BRONCHORELAXANT, MAST CELL STABILIZING, ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF *RANDIA DUMETORUM* (RETZ.) LAMK. EXTRACTS

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Abstract: *Randia dumetorum* (RD) fruits in different form have been ethnopharmacologically reported to possess antiasthmatic property. Therefore, present study was undertaken to evaluate two different extracts of RD i.e., ethyl acetate (RD-EA) and methanol (RD-ME) for bronchorelaxant, anti-inflammatory, mast cell stabilizing and antioxidant effect along with safety margin, according to OECD guidelines for toxicity. RD-ME and RD-EA (1 mg/mL) exhibited 68.75 and 57.39% inhibition of contraction against acetylcholine, while against histamine induced contraction, inhibition observed was 100 and 78.13%, respectively. Moreover, extracts attenuated the experimentally induced inflammation at 200 mg/kg with % inhibition of 41.62 by RD-ME and 30.36 by RD-EA in carrageenan model, while in egg albumin model RD-ME and RD-EA exhibited % inhibition of 48.31 and 33.75, respectively. In addition, RD-ME and RD-EA at 100 µg/mL demonstrated significant decrease in histamine release of 08.31 and 16.71 in C-48/80 induced mast cell degradation. RD extracts also exhibited antioxidant activity in DPPH, reducing power and metal chelation method, along with safe margin for oral administration as observed in acute toxicity evaluation.

Keywords: *R. dumetorum,* asthma, bronchodilation, guinea pig trachea, anti-inflammatory, mast cells, antioxidants

Randia dumetroum (Retz.) Lam., synonym *Catunaregam spinosa* (Thunb.) Tirveng., family Rubiaceae (RD), is a deciduous thorny shrub growing throughout India up to an altitude of 1,350 meters. It is commonly known as emetic nut in English and *Maniphal* in Hindi. Fruit extracts of RD have been widely practiced in the traditional systems of Indian medicine and ethnically reported for the treatment of asthma, bronchitis and inflammation (1).

Ethnopharmacological profile of RD fruits have been attributed to various isolated phytoconstituents viz., randia saponin and randillic acid (2), oleanilic acid based saponin (3), urosaponin (4), dumetoronin A-F (5), randioside A (6), molluscidal tritrpenoid glycoside (7) and randianin (8). Various preclinical research reports on RD extracts claim its antimalarial (9) and antimicrobial properties (10,

11). Moreover, oleanilic based saponin from RD fruit extract has been reported to possess antifertility activity (12, 13).

However, no research has demonstrated the report on comparative evaluation of bronchorelaxant, mast cell stabilization, anti-inflammatory and antioxidant effect of two different extracts of RD fruit i.e., ethyl acetate (RD-EA) and methanol (RD-ME). Therefore, present investigation attempts to explore the bronchorelaxant effect using acetylcholine and histamine precontracted guinea pig tracheal chain, stabilizing activity against mast cell degradation induced by compound 48/80 (C-48/80), scavenging effect against different free radicals and anti-inflammatory effect on carrageenan and egg albumin induced inflammation along with safety evaluation of RD extracts. Outcome of present studies demonstrate that RD-ME as well as RD-EA

induces bronchorelaxation against both acetyl-choline and histamine induced contraction. The bronchorelaxant potential of RD fruit extracts is well supported by anti-inflammatory effect, stabilization of mast cells and scavenging of different free radicals.

EXPERIMENTAL

Collection and extraction of RD fruits

RD fruits were collected from the medicinal garden of R.T.M. Nagpur University campus, Nagpur, India, and authenticated from Botanical Survey of India (BSI), Pune, India. [Reference No. BSI/WC/Identi./Tech./2008/473]. Collected fruits were cleaned, shade dried, coarsely powdered (500 g) and defatted using petroleum ether (60–80ºC) and finally extracted successively with ethyl acetate and methanol using Soxhlet apparatus. Both the extracts were collected and filtered through Whatman filter paper (No. 44), concentrated over thermostat water bath and referred as RD-EA and RD-ME, respectively. Concentrated extracts were stored in vacuum desiccators.

Chemicals

Histamine, acetylcholine, compound 48/80 (C-48/80), ascorbic acid, ethylene diaminetetra acetic acid (EDTA), egg albumin and carrageenan were purchased from Hi- Media, Mumbai, India. 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine [ferrozine], *o*-phthalaldehyde, *n*-heptane, potassium ferricyanide, metrizamide and trichloroacetic acid (TCA) were purchased from Sigma Chemicals, USA. Ferrous chloride, carboxymethyl cellulose (CMC), *n*-butanol, solvents for extraction; petroleum ether (60–80ºC), ethyl acetate and methanol were procured from Loba Chemicals, Mumbai, India. Indomethacin was generously gifted by Wockhardt Pharmaceuticals, Aurangabad, India. Disodium cromoglycate (DSCG) and aminophylline were generously gifted by Cipla Limited, Mumbai, India. All other chemicals used for the present investigation were of analytical grade.

