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ORIGINAL ARTICLE

Pharmacological evaluation for anti-asthmatic and anti-inflammatory potential of *Woodfordia fruticosa* flower extractsMahavir Hiralal Ghante^{1,2}, Kishore P. Bhusari¹, Nandkishore J. Duragkar¹, and Nitin B. Ghiware²

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Abstract

Context: *Woodfordia fruticosa* Kurz. (Lythraceae) flowers are ethnopharmacologically acclaimed in the Indian medicinal system to treat asthma.

Objective: To evaluate *W. fruticosa* flower extracts for anti-asthmatic effect.

Materials and methods: Ethyl acetate, acetone, methanol, and hydro-alcohol extracts of *W. fruticosa* flowers were obtained successively and standardized. Ability of extracts to stabilize free radicals and compound-48/80-induced mast cell degranulation was evaluated. *In vitro* anti-inflammatory potential of extracts at 100 and 200 µg/ml by membrane stabilization and *in vivo* inhibition of rat paw edema up to 5 h (100 and 200 mg/ml; p.o.) was evaluated. *In vitro* bronchorelaxant effect was examined against histamine- and acetylcholine (1 µg/ml; independently)-induced guinea pig tracheal contraction. Extracts were evaluated for bronchoprotection (*in vivo*) ability against 0.1% histamine- and 2% acetylcholine-induced bronchospasm in guinea pigs at 100 and 200 mg/ml; p.o.

Results: Standardization studies revealed that the methanol extract exhibited highest polyphenolic (62.66 GAE), and flavonoid (6.32 RE) content and HPLC fingerprinting confirmed the presence of gallic acid (R_f 1.383). IC_{50} values for DPPH scavenging and metal chelation by the methanol extract were 40.42 and 31.50 µg/ml. Methanol and ethyl acetate extracts at 100 µg/ml exhibited 06.52 and 07.12% of histamine release. Methanol, ethyl acetate, and hydro alcohol extracts at 200 mg/kg demonstrated 32.73, 29.83, 26.75% and 32.46, 9.38, 26.75% inhibition of egg albumin and carrageenan-induced inflammation, respectively. Methanol extract exhibited 100% bronchorelaxation and 48.83% bronchoprotection.

Conclusion: *Woodfordia fruticosa* flower (WFF) extracts exhibited anti-asthmatic effect by demonstrating bronchoprotection, bronchorelaxation, anti-inflammatory, antioxidant, and mast cell stabilization ability.

Keywords

Asthma, antioxidant, bronchoprotection, bronchospasm, HPLC and spectroscopic standardization, mast cells, membrane stabilization

History

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Introduction

Woodfordia fruticosa Kurz. (Lythraceae) is an ethnopharmacologically popular plant in the Indian medicinal system for various disorders. Traditional reports indicate the use of dried *W. fruticosa* flowers to treat disorders of mucus membrane, and the paste of flowers is used for the treatment of asthma and cough in the central Indian province. Flowers are also claimed to suppress kapha and pitta, which are fundamental principles governing activities related to mind and body according to the Ayurvedic system of medicine. These traditional claims noticeably suggest anti-asthmatic potential of flowers. Wound healing, analgesic, antirheumatic, acrid, alexiritic, and anthelmintic properties of flowers were extensively used by tribal people (Khare, 2007).

Preclinical data from various studies indicate that dried *W. fruticosa* flower (WFF) extract possesses antipyretic, granuloma inhibition (Muzaffar et al., 1990), antitumor (Kuramochi et al., 1992), antiviral (Bhatt, 2008) immunomodulatory (Shah & Juvekar, 2010), antifertility (Khushalani et al., 2006), antibacterial (Parekh & Chanda, 2007), hepatoprotective (Baravalia & Chanda, 2011), and antihyperglycemic (Verma et al., 2012) activities. The traditional and preclinical claims of WF dried flowers can be ascribed to its important bioactive phytoconstituents, namely, different types of tannins [woodfordin A-C, and five oligomers woodfordin E-I] (Kuramochi et al., 1992; Yoshida et al., 1989a,b, 1990, 1991, 1992), sterols, anthroquinones, saponins, and flavonoids [quercetin-3 having galactopyranoside, glucopyranoside, arabinoside, and oxylpyranoside glycosides, in addition to myricetin with galactopyranoside and arabinopyranoside glycosides] (Chauhan et al., 1979a,b; Nair et al., 1976).

A literature survey reveals a lack of preclinical and clinical evaluation reports claiming the use of WFF extracts for the

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treatment of asthma. Therefore, the present investigation aimed to validate the traditional claim of WFF for the effectiveness in asthma.

As natural anti-inflammatory, leukotriene inhibitor, and antioxidant products have been proved effective in controlling bronchial asthma (Houssen et al., 2010), the present work was aimed to evaluate anti-asthmatic (both bronchodilatory and bronchoprotective) effect in histamine and acetylcholine-induced bronchoconstriction and bronchospasm in isolated guinea pig tracheal chain and guinea pigs, respectively. Extracts were also evaluated for C-48/80-induced mast cell destabilization in rats. Moreover, the anti-inflammatory potential of WFF extracts was examined in carrageenan- and egg albumin-induced inflammation in rats. *In vitro* anti-inflammatory potential using a membrane stabilization model was also performed with the extracts. Screening of acute toxicity, *in vitro* antioxidant analysis (DPPH scavenging, % relative rate of inhibition of DPPH, reducing power and metal chelation), was performed. All the extracts were standardized using spectroscopic and chromatographic techniques.

