in Table 4, it was found that the methanol fraction of flowers had tremendous immune-stimulating potential in respect of humoral and cell-mediated immune response followed by that of methanol fraction of leaves. The methanol fraction of flowers (RBF1-M) showed a maximum dose-dependent stimulatory effect on humoral response with 125% at 100 mg/mL,

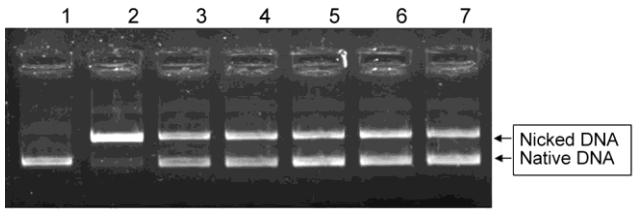

Fig. 4 — Protective effect of fractions of *R. brunonii* flowers on DNA nicking caused by hydroxyl radicals. Lane 1: Plasmid DNA, 2: DNA+FR, 3: DNA+RBF1-C+FR, 4: DNA+RBF1-E+FR, 5: DNA+RBF1-A+FR, 6: DNA+RBF1-M+FR, 7: DNA+RBF1-Aq.+FR.

115% at 50 mg/mL and 95% at 25 mg/mL which were quite comparable to that of standard drug which showed 133% stimulation on humoral response. In the case of leaves, the methanol fraction was found to show dose-dependent stimulating humoral response with 105% activity at 100 mg/mL, 90% at 50 mg/mL and 75% at 25 mg/mL. However, the stimulatory effect of the methanol fraction of flowers was more than that of the leaf methanol fraction. The other fractions of flowers and leaves showed a moderate or negligible stimulatory effect on humoral response (Table 4).

Delayed type hypersensitivity response

Similar results were obtained in the case of modulatory effects on DTH response. The methanol fraction of flowers exhibited remarkable stimulatory effect on DTH response with 166% activity at 100 mg/mL, 140% at 50 mg/mL and 117% at 25 mg/mL. The results were quite comparable to the standard drug Levamisole which showed a 168% stimulatory effect on DTH response. The methanol fraction of leaves also showed significant stimulatory effect with 135% at 100 mg/mL and 117% at 50 mg/mL followed by moderate effect of aqueous fraction with108% at 100 mg/mL and 100% stimulation at 50 mg/mL. However, the stimulatory activity on the cell-mediated response (DTH) of the methanol fraction of flowers was far better than all the other fractions relatively. Therefore, this fraction was also subjected to column chromatography to identify the active phytoconstituents present.

In vitro MTT assay for LPS and Con-A induced murine lymphocytic proliferation

All the fractions of *R. brunonii* when analysed for modulating effects on mitogen-induced lymphocytic proliferation revealed moderate effects (Table 5). However, the chloroform fraction of leaves (RBL1-C) showed significant suppressive effects i.e. by 60.67% on T-cell proliferation at 10^{-4} µg/mL conc. and methanol fraction (RBL1-M) showed suppression on B-cell proliferation as (52.04, 24.48, and 21.42% at 10^{-4}, 10^{-5}, and 10^{-6} µg/mL respectively).

In case of *R. brunonii* flowers, some noteworthy suppressive effects were observed in case of methanol fraction of flowers (RBF1-M) against T-cell proliferation as (31.46, 21.34, and 26.92% at 10^{-4}, 10^{-5}, and 10^{-6} µg/mL) and against B-cell proliferation as (39.79, 33.69, and 43.87% at 10^{-4}, 10^{-5}, and 10^{-6} µg/mL respectively).

Table 4 — Effect of fractions of *R. Brunonii* leaves and flowers on humoral and cell mediated (DTH) immune response in immune suppressed balb/c mice