Animals

Albino rats and guinea pigs were housed under standard 12:12 h light/dark cycle in a temperature controlled (24 ± 1ºC) environment with *ad libitum* access to rodent chow (Lipton, India) and water. All experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) Constituted for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA) by Ministry of Environment and Forests, Government of India, New Delhi (IAEC approval No. 536/02/C/CPCSEA).

Preliminary phytochemical screening and standardization of extracts

Preliminary phytochemical screening

RD fruit extracts were subjected to preliminary phytochemical screening for the presence of various phytoconstituents such as alkaloids, flavonoids, polyphenols, steroids, saponins, sugars and terpenoids (14).

Standardization of extracts for total polyphenols and flavonoid content

In order to standardize, RD extracts were quantized for the content of total phenolics and flavonoids.

Total phenolics analysis

Total amount of phenolics in RD extracts was measured by using Folin-Ciocalteu (F-C) reagent assay method (15). Accordingly, 4 mL of F-C reagent which was diluted with distilled water (1:10), was mixed with 1 mL of test extract. To this mixture 5 mL of 7.5% sodium carbonate solution in distilled water was mixed. The absorbance of colored complex was read at 765 nm after 30 min with a UV-Visible spectrophotometer (Shimadzu, UV-1601). Gallic acid was used as a standard substance for the calibration curve. The total amount of phenolic compounds was calculated and expressed in mg/g of a dry weight of extracts and expressed in gallic acid equivalent (GAE). All the determinations were performed in three replications.

Determination of total flavonoids concentrations

The content of total flavonols in the RD extracts was determined as described previously (16). Test extract solution was added to a 25 mL flask containing 5% $NaNO_2$ (w/w, 0.75 mL) and allowed to react for 6 min, then 10% $Al(NO_3)_3$ (w/w, 0.75 mL) was added, and 6 min later, 4% NaOH (w/w, 4 mL) was added. After mixing, an aqueous solution of 30% ethanol was added to the flask, which was made up to the mark. The solution was allowed to stand for 10 min at room temperature and the absorbance at 500 nm was measured. The total flavonoid concentration was calculated using rutin calibration curve and expressed as total flavonoids (mg/g).

In vitro antioxidant activity

Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The radical scavenging activity of RD extracts was evaluated with the help of DPPH radical according to the method of Chu et al. (17). DPPH solution, 0.5 mL (0.1 mM) was added to a test tube containing 1 mL of RD extracts in different concentrations (µg/mL). Methanol was used instead of extracts and considered as a control. These reaction mixtures were vortexed at room temperature and absorbance was measured after 30 min at 520 nm. Percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the RD extracts or standard.

Determination of percentage residual rate of inhibition (%RRI)

%RRI of DPPH by RD extracts was measured according to the method described by Yamasaki et al. (18), in which decrease in absorbance of reaction mixture containing DPPH and RD extracts were measured after fixed time interval. Accordingly, 1 mL of RD extract, 4 mL of acetate buffer (0.1 M, pH 5.5), and 4 mL DPPH (1.25×10^{-4} M) was mixed with constant stirring and final volume was made up to 10 mL with methanol. After 0.5, 1, 3, 5 and 7 h absorbance was determined at 524 nm. Concentration of RD extracts was selected on the basis of results obtained from above DPPH scavenging results. %RRI was calculated using formula:

$$\%RRI = (Abs\ t_0) - (Abs\ t_x) / (Abs\ t_0) \times 100$$

where, Abs t_0 is the absorbance at 0 min and Abs t_x is absorbance at various time intervals (0.5 to 7 h).

Ferrous metal ion chelating activity

RD extracts were evaluated for its ferrous ions chelating capacity according to the previously described method (17). Accordingly, 2 mL of RD extracts (25–50 µg/mL) were mixed with a solution of 2 mM $FeCl_2$ (0.5 mL) and 0.2 mL of 5 mM ferrozine with vigorous shaking to initiate the reaction. Resultant mixtures were kept standing for 10 min at room temperature to achieve the equilibrium. Absorbances of the solutions were measured at 562 nm. The control contained $FeCl_2$ and ferrozine complex. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated as described under the DPPH scavenging activity.

Reducing power

Reducing capacity of RD extracts was evaluated as per the method of Oyaizu (20). RD extracts (1 mL) in different concentrations (25–125 µg/mL) were added to the mixture containing phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The resultant solution was incubated at 50°C for 20 min and to this tricarboxylic acid (TCA) (2.5 mL, 10%) was added, mixed and centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with $FeCl_3$ (0.5 mL, 0.1%) and distilled water (2.5 mL), the absorbance of final solution was read at 700 nm.

In above methods of *in vitro* antioxidant evaluation ascorbic acid (AA) was used as a standard for DPPH and reducing power assay, while EDTA was used for ferrous metal ion chelating activity.

