ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Phytochemical Screening and Pharmacological Evaluation of Anti-Asthmatic Activity of *Rubia cordifolia* in Asthma Induced Models

Manuvarthi Sushma*, Atluri Bhavana, K. Padmalatha
Department of Pharmacology,
Vijaya Institute of Pharmaceutical Sciences for Women Enikepadu,
Vijayawada, Andhra Pradesh, India, 521108.

Abstract

Asthma is a chronic disease that affects approximately 300 million people worldwide. Although wide range of drug is available, the relief provided by them is mainly symptomatic and short lived. Moreover, the side effects of these drugs are also quite disturbing. Hence, a continuous search is on going to identify effective and safe remedies to treat bronchial asthma. Ayurveda is a great Indian tradition and have an important role in discovery of new medicines. There are many natural herbs that can be used for asthma, treatment. *Rubia cordifolia* is traditionally used to treat asthma.

Key Words: Antiasthmatic, *Rubia cordifolia*, Histamine-induced model and Acetylcholine induced model.

INTRODUCTION

Asthma is a disease of the human respiratory system in which the airways constrict and become narrow, often in response to a "trigger" such as exposure to an allergen, cold air, exercise, or emotional stress. Asthma affects 7% of the total population and approx 300 million worldwide. During attacks (exacerbations), the smooth muscle cells in the bronchi constrict, and the airways become inflamed and swollen with difficulty in breathing. Asthma causes 4,000 deaths a year in the US alone. Attacks can be prevented by avoiding triggering factors and by drug treatment[1].

There is limited information on field epidemiology of bronchial asthma in Indian adults. The results of the study indicated that from 73605 respondents (37682 men, 35923 women), one or more respiratory symptoms were present in 4.3-10.5% subjects. Asthma was diagnosed in 2.28%, 1.69%, 2.05 and 3.47% respondents respectively at Chandigarh, Delhi, Kanpur, and Bangalore, with overall prevalence of 2.38%. Female sex, advancing age, usual residence in urban area, lower socioeconomic status, history suggestive of atropy, history of asthma in a first degree relative, and all forms of tobacco smoking were shown as responsible ailments of asthma. Prevalence estimates of asthma in adults in this study pointed a high overall national burden of disease[2].

Asthma is one of the commonest chronic diseases of affluent societies. The striking increase in prevalence of asthma over recent decades in affluent societies and the rarity of this disease in less affluent populations confirms the importance of environmental and other factors in the cause of asthma, although which environmental and other factors are responsible is still not clear. The studies showed that genetic factors are also important in determining individual susceptibility to asthma. The results of genetic studies suggest that there are many genes with moderate effects rather than a few major genes. Asthmatic airways show inflammation with $CD4^+$ helper cells, mast cells, and eosinophils, characterizing the inflammatory response. Inhaled corticosteroids remain the cornerstone of treatment with the addition of long-acting -agonists as the next step if symptoms continue. Leukotriene antagonists, the only new drugs to reach the market in the past decade, have modest effects. A better understanding of the mechanisms underlying asthma and the genetic and environmental factors that predispose individuals to asthma will play a key role in understanding better preventative strategies and new therapeutic approaches [3].

It has been estimated that about 6000 articles are published every year about asthma in various indexed journals. The increasing prevalence of asthma has led to extensive research into its cause, pathophysiology, and management [3].

A few patients have asthma as part of a wider problem such as allergic broncho pulmonary aspergillosis[4], Churg-Strauss syndrome (Conron & Beynon, 2000) and aspirin-inducedasthma[6]

PLANT PROFILE:

BOTANICAL NAME	:	*Rubia Cordifolia.Linn*
KINGDOM	:	Plantae
DIVISION	:	Angiosperms
CLASS	:	Eudicots
ORDER	:	Gentianales
FAMILY	:	Rubiaceae
GENUS	:	Rubia
SPECIES	:	*R.cordifolia*
COMMON NAME	:	Manjishta, Majith, Tamaralli, Manditti.

Habitat: throughout the India, in hilly districts upto 3500 meters.

