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Original Article

Bioactivity-guided Isolation of Phytoconstituents from *Saraca asoca* Seeds for Anti-asthmatic Potential

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ABSTRACT

Objectives: It is interesting to consider biologically active substances derived from natural sources as potential novel medications for infectious disorders. *Saraca asoca* is an indigenous medicinal plant with lots of traditional importance belonging to the family Caesalpinaceae. As these plants have been ethno-pharmacologically claimed for the treatment of respiratory system-related disorders such as asthma, and cough, in addition, to treating inflammatory conditions. The anti-asthmatic activity is not supported by scientific research on *Saraca asoca* and no data is available for the anti-asthmatic chemical constituent of *Saraca asoca* seeds. In the present study, the scientific evidence-based anti-asthmatic compound was isolated from methanol fractions of crude extract of *Saraca asoca* seeds. The current evaluation attempted to assess the antiasthmatic result of repeatedly achieving *Saraca asoca* seeds extracts using milk-induced leucocytosis as well as eosinophilia in mice Furthermore, extracts were tested for their ability to inhibit guinea pig tracheal chain contraction induced by acetylcholine and histamine. Finally, the extracts were tested for their ability to inhibit acetylcholine and histamine aerosol-induced bronchospasm in guinea pigs. **Results:** In this study, methanolic extracts of *Saraca asoca* (SA) were finally evaluated for antiasthmatic potential, results from this study suggested that methanolic extracts of SA were most active and further subjected to column chromatography to isolate phytoconstituents. The methanol fraction was determined to be mainly composed of 2-(3,4-dihydroxy phenyl)-3,5,7-dihydroxy-4H-1-benzopyran-4-one. All the isolated compounds were subjected to phytochemical analysis and TLC fingerprinting in addition to spectral study viz UV Shift for functional groups position, FTIR, ^{1}H NMR, ^{13}C NMR, and MS. Isolated phytoconstituents were found to exhibit % DPPH scavenging, mast cell stabilization and bronchorelaxent, milk-induced leucocytosis and eosinophilia in mice activity in a concentration-dependent manner. However, further detailed evaluation of the mechanism of action of extracts or phytoconstituents of highly needed. **Conclusion:** These results show the methanol extract of *Saraca asoca* seeds shows a potential natural ingredient for the treatment of anti-asthmatic activity, which is due to bioactive compounds such as Quercetin.

Keywords: Anti-asthmatic, Quercetin, Milk-induced leukocytosis, Eosinophilia.

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INTRODUCTION

An allergic reaction, exposure to cold air, physical activity, or mental stress are just a few examples of "triggers" that can cause airways to narrow and constrict in people with asthma. Approximately 300 million people worldwide, or 7% of the population, suffer from asthma. The smooth muscle cells in the bronchi contract during attacks (exacerbations), causing swelling and inflammation of the airways and making breathing difficult. 4,000 people die from asthma each year in the US alone. Avoiding triggers and receiving medication treatment helps stop attacks.[1] Around 200 million people globally have asthma, and each year, 0.2 million people die from it. According to studies, the prevalence of asthma has risen by approximately 7% over the past three decades in most nations, including India. More than 15 million people in India are thought to suffer from asthma.[2]

Current synthetic drugs belonging to different classes such as α2 agonists (Ephedrine), corticosteroids (Hydrocortisone, Prednisolone), mast cell stabilizers (Sodium chromoglycate, Ketotifen), methylxanthines (Theophylline, Aminophylline), leukotriene antagonists (Montelukast, Zafirlukast) are widely used in the treatment of asthma therapy. Current pharmacotherapy includes bronchodilators, anti-inflammatory agents, mast cell stabilizers, Leukotriene (LT) modifiers, and IgE antibodies, among other things. Current therapies have limitations in that they may not produce a complete cure and may not prevent all bronchial asthma complications. Even though these synthetic drugs are used, they are not completely safe, particularly for long-term use,

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and are associated with several serious side effects such as renal failure, liver failure, skeletal muscle tremor, hypokalemia, intense irritability, compromised immune system, sustained high blood pressure, and so on. This has directed researchers' attention to the potential of medicinal plants and herbal formulations claimed in traditional systems of medicine such as Ayurveda, claiming that these therapies can be successfully integrated with conventional therapy to provide patients with the greatest possible benefit.[3]

Saraca asoca (Caesalpiniaceae) is a traditional medicinal plant growing wild from Assam, Odisha, Tamil Nadu, Karnataka, Kerala, and Meghalaya. Due to the presence of secondary metabolites such alkaloids, terpenoids, flavonoids, steroids, glycosides, anthraquinones, phenolics, tannins, saponins, and other phytochemicals, almost all components, including bark, flowers, and seeds, are regarded as therapeutically beneficial.[4]

Reduced respiratory function and increased eosinophil infiltration into the airways characterize asthma, an inflammatory disease of the lungs. Increased Airway Hyperresponsiveness (AHR), bronchoconstriction, and mucus production are all results of inflammation.[5]

The seeds' extracts have been employed as an anti-pyretic action after undergoing pharmacognostic examination, physio-chemical analysis, and toxicity evaluation.[6]

In the present study, the bioactivity-guided chemical constituent has been isolated from Seeds of *Saraca asoca*, the anti-asthmatic activity studied by milk-induced leukocytosis and eosinophilia in mice, and also the evaluation of antioxidant potential has been carried out.

MATERIALS AND METHODS

Chemicals

All the chemicals used were of analytical grade, n-hexane, ethyl acetate, dichloromethane, and methanol (Merck Life. Sci. Pvt. Ltd., India).