Inhibition of histamine release from rat peritoneal mast cells

Preparation of rat peritoneal mast cell

Effect of RD extracts on percentage histamine release and preparation of rat peritoneal mast cells (RPMCs) was carried out as per the methods described earlier (21). In detail, exsanguinated rats were injected with Hank's balanced salt solution (HBSS) in abdominal cavity. The abdomen was gently massaged for 120 s and cavity was opened to aspirate the fluid containing peritoneal cells. Peritoneal fluid was centrifuged at 2000 rpm to obtain cell components, which were resuspended in HBSS. Resultant cells suspension was treated for separating mast cells from other components (i.e., macrophages and small lymphocytes) by suspending peritoneal cells in 1 mL of HBSS which was layered on 2 mL of 0.225 g/mL metrizamide (density 1.120 g/mL) and centrifuged at room temperature for 15 min at 3000 rpm. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL of HBSS and considered as mast cell suspension for the present study.

Measurement of histamine release from RPMC

RD extracts (25–100 µg/mL) or disodium cromoglycate (DSCG) (10-50 µg/mL) was mixed with mast cell suspension and incubated at 37°C for 15 min in separate test tubes. Each mixture thus obtained was made up to 3 mL with HBSS, to this an equal volume of C-48/80 (10^{-6} g/mL) was added and allowed to incubate at 37°C for 30 min followed by centrifugation at 2500 rpm. Top layer of the resulting solution from each test tube was transferred to a separate tube containing 300 mg NaCl and 1.25 mL *n*-butanol. This solution was alkalized by adding 3 M NaOH (1 mL) to extract histamine

Table 1. Phytochemical study of the RD extracts.

Phytoconstituents	Flavonoids	Alkaloids	Sugars	Saponins*	Tannins	C glycosides	Steroids	Triterpenoids
RD-ME	+++	-	+++	++	++	+	−	++
RD-EA	++	+	+	+	+	++	+	+

RD-ME – *R. dumetorum* methanol extract, RD-EA – *R. dumetorum* ethyl acetate extract. Phytoconstituents present in (+) low, (++) moderate and (+++) high concentration as reflected by color intensity of test or * indication parameter like foam intensity as in saponins test.

Table 2. Standardization of RD extracts for total polyphenols and flavonoids contents.

	Total polyphenols	Total flavonoids
Extracts	mg/g	
RD-EA	3.18 ± 0.81	3.32 ± 0.56
RD-ME	2.66 ± 00.76	8.02 ± 1.04

Values represent the mean ± SEM. of three independent replicates. RD-ME – *R. dumetorum* methanol extract, RD-EA – *R. dumetorum* ethyl acetate extract.

into *n*-butanol. After shaking, the sample was centrifuged for 5 min and 1 mL of the top layer (*n*-butanol) was pipetted into a tube containing 2 mL of *n*-heptane and 0.4 mL of 0.12 M HCl. The test tubes contents were mixed by inverting them several times and were allowed to stand for phase separation; 0.5 mL of the aqueous phase was transferred to another test tube. To each tube 1 M NaOH (100 µL) and 0.2% *o*-phthalaldehyde (100 µL) solution was added immediately under constant stirring. Further, to impede *o*-phthalaldehyde complexing reaction, 3 M HCl (50 µL) was added after 2 min and finally histamine concentration was determined from resultant reaction mixtures by using a fluorescence detector at excitation and emission wavelengths of 350 and 450 nm, respectively (Spectrofluorometer-530, Shimadzu). The control solutions were prepared in the following manner:

1) Spontaneous histamine release: containing mast cells and solutions used to determine baseline,

2) Histamine release: which contains mast cells and calcium-ionophore (10^{-6} g/mL),

3) Test compound control: contains solutions and test compound and

4) Solution control: contains only solutions used in the test to determine baseline.

Percent histamine release (HR) inhibition from mast cells was determined by the following formula:

$$(\text{sample HR} - \text{spontaneous HR}) / (100\% \text{ HR} - \text{spontaneous HR}) \times 100$$

Acute toxicity study

The acute oral toxicity study was carried out for RD extracts, as per the limit dose test of up and down system mentioned in OECD test guidelines No. 423 at a limit dose of 2 g/kg body weight (*p.o.*) (22). Three rats (one male and two female) were selected for each group, in such a way that the weight differences were not exceeding ± 10% of the mean initial weight of the population. Rats were fasted for food but water was provided *ad libitum* overnight prior to RD extract administration (2000 mg/kg, *p.o.*), suspended in 1.0%, w/v, carboxymethyl cellulose (CMC) and the access to food was reinstated after 3–4 h. After dosing, individual rat was observed at least once during the first 30 min, periodically during the initial 24 h, with special attention given during the first 4 h and daily thereafter, for a total of 14 days. The systemic and behavioral toxicity patterns were studied as described in OECD test guidelines. At the end of toxicity study, all surviving animals were sacrificed.