Chemical constituents: Characteristic chemical constituents of the rootstock of this plant are anthraquinones and cyclic peptides. Main anthraquinone constituents are munjistin, purpurin, and pseudopurpurin. New anthraquinones namely 1-hydroxy-2,7-dimethylanthraquinone, 2-hydroxy-6-

methylanthraquinone, 2,6-dihydroxyanthraquinone, 1-hydroxy 2-methylanthraquinone, nordamnacanthal, physcion, 1,4-dihydroxy 6-methyl-anthraquinone, 1,4-dihydroxy 2-methylanthraquinone, 1,5-dihydroxy 2-methylanthraquinone, 3-prenyl methoxy 1,4-naphthoquinone, 1-hydroxy 2-methoxy anthraquinone, 1,4-dihydroxy 2-methyl 5-methoxy anthraquinone or 1,4-dihydroxy 2-methyl 8-methoxy anthraquinone, 1,3-dimethoxy 2-carboxy anthraquinone and rubiadin have been isolated from Rubia cordifolia roots [14, 15, 16, 17]. Three new anthracene derivatives, rubiasins A–C, were isolated from the combined roots and stems of *Rubia cordifolia* [18].

Naphthoquinones, naphthohydroquinones and naphthohydroquinones dimmers have also been identified in the roots. Two new naphthoquinones, 2-carbamoyl-3-methoxy-1,4- naphthoquinone and 2-carbamoyl-3-hydroxy-1,4-naphthoquinone, isolated from the methanol extract of *Rubia cordifolia* [19].Anthraquinones and Naphtho-quinones in chloroform extract of roots constitute around 3-5 % of the roots [6]. Two pentacyclic triterpenes, Rubicoumaric acid and rubifolic acid isolated from *Rubia cordifolia* [20]. A new iridoid glucoside, 6-Methoxygeniposidic acid was isolated from the roots of *Rubia cordifolia* [21]. Mollugin, furomollugin and dehydro-a-lapchone is isolated from the chloroform fraction of *Rubia cordifolia (Linn.)* roots [22]. Four naphthoic acid esters namely rubilactone, 3'-carbomethoxy-4'-hydroxy-naphtho [1',2'-2,3] furan, dihydromollugin and 3-carbomethoxy-2-(3'-hydroxy)isopentyl-1,4-naphthohydroquinone-1-O -beta-D-glucoside were isolated from the roots of *Rubia cordifolia* [23].

Uses:

It is highly valuable plant in Ayurvedic system of medicine used for,

1. Powdered dried roots and fruits are taken internally for the treatment of skin diseases and disorder of spleen [5, 6].
2. It is used for the treatment of major burns, ulcers and bone fractures [6].
3. It is considered tonic, antitussive, and useful in chronic low fevers [6].
4. The roots are used internally in the treatment of abnormal uterine bleeding, internal and external haemorrhage, bronchitis, rheumatism, stones in the kidney, bladder and gall, dysentery etc. The plant is used in the treatment of blood disorders [7].
5. The roots are alterative, anodyne, antiphlogistic, astringent, diuretic, expectorant, styptic and vulnerary [12].

Reported pharmacological activities are:

1. Root powder has been shown to exhibit haemostatic action by its application on rabbits with severed femoral artery [6].
2. This plant is found to be effective in wound healing activity [24, 25].
3. Anti-inflammatory activity of petroleum ether and water extract of *Rubia cordifolia* roots [26, 27].
4. Immunomodulatory activity of alcoholic extract of *Rubia cordifolia* Linn [28].
5. In vivo evaluation of antioxidant activity of alcoholic extract of *Rubia cordifolia* Linn. and its influence on ethanol-induced immunosuppresion [29].
6. Antioxidant property of *Rubia cordifolia* extract and its comparison with vitamin E and parabenzoquinone [30].
7. Anticonvulsant and behavioral action of triterpene isolated the acetone soluble part of petroleum ether extract of *Rubia cordifolia* [31].
8. Antihyperglycemic, antistress and nootropic activity of alcoholic extract of roots of *Rubia cordifolia Linn* [32].
9. Antihyperglycemic activity of aqueous root extract of Rubia cordifolia in streptozotocin-induced diabetic rats [33].
10. Hepatoprotective effects of rubiadin, a major constituent of *Rubia cordifolia* Linn. were evaluated against carbon tetrachloride (CCl_4)-induced hepatic damage in rats [34].
11. Effect of aqueous-methanol extract of *Rubia cordifolia* on acetaminophen and CCl_4-induced hepatotoxicity [35].
12. Novel antitumor bicyclic hexapeptide dimer, named RA-dimer A, RA-VI, RA–VIII, RA-XV and RA-XVI were isolated from the roots of *Rubia cordifolia*[36, 37, 38].
13. Evaluation of the anti-proliferative properties of selected psoriasis-treating Chinese medicines on cultured HaCaT cells [39].
14. Evaluation of nitric oxide scavenging activity of *Rubia cordifolia Linn* [40].
15. Mitodepressive effect of Rubia cordifolia extract on the bone marrow cells of mice [41].
16. Evaluation of the antibacterial activity of root extracts of *Rubia cordifolia Linn.*[42].
17. Protective effect of alcoholic extract of *Rubia cordifolia* on lipid peroxide formation in isolated rat liver homogenate [43].
18. Role of alcoholic extract of root of *Rubia cordifolia Linn.* in radiation protection[44].
19. Anti-platelet activating factor property of *Rubia cordifolia Linn* [45].