Plant material

Seeds of *Saraca asoca* along with other identifiable parts such as the fruit (pod), and leaves were collected in August 2019, from Nanded Localities, (Maharashtra, India), and presented to a taxonomist. Taxonomical details of the plant materials were authenticated from the Botanical Survey of India, Pune, India

(Reference No. BSI/WRC/Iden.Cer./2021/1405210002949 Date 17/05/2021). Plant materials were also identified and authenticated by Dr. S. S. Bodke HOD, Department of Botany, Yeshwant Mahavidyalaya, Nanded. A voucher specimen (CPR1PL and CPR2CG) was deposited in the herbarium for further use.

Extraction

SA seeds were shade dried, cleaned, and pulverized using a milling machine to obtain coarse powder which was separated on sieves. The coarse powder (500 g) of SA was extracted successively with different solvents at 40°C, namely Hexane (HE-SA), Ethyl acetate (EA-SA), Dichloromethane (DCM-SA), Methanol (ME-SA), and Water (WE-SA) using soxhlet apparatus. All the extracts were collected, filtered through Whatman filter paper (No.44), concentrated in a rotary evaporator under reduced pressure and at a low temperature to prevent the degradation of plant materials, and stored in a tight desiccator until further use.

Animals

Albino mice (25-30 g) Wistar rats (200-250 g) and Guinea pigs (300-350 g) of either sex was housed under standard husbandry conditions of 12;12 hr light/dark cycle in a temperature controlled (24±1°C). The animals had free access to food and water. All experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) constituted for the control and supervision of experiments on animals, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) by Ministry of Environment and Forests, Government of India. Institute approved all the protocols of the study (Proposal no. SNIOP/CPCSEA/IAEC/CP-PL/15-2021).

Thin Layer Chromatography (TLC) analysis of extracts

Chromatographic fingerprinting was carried out using silica gel G as the stationary phase and different solvent combinations as mobile phase to identify and confirm the presence of major phytoconstituents (polyphenols, triterpenoids and steroid etc.,). Solutions of test extract 1 mg/mL in the required amount (4-8 µL) were applied on the TLC plates. Developed TLC plates were observed with various derivatizing reagents (FeCl3, iodine, 10% H_2SO_4 and vanillin- H_2SO_4 for analysis of various compounds and R_f values of observed spots were recorded.[7]

TLC analysis was done with each of the solvent extracts to identify the best solvent system for the further separation process.

Fingerprinting profiles of SA and CP extracts confirmed the presence of flavonoids, polyphenols as well as triterpenoids, as observed by blue and greenish blue color when sprayed with 5% $FeCl_3$, pink color with 10% H_2SO_4 and violet color when sprayed with vanillin H_2SO_4 respectively.

Inhibition of Histamine Release from Rat Peritoneal Mast Cells

Preparation of Rat Peritoneal Mast Cell (RPMCs)

Rats were exsanguinated and injected with Hank's Balanced Salt Solution (HBSS) in the abdominal cavity. After gently massaging the abdomen for 120 sec, the cavity was opened to

aspirate the fluid containing the peritoneal cell. Peritoneal fluid was centrifuged at 2000 rpm to extract cell components, which resulted in a buffy mass that was resuspended in HBSS. The resulting cell suspension was used to separate mast cells from other components (such as macrophages and small lymphocytes) by suspending peritoneal cells in 1 mL of HBSS, which was then 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 at the buffer-metrizamide interface were aspirated and discarded, while the cells in the pellet were washed and resuspended in 10 mL of HBSS. The obtained suspension was considered as mast cell suspension.[8]

Measurement of histamine release from RPMC

To test extract in different concentrations or Disodium Cromoglycate (DSCG) (1 mL; 10-100 µg/mL) in separate test tubes, was mixed with mast cell suspension and incubated for 15 min at 37°C. Each mixture was made up to 3 mL with HBSS, to which an equal volume (3 mL) of compound 48/80 [C-48/80] (10-6 g/mL) was added and incubated at 37°C for 30 min before centrifugation at 2500 rpm for 5 min. The top layer of the resulting solution from each test tube was transferred to the tube containing 300 mg NaCl and 1.25 mL n-butanol. This solution was alkalized by adding 3 N NaOH (1 mL) to extract histamine into n-butanol. After shaking, the sample was centrifuged for 5 min at 2000 rpm and 1 mL of the top layer (n-butanol) was separated and mixed well with 2 mL of n-heptane and 0.4 mL of 0.12 N HCl. The test tube content was mixed and allowed to stand for obtaining organic and aqueous phase, 0.5 mL of the aqueous phase was then transferred to another test tube. To each tube containing the aqueous phase, 1 N NaOH (100 µL) and 0.2% O-Phthaladehyde (OPT) (100 µL) were added immediately under constant stirring. To this mixture, 3 N HCl (100 µL) was added after 2 min and finally, histamine concentration was determined from resultant reaction mixtures by using a spectrofluorometer with excitation and emission wavelengths of 350 and 450 nm respectively.[9] Results are given in Table 1.

The different test and control solutions required for analysis were prepared in the following manner.

1. Spontaneous histamine release-containing mast cells and solutions used to determine baseline;

2. Histamine release: solution contains mast cells and C-48/80 (10^{-6} g/mL);

3. Test compound control: contains solutions and test compound and percent Histamine Release Inhibition (% HRI) from mast cells was determined by the following formula;

% HRI = (Sample HR-spontaneous HR) / (100% HR-spontaneous HR) X 100.

Where, % HRI - Histamine release inhibition HR - Histamine release.

The inhibitory effect of SA extracts and standard drug Disodium Cromoglycate (DSCG) on C-48/80-induced histamine release from Rat Peritoneal Mast Cells (RPMC).

SA extracts and DSCG in the concentration range of 10-100 µg/mL exhibited a dose-dependent increase in the stabilization of mast cells along with a decrease in histamine from RPMC.

At 100 µg/mL, DSCG and ME-SA exhibited 14.67 and 9.36 respectively. The least amount of % histamine release suggests the better potential to stabilize mast cells. Percent histamine release inhibition from mast cells was found least with ME-SA (09.36) as compared to other extracts.