Evaluation of anti-inflammatory effect
Carrageenan induced rat paw edema

The anti-inflammatory activity of RD-ME and RD-EA was determined by carrageenan induced inflammation test in hind paw of rats (23). Rats were fasted for 24 h before the commencement of experiment. Edema was induced by injecting 0.1 mL of 1% (w/v) carrageenan in saline. Rats were divided into different treatment groups (n = 5) viz.,

group I (vehicle, 10 mL/kg), group II (indomethacin, 10 mg/kg), group III (RD-ME, 100 mg/kg), group IV (RD-ME, 200 mg/kg), group V (RD-EA, 100 mg/kg), and group VI (RD-EA, 200 mg/kg). RD extracts and indomethacin (IM) were suspended in 1% CMC and administered orally by intragastric tube 1 h before the carrageenan injection. After the administration of the phlogistic agent, paw volume (in mm) of individual rat was measured at 1, 3 and 5 h.

Egg albumin induced rat paw edema

Egg albumin is reported to induce inflammation in the hind paw of rats (24) and therefore, this model was employed to evaluate the anti-inflammatory activity of RD-ME and RD-EA. Acute inflammation was induced by injecting 0.1 mL/kg of fresh egg albumin into the plantar region of the hind paw of rats. Separate sets of rats (n = 5) were employed for control, standard and treatment groups as described in carrageenan induced inflammation experiment. The change in paw volume (mm) was measured up to 120 min, at 20 min intervals after egg albumin injection.

Effect of RD extracts on the spasmogen induced contraction on guinea-pig isolated trachea

Bronchorelaxant effect of RD extracts was evaluated according to the method described by Vogel (21); guinea pigs were sacrificed by a deep ether anesthesia and exsanguinated. The section of trachea was separated from adjacent tissue to obtain tracheal rings which were tied to get chain of 3–4 individual tracheas. The chain was mounted in a 20 mL double unit Students organ bath (AVI-PH-4540) containing Krebs-Henseleit (K-H) solution of the composition (mM): NaCl 118.4, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 11.1, pH 7.4 ± 0.05 and temperature of bath was maintained at 37 ± 1°C. Tracheal chain was suspended under isotonic tension of 0.5 g and allowed to equilibrate for at least 1 h before commencing the experiment. During the experiment K-H solution was replaced after every 10 min. After equilibrium period the contraction was induced by adding the acetylcholine or histamine (1 μg/mL). Thereafter, the RD extracts (1 mg/mL) were added serially in increasing doses to the tissue bath until complete relaxation (baseline reading) was observed, tracings of observation were recorded using Digital Kymograph. At the end of experimentation, the effect of RD extracts on pre-contracted tracheal chain was expressed as percent bronchorelaxation.

Statistical analysis

All results were expressed as the mean ± SEM. The data of bronchorelaxation study were analyzed by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test. The results of anti-inflammatory activity were analyzed by two-way ANOVA followed by *post hoc* Bonferroni tests (Factor I: Treatment; Factor II: Time). A value of p < 0.05 was considered to be statistically significant in all the cases.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

This study revealed that alkaloids, flavonoids, saponins, tannins and cardiac glycosides were present in RD-EA while RD-ME contains flavonoids, saponins, steroids, tannin and triterpenoids (Table 1). In order to standardize, the RD extracts were quantized for the content of total of phenolics and flavonoids; results of standardization revealed that RD-EA and RD-ME showed the presence of 2.66 and 3.18 mg/g of polyphenols, while total flavonoids found were 3.32 and 8.02 mg/g, respectively (Table 2). Polyphenols (tannins, flavonoids and flavonols) were reported to possess anti-inflammatory, antioxidant, mast cell stabilization and bronchorelaxant effect. Saponins are also reported to have antiasthmatic and anti-inflammatory activity (25, 26).

Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The %DPPH scavenging activities possessed by standard antioxidant ascorbic acid, RD-EA and RD-ME were found to be 86.23, 72.87 and 88.26, respectively at the concentration of 50 μg/mL. RD-ME exhibited better antioxidant activity than RD-EA as well as standard antioxidant ascorbic acid. These results of %DPPH scavenging by RD extracts and standard antioxidant ascorbic acid at different concentrations are shown in Table 3.

Percentage residual rate of inhibition (% RRI) of DPPH

RD-ME and RD-EA showed %RRI of 43.21 and 38.10, respectively at 7th h, suggesting the better ability of RD-ME as compared to RD-EA to scavenge the free radical for longer duration. AA exhibited 59.16 of %RRI. The results of %RRI by 100 μg/mL of RD extracts and standard at different time intervals are shown in Table 3.

Reducing power of RD extracts

RD extracts and ascorbic acid exhibited reducing power in the order: ascorbic acid > RD-ME >

Table 3. Effect of different RD extracts on DPPH, % RRI DPPH, reducing power and metal chelation.