MATERIALS AND METHODS

The designing of methodology involves a series of steps taken in a systematic way in order to achieve the set goal(s) under the prescribed guidelines and recommendations. It includes in it all the steps from field trip to the observation including selection and collection of the medicinal plant, selection of dose value, standardization of protocol, usage of instruments, preparation of reagents, selection of specific solvents for extraction, formation of protocol and final execution of the standardized protocol. All this requires good build of mind and a good and soft technical hand to handle the materials and procedure in a true scientific manner.

Drugs and Chemicals:

Histamine dihydrochloride, acetylcholine chloride, atropine sulfate, mepyramine meleate, carrageenan were purchased

from Sigma (St. Louis, MO, USA). Histamine solution was freshly prepared in normal saline (NaCl, 8.5 g/l). All the other drugs were of analytical grade and they were dissolved in distilled water and desired concentration was prepared.

Extraction:

Leaves of Rubiaw Cordifolia were washed with distilled water to remove dirt and soil, and shade dried. Routine pharmacognostic studies including macroscopic and microscopic observations were carried out to confirm the identity of the materials. The dried material of each plant was coarsely powdered (500 g) and defatted with aqueous (60-800C), and then extracted separately with alcohol (95% v/v) in Soxhlet apparatus (Figure 4.5). The extracts were filtered, and concentrated by distilling off the solvents and evaporated to dryness using water bath to get crude ethanol extract. The yield values for aqueous extract of *R.cordifolia* (AQERC) and alcoholic extract of *R.cordifolia* (ALERC) were 20.48% and 10.96% w/w, respectively. Now these extracts were subjected to phytochemical screening and screened for possible pharmacological actions. For the pharmacological tests, the extracts were suspended in double distilled water containing carboxy methyl cellulose (CMC, 0.5% w/v).

Histamine dihydrochloride, acetylcholine chloride, atropine sulfate, mepyramine meleate, carrageenan were purchased from Sigma (St. Louis, MO, USA). Histamine solution was freshly prepared in normal saline (NaCl, 8.5 g/l). All the other drugs were of analytical grade and they were dissolved in distilled water and desired concentration was prepared.

Experimental animals:

Anti-asthmatic studies were conducted on guinea pigs (350-500 g) of either sex fed on commercial pellet diet (Amrut, Pranav Agro Industries Ltd, India). They were group housed in polypropylene cages (640 x 410 x 250 mm high) under standard conditions of temperature (22 ± 20C), relative humidity (60 ± 5%). They were divided in groups of ten animals each. The saline fed group served as control and one group was treated with a standard drug.

Before experimentation, the animals were kept on fast for 24 h but water was given ad libitum, Animals receiving different doses of plant extracts were also observed for any alteration in their general behavior.

They were provided with standard rodent pellet diet (Amrul, Pranav Agro Industries Ltd, Baroda, Gujarat, India) and tap water ad libitum except the food was withdrawn 18–24 h before the experiment. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC)) with approval number VIPW/IAEC/1581/PO/Re/11/CPCSEA/M.Ph/007/2020-21. All the experiments and the care of the laboratory animals were according to current ethical guidelines by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

Habituation to animals:

Guinea pigs were habituated to handling by holding them and injecting vehicle through oral/i.p. routes to minimize non-specific stress and to simulate the actual protocol conditions. Handling also consisted of weighing and restraining animals on platform for 1 min, and gently massaging on dorsal site as was done in the actual protocols. The same platform was used during drug administration. Moreover, the animals were familiarized with the diet in their home cage-environment and laboratory environments before subjecting them to the tests. All the experiments commenced 24 hours following the final habituation and were conducted according to the protocols mentioned below.