Bronchodilation study

Guinea pigs of either sex was sacrificed by a blow to the head and exsanguinated. The trachea was separated from adjacent tissue to create tracheal rings, which were then tied together to form a chain of 3-4 individual tracheas.[10] The chain was mounted in a 20 mL organ bath 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], the temperature of bath was maintained at 37±1°C. Before beginning the experiment, the tracheal chain was suspended under isotonic tension of 0.5 g and allowed to equilibrate for at least 1 hr. During the experiment, the K-H solution was replaced every 10 min. After the equilibrium period contraction was induced by adding the acetylcholine or histamine Thereafter, the test extracts (1 mg/mL) were added serially (0.1, 0.2 up to 0.6 mL) in increasing doses and observed for bronchodilation. Results are given in Table 2. At the conclusion of the experiment, the percentage inhibition of contraction caused by the test extract on the tracheal chain was calculated (bronchodilation) as shown in Figure 7.

In the current study, isolated guinea pig tracheal chains were successfully contracted by the well-known bronchoconstrictors histamine and acetylcholine at a concentration of 1 µg/mL.

Since ME-SA extract showed significant in mast cell stabilization and bronchodilatory effect hence, these extracts were selected for further studies. Before evaluation of *in vivo* effects, active extracts were evaluated for acute toxicity study.

The acute oral toxicity study

According to the limit dosage test of the up and down system described in OECD test recommendations No. 423, the acute oral toxicity study for extracts was conducted at a limit dose of 2000 mg/kg body weight (p.o.). Three rats-one male and two females-were chosen for each group so that the weight discrepancies did not surpass ± 10% of the population's mean beginning weight.[11] Rats were fasted for food but the water was provided *ad libitum* overnight before SA extracts administration

Table 1: Effect of different SA extracts on percentage histamine release inhibition.

Treatment				
Control-Normal	17.26 ±1.25	17.46 ±2.75	13.19 ±1.35	14.52 ±1.57
C-48/80-HR	95.22 ±1.18	97.46 ±2.24	94.59 ±1.10	95.84 ±1.09
Effect on % HRI at different concentration (µg/mL)				
	10	**25**	**50**	**100**
DSCG	97.46 ±2.24	75.54 ±2.86*	34.12 ±2.10**	14.67 ±1.07**
HE-SA	95.16 ±2.02	56.56 ±2.20*	36.16 ±1.63**	27.50 ±1.64**
EA-SA	88.13 ±3.21*	61.42 ±1.62*	36.58 ±1.72**	17.66 ±1.89**
DCM-SA	92.56 ±1.42	78.61 ±1.32*	43.42 ±1.42**	40.13 ±2.07**
ME-SA	82.25 ±3.43*	58.56 ±1.62*	38.12 ±1.71**	09.36 ±0.19**
WE-SA	94.51 ±1.50	89.56 ±2.13*	78.84 ±1.52*	67.46 ±1.1*

Each value is presented as the mean ± S.D. of three independent determinations. Statistical significance was assessed using a one-way ANOVA with Bonferroni multiple comparison test: * $p<0.05$, ** $p<0.01$ vs control group i.e., compound 48/80-induced histamine release level.

Table 2: Effect of SA extracts and aminophylline on acetylcholine and histamine preconcentrated guinea pig tracheal chain.

Drug added 1 mg/mL (mL)	Percent inhibition of contraction (bronchodilation)	
	Acetylcholine (1mg/mL) induced contraction Vs treatment	
	AMN	ME-SA
0.1	18.17±3.42	11.38±0.78
0.2	41.98±2.75	25.20±0.83
0.4	68.25±3.56	36.45±1.26
0.8	97.83±3.09	85.75±3.52
1.6	100±2.73	92.36±2.43
Histamine (1mg/mL) induced contraction Vs treatment		
0.1	18.82±2.31	16.11±2.48
0.2	44.76±3.78	43.7±2.74
0.4	89.62±2.85	76.10±3.97
0.8	100±2.82	89.28±3.61
1.6	100±3.89	100±4.48

Values represent the mean ± S.D. of three independent replicates for each group. (n=6), $p< 0.05$, compared with the data of negative control, after drug administration $p< 0.05$ compared to before drug administration.

(2000 mg/kg, p.o.), suspended in 1.0%, w/v, Carboxy Methyl Cellulose (CMC), and the access to food was reinstated after 3-4 hr.

Following administration, each rat was watched at least once during the first 30 min, periodically during the first 24 hr, with special attention given during the first 4 hr and every day, then sporadically up to 48 hr, for the appearance of any toxic sign or symptom, such as behavioral changes, locomotion, loss of righting reflex, convulsions, etc.

Thereafter, for a total of 14 days for the occurrence of any significant changes in the autonomic or behavioral responses and mortality. The systemic and behavioral toxicity patterns were studied as described in OECD test guidelines. At the end of the toxicity study, all surviving animals were sacrificed. all the tested extracts exhibited a safety margin as indicated by lack of systemic and behavioral toxicity up to 2000 mg/kg.

Oral administration of different extracts of seeds of *Saraca asoca* up to 2000 mg/kg did not produce any toxic effects in mice during the initial 30 min, 24 hr, and even at 14th day of the extract administration. No mortality was observed and all selected extracts were found to be safe at the given doses.

Accordingly, one-tenth of this dose was considered as an experimental safe dose and hence, doses of each extract were selected, i.e., three doses of each extract-100, 200, and 300 mg/kg-were chosen at random for the subsequent investigations for the *in vivo* bronchoprotective studies.