Plant part	Samples	Conc. (mg/mL)	Antibody Titre Mean±S.E.	% Activity	DTH response Mean±S.E.	% Activity
Standard drugs	Control	-	6.5 ± 0.21	-	0.80 ± 0.16	-
	Cyclophosphamide	200	4.5 ± 0.21	- 30.7	-	-
	Cyclosporin	5	-	-	0.35 ± 0.10	- 56.25
	Levamisole	2.5	7.16 ± 0.22	+ 133	1.11 ± 0.22	+ 168
*R. brunonii*leaves	Chloroform (RBL1-C)	100	6.01 ±1.16	+ 75	0.63 ± 0.21	+ 63
		50	5.81 ± 0.16	+ 67	0.60 ± 0.20	+ 56
		25	4.8 ± 0.31	+ 15	0.54± 0.24	+ 42
	Ethyl acetate (RBL1-E)	100	5.8 ± 1.12	+ 65	0.75± 1.17	+89
		50	5.5 ± 0.57	+ 50	0.70 ± 0.32	+78
		25	4.7 ± 0.28	+10	0.65 ± 0.26	+67
	Acetone (RBL1-A)	100	6.1 ± 0.41	+ 80	0.52 ±1.17	+38
		50	5.72 ± 0.22	+ 61	0.48 ± 1.25	+29
		25	5.1± 0.52	+ 25	0.45 ±1.19	+23
	Methanol (RBL1-M)	100	**6.6 ±0.56**	**+105**	**0.96 ±0.71**	**+135**
		50	**6.3 ± 1.57**	**+90**	**0.88 ± 0.12**	**+117**
		25	6.00 ± 0.74	+75	0.75 ±0.18	+89
	Water (RBL1-W)	100	6.12 ±1.16	+81	0.84 ±0.52	+108
		50	6.00 ±1.55	+ 75	0.80 ±1.38	+100
		25	4.9 ±1.25	+ 20	0.74 ± 0.98	+87
R.. brunonii flowers	Chloroform (RBF1-C)	100	6.3± 1.25	+90	0.65±0.18	+67
		50	5.75 ± 0.72	+ 62	0.60±1.17	+56
		25	5.5 ± 0.68	+ 50	0.57±1.32	+49
	Ethyl acetate (RBF1-E)	100	6.2 ± 0.55	+80	0.72±0.65	+83
		50	5.9 ± 0.72	+70	0.68±0.17	+74
		25	5.5 ± 0.69	+ 50	0.63±0.62	+63
	Acetone (RBF1-A)	100	6.4± 1.16	+95	0.75 ±0.18	+89
		50	6.0±0.94	+75	0.70 ± 0.35	+78
		25	5.72±1.52	+60	0.65±0.87	+67
	Methanol (RBF1-M)	**100**	**7.00±054**	**+125**	**1.10±1.87**	**+166**
		50	**6.8±0.43**	**+115**	**0.98±1.25**	**+140**
		25	**6.4±1.18**	**+95**	**0.88 ± 0.19**	**+117**
	Water (RBF1-W)	100	5.5±0.76	+65	0.63±1.27	+63
		50	5.2±0.54	+35	0.54± 0.17	+ 42
		25	5.00±1.15	+25	0.5±1.37	+34

Values are expressed as mean±standard deviation. + indicates immunostimulation; - indicates immunosuppression

Acute toxicity studies

General observations

The exposure to methanol fraction of leaves (RBL1-M) as well as to the methanol fraction of flowers (RBF1-M) did not reveal any significant changes in the physiological or behavioural patterns of mice (Table 6). Weekly observations in the body weight did not show any significant changes between the control and treated groups.

Biochemical analysis

The biochemical analysis also did not reveal any significant changes in the Glucose, ALT, SGOT, SGPT, Uric acid analysis, Bilirubin, Biliverdin etc. in the treated and control groups (Table 6).

Hematology

The blood analysis did not reveal any noteworthy changes in RBC and WBC count and other parameters like Hb, HCT, PTC, NC, and BT etc. (Table 6) between the control and treated groups.

Identification of active phytoconstituents

To identify the phytoconstituents responsible for remarkable antioxidant activity, the methanol fraction of leaves was subjected to column chromatography and RP-HPLC which led to the identification and isolation of active phytoconstituent i.e. myricetin (Fig. 5 a-c). The presence of this flavonoid may have been attributed to the remarkable antioxidant potential of the methanol fraction of *R. brunonii* leaves. In the

Table 5 — Effect of different concentrations of fractions of *R. brunonii* flowers on Con A and LPS induced murine lymphocyte proliferation