Determination of antioxidant activity in various free radical generating system														
% DPPH scavenging %				RRI DPPH at 100 µg/mL				Reducing power (Absorbances)				% Metal chelating ability		
Conc. (µg/mL)	AA	RD-EA	RD-ME	Time (h)	AA	RD-EA	RD-ME	Conc. (µg/mL)	AA	RD-EA	RD-ME	EDTA	RD-EA	RD-ME
10	22.57 ± 0.99	11.34 ± 0.78	15.44 ± 1.05	0.5	98.73 ± 1.44	81.09 ± 2.48	89.64 ± 2.17	25	0.294 ± 0.076	0.235 ± 0.088	0.142 ± 0.066	28.12 ± 0.19	16.09 ± 2.11	19.74 ± 1.68
20	36.41 ± 1.26	27.99 ± 0.89	31.09 ± 1.48	1	83.45 ± 1.73	67.98 ± 1.18	62.29 ± 1.62	50	0.413 ± 0.098	0.307 ± 0.102	0.286 ± 0.074	57.22 ± 1.04	37.14 ± 1.92	33.12 ± 1.74
30	49.33 ± 1.54	35.41 ± 0.92	47.26 ± 0.98	3	71.19 ± 0.78	53.45 ± 1.89	51.37 ± 1.43	75	0.682 ± 0.128	0.460 ± 0.128	0.536 ± 0.108	79.16 ± 1.29	50.43 ± 1.33	54.38 ± 2.65
40	61.89 ± 1.08	49.56 ± 1.29	61.92 ± 0.75	7	59.16 ± 1.29	38.10 ± 1.37	43.21 ± 1.28	100	0.841 ± 0.109	0.602 ± 0.116	0.784 ± 0.092	91.81 ± 2.18	72.79 ± 1.71	78.16 ± 1.03
50	86.23 ± 0.84	72.87 ± 1.47	88.26 ± 1.10					125	1.095 ± 0.153	0.807 ± 0.147	0.912 ± 0.110	98.68 ± 2.11	89.37 ± 1.89	94.07 ± 2.27

Values represent the mean ± SEM of three independent replicates. AA – ascorbic acid, RD-ME – *R. dumetorum* methanolic extract; RD-EA – *R. dumetorum* ethyl acetate extract. % Inhibition for DPPH and metal chelation was calculated using formula: % Inhibition = $[(A_0 - A_1)/A_0] \times 100$. % RRI of DPPH was calculated by formula: % RRI = (Abs t_0) – (Abs t_x) / (Abs t_0) × 100. The reducing capacities were determined by reading the absorbance at 700 nm; increased absorbance reflects increased inhibition of $Fe^{3+} \rightarrow Fe^{2+}$ transformation.

RD-EA, which showed 1.095, 0.912 and 0.807 absorbances, respectively, at the concentration of 125 µg/mL. The highest absorbance was exhibited by RD-ME indicating the greater reducing power as compared to RD-EA (Table 3).

Metal chelating capacity

The percentages of metal chelating capacity of EDTA, RD-EA and RD-ME at 125 µg/mL concentration were found to be 98.68, 89.37 and 94.07%, respectively. The metal chelating capacity of RD-ME was found better than RD-EA but inferior to standard metal chelating agent – EDTA. The results of %metal chelating ability exhibited by RD extracts and EDTA at different concentrations are shown in Table 3.

Oxidative stress involves a cascade of complex reactions, which encompasses excessive production of reactive species in biological systems. This might be observed due to imbalance between oxidant and/or prooxidant and defense systems, excess production of radicals, degeneration of defense system, diseased conditions and immunological factors. Such excessive accessibility of reactive species in the body is thought to lead to a number of pathological conditions including rheumatic or pulmonary diseases, atherosclerosis, cardiac and cerebral ischemias (27). Oxidative stress can be counteracted by antioxidant effect produced by mediating one of the mechanism or in combination; by capacity to cause reduction, inhibition of continued hydrogen abstraction, perturbation of chain initiation, decomposition of peroxides and chelation of catalytic transitional metal ions. To understand the means of antioxidant behavior, single method can not be helpful to depict the mechanism. Therefore, among the several methods reported, antioxidant effect of RD extracts has been evaluated using different free radical generating systems viz., DPPH, %RRI of DPPH, reducing power and metal chelating effect. DPPH assay involves the formation of stable diamagnetic molecule by accepting an electron or hydrogen radical, which alters color intensity of the reaction mixture. The change in color intensity is proportional to the reduction of pink colored 2,2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow-colored diphenylpicrylhydrazine (17). Measurement of %RRI is another method, which measures the ability of test compounds and extracts to scavenge radicals, which may indicate the potential to protect biological system at different time intervals (18). Results obtained from both the studies suggest that RD extracts exhibits ability to scavenge the DPPH radicals at different concentrations as well as at dif-

Table 4. Effect of different RD extracts on % histamine release.