Materials and methods

Chemicals and reagents

Acetylcholine, ascorbic acid, carrageenan, compound 48/80, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), and histamine were purchased from Sigma Chemicals (St. Louis, MO). 3-(2-Pyridyl)-5, 6-bis (4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), *o*-phthalaldehyde, and metrizamide were purchased from Hi-Media, Mumbai, India. Silica gel TLC plates F 254 grade, 20 × 20 cm, 0.25 mm were from Merck (Whitehouse Station, NJ). Acetyl salicylic acid and indomethacin were generously gifted by Wockhardt Pharmaceuticals, Aurangabad, India. Disodium cromoglycate (DSCG) and aminophylline were obtained as gift samples from Cipla Limited, Mumbai, India. All other chemicals and reagents of analytical grade were procured from Loba Chemicals, Mumbai, India.

Instruments used

Biopac MP 45 data acquisition system, Biopac Systems Inc, (Goleta, CA); Plethysmometer-PLM01, Orchid, India; Spectrofluorometer-530, Shimadzu (Fujian, China); Agilent-1200 HPLC with UV-Vis detector; UV-Vis spectrophotometer-1601, Shimadzu.

Collection and authentication of *W. fruticosa*

Woodfordia fruticosa flowers were collected from the outskirts of Nagpur, Maharashtra, India, during the period of January–July 2008 and authenticated by Dr. P. G. Diwakar, Botanical Survey of India, Pune, Maharashtra, India; authentication number BSI/WC/Identi./Tech./2008/272.

Extraction of *W. fruticosa* flowers

Coarse powder (500 g) of WFF was exhaustively defatted using petroleum ether (60–80 °C) (WFF-PE) and extracted successively with ethyl acetate (WFF-EA), acetone (WFF-AC), and methanol (WFF-ME) using a Soxhlet apparatus (Borosil, Mumbai, India). The remaining mark was macerated to obtain hydro alcoholic [distilled water: absolute ethanol (1:1)] (WFF-HA) extract. All the extracts were collected, filtered through Whatman filter paper, concentrated, stored in an air-tight container, and finally in dessicator. The yield of WFF extracts is given in Table 1.

Animals

Albino rats of Wistar and Sprague Dawley strain (200–250 g), Swiss Albino mice, and Albino guinea pigs (300–400 g) were kept under standard 12:12 h light/dark cycle in a temperature-controlled (24 ± 1 °C) environment with *ad libitum* access to rodent chow (Lipton, India) and water. All employed experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) Constituted for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) by Ministry of Environment and Forests, Government of India, New Delhi (IAEC approval no. 536/02/C/CPCSEA).

Table 1. Percentage yield, phytochemical screening, and standardization of WFF extracts.

Phytochemical tests	Extracts obtained			
	WFF-EA	WFF-AC	WFF-ME	WFF-HA
Weight of extracts (g) and % yield	17.24 (3.27)	12.58 (2.34)	20.26 (3.88)	8.46 (1.66)
Flavonoids	+	–	+	–
Alkaloids	–	–	–	–
Saponins	–	–	+	+
Tannins	–	+	+	+
Cardiac glycosides	+	–	–	–
Steroids	–	–	+	–
Triterpenoids	+	+	+	–
Carbohydrates	+	+	+	+
Standardization for content of phytochemicals				
Total polyphenols	13.48 ± 1.02	47.66 ± 0.82	62.66 ± 1.89	56.00 ± 1.33
Total flavonoids	5.02 ± 0.29	ND	6.32 ± 0.76	ND
Total saponin	–	–	–	0.96 ± 0.17 ^a

For phytochemical screening: (+) presence of phytoconstituents and (–) absence of phytoconstituents; values of standardization for the content of phytochemical represent the mean ± SD of three independent replicates; ND, not determined due to absence of phytoconstituents in extracts.

^aDetermined for crude WFF powder.

Preliminary phytochemical screening

All the extracts were screened for the presence of various phytoconstituents, namely alkaloids, flavonoids, tannins, steroids, saponins, triterpenoids, proteins, and sugars (Evans, 1989).

Phytochemical standardization of extracts

Determination of total polyphenolic content

The Folin–Ciocalteu (F–C) method was used for the determination of total polyphenols in the test extract which showed positive test for polyphenols (Kim et al., 2003). Total polyphenolic content of the extract was expressed as gallic acid equivalent (GAE). GAE was calculated using the following equation obtained from standard gallic acid graph, absorbance (y) = 0.0039 [gallic acid (μg)] – 0.1077, $R^2 = 0.9764$ (Table 1).

Determination of total flavonoid content

The content of total flavonoids in extracts which showed positive tests for flavonoids was measured with the aluminum trichloride complex colorimetric assay (Zhishen et al., 1999). The total flavonoid concentration was expressed as rutin equivalent (RE), which was calculated by the equation obtained from calibration curve of rutin, absorbance (y) = 0.0012 [rutin (μg)] x + 0.0036, with $R^2 = 0.9764$ (Table 1).

Determination of total saponins

Saponin was isolated from crude WFF and total saponin content was determined in accordance to the methodology described previously (Ukpabi et al., 2003), based on the measurement of absorbance of complex formed between vanillin H_2SO_4 and saponin at 472 nm (Table 1). The saponin content was calculated and expressed as purified saponin equivalent (PSE) from the equation of calibration curve drawn using purified saponin. Absorbance (y) = 0.0027 [PSE (μg)] x + 0.0007, $R^2 = 0.9974$.

Qualitative high-performance liquid chromatography analysis of extract for the determination of gallic acid

Sample preparation. The WFF-ME was subjected to study high-performance liquid chromatography (HPLC) fingerprinting for the determination of gallic acid in the extract. WFF-ME and gallic acid were initially dissolved in methanol to prepare to stock solutions; prepared stock solutions were sonicated and filtered through membrane filter. Further dilutions of stock solutions were prepared in mobile phase to obtain a concentration of 10 $\mu\text{g}/\text{ml}$. Required amount of 5 μl of both WFF-ME and gallic acid were injected to obtain chromatogram (Althaf et al., 2012).