Samples	Conc. mg/kg p.o.	Antibody titre Mean±S.E.	% Activity	DTH Mean±S.E.	% Activity
Control	-	0.89±0.00	-	1.11±0.05	-
RBL1-C	10^{-4}	0.35±0.05	- 60.67	1.09±0.38	+ 11.22
	10^{-5}	0.94±0.10	+ 5.61	0.92±0.02	- 6.12
	10^{-6}	0.78±0.38	+ 12.35	0.74±0.02	- 24.48
RBL1-E	10^{-4}	0.98±0.09	+ 10.11	0.85±0.09	- 13.26
	10^{-5}	0.56±0.09	- 37.07	0.77±0.03	- 21.42
	10^{-6}	0.55±0.13	- 38.20	0.77±0.27	- 21.42
RBL1-A	10^{-4}	0.61±0.10	- 31.46	0.47±0.12	- 22.10
	10^{-5}	0.60±0.05	- 32.58	0.74±0.01	- 27.36
	10^{-6}	0.52±0.05	- 41.57	0.77±0.06	-21.42
RBL1-M	10^{-4}	1.27±0.29	+ 69.33	0.74±0.20	- 52.04
	10^{-5}	0.73±0.15	- 2.66	0.69±0.02	- 24.48
	10^{-6}	0.60±0.10	- 20.00	0.99±0.05	+ 4.21
RBL1-W	10^{-4}	0.85±0.04	+ 13.33	0.58±0.09	- 38.94
	10^{-5}	0.63±0.14	- 16.00	0.74±0.07	- 22.10
	10^{-6}	0.72±0.05	+ 4.00	0.98±0.07	+3.15
RBF1-C	10^{-4}	0.84±0.09	- 5.61	0.78±0.03	- 20.40
	10^{-5}	0.66±0.08	- 25.84	0.67±0.15	- 31.63
	10^{-6}	0.65±0.11	- 26.96	0.83±0.17	- 15.30
RBF1-E	10^{-4}	0.98±0.09	+ 10.11	0.85±0.09	- 13.26
	10^{-5}	0.56±0.09	- 37.07	0.77±0.03	- 21.42
	10^{-6}	0.55±0.13	- 38.20	0.77±0.27	- 21.42
RBF1-A	10^{-4}	0.72±0.17	- 19.10	1.06±0.16	+ 8.16
	10^{-5}	0.60±0.06	- 32.58	0.74±0.13	- 24.48
	10^{-6}	0.61±0.04	- 31.46	0.76±0.21	- 22.44
RBF1-M	**10^{-4}**	**0.61±0.12**	**- 31.46**	**0.59±0.11**	**- 39.79**
	10^{-5}	**0.70±0.08**	**- 21.34**	**0.65±0.04**	**- 33.67**
	10^{-6}	**0.65±0.08**	**- 26.92**	**0.55±0.14**	**- 43.87**
RBF1-W	10^{-4}	1.07±0.06	+ 20.22	0.78±0.25	- 20.40
	10^{-5}	0.82±0.17	- 7.86	0.69±0.07	- 29.59
	10^{-6}	0.58±0.24	- 34.83	0.82±0.02	- 16.32

Values are mean±S.E. of triplicate experiments. + indicates immune stimulantion; - indicates immunosuppression.

case of flowers, the methanol fraction demonstrated noteworthy immune-modulating potential. Therefore, it was also subjected to column chromatography and RP-HPLC, which led to the identification of active phenolic compound i.e. *p*-coumaric acid (Fig. 5a & d).

Characterisation of active compound isolated from methanol fraction of R. brunonii leaves

The active compound was isolated from the methanol fraction of *R. brunonii* leaves, which proved to be the most effective antioxidant agent. The compound was identified as Myricetin through RP-HPLC analysis using standard drugs. The characterization of the compound was done using ^{1}H-NMR and Infrared analysis. The compound was obtained as yellow needles (melting point- 357 °C). Its chemical formula was identified as $C_{15}H_{10}O_8$ and molecular weight was found to be 318.235 g/mol from its mass spectrum which gave molecular ion peak at *m/z*: 318.

^{1}HNMR ($CDCl_3$, 400MHz)

A broad singlet appeared in its ^{1}H NMR spectrum at δ 12.49 appeared due to an intramolecular hydrogen-bonded phenolic proton.^{1}H-NMR spectrum showed the presence of four methine protons with two doublet peaks at 6.12 d, 6.32 d, and one singlet at 6.84 s; 12.9s (H-bonded OH) (Fig. 6). This data was found to be as per the earlier reports available regarding the structural characterisation of Myricetin.

FTIR Spectra

Infra Red (IR) peaks were observed at 3384/cm (OH). The bands around 1678 cm^{-1} and 1631^{-1} (C-O), 1593^{-1} (C-C) in the hexatomic ring. The 1536 cm^{-1} indicates an aromatic group and the bands 1359 cm^{-1}, and 1165 cm^{-1} indicate C-O-C vibration. The IR

Table 6 — Acute toxicity studies of the active methanol fractions of *R. brunonii* leaves and flowers on female Swiss mice