The broncoprotective tests

Before the experiment, guinea pigs were fasted for 12-24 hr and only water was given to them *ad libitum*. Animals were tested for sensitivity and appropriateness for research by confronting them with an equal amount of 0.1% histamine hydrochloride and 2% acetylcholine chloride under an average pressure of 45 mm Hg for 15 sec in a plexiglass chamber (histamine chamber). The time to onset of respiratory distress (pre-convulsive time in sec) during the aerosol challenge was measured. Guinea pigs were considered to be insensitive and discarded with pre-convulsive time of more than 120 sec. The adequate and sensitive guinea pigs were randomly allotted to different groups (control, treatment and standard, describe below) with 4 per each. The negative control of animals administered 0.1% 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 (200 and 300 mg/kg suspended in 0.1% CMC).

All animals received a single dosage of extracts and Aminophylline (AMN), given every day for three days before to the challenge with the last dose given 1 hr before the bronchial challenge. The delitescence of convulsion for each animal and tumble numbers for each group during challenge within a 6 min exposure period were recorded. Aerosol provoked a broncospastic reaction in all animals within 3 min. The delay in the appearance of the bronchospastic reaction was considered as bronchoprotective effect and bronchoprotection from convulsion was expressed related to control.[9] Results are shown in Table 3.

$$\text{Percentage protection} = [1-(T_1/T_2)] \text{ X } 100$$

Where T_1 = preconvulsive breathing time (sec) in control group

T_2 = preconvulsive breathing time (sec) in treatment and standard group

Results of bronchoprotective assay suggest that ME-SA exhibited better activity hence further selected for isolation of phytoconstituents.

Evaluation of inhibition milk-induced leukocytosis and eosinophilia in mice

The extracts were dissolved in DMSO and different doses of the extracts 100 mg/kg, 200 mg/kg, and 300 mg/kg were used for the milk-induced leukocytosis and eosinophilia in mice.[12]

Albino mice were divided into five groups (n=6). Before any treatment blood samples of all grouped animals were collected by retro-orbital plexus method to record total leukocyte and eosinophil count. Group I (control) received distilled water only, 10 mL/kg; p.o. Group II received, boiled and cooled milk (4 mL/kg), subcutaneously. Group III Standard control, Dexamethasone (50 mg/kg i.p.), Group IV-VIII were received hexane, ethyl acetate, dichloromethane, methanol and water extracts of *Saraca asoca* seeds 100, 200, and 300 mg/kg, p. o., resp. Exactly after 1 hr except for the control group, all groups were subcutaneously injected (4 mL/kg) boiled and cooled milk. Group II was observed as an intoxicant. After 24 hr of extract or ref drug administration blood samples were collected from all animal groups and total leukocyte and eosinophil count was recorded to estimate any difference before and after drug treatment.[13,14] Results are shown in Table 4.

A significant (p<0.01) increase in total leucocyte and eosinophil count was observed by administration of subcutaneous injection after 24 hr. Mice in treatment groups of 100, 200, and 300 mg/kg, p. o. of methanol extract of *Saraca asoca* revealed a significant inhibition of milk-induced leukocytosis (p<0.01) and eosinophilia (p<0.05 and p<0.01)

The ME-SA extract showed maximum inhibition of milk-induced leukocytosis and eosinophilia.

Results of bronchoprotective assay suggest that ME-SA exhibited better activity and hence further selected for isolation of phytoconstituents.

Among the above extracts, ME-SA 300 mg/kg dose has shown significant activity as compared to standard Dexamethasone (50 mg/kg) in a dose-dependent manner.

The methanol extract shows the maximum reduction in leukocyte as well as eosinophil count, so that the fractionation of methanol extract has been carried out and the most active fraction was purified and subjected to structural elucidation.

Isolation pharmacological evaluation and structural elucidation of phytoconstitutes from *Saraca asoca* seeds.

Preparation of column and stationary phase

A cleaned glass column (4 cm X 72 cm) with sintered disk was used for the separation of phytoconstituents. Silica gel for column chromatography (62-120 mesh) was kept in the oven for 12 hr at 110°C so as to remove all the moisture content present in it (activation).

The slurry of the activated stationary phase was prepared by uniform mixing with a proportional and required amount of petroleum ether (60-80°C)

Packing of column

The column was aligned in a vertical position with the help of clamps attached to a metal rod stand and concrete support. The column was filled to about 1/3 volume by the mobile phase. The column was slowly and evenly filled to about 5/6 volumes full, by the gradual addition of slurry with tapping and the stopcock was opened to drain the excess mobile phase. The side of the chromatographic column was gently tapped with a cork

Table 3: Bronchoprotective effect of ME-SA and aminophylline on acetylcholine and histamine aerosol induced bronchospasm in guinea pigs.

Group	Treatment group	Dose (mg/mL)	Tumble No.	Latency		% Protection
				Before treatment	After treatment	
I	Control	1% CMC	14	88±13	89±14	
II	ME-SA	200	10	87±12	129±16*	31
III		300	07	89±14	157±14**	43
IV	AMN	10	08	90±26	163±23**	45

Values represent the ± S.E.M of four independent replicates * p<0.05 and ** p<0.01 indicates the different levels of Significance when compared against control group.

during the filling to ensure the uniform packing and to remove air bubbles, if any trapped while packing of silica gel. A piece of cotton soaked in the mobile phase was placed at the top of the column and gently tamped down with a glass rod.

Loading of test extract

The uniform slurry of test extract (ME-CP; 25 g) with an equal amount of silica gel for column chromatography was prepared using pet ether (200 mL). The obtained slurry was loaded slowly and uniformly over a previously prepared column. A piece of cotton soaked in the mobile phase was placed at the top of the sample in the column and gently tamped down with the glass rod. After completion of packing, the excess mobile phase was drained until a small quantity of eluent was allowed to remain at the top of the column (about 4 cm/ about 10-15 mL) in order to prevent the drying and possible cracking of the packed column.

Elution pattern, collection of fraction and TLC of fractions

The column was eluted successfully with different combinations of n-hexane, ethyl acetate, dichloromethane, and methanol to obtain different fractions and phytoconstituents. Obtained fractions were collected, the required amount was concentrated for TLC and R_f values were recorded in the different mobile phase. The details of the elution pattern, fractions collected and TLC of fractions are given in Table 5. Change in solvent ratio and increment of 5-10 mL was decided based on the TLC pattern.