Instrumentation and chromatographic condition. The Agilent 1200 HPLC with UV–Vis detector (Morges, Switzerland), isocratic gradient pump was used having a column 4.6 $\times$ 250 mm, particle size packing of 5 μm , and stationary phase of C_{18} (Grace). The flow rate was kept 1.2 ml/min, with a detection wavelength of 268 nm. A combination of

acetonitrile and methanol (30:70) was used as a mobile phase. Chromatograms were compared for the first 5 min for the presence of peaks. Retention time along with UV spectra was considered as the basis for the peak assignment of gallic acid and WFF-ME. The chromatogram was compared for retention time and relative retention time. Before running the sample, the HPLC system was equilibrated for 15 min with a flow rate of 0.5 ml/min with the mobile phase. After running gallic acid, system was washed with acetonitrile.

Evaluation of antioxidant activity

Evaluation of scavenging activity on DPPH radicals

DPPH radical scavenging of WFF extracts and positive control ascorbic acid were evaluated by the method of Copland et al. (2003) and expressed in terms of IC_{50} (Table 2).

Determination of percentage residual rate of inhibition (% RRI) of DPPH

Percentage RRI was determined in accordance to the reported method, and absorbance of the mixture containing test extract, phosphate buffer (4 ml, 1 M, pH 5.5), and DPPH (4 ml, 1.25×10^{-4} M in methanol) was recorded at 520 nm after 0.5, 1, 3, and 7 h (Table 2) (Yamasaki et al., 1994). The percent residual rate of inhibition (% RRI) was calculated using the formula $\text{RRI} = (\text{Abs } t_0) - (\text{Abs } t_x) / (\text{Abs } t_0) \times 100$, where $\text{Abs } t_0$ is the absorbance at time interval 0 min and $\text{Abs } t_x$ is the absorbance at specific time interval.

Determination of reducing power

The method of Meir et al. (1995) was used to evaluate the reducing power of the test extract. Accordingly, changes in absorbance of the reaction mixture were recorded (Table 2).

Determination of ferrous metal ion chelating activity

The method is based on measurement of the inhibition of ferrozine– Fe^{2+} complex according to a previously reported method (Ilhami et al., 2003) and expressed in terms of IC_{50} (Table 2).

Evaluation of inhibition of histamine release from rat peritoneal mast cells (RPMCs)

Measurement of inhibition of histamine release from RPMC was performed according to the method of Kim, i.e., spectrofluorometric determination of histamine *o*-phthaldehyde complex at excitation and emission wavelengths of 350 and 450 nm, respectively, and expressed as percent histamine release (% HR) (Ghante et al., 2012; Kim et al., 2006) (Table 2).

Acute toxicity study

Extract doses of 2000 mg/kg of body weight (p.o.) (in 1.0%, w/v, carboxy methyl cellulose) were used for the evaluation of acute toxicity study (OECD guidelines no. 423). Mice (five groups – three animals/group, one male and two females/group) – were fasted overnight but water was provided *ad libitum* prior to test extract administration only. Individual mice were observed for a period of 14 d. Observations were at

Table 2. Effect of different WFF extracts on percentage DPPH inhibition, percentage metal chelation, percentage RRI of DPPH, reducing power, and percent histamine release.

Treatment	IC ₅₀ (µg/ml)		% RRI of DPPH					Reducing power (absorbances at 700 nm)					% Histamine release				
	DPPH scavenging		At time (h) for 100 µg/ml of treatment					Concentration of extracts (µg/ml)					Concentration of extracts (µg/ml)				
	Metal chelation		0.5	1	3	7	25	50	75	100	125	10	25	50	100		
Standard	31.69	43.85	98.73 ± 1.44	83.45 ± 1.73	71.19 ± 0.78	59.16 ± 1.29	0.294 ± 0.076	0.413 ± 0.098	0.682 ± 0.128	0.841 ± 0.109	1.095 ± 0.153	97.56 ± 2.24	75.64 ± 2.86*	34.22 ± 2.10*	01.68 ± 1.07**		
WFF-EA	44.44	38.12	78.24 ± 1.24	54.33 ± 0.86	38.66 ± 1.18	19.88 ± 1.45	0.291 ± 0.086	0.328 ± 0.092	0.419 ± 0.070	0.531 ± 1.10	0.765 ± 0.092	89.33 ± 1.09	46.67 ± 1.12*	13.57 ± 0.97*	07.12 ± 1.48**		
WFF-ME	40.42	31.50	72.18 ± 1.18	57.43 ± 1.37	45.31 ± 1.73	25.37 ± 1.98	0.183 ± 0.067	0.349 ± 0.081	0.632 ± 1.08	0.747 ± 0.122	0.991 ± 0.093	77.39 ± 0.98*	33.42 ± 0.96*	12.78 ± 1.24*	06.52 ± 1.72**		
WFF-AC	68.27	45.28	57.23 ± 2.03	25.41 ± 1.42	18.34 ± 1.12	06.73 ± 1.38	0.129 ± 0.034	0.273 ± 0.062	0.487 ± 0.09	0.591 ± 0.06	0.704 ± 0.10	96.85 ± 1.11	72.49 ± 0.93	45.39 ± 1.02	17.32 ± 1.93		
WFF-HA	56.77	41.15	56.27 ± 1.65	42.71 ± 1.09	33.56 ± 1.39	21.92 ± 2.71	0.234 ± 0.17	0.395 ± 0.97	0.526 ± 0.12	0.796 ± 0.18	0.913 ± 1.02	80.33 ± 0.89*	58.67 ± 1.72*	19.86 ± 1.07*	10.13 ± 2.07*		

All values represent the mean ± SD of three independent replicates. For percent histamine release determination, statistical significance was assessed using a one-way ANOVA with Bonferroni multiple comparison test. Ascorbic acid was used as a standard for percent DPPH inhibition, percentage RRI of DPPH, and reducing power; EDTA for percentage metal chelation and disodium cromoglycate (DSCG) for percent histamine release determination.

* $p < 0.05$, ** $p < 0.001$.