Parameters	Control group	Treated groups	
		(RBL1-M)	(RBF1-M)
Body wt. changes			
0 day	22.3±1.1	23.4±1.9	24.5±1.10
7^{th} day	25.4±4.7	25.1±2.6	25.0±2.11
14^{th} day	29.4±3.5	28.4±1.6	27.6±0.98
Biochemical parameters			
Triglyceride (mg/dL)	68.95±2.56	58.34±2.79	62.35±1.26
SGOT (IU/L)	169.4±8.68	132.3±9.89	145.3±2.15
SGPT (IU/L)	68.95±2.56	58.34±2.79	62.27±2.27
Glucose (mg/dL)	79.07±5.56	90.84±8.21	80.38±1.18
Cholesterol (mg/dL)	73.17±7.14	79.95±12.62	72.56±5.67
Protein (g/dL)	6.44±0.73	7.01±0.72	6.87±1.15
ALP (IU/L)	224.3±21.64	249.5±22.95	210±33.21
Hematology parameters			
WBC (10^3/uL)	10.12±1.016	14.49±2.128	12.20±1.17
RBC (10^6/uL)	6.68±0.306	6.868±0.235	7.25±0.24
HCT (%)	38.5±1.126	39.7±0.451	37.60±2.16
MCH (pq)	18.46±0.314	18.7±0.638	17.75±1.10
MCHC (g/dL)	31.96±0.64	32.2±0.184	30.25±2.25
PLT (10^3/uL)	823.2±115.7	523.8±113.2	725.0±103
LYMPH (%)	76.88±2.319	77.02±1.532	73.26±2.17
NEUT (%)	15.8±1.954	16.72±1.349	17.20±3.34
MONO (%)	5.2±0.570	4.58±0.428	6.75±1.13
EO (%)	2±0.640	1.54±0.191	2.16±0.67
BASO (%)	0.12±0.02	0.14±0.02	0.10±0.03

Values are Mean±SEM

bands at 1368 cm^{-1}, 1625 cm^{-1}, 1455 cm^{-1} are assigned to hydroxyl and quinoid moiety respectively. Aromatic absorptions appeared at 3390 and 920 cm^{-1} (Fig. 7). This data was as per the earlier reports on the Infrared spectra of Myricetin.

Discussion

In the past few years, intense focus has been given to medicinal plants as nutraceuticals due to their phytoconstituents which have many health benefits and medicinal properties. In living systems, oxidation is a basic part of the normal metabolic process, in which reactive oxygen species and many free radicals like hydroxyl radicals and superoxide anions are produced[22]. Rapid production of free radicals may cause alteration in the structure and function of cell constituents and membranes and can result in human neurologic and other disorders such as cancer, diabetes, inflammatory disease, asthma, cardiovascular, neurodegenerative diseases, and premature ageing[23]. There are numerous synthetic drugs available in the market for curing the above conditions. But, they are also associated with diverse side effects. Therefore, the prevention of the above conditions necessitates the use of medicinal plants as a natural source of antioxidants.

The Himalayan musk rose i.e., *Rosa brunonii* (Rosaceae) is a gregariously growing shrub in the Northwest Himalayan region. Members of the Rosaceae family have long been used for food and medicinal purposes. Many species of rose have been scientifically explored to identify their medicinal potential and phytochemical analysis. The pharmacological potential of *R. damascene* species has also been established in various scientific reports for its anticonvulsive, anti-HIV, antibacterial, antioxidant and various other biological potentials[24]. The pharmacological potential of many other rose species like *R. canina, R. hemispherica, R. centifolia* etc. has also been reported[25]. Phenolic compounds have been identified in *R. damascena* along with its antioxidant and anti-microbial activities[26]. *R. brunonii* is used by local indigenous people for curing eye and skin infections, wound healing, diarrhoea and as an aphrodisiac[8-9]. Some phenolic compounds have also been identified in the crude extracts of *R. brunonii* flowers[27]. However, scientific reports on the pharmacological potential of wild rose species, *R. brunonii* is hardly available. Therefore, the current