Concentration (µg/mL)	DSCG	RD-EA	RD-ME
10	97.56 ± 2.24	95.54 ± 2.09	92.25 ± 3.28*
25	75.64 ± 2.86*	78.25 ± 1.91*	61.38 ± 2.58**
50	34.22 ± 2.10**	43.41 ± 1.43**	36.41 ± 1.72**
100	01.68 ± 1.07**	16.71 ± 2.49**	08.37 ± 1.33**

DSCG – Disodium cromoglycate; RD-ME – *R. dumetorum* methanolic extract; RD-EA – *R. dumetorum* ethyl acetate extract. Each value is presented as the mean ± SEM. of three independent determinations. % Histamine release from rat peritoneal mast cells was calculated by the formula: (sample HR – spontaneous HR) / (100 % HR – spontaneous HR) × 100. Statistical significance was assessed using a one-way ANOVA with Bonferoni multiple comparison test: *p < 0.05 and **p < 0.001 *vs.* control group i.e., compound 48/80-induced histamine release level.

ferent time intervals, which implies the role of RD extracts as an effective antioxidant.

The reducing capacity of test compounds or extracts grants a significant indicator of its potential antioxidant activity (28). Reducing ability of RD extracts was evaluated on the basis of inhibition of Fe^{3+}–Fe^{2+} transformation system and monitored on the basis of change in absorbance of reaction mixture as increase in reducing power was indicated by an an increase in absorbance. RD-ME exhibited more absorbance as compared to RD-EA but less than standard antioxidant ascorbic acid. RD extracts may act by donating electrons, which reduce the oxidized intermediates of lipid peroxidation processes and thus are considered as primary and secondary antioxidants.

In addition to above methods of evaluation, metal chelation is believed to be important for protection against various diseases. Chelating agents are considered as effective antioxidants as they reduce the redox potential of system and diminish the oxidative stress by stabilizing the oxidized form of the metal ions (29). DPPH scavenging, reducing power and metal chelating activity of extracts may relate to the marked antioxidant potential, which may provide improved protection to biological system. In all the *in vitro* antioxidant assays RD-ME was found superior to RD-EA.

Mast cell stabilization activity of RD extracts

The inhibitory effect of RD extracts and standard drug disodium cromoglycate (DSCG) on C-48/80 induced histamine release from rat peritoneal mast cells (RPMC) are depicted in Table 4. RD extracts and DSCG in the concentration range of 10–100 µg/mL exhibited a dose dependent increase in stabilization of mast cells along with a decrease in histamine release from RPMC. At 100 µg/mL,

DSCG, RD-EA and RD-ME exhibited 1.68, 16.71 and 8.37% histamine release, respectively. Least amount of % histamine release suggests better potential to stabilize mast cells. RD-ME exhibited superior potential by stabilizing C-48/80 activated RPMC along with decrease in % histamine release as compared to RD-EA.

Mast cells are the predominant inflammatory cell type localized in the airway smooth muscles in asthmatic patients. The interaction of mast cells constituents and airway smooth muscles suggest that mast cell contents are imperative contributors to airway hyper responsiveness in asthma (30). The effect of test compound/extract on mast cell mediators such as histamine can best be studied with the help of interaction between C-48/80 and rat peritoneal mast cell (RPMC). C-48/80 is a cationic amphiphile prepared from condensation of *N*-methyl-*p*-methoxyphenethylamine and formaldehyde, which is used as a direct and convenient reagent to study mast cell related effects (31).

According to the results obtained from *in vitro* studies, an attempt has been executed to investigate the *in vivo* effect of the extracts using acute toxicity, anti-inflammatory and bronchorelaxation activity.

Acute toxicity studies

Acute toxicity studies of RD-ME and RD-EA were performed according to OECD guidelines (No. 423), where RD-ME and RD-EA exhibited significant safety margin as indicated by a lack of systemic and behavioral toxicity up to 2000 mg/kg. No adverse effects were observed at 2000 mg/kg during first 30 min, 24 h and even up to 14 days after administration of RD extract. Therefore, randomly two doses of each extract were selected i.e., 100 and 200 mg/kg for the anti-inflammatory studies.

Table 5. Anti-inflammatory activity of RD-ME and RD-EA in carrageenan and egg- albumin induced inflammation model.