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, after extract administration. The systemic and behavioral toxicity patterns were studied as described in the OECD guideline.

Membrane stabilization study

Membrane stabilization potential of extracts was evaluated as an effect of the inhibition of hypotonic solution and heat-induced haemolysis, according to a previously reported method (Shinde et al., 1999).

Evaluation of anti-inflammatory effect

Carrageenan-induced rat paw edema

Assessment of anti-inflammatory activity in SD rats using carrageenan suspension (0.1 ml; 1% w/v)-induced paw edema was carried out as previously reported (Winter et al., 1962). Change in the paw volume was recorded using a digital plethysmometer – PLM01, at 0, 1, 3, and 5 h after the carrageenan injection and compared against the control group (Table 3) to calculate percent inhibition by the formula: [percent inhibition of inflammation = (paw edema volume control – sample/control) × 100].

Egg albumin-induced rat paw edema

Evaluation of anti-inflammatory activity in Albino rats of Wistar strain using 0.1 ml/kg of fresh egg albumin-induced paw edema was carried out as previously reported (Winter et al., 1963). Change in the paw volume was measured after egg albumin injection up to 120 min, at 20 min intervals, starting at 0 min. Percent inhibition of inflammation was calculated as described above. For both the studies, extracts were administered p.o. at two different doses of 100 and 200 mg/kg, while indomethacin (10 mg/kg; p.o.) was used as a standard drug (Table 3). Separate sets ($n = 6$) of rats were employed for control, standard, and treatment groups for both anti-inflammatory evaluation experiments.

Bronchodilation study

Guinea pigs of either sex were selected and tracheal chain was separated from adjacent tissue to obtain tracheal rings that were tied to get a chain of 3–4 individual tracheas. The chain was mounted in a 20 ml organ bath containing Krebs–Henseleit (K–H) solution [(mM): NaCl, 118.4; KCl, 4.7; KH₂PO₄, 1.2; NaHCO₃, 25.0; CaCl₂, 2.5, MgSO₄, 1.2; glucose, 11.1; pH 7.4 ± 0.05], the temperature of bath was maintained at 37 ± 1 °C. Prepared tracheal chain was suspended under isotonic tension of 0.5 g and allowed to equilibrate at least for 1 h before commencing the experiment. During the experiment, the K–H solution was replaced after every 10 min. After the equilibrium period, contraction was induced by adding acetylcholine or histamine separately. Thereafter, the test extracts (1 mg/ml) was added serially 0.1, 0.2 up to 0.6 ml in cumulative doses and observed for bronchodilation using a Biopac MP 45, data acquisition system, respiratory transducers, and expressed as percent bronchorelaxation (Table 4) (Xiangping et al., 2007).

Table 3. *In vivo* anti-inflammatory activity of different WFF extracts in egg albumin and carrageenan-induced inflammation model and *in vitro* heat and hypotonic solution induced hemolysis of erythrocyte membrane.

		% Inhibition of hemolysis		Measurement of paw volume and % inhibition of inflammation											
				Egg albumin-induced inflammation (min)								Carrageenan-induced inflammation (h)			
Treatment	Dose (mg/kg) & $\mu\text{g/ml}$	Heat-induced	Hypotonic solution-induced	0	20	40	60	80	100	120	0	01	03	05	
Control	–	–	–	4.48 $\pm$ 0.10	5.45 $\pm$ 0.12	5.96 $\pm$ 0.23	6.62 $\pm$ 0.17	7.67 $\pm$ 0.21	7.30 $\pm$ 0.33	7.14 $\pm$ 0.19	4.20 $\pm$ 0.14	6.23 $\pm$ 0.13	7.45 $\pm$ 0.14	6.50 $\pm$ 0.19	
	100	6.71 $\pm$ 1.52 ^{ns}	5.38 $\pm$ 1.25 ^{ns}	4.42 $\pm$ 0.21	5.40 $\pm$ 0.81 [0.91]	5.36 $\pm$ 0.77 [10.06]*	6.01 $\pm$ 0.61 [9.21]	5.98 $\pm$ 0.32 [22.03]*	5.67 $\pm$ 0.78 [22.32]*	6.37 $\pm$ 1.03 [10.78]*	4.43 $\pm$ 0.52	5.53 $\pm$ 0.87 [11.23]*	5.24 $\pm$ 0.65 [29.66]**	5.22 $\pm$ 0.32 [19.69]*	
	200	18.92 $\pm$ 1.33*	16.31 $\pm$ 1.8*	4.30 $\pm$ 0.15	4.98 $\pm$ 0.72 [8.62]	5.72 $\pm$ 0.93 [4.02]	5.94 $\pm$ 0.82 [10.27]*	6.13 $\pm$ 0.50 [20.07]*	5.56 $\pm$ 0.97 [23.83]*	5.01 $\pm$ 0.63 [29.83]**	4.36 $\pm$ 0.36	5.86 $\pm$ 0.48 [5.93]	5.22 $\pm$ 0.81 [29.93]**	5.89 $\pm$ 0.45 [9.38]	
WFF-ME	100	11.82 $\pm$ 1.77*	17.59 $\pm$ 1.07*	4.82 $\pm$ 0.13	5.33 $\pm$ 0.75 [2.20]	5.83 $\pm$ 0.56 [2.18]	5.14 $\pm$ 0.39 [22.35]*	6.27 $\pm$ 0.94 [18.25]*	5.86 $\pm$ 0.32 [19.72]*	6.43 $\pm$ 0.51 [10.08]*	4.68 $\pm$ 0.72	6.07 $\pm$ 0.24 [2.56]	5.88 $\pm$ 0.52 [21.07]*	5.16 $\pm$ 0.46 [20.61]*	
	200	49.16 $\pm$ 2.48**	45.22 $\pm$ 1.46**	4.21 $\pm$ 0.17	4.84 $\pm$ 0.69 [11.19]*	5.51 $\pm$ 0.91 [7.55]	5.41 $\pm$ 0.43 [18.27]*	5.43 $\pm$ 0.38 [29.20]**	5.32 $\pm$ 0.61 [27.04]**	4.80 $\pm$ 0.75 [32.73]**	4.18 $\pm$ 0.26	5.06 $\pm$ 0.36 [18.78]*	4.58 $\pm$ 0.23 [38.52]**	4.39 $\pm$ 0.46 [32.46]**	
	100	8.73 $\pm$ 1.32 ^{ns}	5.27 $\pm$ 2.15 ^{ns}	5.10 $\pm$ 0.12	5.30 $\pm$ 0.45 [2.75]	5.61 $\pm$ 0.66 [5.87]	5.57 $\pm$ 0.75 [15.86]*	6.78 $\pm$ 0.71 [11.60]	6.81 $\pm$ 1.12 [6.71]	6.92 $\pm$ 0.99 [3.08]	4.74 $\pm$ 0.64	6.17 $\pm$ 0.42 [0.96]	6.81 $\pm$ 0.86 [8.59]	5.66 $\pm$ 0.63 [12.92]*	
WFF-HA	200	23.89 $\pm$ 1.96**	19.26 $\pm$ 1.83*	4.60 $\pm$ 0.18	5.13 $\pm$ 0.88 [5.87]	5.45 $\pm$ 0.67 [8.55]	5.62 $\pm$ 0.86 [15.10]*	5.76 $\pm$ 0.79 [26.07]**	5.94 $\pm$ 1.09 [18.63]*	5.23 $\pm$ 0.71 [26.75]**	4.38 $\pm$ 0.41	5.64 $\pm$ 0.23 [9.47]	5.85 $\pm$ 0.27 [21.47]*	5.20 $\pm$ 0.58 [20]*	
	10/50	19.46 $\pm$ 1.45*	18.13 $\pm$ 2.54*	4.12 $\pm$ 0.17	4.99 $\pm$ 0.13 [8.41]**	5.27 $\pm$ 0.12 [11.58]**	5.53 $\pm$ 0.23 [16.43]**	5.76 $\pm$ 0.16 [24.96]**	3.93 $\pm$ 0.18 [46.15]**	3.25 $\pm$ 0.14 [54.48]**	3.10 $\pm$ 0.12	5.05 $\pm$ 0.17 [18.97]*	3.15 $\pm$ 0.15 [57.72]**	3.67 $\pm$ 1.02 [43.53]**	