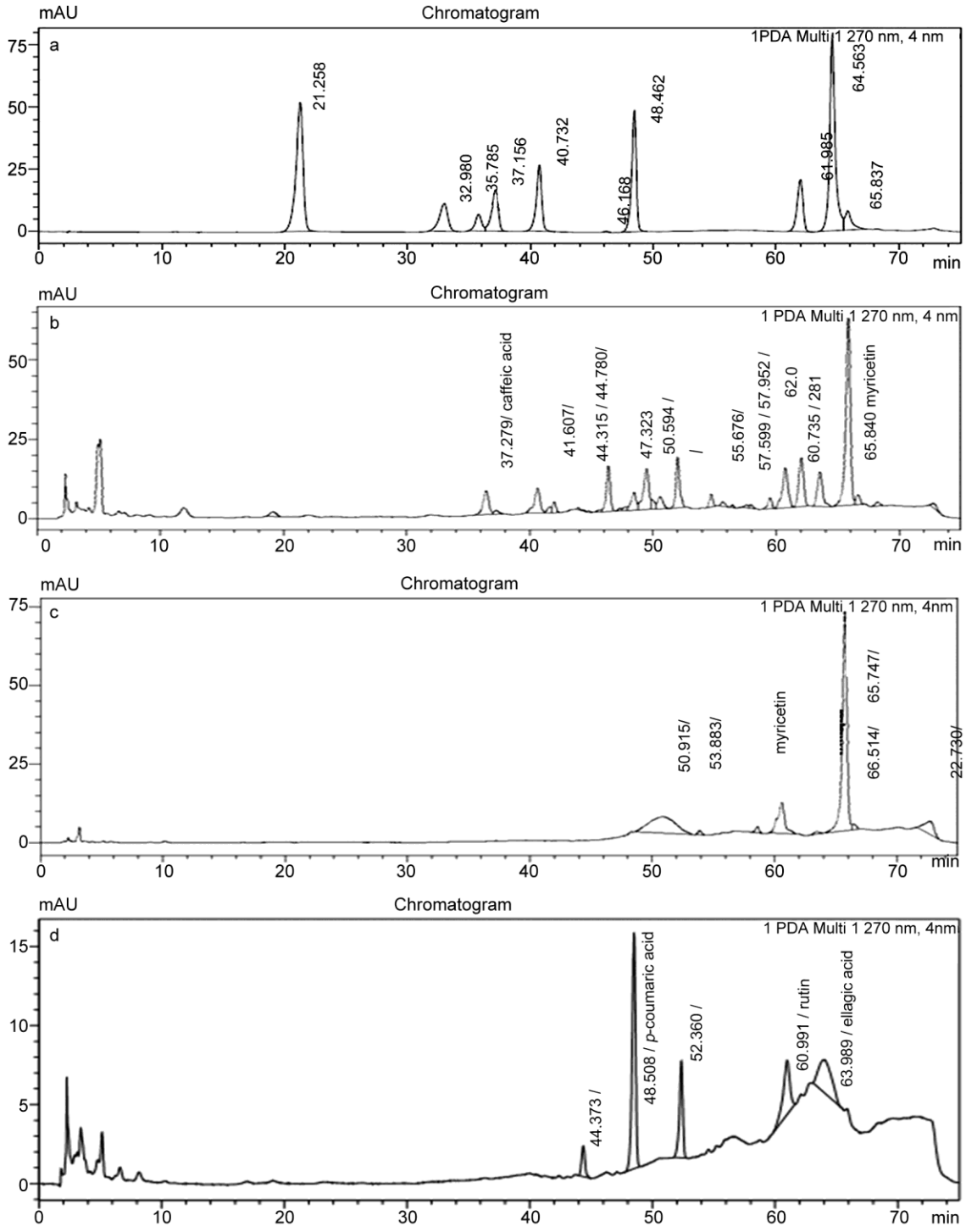

Fig. 5 —a) RP-HPLC chromatogram of standard phenolic compounds, b) RP-HPLC chromatogram of active methanol fraction of leaves of *R. Brunonii*, c) RP-HPLC chromatogram of the purified sub-fraction which lead to the isolation of active constituent (Myricetin) from *R. brunonii* leaves, d) RP-HPLC chromatogram of the purified sub-fraction which lead to the identification of active constituent (p-coumaric acid) from *R. brunonii* flowers.

work focussed on assessing the antioxidant and immunomodulatory potential of wildly growing species of rose *viz. Rosa brunonii*.

The DPPH radical scavenging assay provides the rapid, simplest and widely used approach to determine the antioxidant activity of compounds as

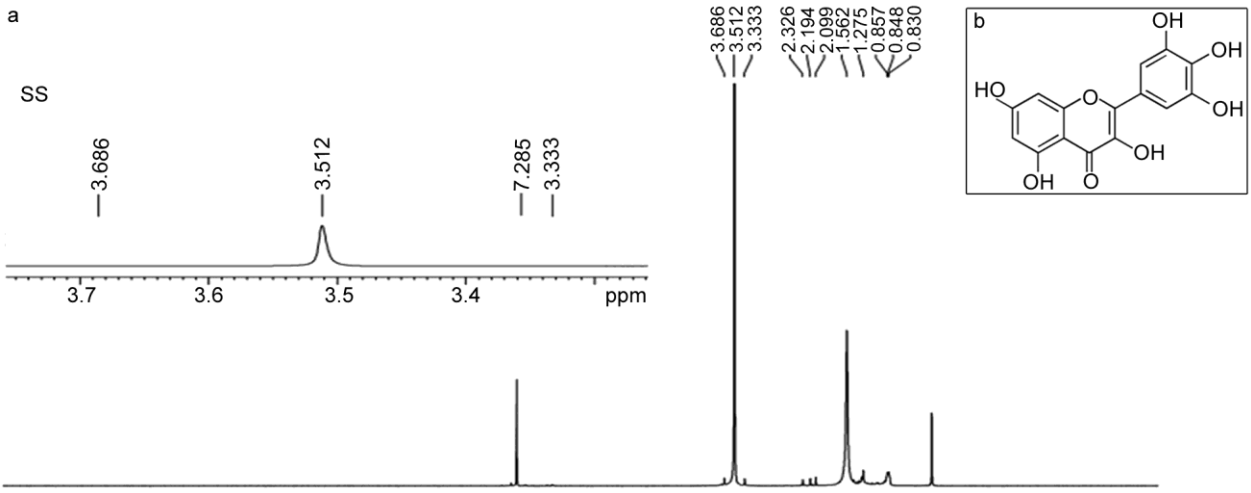

Fig. 6 — a) ^{1}H NMR of isolated compound Myricetin from *R. brunonii* leaves, b) Myricetin.