Acute toxicity test (Determination of LD50):

The acute toxicity tests (LD50) for the different plant extracts were determined according to the procedure described by Lorke (1983). The crude aqueous and alcoholic extracts were used for the test. Guinea pigs (300–350 g) of either sex were used. This method involved an initial dose finding procedure, in which the animals were divided into three groups of three animals per group. Doses of 10, 100, and 1000 mg/kg were administered orally, one dose for each group. The treated animals were monitored for 24 h for mortality and general behavior.

From the result of the above step, four different doses of 200, 400, 800, and 1600 mg/kg were chosen and administered (p.o.) respectively to four groups of one guinea pig per group. The treated animals were again monitored for 24 h. The LD50 was then calculated as the geometric mean of the lowest dose showing death and the highest dose showing no death.

Preparation of plant extracts

Preparation of Aqueous Extract:

Fresh leaves of *R.cordifolia* were collected and washed under tap water. The leaves extract used was prepared by taking 20gms of finely cut leaves into 250ml beaker containing 200ml of water. The contents were mixed well and then the mixture was boiled upto 80-1000C for 4-5hrs. Further the extract was filtered with whatmann filter paper. The filtrate was boiled until the concentrated residue is formed. The concentrated product was sealed in sample covers and stored under room temperature and used for further experiment to check the activities.

Preparation of Alcoholic Extract:

Fresh leaves of *R.cordifolia* were collected and washed under tap water. The leaves extract used was prepared by taking 20gms of finely cut leaves into 250ml beaker containing 200ml of alcohol. The contents were mixed well and then the mixture was boiled upto 50-600C for 4-5hrs. Further the extract was filtered with whatmann filter paper. The filtrate was boiled until the concentrated residue is formed. The concentrated product was sealed in sample covers and stored under room temperature and used for further experiment to check the activities.

Phytochemical screening of aqueous and ethanolic extracts of *Rubia cordifolia*:

Aqueous and Alcoholic extracts were chemically tested to know the presence of different primary, and secondary metabolites present in them as per the following scheme:

Tests for sterol/steroids:

Salkowaski test:

Few mg of the sample was taken in 2 ml of chloroform and 2 ml of concentrated sulphuric acid and shaken. The development of red color in the chloroform layer indicates the presence of sterols/steroids.

Test for terpenoids:

Liebermann–Burchard test:

Few mg of the sample was dissolved in 1 ml of chloroform and few drops of acetic anhydride. Concentrated sulphuric acid was added by the side of the test tube. Production of purple color indicates the presence of triterpenoids and blue–green color indicates the presence of sterols.

Test for alkaloids:

Few mg of the sample was taken in 5 ml of 1.5% v/v hydrochloric acid and filtered. The filtrate was then tested using following reagents:

Dragendorff's reagent:

It is a solution of potassium bismuth iodide. It was prepared by dissolving bismuth nitrate (8 gm) in nitric acid (20 ml), and separately dissolving potassium iodide (27.2 gm) in water (50 ml), mixing the two solutions, and making up the volume to 100 ml. Above Dragendorff's reagent was sprayed on Whatman No. 1 filter paper then the paper was dried. The test filtrate after basification with dilute ammonia was extracted with chloroform and the chloroform extract was applied on the filter paper, impregnated with Dragendorff's reagent, with the help of a capillary tube. Development of an orange red color on the paper indicated the presence of alkaloids.

Mayer's Reagent:

1.36 gm of mercuric chloride, and 3 gm of potassium iodide were dissolved in water to make 100 ml. To a little of each extract taken in dilute hydrochloric acid in a watch glass, few drops of the reagent was added, formation of cream colored precipitate shows the presence of alkaloid.

Hager's reagent:

It is a saturated solution of picric acid in water. When the test filtrate was treated with this reagent, yellow precipitate was obtained indicating the presence of alkaloids.