For inhibition of hemolysis, values represent the mean $\pm$ SD of three replicates; standard used for percent inhibition of hemolysis was acetyl salicylic acid (50 $\mu\text{g/kg}$); treatment dose concentration used was $\mu\text{g/ml}$. For percent inhibition of inflammation, values represent the mean $\pm$ SD of six animals for each group; values in parenthesis indicate the percentage inhibition rate of inflammation, standard used was indomethacin (10 mg/kg) for egg albumin, and carrageenan-induced inflammation model, treatment dose concentration used was mg/kg ; ns, non-significant.

* $p < 0.01$ and ** $p < 0.001$ indicate different levels of statistically significant values against control.

Table 4. Evaluation of different WFF extracts and aminophylline for maximal bronchorelaxation in acetylcholine and histamine precontracted guinea pig tracheal chain and histamine aerosol-induced bronchospasm in guinea pigs.

Treatment groups	Bronchorelaxation in acetylcholine and histamine precontracted guinea pig tracheal chain										Bronchoprotective effect on acetylcholine and histamine aerosol-induced bronchospasm in guinea pigs				
	Acetylcholine-induced contraction versus treatment in ml										Treatment groups	Dose (mg/kg)	Tumble no.	Latency (s)	
	0.1	0.2	0.3	0.4	0.5	0.6	0.1	0.2	0.3	0.4				Before treatment	After treatment
Standard	18.17 ± 3.42	41.98 ± 2.75	68.25 ± 3.56	97.83 ± 3.09	97.83 ± 3.09	97.83 ± 3.09	18.82 ± 2.31	44.76 ± 3.78	78.25 ± 3.56	100 ± 3.85	Control	10	08	88 ± 13	162 ± 27**
WFF-EA	11.32 ± 3.12	26.55 ± 4.06	44.87 ± 2.33	63.52 ± 2.65	80.95 ± 2.83	96.27 ± 2.88	18.7 ± 2.74	33.48 ± 3.57	58.93 ± 2.77	70.56 ± 3.02	WFF-EA	100	12	87 ± 14	113 ± 19
WFF-ME	4.76 ± 1.34	19.04 ± 2.16	42.81 ± 3.02	66.66 ± 3.61	82.03 ± 3.34	100 ± 3.13	11.36 ± 3.12	36.73 ± 2.95	53.81 ± 2.83	81.68 ± 2.14	WFF-ME	200	08	81 ± 17	144 ± 16
WFF-HA	9.38 ± 1.21	19.3 ± 1.48	44.89 ± 2.46	75.51 ± 4.13	91.81 ± 4.72	91.81 ± 3.96	7.35 ± 2.06	22.75 ± 1.31	42.05 ± 3.27	69.35 ± 2.43	WFF-HA	200	05	85 ± 16	172 ± 22**
												100	08	86 ± 21	119 ± 12
												200	06	92 ± 20	167 ± 21

For bronchorelaxation study, values represent the mean ± SD of four replicates for each group. * $p < 0.01$ versus acetylcholine- (at 0.4 ml of 1 µg/ml, 32 mm was considered 100%) or versus histamine (at 0.4 ml of 1 µg/ml, 29 mm was considered 100%) induced contractions (a one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test. For bronchoprotective test, values represent the mean ± SD of six independent replicates.

* $p < 0.05$ and ** $p < 0.001$ indicate the different levels of significance when compared against control group; standard – aminophylline.