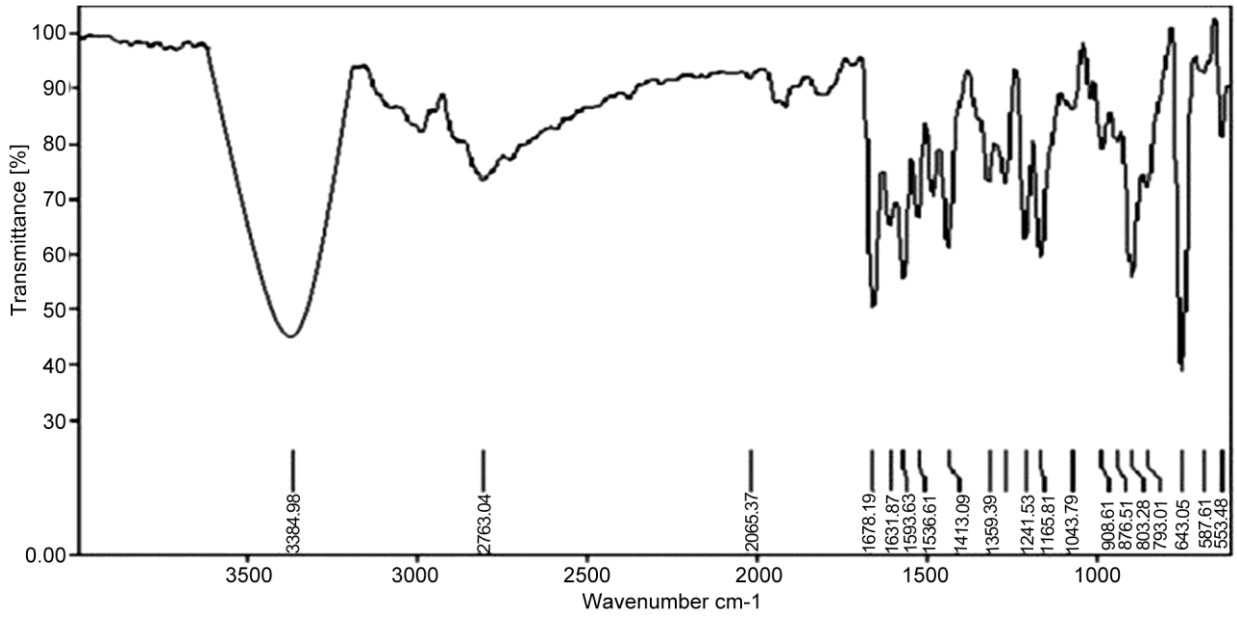

Fig. 7 — Infra Red spectrum of Myricetin isolated from *R. brunonii* leaves.

radical scavengers or hydrogen donors. The antioxidants donate a hydrogen atom to the odd electron of the nitrogen atom of the DPPH molecule thereby reducing it to hydrazine form. Kedare and Singh elaborated the advantage of this method is that DPPH free radical is scavenged even by weak antioxidants and also it can be used to study both hydrophilic and lipophilic antioxidants[28]. The DPPH radical scavenging studies showed that the crude methanol extract of *R. brunonii* leaves revealed remarkable DPPH radical scavenging potential in comparison to the other crude extracts with the lowest IC_{50} value of 62 µg/mL followed by that of *R. brunonii* flowers (IC_{50} 71.3 µg/mL). Therefore, the crude methanol extracts of *R. brunonii* leaves and flowers were subjected to sequential fractionation using five solvents i.e., chloroform, ethyl acetate, acetone, methanol and water. The obtained fractions when again analysed for DPPH radical scavenging activity showed that the methanol fraction of crude methanol extract of *R. brunonii* leaves had noteworthy radical scavenging potential with the lowest IC_{50} value of 32.2 µg/mL followed by that of methanol fraction of crude methanol extract of flowers (IC_{50} 49.82 µg/mL). Some reports are available authenticating the radical scavenging

potential of other rose species. Sharafi *et al.*, 2010 have reported the antioxidant potential of *R. Damascena*[29]. DPPH radical scavenging potential of three rose species has been reported *viz., R. damascena, R. bourboniana* and *R. brunonii,* wherein they also identified the phenolic compounds present in these species like glycosides of quercetin, kaempferol, myricetin, gallic acid, rutin and tannins[27].