Wagner's Reagent:

It is a solution of potassium triiodide in water which was prepared by dissolving 1.3 gm iodine in a solution of potassium iodide (2 gm) in water to make 100 ml. Formation of brown precipitate after addition of this reagent in extract indicates the presence of alkaloids.

Test for tannins:

Sample was taken separately in water, warmed, and filtered. Tests were carried out with the filtrate using following reagents:

4.12.4.1 Ferric chloride test

A 5% w/v solution of ferric chloride in 90% alcohol was prepared. Few drops of this solution were added to a little of the above filtrate. Dark green or deep blue color shows the presence of tannins.

Lead acetate test:

A 10% w/v solution of basic lead acetate in distilled water was added to the test filtrate. Precipitate indicates the presence of tannins.

Test for flavonoids

Shinoda test

To the sample, 5 ml of 95% ethanol, few drops of HCl, and few magnesium turnings were added. The pink color shows the presence of flavonoids.

Test for carbohydrates:

Molisch's test:

About 0.1 gm of the sample was dissolved in 2 ml of water, and added 2-3 drops of 1% ethanolic solution of alpha naphthol, and then carefully poured 2 ml of concentrated sulphuric acid down the side of the test tube so that it forms a heavy layer at the bottom. A deep violet color is produced if carbohydrates are present.

Fehling's test:

1 ml of Fehling's A and 1 ml of Fehling's B solutions were mixed and boiled for 1 minute. Equal volume of sample was then added, heated in boiling water bath for 5-10 minutes. First a yellow then brick red color shows the presence of carbohydrates.

4.12.6.3 Benedict's test

Equal volumes of the reagent, and sample were added. Mixture was then boiled for five minutes. Depending on the reducing sugar present a range of colors develops.

Test for proteins:

Biuret test:

To the sample 4% NaOH, and few drops of 1% $CuSO_4$ were added. Violet or pink color indicates the presence of proteins.

Xanthoproteic test:

Sample was mixed with 1 ml of concentrated sulphuric acid, formation of precipitate shows positive test.

Millon's test:

3 ml of sample was mixed with Millon's reagent, formation of precipitate indicates the presence of proteins.

Test for saponins

Foam test:

A few mg of the sample was shaken vigorously with water. Honeycomb like foam indicates the presence of saponins.

Heamolytic test:

Sample was added to one drop of blood placed on glass slide. Hemolytic zone indicated the presence of saponins.

Test for anthraquinone glycosides

Borntrager's test:

To 3 ml of the sample, dilute sulphuric acid was added, boiled, and flittered. To the filtrate equal volume of chloroform was added, and shaken. After separating the organic layer, ammonia was added. Turning pink of ammonical layer indicates the presence of said glycosides.

Test for cardiac glycosides:

Keller-Kiliani test:

The sample was dissolved in acetic acid containing trace amounts of ferric chloride. It was then transferred to the surface of concentrated sulphuric acid. A reddish brown ring will be formed at the junction, and the color slowly changes to blue.

Legal's test:

A few drops of pyridine, and sodium nitroprusside were added to the sample, and made alkaline. A pink or red color is obtained.

Screening for antiasthmatic activity:

Histamine-induced bronchospasm in guinea pigs(Armitage et al., 1961)

The guinea pigs fasted for 24 h were exposed to an atomised fine mist of 2% histamine dihydrochloride aerosol (dissolved in normal saline) using nebulizer at a pressure of 300 mm Hg in the histamine chamber (24 x 14 x 24 cm, made of perplex glass) (Figure 4.7). Guinea pigs exposed to histamine aerosol showed progressive signs of difficulty in breathing leading to convulsions, asphyxia, and death. The time until signs of convulsion appeared is called pre-convulsion time (PCT/PCD). By observation experience was gained so that the pre convulsion time can be judged accurately. As soon as PCD commenced, animals were removed from the chamber and placed in fresh air to recover. In the present experiments the criterion used was time for onset of dysponea and percent protection was calculated. Those animals which developed typical histamine asthma within 3 min were selected out three days prior to the experiment and were given habituation practice to restrain them in the histamine chamber. They were divided in groups of six animals each. Mepyramine 8.0 mg/kg, i.p., and different doses of plant extracts or polyherbal formulations intraperitonially were administered 30 min prior to exposure. Animals, which did not develop typical asthma within 6 min, were taken as protected.