Bronchoprotective test

Guinea pigs were fasted overnight and only water was provided *ad libitum* before commencement of the experiment. Sensitivity as well as suitability of animals for the study were screened by challenging animals with an equal volume mixture of 0.1% histamine hydrochloride and 2% acetylcholine chloride, under the pressure of 450 ± 50 mmHg for 15 s in a histamine chamber. Preconvulsive time in seconds, that is time for onset of respiratory distress during the aerosol challenge, was measured. Guinea pigs with preconvulsive time of more than 120 s were considered insensitive, unsuitable, and removed from the study groups. Adequate and sensitive guinea pigs were randomly allotted to different groups (control, treatment, and standard) with six per each group. The negative control of animals administered 0.1% carboxymethylcellulose (CMC), 5 ml/kg, the positive control animals administered aminophylline (10 mg/kg) suspended in 0.1 CMC and test extract groups were administered with 100 and 200 mg/kg, suspended in 0.1% CMC. All animals were treated with a single dose of extracts and aminophylline, daily for 3 d prior to bronchial challenge while the last dose of extract or standard drug was administered 1 h before the bronchial challenge. The delitescence of convulsion and tumble numbers for each animal were recorded within a 6 min interval of exposure. Bronchial challenge by aerosol provoked a bronchospastic reaction in all sensitive animals within 3 min. The delay in the appearance of the bronchospastic reaction was considered as bronchoprotective effect and expressed as percent protection relative to the control group (Xiangping et al., 2007); percent protection = $[1 - (T_1/T_2)] \times 100$, where T_1 is the preconvulsive breathing time (s) in the control group; T_2 is the preconvulsive breathing time (s) in the treatment or standard group, results are depicted in Table 4.

Statistical analysis

All experimental results were expressed as the mean ± SD. Results of anti-inflammatory activity were analyzed by a two-way ANOVA followed by the *post hoc* Bonferroni tests (factor I: treatment; factor II: time). Data of bronchorelaxation study were analyzed by a one-way ANOVA followed by *post hoc* Dunnett's multiple comparison tests. A value of $p < 0.05$ was considered to be statistically significant in all the cases.

Results

Preliminary phytochemical evaluation of WFF extracts showed the presence of flavonoids, tannins, cardiac glycosides, steroids, triterpenoids, and absence of alkaloids. Spectrophotometric standardization of WFF extracts reflects the presence of highest polyphenolic contents in WFF-ME 62.66, followed by 56, 47.66, and 13.48 GAE in WFF-HA, WFF-AC and WFF-EA, respectively. Total flavonoid contents were found to be 6.32 and 5.02 RE in WFF-ME and WFF-EA, respectively, while the total saponin content found in WFF was 1.96 PSE (Table 1).

The IC₅₀ values observed for DPPH by ascorbic acid, WFF-EA, WFF-ME, WFF-AC, and WFF-HA were found to be 31.69, 35.44, 32.07, 42.39, and 38.71 µg/ml,

respectively. WFF-ME exhibited better antioxidant activity to scavenge DPPH radical than the other WFF extracts as reflected by lower IC₅₀ value (Table 2). WFF-ME showed highest percentage RRI of 25.37, followed by WFF-HA (21.92), WFF-EA (19.88), and WFF-AC (06.73) at the seventh hour (Table 2). The metal-chelating capacity was also expressed as IC₅₀ and found to be 43.85, 38.12, 31.50, 45.28, and 41.15 µg/ml for EDTA, WFF-EA, WFF-ME, WFF-AC and WFF-HA, respectively (Table 2). The reducing ability observed was in the order of ascorbic acid > WFF-ME > WFF-HA > WFF-EA > WFF-AC with absorbances of 1.095, 0.991, 0.913, 0.765 and 0.704, respectively, at the concentration of 125 µg/ml (Table 2).

WFF extracts and DSCG in the concentration range of 10–100 µg/ml exhibited dose-dependent stabilization of mast cells as measured by change in histamine release from RPMC. At 100 µg/ml DSCG, WFF-EA, WFF-ME, WFF-HA, and WFF-AC exhibited 1.68, 7.12, 6.12, 10.13 and 17.32% histamine release (Table 2). The least amount of percent histamine release suggests better potential to stabilize mast cells. WFF-AC extract exhibited comparatively poor antioxidant and mast cell stabilization potential, thus the extract was not screened for the further studies.

Acute toxicity studies of WFF-EA, WFF-ME and WFF-HA were performed according to the OECD guideline (no. 423), where all the tested extracts exhibited a safety margin as indicated by lack of systemic and behavioral toxicity up to 2000 mg/kg. No adverse effects were observed at 2000 mg/kg during the initial 30 min, 24 h, and even at 14th day of the extract administration. Accordingly, one-tenth of this dose was considered as an experimental safe dose and hence, doses of each extract were selected, i.e., 100 and 200 mg/kg for the *in vivo* anti-inflammatory and bronchoprotective studies.

When evaluated for cell membrane stabilization ability, WFF-EA inhibited 18.92 and 16.31; WFF-ME inhibited 49.16 and 45.22, WFF-HA 23.89 and 19.26 at 200 µg/ml, while acetyl salicylic acid inhibited 19.46 and 18.13% of heat-induced and hypotonic solution-induced haemolysis, respectively (Table 3).

In the carrageenan-induced inflammation study, WFF-ME at 100 mg/kg inhibited only the initial phase of inflammation, whereas the initial and late phases of inflammation were significantly inhibited by WFF-ME at 200 mg/kg with an inhibition of inflammation of 18.78, 38.52 and 32.46%, respectively, at 1, 3, and 5 h. WFF-EA was found effective at 100 mg/kg by inhibiting inflammation by 11.23, 29.66, and 19.69%, at 200 mg/kg, the inhibition observed was 05.93, 29.93 and 09.38%, respectively, at 1, 3, and 5 h. WFF-HA at 100 mg/kg attenuated the lateral phase of inflammation with 12.92% inhibition of inflammation at 3 h and at 200 mg/kg exhibited 21.47 and 20% inflammation inhibition at 2 and 3 h, respectively (Table 3).