Most reactive oxygen species (ROS) are generated as by-products during mitochondrial electron transport and other metabolic reactions. In addition, ROS are formed as necessary intermediates of metal-catalyzed oxidation reactions. The transition metal ion, Fe^{2+} possesses the ability to propagate the formation of free radicals by gain or loss of electrons. Therefore, the reduction of the formation of reactive oxygen species can be achieved by the chelation of metal ions with chelating agents. Various chelating agents have been identified for chelation therapy against metal intoxication like BAL, DMPS, DMSA, and EDTA but they are associated with several side effects like renal necrosis etc[30]. Therefore, chelation therapy using natural chelating compounds from medicinal plants have been of interest and combined administration of antioxidants along with chelating agents has been reported to reduce metal toxicity[31]. Chelation of iron is an indirect mechanism of prevention of free radical production thus chelation capacity of the crude extracts and fractions illustrated that the crude methanol extract of leaves of *R. brunonii* and its methanol fraction possessed the highest chelating capacity on ferrous ions (71.2±0.44 and 66±0.22% respectively at 100 µg/mL) (Table 1, Fig. 3). Excess of metal ions can lead to various anomalies in the body. The iron (II) chelating activity of plant extracts is of great significance because it has been proposed that the transition metal ions contribute to oxidative damage in neurodegenerative disorders like Alzheimer's and Parkinson's diseases[32]. *R. brunonii* proved to be a good source of chelators of ferrous ions which could be explored further for their chelating potential.

Another assay i.e., Ferric reducing antioxidant power (FRAP) was conducted on all the extracts and fractions of *R. brunonii*. In this assay, reduction of ferric tripyridyltriazine (Fe^{3+}- TPTZ) complex to ferrous form which has an intense blue colour is monitored by measuring the change in absorption at 593 nm. Test conditions favour reduction of the complex and, thereby, colour development provided that a reductant (antioxidant) is present. In the FRAP assay, excess Fe (III) is used, and the rate-limiting factor of Fe(II)-TPTZ and hence colour formation is the reducing ability of the sample. Ferrous ions are more potent in decomposing hydrogen peroxides than Ferric ions. Therefore, the reduction can be marked as a measure of antioxidant potential. Polyphenols present in the fractions may bind to metal ions and form complexes which can be considered as another antioxidant mechanism apart from free radical scavenging[33]. The results of this assay showed the remarkable ferric reducing potential of the crude methanol extract of leaves as compared to that of the methanol fraction of flowers with FRAP values of 1890±1.22 and 1256±0.32 µmol/g dry wt, respectively. Further, methanol fractions of both leaves and flowers showed the best FRAP activity as compared to other fractions with FRAP values of 3996±2.17 and 3616±1.83 µmol/g dry wt, respectively.

The reducing power activity on ferric ions was again measured by using potassium ferricyanide for both the plant species. However, crude methanol extracts of both *R. brunonii* leaves and flowers showed significant reducing potential on ferric ions with EC_{50} values of 0.199 and 0.351 mg/mL respectively. The subsequent fractions also showed good results with methanol fractions of both leaves and flowers showing the lowest EC_{50} values of 0.124 and 0.265 mg/mL.

The fractions of *R. brunonii* leaves and flowers were also analysed for their protective effect against free radical-induced DNA damage. It was observed that the methanol and aqueous fractions of *R. brunonii* leaves had maximum shielding effects on the hydroxyl radical-induced DNA damage. In the case of *R. brunonii* flowers, acetone, methanol and aqueous fractions showed a considerable protective effect. The Fenton-reaction generates hydroxyl radicals which introduce nicks in the DNA by breaking the phosphorous linkages among the nitrogenous bases of DNA. The antioxidants or polyphenols present in the extracts/fractions may scavenge the free radicals, which in turn leads to the protection of DNA from nicking[34].

The phytochemical analysis was also conducted on all the fractions of both plant species. The quantitative analysis was done for the assessment of TPC and TFC and the qualitative analysis was done to detect different phytochemicals like terpenoids, coumarins,

glycosides, saponins, anthraquinones etc. The methanol fraction of *R. brunonii* leaves showed high content of flavonoids (344±1.76 mg QE/g dry wt.) and phenols (250±0.54 mg GAE/g dry wt.) followed by methanol fraction of *R. brunonii* flowers which showed flavonoid content of 204±0.79 mg QE/g dry wt. and total phenolic content of 188±1.26 mg GAE/g dry wt. The qualitative phytochemical analysis confirmed tannins in the methanol fractions of leaves and flowers and saponins in the aqueous fractions. Some coumarins and terpenoids were also detected in fractions of flowers. The presence of the phenols and flavonoids in the active methanol fractions of *R. brunonii* leaves and flowers is evident of the fact that such phytochemicals possess antioxidant properties. The antioxidant potential of plant extracts and fractions is directly correlated to the presence of a high amount of polyphenolic compounds. Phytochemicals have been known to work synergistically to exert health benefitting effects like antimicrobial and many other preventive effects against lipid peroxidation, DNA damage, cardiovascular diseases, neurological diseases and cancer[35]. It is also pertinent to mention that the human body cannot synthesize polyphenols. Therefore, uptake of such phenolic compounds and flavonoids from external sources becomes essential[36]. Earlier reports are available authenticating the presence of phenols and flavonoids in some other rose species. *Rosa damascena* was observed to possess significant amounts of gallic acid, quercetin and syringic acid which were believed to be responsible for its antioxidant potential. Roman *et al.* have also reported a direct correlation of phenolic content and free radical scavenging potential of *Rosa canina* hips[37].