Acetylcholine-induced bronchospasm in guinea pigs (Kumar & Ramu, 2002)

The guinea pigs fasted for 24 h were exposed to an atomized fine mist of 0.5% acetylcholine aerosol (dissolved in normal saline) using nebulizer at a pressure of 300 mm Hg in the histamine chamber (24 x 14 x 24 cm, made of perplex glass) (Figure 4.7). Guinea pigs exposed to acetylcholine aerosol showed progressive signs of difficulty in breathing leading to convulsions, asphyxia, and death. The time until signs of convulsion appeared is called pre-convulsion time (PCT/PCD). By observation experience was gained so that the pre convulsion time can be judged accurately. As soon as PCD commenced, animals were removed from the chamber and placed in fresh air to recover. In the present experiments the criterion used was time for onset of dysponea and percent protection was calculated. Those animals which developed typical acetylcholine asthma within 3 min were selected out three days prior to the experiment and were given habituation practice to restrain them in the histamine chamber. They were divided in groups of six animals each. Atropine sulphate 2 mg/kg, i.p., was taken as standard and different doses of plant extracts intraperitonially were administered 30 min prior to exposure. Animals, which did not develop typical asthma within 6 min, were taken as protected.

Data analysis:

The data are presented as mean ± S.E.M. Statistical significance was determined using One-way Analysis of Variance (ANOVA) followed by Dunnett's t-test or Chi-square test with Yate's correction factor. Differences were considered significant at $P < 0.05$.

RESULTS:

Extraction:

Dried leaf powder of R.Cordifolia (500 gm) were extraced with water and alcohol (95% w/v) to get concentrated aqueous and alcoholic extract. The yields of extracts were as follows: AQERC (20.48% w/w), ALERC (11.45% w/w).

Acute toxicity test (Determination of LD50)

The LD50 of the AQERC and ALERC was calculated to be 2262.7 and 1131.4mg/kg by oral route. During the acute toxicity study, animals were observed for gross behavioral and morphological changes (respiratory distress, immobility, convulsion, loss of righting reflex etc.). Based on the results of acute study, doses were then selected. The plant extracts did not produce any significant changes in the normal behavior of the animals, and no toxic symptoms were seen at the dose levels studied.

Phytochemical screening of different plant extracts:

Table 1 depicts results of screening of different plant extracts for various phytochemical constituents. AQERC showed the presence of alkaloids, proteins, anthraquinone glycosides, flavonoids, saponins, sterols, tannins, and carbohydrates. ALERC was found to contain alkaloids, flavonoids, saponins, steroids, and tannins.

Histamine-induced bronchospasm in guinea pigs:

The results are summarised in Tables 2. Histamine aerosol produced bronchoconstriction in all control group animals. All control animals showed convulsion during the first 3 min of the experiment. The aqueous and alcoholic extracts were significantly protected animals ($P < 0.01$) from histamine-induced bronchoconstriction. Prior treatment of AQERC (200, and 300 mg/kg, i.p.) offered significant protection to guinea-pigs against histamine aerosol-induced bronchospasm ($P< 0.01$). ALERC at the doses 200, and 300 mg/kg, i.p. significantly protected guinea-pigs against histamine aerosol-induced bronchospasm ($P < 0.01$). Mepyramine (a standard anti-histaminic drug, 8 mg/kg, i.p.) significantly protected 90% of animals from asphyxia ($P < 0.001$). However, prior treatment with AQERC (100 mg/kg), and ALERC (100 mg/kg) could not offer protection from the development of asphyxia produced by histamine aerosol.

Table 1

Compounds	Name Of The Test	Leaf Extracts	
		Aqueous	Alcoholic
Alkaloids	Dragendroff's test	+	-
	Mayer's test	-	+
	Wagner's test	-	-
	Borntrager's test	+	+
Anthraquinone	Modified Borntrager's test	-	-
	Molisch's test	-	+
Carbohydrates	Anthrone reagent test	+	+
	Benedict's reagent test	+	+
Cardiac Glycosides	Kellar Kilani test	-	-
	Potassium hydroxide test	-	-
Flavonoids	Shinoda test	+	-
	Ammonia test	-	+
Glycosides	Benodict's test	+	-
	Fehling's test	-	-
Phenols	Ferric chloride test	-	-
	Phosphoimolybdic acid test	-	-
Phlobatannins	Hydrochloric acid test	-	-
	Xanthoprotein teswt	-	-
	Biurete test	-	-
Proteins	Million's reagent test	+	-
Quinines	Sodium hydroxide test	-	-
Reducing sugar	Benedict's reagent test	-	-
Resins	Turbidity test	+	+
Saponins	Foam test	+	+
Steroids/Terpenoids	Salkowski test	+	+
	Liberman-Burchard test	-	-
Tannins	Bramer's test	+	+