In the egg albumin-induced inflammation study, WFF-ME (100 mg/kg, p.o.) failed to exhibit anti-inflammatory effect ($p > 0.05$) at the 20 and 40 min time interval (initial phase) but showed significant inhibition of inflammation at 60, 80, 100, and 120 min (late phase) after egg albumin administration as compared to control. In contrast, the WFF-ME (200 mg/kg, p.o.) significantly inhibited ($p < 0.05$) egg albumin-induced inflammation at all time intervals (initial as well as late

phases). The effect of WFF-EA (100 mg/kg, p.o.) was found quite similar to that of WFF-ME, as it also fails to inhibit the inflammatory effects at time interval 20, 40, 60, and 80, but significantly inhibited the inflammation at the 100 and 120 min interval (with % inhibition of 19.72 and 10.08). At 200 mg/kg, WFF-EA exhibited significant inhibition of inflammation of 32.73% at 120 min. WFF-HA also produced dose-related inhibition of paw edema, at 200 mg/kg with 26.75% inhibition of inflammation at 120 min. WFF-ME showed a superior anti-inflammatory effect as compared to other WFF extracts (Table 3).

WFF extracts, when added cumulatively to organ bath containing either acetylcholine or histamine precontracted tracheal chain, exhibited varying degrees of bronchorelaxation. WFF-EA, WFF-ME, and WFF-HA exhibited maximum relaxation of 96.27, 100, and 91.81% against acetylcholine, while 90.23, 100, and 93.58% against histamine-induced tracheal chain contraction (Table 4).

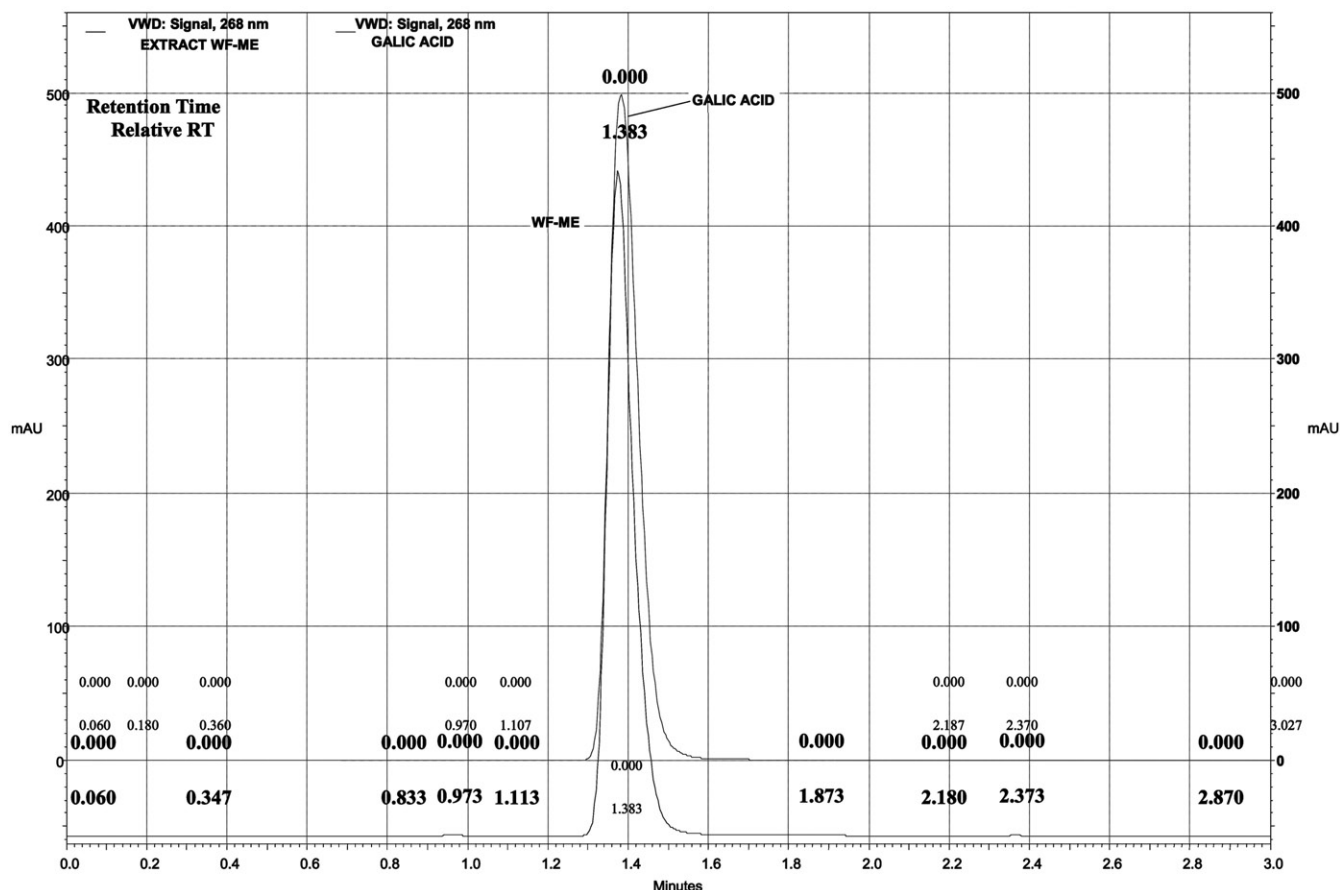
At 100 and 200 mg/kg, WFF-ME showed bronchoprotection against aerosol-induced bronchospasm which was 29.03 and 48.83%, respectively. WFF-EA and WFF-HA exhibited 43.75 and 44.91% bronchoprotection, respectively. The result of percentage bronchoprotection exhibited by WFF-ME at 200 mg/kg was comparable to the standard drug aminophylline 45.44 (Table 4). This suggests the following order of potency in terms of bronchoprotective effects as WFF-ME > aminophylline > WFF-EA > WFF-HA. HPLC chromatogram of the most active extract WFF-ME shows the presence of gallic acid with R_f 1.383 along with 0.00 relative retention time (Figure 1).