Treatment	Dose (mg/kg)	Edema inhibition against carrageenan-induced inflammation (h)			Edema inhibition against egg albumin-induced inflammation (min)					
Measurement time interval →		1	2	3	20	40	60	80	100	120
Control		6.23 ± 0.13	7.45 ± 0.14	6.50 ± 0.19	5.45 ± 0.12	5.96 ± 0.23	6.62 ± 0.17	7.67 ± 0.21	7.30 ± 0.33	7.14 ± 0.19
RD-ME	100	5.10 ± 0.21 [17.30]#	7.11 ± 0.15 [4.45]ns	6.33 ± 0.10 [3.35]ns	5.28 ± 0.23 [3.11]ns	5.76 ± 0.26 [3.45]ns	6.31 ± 0.32 [4.60] *	5.27 ± 0.34 [31.30] *	4.86 ± 0.23 [33.29]*	5.32 ± 0.22 [25.59]*
	200	4.26 ± 0.25 [31.69]*	4.83 ± 0.18 [35.11]*	3.79 ± 0.11 [41.62]*	4.16 ± 0.15 [21.14]*	4.69 ± 0.16 [21.27]*	5.41 ± 0.16 [18.27]*	5.43 ± 0.27 [29.18]*	4.80 ± 0.21 [34.14]*	3.69 ± 0.11 [48.31]*
RD-EA	100	6.21 ± 0.21 [3.28]ns	7.13 ± 0.25 [4.29]ns	6.08 ± 0.38 [6.38]ns	5.34 ± 0.19 [2.01]	5.67 ± 0.11 [4.86]	6.38 ± 0.13 [3.62]	7.29 ± 1.02 [4.95]	6.43 ± 1.18 [11.91]*	6.22 ± 0.41 [12.88]*
	200	4.75 ± 0.34 [23.75]*	5.28 ± 0.41 [29.12]*	4.52 ± 0.21 [30.36]*	3.85 ± 0.17 [29.35]*	4.73 ± 0.29 [20.63]*	5.12 ± 0.28 [22.61]*	6.13 ± 0.25 [20.00]*	5.38 ± 0.19 [26.30]*	4.73 ± 0.24 [33.75]*
IM	10	5.05 ± 0.17 [18.97]*	3.15 ± 0.15 [57.72]*	3.67 ± 1.02 [43.53]*	4.99 ± 0.13 [8.41]*	5.27 ± 0.12 [11.58]*	5.53 ± 0.23 [16.43]*	5.76 ± 0.16 [24.96]*	3.93 ± 0.18 [46.15]*	3.25 ± 0.14 [54.48]*

IM – Indomethacin; RD-ME – R. dumetorum methanolic extract; RD-EA – R. dumetorum ethyl acetate extract. Values represent the mean ± SEM. of five animals for each group; Values in square brackets indicate the percentage inhibition rate of inflammation. *p < 0.01 and #p < 0.001 indicate different levels of statistically significant values against control and ns stands for non significant. % Inhibition calculated using formula: paw edema volume control-sample/control.

According to Bousquet et al. (32), two important targets that should be controlled in the treatment and management of asthma are bronchoconstriction and exacerbated inflammatory conditions. Therefore, present study also endeavored to evaluate bronchodilatory and anti-inflammatory potential of RD-ME and RD-EA.

Anti-inflammatory activity against carrageenan-induced inflammation

RD-ME at the dose of 100 mg/kg inhibited only the initial phase of inflammation, while initial as well as lateral phases of inflammation were inhibited by RD-ME at the dose of 200 mg/kg with percent inhibition of inflammation 31.69, 35.11 and 41.62 %, respectively, at 1, 3 and 5 h. On the other hand, RD-EA was devoid of any effect on all the phases of inflammation at the lower dose (100 mg/kg), but effectively inhibited both the phases of inflammation at higher dose (200 mg/kg) with % inhibition of 23.75, 29.12 and 30.36%, respectively, at 1, 3 and 5 h. Standard drug, indomethacin attenuated the inflammation at 10 mg/kg with 18.97, 57.72 and 43.53% inhibition, respectively, at 1, 3 and 5 h (Table 5). These results were found to be dose dependent.

Anti-inflammatory activity against egg albumin-induced inflammation

To substantiate the anti-inflammatory activity of the RD-ME and RD-EA, the efficacy of these two extracts was also studied against egg albumin-induced inflammation. The RD-ME (100 mg/kg, *p.o.*) failed to exhibit anti-inflammatory effect (p > 0.05) at 20 and 40 min time interval (initial phase) but showed significant inhibition of inflammation at 60, 80, 100 and 120 min (lateral phase) after egg albumin administration as compared to control. On the other hand, the RD-ME (200 mg/kg, *p.o.*) significantly inhibited (p < 0.05) egg albumin-induced inflammation at all time interval (initial as well as lateral phase). The effect of RD-EA (100 mg/kg, *p.o.*) exhibited quite similar anti-inflammatory profile to that of RD-ME as it also failed to inhibit the inflammation at time interval 20, 40, 60 and 80 min, but significantly inhibited the inflammation in the last two time intervals i.e., 100 and 120 min, respectively. At 200 mg/kg RD-EA exhibited

Table 6. Evaluation of RD extracts and aminophylline for maximal bronchorelaxation in acetylcholine and histamine precontracted guinea pig tracheal rings.