Isolation and phytochemical evaluation of fractions, phytoconstituents from ME-SA

Among the obtained fractions, fractions with the similar TLC pattern were combined and subjected to phytochemical screening. Fractions with similar TLC patterns and phytochemical profiles were combined. The phytochemical nature of elute was evaluated by spraying TLC of column elute with ferric chloride.

In addition, TLC was also exposed to UV/examined in UV chamber for fluorescence determination in the presence or absence of NH_3 vapors for the detection of flavonoids and related phytoconstituents. Among the collected fractions, fractions that showed a single spot in TLC were combined and concentrated, evaluated for purity by TLC single spot. Fractions were concentrated separately to obtained a brown cream-colored compound, which was further purified to obtain 75 mg.

TLC and phytochemical evaluation of isolated compounds

Thin layer chromatography was used for the resolution of compounds present in the crude extract and was performed to optimize the best solvent system to proceed with column purification. The thin layer chromatogram was resolved in

Table 4: Effect of crude extract of *Saraca asoca* on milk-induced leucocytosis and eosinophilia in mice.

Group	Treatment and Dose (mg/kg, p.o)		Difference in number (mm^3)	
			Leucocytes count	Eosinophil count
1	Control Gr.		82.6 ± 0.58	18.7 ± 0.88
2	Milk		4814 ± 1.17**	151.5 ± 1.47**
3	Dexamethasone		2108±158.06***	56±2.36***
4	HE-SA	100	4764 ± 8.67*	149.17 ± 0.60
5		200	4759 ± 6.67*	148.50 ± 0.76
6		300	4763 ± 8.45*	146.33 ± 1.17*
7	EA-SA	100	4760 ± 6.25*	149.17 ± 1.02
8		200	4764± 6.50*	146.50 ± 1.48*
9		300	4765 ± 9.94*	146.67 ± 0.49*
10	DCM-SA	100	4759 ±12.05*	146.67 ± 0.49*
11		200	4764± 9.44*	146.50 ± 0.76*
12		300	4763± 8.15*	146.67 ± 0.88*
13	ME-SA	100	4113 ± 35.87**	135.17 ± 1.35**
14		200	3570 ± 10.39**	111.00 ± 0.96**
15		300	3217 ± 1.76**	90.50 ± 1.31**
16	WE-SA	100	4759 ± 6.22*	148.50 ± 0.67
17		200	4760 ± 5.41*	146.67 ±1.74*
18		300	4758 ± 9.28*	146.8 ± 0.72 *

The values are represented as mean ± S.E.M (n=6) for all groups and statistical significance between treated and control groups was analyzed using one-way ANOVA, followed by Dunnett's test. * p<0.05-Significant difference when compared to control, ** p<0.01- Highly significant difference when compared to control.

95% dichloromethane in Methanol for the better resolution of compounds for the crude methanol extract of *Saraca asoca* (Figure 1, Crude). The same solvent system was used to analyze the fractions and the pure compounds of *Saraca asoca* (Figure 1, Pooled fractions, Pure).

Thin layer chromatography of *Saraca asoca* methanol crude, pooled column fractions 1-5 and the bioactive pure isolated from pooled fraction 4. The TLC plates were subjected to ferric chloride spray test and were documented. Results are shown in Table 6.

In vivo evaluation of fractions of methanol extract

The active fraction identified by milk-induced leukocytosis and eosinophilia was subjected to co-crystallization techniques and repeatedly rinsed using methanol to remove polar impurities. The final crystalline fraction was subjected to structural elucidation. Results are shown in Table 7.

The fraction IV of ME-SA extract showed maximum inhibition of milk-induced leukocytosis and eosinophilia.

Structural Elucidation of isolated compounds

Isolated compounds were subjected to UV, FTIR, MS, and NMR (^{1}H and ^{13}C) analysis.

UV analysis of isolated compounds

UV analyses of isolated compounds were recorded with different UV shift reagents, in the case of the flavonoids. Isolated compounds were dissolved in absolute ethanol to obtain the required concentration and the spectrum was recorded. To the same solution 5% $AlCl_3$ was added and changes in the spectrum were recorded, the effect of successive addition of HCl was also recorded. In addition, the solution of the isolated compound was successively mixed with boric acid (1-2 mg powder directly added) and sodium metal in methanol. Spectra were recorded and compared to the expected effects of various shift reagents on the UV absorption Spectra. The UV spectrum of this compound exhibited two major absorption peaks in the region 360 nm and 258 nm, which indicates the presence of a flavonol structure.

Recording of FTIR, Mass, ^{1}H and ^{13}C NMR Spectra.

FT-IR of the isolated compound was recorded by preparing the KBR pellet method are shown in Figure 2

The NMR experiments were done in CdCl3 solution on a Bruker Avance 400 MHz NMR spectrometer. Chemical shifts of 13C and 1H were measured relative to tetramethyl silane as an internal reference and confirmed the elemental position of the bio-active lead structure from *Saraca asoca* (Figures 3 and 4). Mass Spectroscopy (MS) analysis was performed on Waters Quattro micro-API with Electrospray Ionization (ESI) interface

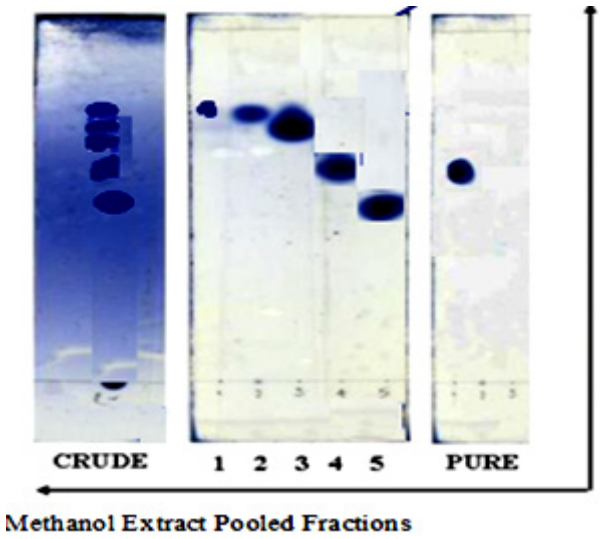


Figure 1: TLC analysis of *Saraca asoca* crude extract and fractions.