Apart from showing remarkable antioxidative effects, *R. brunonii* flowers and leaves also exhibited significant immunomodulatory effects. The use of natural immunostimulants and immunosuppressants, both play a vital role in immunomodulation. Diverse synthetic compounds like levamisole, isoprinosine, pentoxifilline, and thalidomide are some of the most significant immunomodulators that have shown promising results. Also, the discovery of immune-suppressant synthetic drugs like cyclosporine and cyclophosphamide that prevented the rejection of graft and had been used in other auto-immune diseases was a breakthrough achievement. A recent review by Patil *et al.* provides useful information about various synthetic immunomodulators, explaining their adverse side effect profiles and generalized effects which pose a major limitation to their use and emphasises the need for search of more effective and safer immunomodulatory agents[38]. Therefore, the identification and isolation of immunomodulators of natural origin hold enormous importance.

The fractions of *R. brunonii* flowers and leaves when analysed for immunomodulatory potential revealed some interesting results. Among the fractions of crude methanol extracts of *R. brunonii* leaves and flowers, the methanol fraction of flowers showed noteworthy stimulating effects on both the immune responses i.e., HA Titre (125%) and DTH response (166%) which was quite comparable to the standard drug Levamisole. Also, the methanol fraction of flowers showed a significant suppressive effect on the LPS and Con-A induced B-cell and T-cell lymphocytic proliferation as compared to other fractions. The methanol fraction of flowers, when subjected to column chromatography, revealed the presence of *p*-coumaric acid upon HPLC analysis in the active sub-fraction. *p*-coumaric acid is a hydroxycinnamic acid, a phenolic compound that is found in peanuts, navy beans, tomatoes, carrots, basil and garlic. Pragasam *et al.* have also confirmed the stimulating effects of *p*-coumaric acid on immune responses[39]. In addition, they also reported the suppressive potential of *p*-coumaric acid on lymphocytic proliferation. *p*-coumaric acid exhibited cytoprotective effect on Natural Killer (NK) cells and cytotoxic T lymphocytes (CTLs) via their antioxidant capacity[40] *R. brunonii* have been identified to be a rich source of this beneficial phenolic acid which has been proven to possess antioxidant and immunomodulatory potential. Therefore, *R. brunonii* can be exploited to harbour these phytochemicals for the benefit of human health. The acute toxicity studies conducted on the active methanol fractions of both leaves and flowers of *R. brunonii* also authenticated the non-toxic nature of the fractions and their safety for human consumption.

The findings of this work help in concluding that *Rosa brunonii* holds immense biological potential in terms of protection against oxidative stress and also for enhancing immunity especially in the case of immune-compromised patients. This plant can also be further investigated for understanding the underlying mechanisms of action and its effectiveness against

other medical ailments and also for the scientific establishment of its pharmacological potential.

Conclusion

This research work proposed the bioactivity prospection of traditionally used *Rosa brunonii* through scientific experimentation. The findings have authenticated that *Rosa brunonii* holds immense biological potential in terms of protection against oxidative stress as its leaves showed the highest DPPH free radical scavenging (IC_{50} 32.3±1.15 µg/mL), chelation capacity on ferrous ions (66±0.22%), reducing power (EC_{50}124.4±0.17 µg/mL), FRAP (3996±2.17 µM/g dry wt.). as well as highest amounts of total phenols (250±0.54 GAE mg/g dry wt.) and flavonoids (344±1.76 QE mg/g dry wt.). Moreover, a significant reduction in plasmid DNA damage was also observed. Also, the immunomodulatory studies revealed that *Rosa brunonii* flowers possess immense immune stimulation with 125% on humoral response, 166% on cell-mediated response and significant suppressive effect on mitogen-induced lymphocytic proliferation and these findings are suggestive of their use in the case of immune-compromised patients. Additionally, *R. brunonii* harbours natural antioxidant and immunity enhancing compounds, which can be further investigated in detail to understand the underlying mechanisms of action and also to establish its pharmacological potential.

Conflict of interest

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

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