Discussion

The present evaluation was attempted to evaluate the anti-asthmatic effect of successively obtained WFF extracts. Therefore, evaluation was undertaken for antioxidant, mast cell stabilization, inhibition of experimentally induced paw edema, and membrane stabilization activities. In addition, extracts were evaluated for inhibition of acetylcholine- and histamine-induced guinea pig tracheal chain contraction. Finally, the ability of the extracts to inhibit acetylcholine and histamine aerosol-induced bronchospasm in guinea pigs has been evaluated.

Oxidative stress can trigger chronic inflammatory disorders including pulmonary and rheumatic diseases which can be counteracted by various antioxidant effects (Halliwell, 1994). However, to understand the means of antioxidant potential, a single evaluation method cannot be helpful; therefore, effects of WFF extracts have been evaluated using DPPH scavenging, percent residual rate of inhibition (% RRI) of DPPH, reducing power, and metal-chelating effect. The metal-chelating capacity of WFF-ME was found to be better as compared to other extracts of WFF. Reducing ability of extracts was measured as a change in the absorbance of reaction mixture and an increase in reducing power was indicated by an increase in absorbance (Meir et al., 1995).

C-48/80 is a cationic amphiphile used to study effects on mast cell stabilization, due to its ability to induce degranulation of mast cells (Wu et al., 1993). One of the predominant cell types localized in the airways is mast cells and its



released constituents (histamine, cytokines, etc.) may precipitate airway hyper-responsiveness in asthma. Therefore, stabilization of the mast cell is vital in limiting the episodes of asthma and substances that exhibit mast cell stabilizing ability that may be useful to circumvent resulting pathological conditions related to asthma (Brightling et al., 2002). WFF extracts exhibited this potential to decrease % histamine release by stabilizing C-48/80 activated RPMC, which might be due to the presence of flavonoids, polyphenols, and triterpenoids as these phytoconstituents have been reported to exhibit mast cell stabilization ability (Kim et al., 2006). As WFF-AC extract exhibited comparatively poor antioxidant and mast cell stabilization potential, the extract was not screened for further pharmacological evaluations.

In asthma, bronchoconstriction and inflammation induce airway remodeling (Grainage et al., 2011). Therefore, WFF extracts were further evaluated for anti-inflammatory, bronchorelaxation, and bronchoprotective potential. The erythrocyte membrane is analogous to the lysosomal membrane and its stabilization is considered vital for limiting the inflammatory responses (Chou, 1997). In the membrane stabilization study, the most active extract found was WFF-ME as compared to other extracts as well as standard acetyl salicylic acid.

The carrageenan-induced rat paw edema is tagged as a biphasic phenomenon, where the first hour after carrageenan injection is attributed to the release of histamine and 5-HT (initial phase), while 3–5 h after carrageenan is contributed by

induction of prostaglandins, bradykinins, protease, and lysosomes, which provokes edema formation, i.e., late phase (Crunkhon & Meacock, 1971). The phenomenon of inflammation induced by egg albumin is considered similar to the carrageenan, the only difference and advantage of the egg albumin model is the ability to facilitate the measurement of inflammation and inhibition of same between 20 and 120 min at 20 min intervals (Winter et al., 1963). The plausible mechanisms for the observed anti-inflammatory activity of WFF extracts might be attributed to inhibition of synthesis or release of major inflammatory mediators as well as stabilization of cell membranes. Observed effects can be correlated with the presence of important bioactive phytoconstituents of these extracts, namely flavonoids, polyphenols, and triterpenoids which have been reported to possess anti-inflammatory activity (Minky & Ankush, 2013).

Histamine is an important mediator of bronchial muscle contraction and the obstruction of these may occur via H_1 receptors. In addition, acetylcholine released from efferent nerve endings of the inner bronchus results in the excessive formation of inositol 1,4,5-triphosphate (IP_3) in bronchial muscles that lead to the intracellular release of calcium and initiate bronchoconstriction. It has been reported that bronchial acetylcholine and H_1 receptor blockade results in bronchodilation, which is considered as vital in the treatment of asthma (Matsumoto et al., 1994). A prominent effect caused by both leads to varied degree of bronchoconstriction

that causes asphyxia and death. Bronchodilators can delay the occurrence of these symptoms (Kumar et al., 2010).

Results of the inhibition of Ach and histamine-induced tracheal contraction as well as bronchospasm demonstrate the bronchorelaxant and bronchoprotective effects of WFF extracts. These data also suggest concentration-dependent relaxant effect of WFF extracts on acetylcholine and histamine-contracted guinea pig tracheal muscles possibly via its cholinergic as well as histamine receptor antagonistic activity. The observed anti-asthmatic activity can be correlated with the presence of polyphenols and saponins in WFF extracts (Minky & Ankush, 2013).

WFF-ME, the most bioactive extract, when standardized by means of HPLC fingerprinting for the content of gallic acid, HPLC chromatogram clearly demonstrates the presence of gallic acid with a single peak at retention time of 1.383 along with 0.00 relative retention time (Figure 1).

To conclude, above results suggest the possible role of *W. fruticosa* flower extracts for the treatment of asthma. Standardized WFF extracts not only protected guinea pigs from histamine and acetylcholine-induced bronchospasm but also inhibit bronchoconstriction induced by histamine or acetylcholine. WFF extracts also exhibited anti-inflammatory, mast cell stabilization, and antioxidant potential that strongly corroborate its traditional claim for the treatment of asthma.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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