Table 5: Isolation of Phytoconstituents from ME-SA.

Sl. No.	Solvent ratio eluted	Fraction and Yield (mg)	Mobile Phase for TLC	R_f Values
1	HE (100)	1	BE:EA (9:1)	No Spot
2	HE:DCM (80:20)	2(18)	BE:EA (8:2)	0.28
3	DCM (100)	3	BE:EA (9:1)	0.57
4	EA:ME (50:50)	4 (75)	BAW (4:1:1)	0.98
5	DCM:EA (50:50)	5(27)	BE:ME (9:1)	No Spot
6	ME (100)	6(50)	TO:EA:ME (6:4)	0.84

Table 6: TLC and phytochemical details of isolated compounds.

Isolated Compound	Phytochemical Test			R_f Value
SA-1	5% $FeCl_3$	Molish Test	Shinoda Test	
	+	+	++	0.98

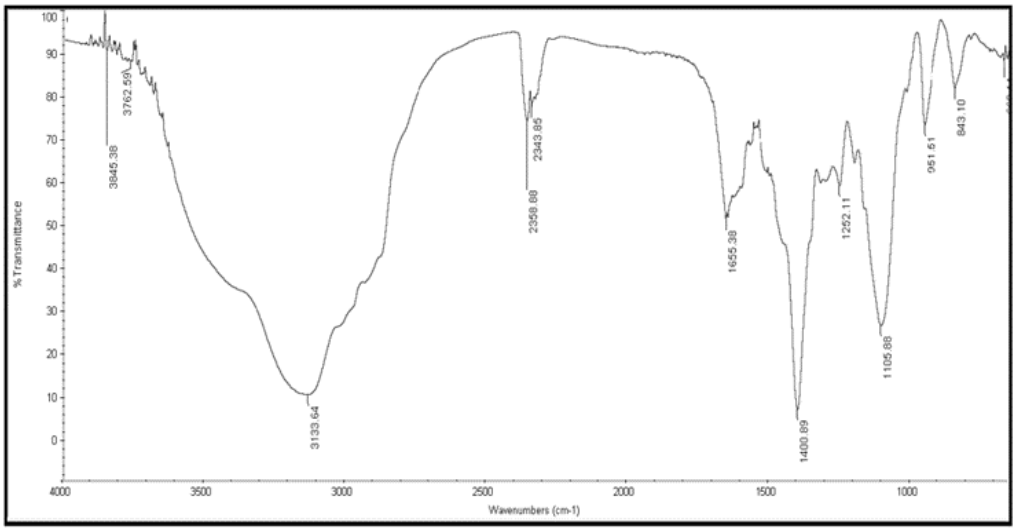


Figure 2: FTIR Spectrum of SA-1.

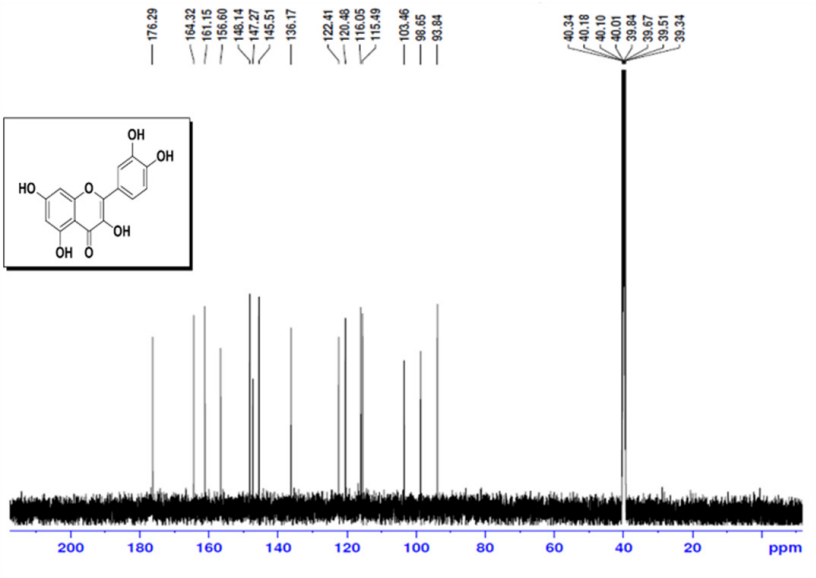


Figure 3: ^{13}C NMR spectrum of SA-1.

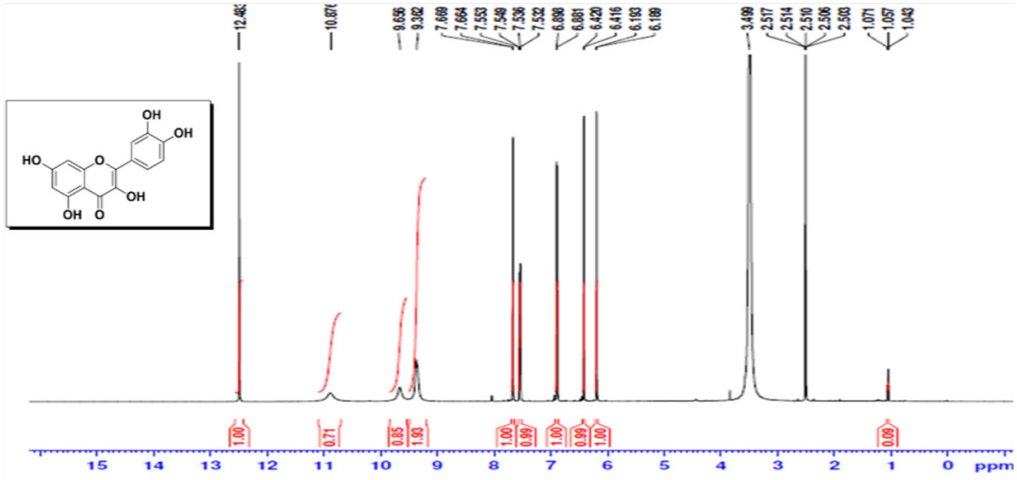


Figure 4: ^{1}H NMR spectum of SA-1.