Amount of drug added (mL)	Effect of RD extracts and amino-phylline in acetylcholine precontracted guinea pig tracheal chain			Effect of RD extracts and amino-phylline in histamine precontracted guinea pig tracheal chain		
	RD-ME	RD-EA	Aminophylline	RD-ME	RD-EA	Aminophylline
0.2	—	03.92 ± 1.64	09.17 ± 3.42*	11.1 ± 2.48*	–	13.82 ± 2.31**
0.4	05.20 ± 0.83*	14.71 ± 2.12**	32.94 ± 2.75**	23.7 ± 2.74**	6.39 ± 1.75*	34.76 ± 3.87**
0.8	36.45 ± 1.26**	45.29 ± 2.34**	68.25 ± 3.56**	49.45 ± 3.47**	26.48 ± 2.31**	82.43 ± 4.23**
1.2	64.58 ± 2.19**	57.39 ±2.45**	97.83 ± 3.09**	76.11 ± 3.98**	51.89 ± 2.86**	100 ± 3.85**
2.4	68.75 ± 3.52**	–	–	89.25 ± 3.61**	78.13 ± 2.08**	–
4.8	68.75 ± 2.43**	–	–	100 ± 4.48**	–	–

Values represent the mean ± SEM. of three replicates for each group. *p < 0.01 and ** p < 0.001 indicates different level of significance *vs.* histamine (55 mm was considered 100%) or Ach (45 mm was considered 100%) induced contractions. Significance was calculated using one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test.

significant inhibition of inflammation at all time intervals (initial as well as lateral phase). These results are shown in Table 5. The anti-inflammatory effect of RD-EA and RD-ME was comparable to the reference standard i.e., indomethacin, against both carrageenan and egg albumin inflammation.

Anti-inflammatory potential of RD-ME and RD-EA were evaluated in acute inflammatory models, which involve biphasic mechanism viz., synthesis and release of phlogistic mediators (33). RD-ME and RD-EA at two different doses (100 and 200 mg/kg) inhibited carrageenan as well as egg albumin induced rat paw inflammation in both time and in dose dependent manner. Thus, RD-ME as well as RD-EA at higher dose (200 mg/kg) exhibited significant anti-inflammatory profile in both the models of inflammation; the plausible mechanisms for the observed anti-inflammatory activity of RD-ME and RD-EA might be attributed to inhibition of synthesis and/or release of major inflammatory mediators like histamine, serotonin, bradykinins and prostaglandins.

Bronchorelaxent effect against acetylcholine and histamine

In the present investigation, two well known bronchoconstrictors, histamine and acetylcholine, at 1 µg/mL successfully contracted isolated guinea pig tracheal chain. RD-ME (1 mg/mL) when added cumulatively to tissue bath containing precontracted tracheal chain, at 0.8 mL exhibited maximum relaxation of 68.75% in acetylcholine-induced contraction and 100% relaxation was achieved against histamine-induced contraction at 3.2 mL. Whilst, cumulative addition of RD-EA (1 mg/mL) at 1.2 mL exhibited maximum relaxation of 57.39 and 78.23%

against acetylcholine and histamine induced contraction, respectively. However, further addition of RD-EA failed to relax precontracted tracheal chain. The bronchorelaxant effect of standard drug aminophylline (1 mg/mL), when added cumulatively at 1.2 mL, was 97.83% against acetylcholine and 100% against histamine (Table 6). In the pursuit to assess the bronchodilatory potential of RD-ME and RD-EA, guinea pig tracheal chain was contracted by histamine or acetylcholine (1 µg/mL). Both the mediators significantly induced contraction in guinea pig tracheal chain. RD-ME and RD-EA showed significant (p < 0.001) bronchodilatory activity in the precontracted tracheal chain preparation, as indicated by attenuation of the histamine as well as acetylcholine induced contractions in concentration dependent manner. Phytochemical evaluation of RD-ME and RD-EA exhibited the presence of saponins, tannins and flavonoids, which might participate in the observed bronchodilating effect of RD-ME and RD-EA (25, 26). Therefore, it can be contemplated that RD extracts may possess both anticholinergic and antihistaminergic activity, suggesting it as an effective bronchorelaxant. The premise of the present investigation projects that RD-ME and RD-EA may acts as a promising target for the control and management of the complications contributed by the oxidative stress, mast cell degradation, inflammation and bronchoconstriction, as evident in asthma.

CONCLUSION

According to these results, RD extracts have variety of potential in the following ways: (a) as an antioxidant by acting as DPPH free radical scav-

engers, reducing agent and as complexes of pro-oxidant metals, (b) mast cell stabilizing agent against C-48/80 induced destabilization, (c) as an anti-inflammatory agent by attenuating carrageenan- as well as egg albumin-induced inflammation and (d) bronchorelaxant which inhibits contraction in acetylcholine and histamine precontracted tracheal chain. These multifunctional properties of RD extracts might be helpful to explain the primary evidence that RD fruit extracts have anti-asthmatic effect, which bolsters its ethnopharmacological claim as useful medicine for asthma, bronchitis and inflammation, having safety margin for oral administration

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