'+' found to be present, '-' found to be absent

Effect of aqueous and alcoholic extracts of *R. cordifolia* leaves on acetylcholine-aerosol-induced bronchospasm in guinea pigs:

Table 2

Treatment	Dose (mg/kg, i.p.)	Protection (%)
Control	Saline, 1ml/kg	-
Mepyramine	8	90
AQERC	100	60
	200	80
	300	85
ALERC	100	64
	200	80
	300	80

*Values are mean ± S.E.M. (n = 5); ns Not significant, **$P < 0.01$ vs. control; Dunnett's *t*-test after One-way ANOVA.

Acetylcholine-induced bronchospasm in guinea pigs:

The results are summarised in Table 3. Acetylcholine aerosol produced bronchoconstriction in all animals of control group. The aqueous and alcoholic extracts were significantly protected animals ($P < 0.01$) from acetylcholine-induced bronchoconstriction. Prior treatment of AQERC (200, and 300 mg/kg, i.p.), and ALERC (200 and 300mg/kg, i.p.) has offered significant protection ($P < 0.01$) to guinea-pigs against acetylcholine aerosol-induced bronchospasm. AQERC (100 mg/kg, i.p.), and ALERC (100 mg/kg, i.p.) could not offer protection from the development of asphyxia produced by acetylcholine aerosol. Atropine sulfate (a standard anti-muscarinic drug, 2 mg/kg, i.p.) significantly prolonged pre-convulsion time to 460 ± 4.33 sec ($P < 0.01$) and protected animals from asphyxia

Table 3

Treatment	Dose (mg/kg, i.p.)	Preconvulsion time(sec)	Protection (%)
Control	Saline, 1ml/kg	128±2.72	-
Standard	2	460±4.33	72.17
AQERC	100	193±1.38	33.67
	200	287±2.98	55.4
	300	280±2.32	54.28
ALERC	100	203±3.74	36.94
	200	298±5.03	57.04
	300	295±5.05	56.61

*Values are mean ± S.E.M. (n = 5); ns Not significant, **$P < 0.01$ vs. control; Dunnett's *t*-test after One-way ANOVA.

DISCUSSION

In this study, an attempt has been made to evaluate antiasthmatic activity of *Rubia cordifolia* in the experimental animals. The results of the study indicated that antiasthmatic effect has shown significant protection of *R.cordifolia* against Histamine and Acetyl Choline induced in rodents.

The acute toxicity was determined by the method of Lorke (1983), which utilizes less number of mice and provides a 24-h LD50 value, which is adequate for most practical purposes. The LD50 values for AQERC were found to be 2262.7 mg/kg, p.o. The LD50 values ALERC were found

to be 1131.4 mg/kg. These LD50 values fall within the practically non-toxic range (Loomis, 1978). Based on these results, doses of 100, 200, 300 mg/kg of different plant extracts were selected for various animal models. The antiasthmatic action was evaluated by sub-effective doses of *R.cordifolia* extracts.

Histamine and acetylcholine antagonists can be conveniently recognized and assayed by their ability to protect guinea pigs against lethal effects of bronchospasm induced by histamine and acetylcholine, respectively (Broadbent & Bain, 1964). The results of the present study showed that prior treatment of aqueous and alcoholic extracts of *R.cordifolia* protected the animals to a significant extent from the development of asphyxia produced by both the spasmogens. This is indicative of antihistaminic and anticholinergic activities of the *R.cordifolia*. Interestingly, the effect was significantly higher in both aqueous and alcoholic plant extracts when compared to control.