Table 7: Effect of fraction I-VI of methanol extract of *Saraca asoca* on milk-induced leucocytosis and eosinophilia in mice.

Group	Treatment and Dose (mg/kg, p.o.)		Difference in no. of (mm^3)	
			Leucocytes count	Eosinophil count
1	Control		82.6 ± 2.48	18.9 ± 2.16
2	Milk		4816 ± 64.12	153.3± 6.09
3	Dexamethasone		2108±158.06***	56±2.36***
4	Fraction I	100	4612 ± 7.12*	151.1 ± 7.3*
5		200	4327 ± 16.10*	147.3 ± 4.2*
6		300	4150± 26.15*	142.4± 6.12*
7	Fraction II	100	4592 ± 8.14*	151.3 ± 8.1*
8		200	4310 ± 9.12*	147.2± 9.4*
9		300	4050 ± 13.2*	141.5± 5.2*
10	Fraction III	100	4512 ± 7.8*	150.4 ± 3.1*
11		200	4310 ± 9.12*	149.1± 2.1*
12		300	4112 ± 13.2*	145.3 ± 3.12*
13	Fraction IV	100	4051 ± 8.12**	130.1 ± 1.91**
14		200	2978 ± 6.18**	104.3± 4.15**
15		300	1812 ± 5.12**	85.8 ± 3.12**
16	Fraction V	100	4516 ± 12.10*	152.4 ± 4.16*
17		200	4612 ± 27.10*	150.9 ± 8.2*
18		300	4320 ± 17.08*	149.3± 9.16*

The values are represented as mean ± S.E.M (n=6) for all groups and statistical significance between treated and control groups were analyzed using one-way ANOVA, followed by Dunnett's test. * p<0.05-Significant difference when compared to control, ** p<0.01- Highly significant difference when compared to control.

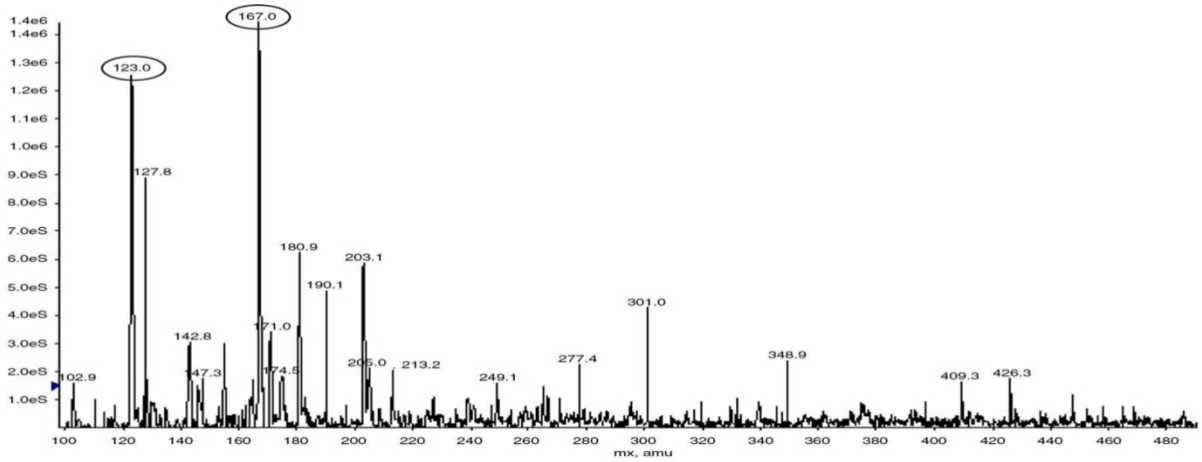


Figure 5: Mass spectrum of SA-1.

working in the positive and or negative mode. The 13C NMR data of compound SA-1 was compared with the published ^{13}C NMR data of quercetin. The mass spectra confirmed the elemental composition of the compounds. As shown in Figure 5. Using mass spectroscopy elemental formula of the active compound from *Saraca asoca* was identified as $C_{15}H_{10}O_7$ and the molecular weight was found to be 302 g/mol and the name of the compound was 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one as shown in Figure 6.

Pharmacological Screening of isolated compounds

Fraction and isolated compounds were subjected to pharmacological screening in terms of % DPPH inhibition, Mast cell stabilization (% HRI) and bronchodilation activity. Results are shown in Tables 8 and 9.

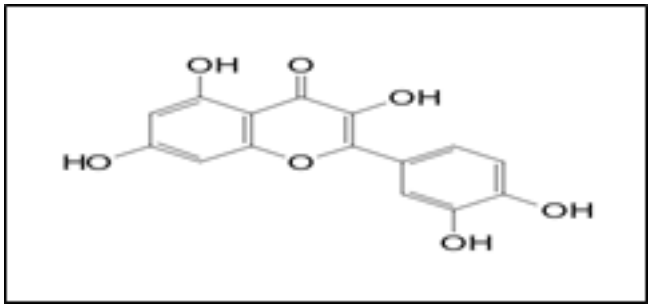


Figure 6: Structure of the isolated compound (SA-1) from *Saraca asoca* seeds. 2-(3, 4-dihydroxyphenyl) – 3,5, 7-dihydroxy-4H-1-benzopyran-4-one.

Table 8: Effect of isolated compounds from ME-SA on % DPPH inhibition and % Histamine release inhibition.

Conc (µg/mL)	% DPPH inhibition	% Histamine release inhibition
05	17..09 ±1.18	95.78±2.13
10	48.32 ±1.92	74.35±2.44
15	74.32±2.61	46.14±1.98
20	94.83±2.03	07.05±1.35

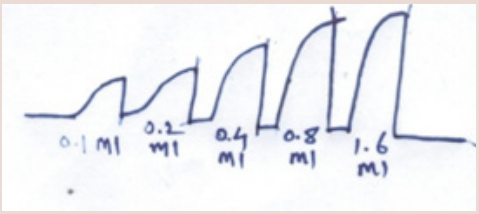
Values are representatives of mean±S.D. All the determination was carried out in three replicates.

Table 9: Effect of isolated compounds from ME-SA on % inhibition of contraction induced by acetylcholine and histamine.

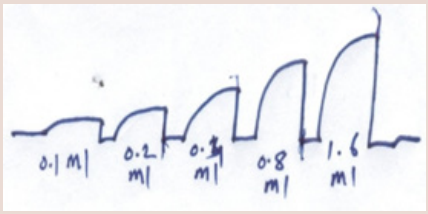
Amount added (mL) Conc.	0.1	0.2	0.3	0.4	0.5	0.6
Compound	% Inhibition of contraction induced by acetylcholine					
SA-1	7.33±0.62	15.63±2.32	38.09±1.27	57.25±1.54	84.62±1.76	97.65±2.19
Compound	% Inhibition of contraction induced by histamine					
SA-1	6.42±1.58	20.11±1.72	46.56±1.43	65.25±2.30	80.12±1.31	93.57±1.28

Values are representatives of mean ±S.D. All the determination was carried out in three replicates. ^{13}C NMR spectrum of SA-1.

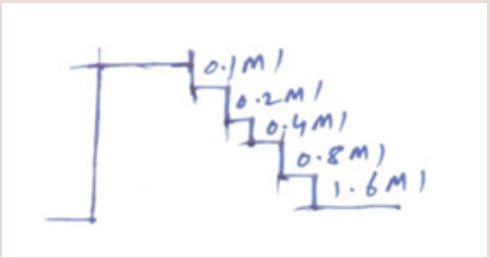
Figure 7: Representative tracing of maximum contraction by tracheal chain in presence of agonist (acetylcholine and histamine added separately) and effect of antagonist (extracts) after cumulative addition which was measured as % inhibition of contraction.



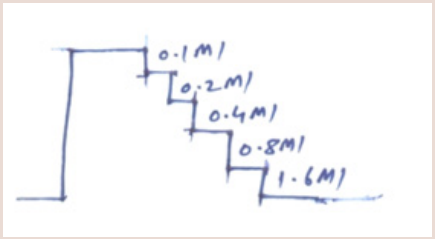
(A) Cumulative response curve of histamine in guinea pig trachea



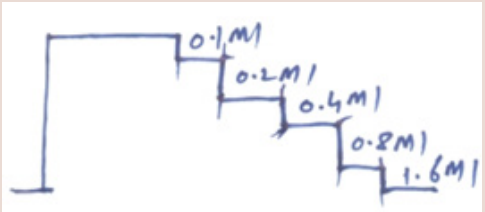
(B) Cumulative response curve of acetylcholine in guinea pig trachea



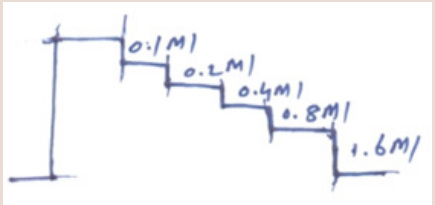
Ach Vs ME-SA



(b) Histamine Vs ME-SA



(e) Ach Vs AMN



(f) Histamine Vs AMN

CONCLUSION

In the present study, the successive extraction of *Saraca asoca* seeds powder has been carried out in hexane, ethyl acetate, dichloromethane, methanol and water.

Results of broncho-relaxant (*in vitro*) suggest that ME-SA is more potent as compared to the other extracts. Therefore ME-SA were evaluated for milk-induced leucocytosis and eosinophilia and bronco protective (*in vivo*) effect and only active extracts were subjected to bioactivity-guided isolation of antiasthmatic phytoconstituents.

The activity of each crude extract is observed in milk-induced leukocytosis and eosinophilia in albino mice. The methanol extract shows a significant decrease in leukocyte as well as eosinophil count in albino mice. So, the methanol extract was subjected to further fractionation by TLC and Column chromatography. The obtained fractions are further evaluated for the *in vivo* effect in albino mice, the fourth fraction shows a significant reduction in leukocyte and eosinophil count. Thus, the fourth fraction is further purified and its structural elucidation is carried out by NMR and Mass spectroscopy. The molecular weight, molecular formula and structure are determined. This study concludes that 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one is present in seeds of *Saraca asoca* and it is responsible for anti-asthmatic-activity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

^{1}H NMR: Proton Nuclear Magnetic Resonance; **^{13}C NMR:** Carbon Nuclear Magnetic Resonance; **DCM-SA:** Dichloromethane extract *Saraca asoca*; **EA-SA:** Ethyl Acetate extract of *Saraca asoca*; **FT-IR:** Fourier transform infrared radiation spectroscopy; **H_2SO_4:** Sulphuric acid; **HCl:** Hydrochloric acid; **HE-SA:** Hexane extract of *Saraca asoca*; **ME-SA:** Methanol extract of *Saraca asoca*; **MS:** Mass Spectroscopy; **TLC:** Thin Layer Chromatography; **UV-vis:** Ultraviolet-visible spectrophotometer.

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