In the present study, guinea pigs were used because of the extreme sensitivity of their airways to the primary mediators of bronchoconstriction, including histamine and leukotrienes, and their ability to be sensitized to foreign proteins. Moreover, the resemblance of pulmonary responses and anaphylactic sensitization to histamine challenge in both guinea pigs and humans made this species the model of choice. Although there is no perfect model of asthma which simulates asthmatic patients, guinea pig airways react to histamine, acetylcholine, leukotrienes, and other bronchoconstrictors in a manner similar to that seen in humans (Popa et al., 1973; Agrawal et al., 1991). Another similarity between the guinea pig model and asthmatic patients is that enhanced bronchoconstriction occurs in both species following sensitization, in response to α-adrenergic antagonists (Matsumoto et al., 1994). Thus, the guinea pig model resembles the human allergic pathology in several aspects, especially in terms of mediator release.

The role of histamine and acetylcholine in asthma is well established (Nelson, 2003). In the early stage of asthma, release of inflammatory mediators like histamine, tryptase, acetylcholine, leukotrienes, and prostaglandins are triggered by exposure to allergens, irritants, cold air or exercise (Bosquet et al., 2000). Some of these mediators directly cause acute bronchoconstriction. Spasmolytic drugs like α-adrenergic agonists, xanthine derivatives, and anticholinergics are used as quick relief medications in such acute asthmatic attacks (Horwitz & Busse, 1995).

In the present study, we have used histamine and acetylcholine as spasmogens in the form of aerosols to cause immediate bronchoconstriction in guinea pigs. Mepyramine (8 mg/kg, i.p.) and atropine sulphate (2 mg/kg, i.p.) were used as reference standard against histamine and acetylcholine-induced bronchospasm respectively (Shah & Parmar, 2003). The *R.cordifolia* have shown significant bronchoprotection against both the types of spasmogens as compared to control. AQERC and ALERC showed significantly protection from histamine-induced bronchoconstriction and anticholinergic action in the acetylcholine-induced bronchoconstriction. Ayurveda has recommended a number of plants for the treatment of asthma and other allergic disorders and has been successful in controlling the disease as well (Zhimmet & Tashkin, 2000). Large numbers of medicinal plant preparations have been reported to possess bronchodilatory effects; some of these include *Adhatoda vasica* (Amin & Mehta, 1959), *Benincasa hispida* (Kumar & Ramu, 2002), *Albizzia lebbeck* (Tripathi & Das, 1977), *Cissampelos sympodialis* (Thomas et al., 1997), and *Sarcostemma brevistigma* (Saraf & Patwardhan, 1988). Phytoconstituents like alkaloids and flavonoids are attributed to possess bronchodilatory activity (Amin & Mehta, 1959; Saraf & Patwardhan, 1988). Qualitative phytochemical investigation of

ethanolic extracts which are present as ingredients in the aqueous and alcoholic extracts of *R.Cordifolia*, have shown presence of these constituents. Therefore, the results of present investigation suggested that, leaves extracts have significant bronchodilatory activity.

CONCLUSION

Among several respiratory diseases affecting man, bronchial asthma is the most common disabling syndrome. The therapy of asthma usually employs steroids or disease modifying drugs. The long-term use of these, however, may not limit the disease progression. All of these drugs have side effects and the search for a novel anti-asthmatic drug continues. There is a need to identify effective and safe remedies to treat bronchial asthma (Govindan et al., 1999). The herbal medicines are rich for the treatment of asthma, selected plant might prove to be the most potent for the treatment of asthma and the side effects of the allopathic treatment can be reduced. For this purpose, *Rubia cordifoolia* has been used as ayurvedic medicine to cure asthma and other disease.

The present study demonstrates the potent mast cell stabilizing activity, anti-histaminic activity, anti-cholinergic activity of leaves of *Rubia cordifoolia* medicinal plants in different models of asthma thereby indicating the possibility of developing a herbal drugs as cheaper, safer and potent anti-asthmatic therapeutic agent. The AQERC and ALERC have produced protective effect against asphyxia. The selected plant species have been used in the traditional systems of medicine for treating various ailments. Importantly, there was no scientific evidence for the anti-asthmatic activity of these plants.

The present study validates traditional claims of these plants. Although the results from this study are quite promising for the use of *Rubia cordifoolia* as a medicinal agent for asthma, several limitations exist in the current literature. Further studies are suggested to establish the antiasthmatic activity by conducting clinical trials and also to isolate and characterize the active principle/s responsible for the action.

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