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# *IN VITRO* AND *IN VIVO* EVALUATION OF ANTI-ASTHMATIC ACTIVITY OF *EUGENIA JAMBOLANA BARK*

**Chichula Gouthami*[1], Dr. Neelam I[2], Dr. Nagaraj.M Kulkarni[3], G Vijaya Kumar[4]**

[1]Department Of Pharmacology, Kgr Institute Of Technology And Management, Rampally, Secunderabad, Telangana 501302.

[2]M Pharm Phd, Associate Prof,Kgr Institute Of Technology And Management, Rampally, Secunderabad, Telangana 501302.

[3]M Pharm Phd, Prof,Kgr Institute Of Technology And Management, Rampally, Secunderabad, Telangana 501302.

[4]B. Pharm ,M Tech Phd Prof And Principal Of Kgr Institute Of Technology And Management, Rampally, Secunderabad, Telangana 501302.

**Abstract:**

*Asthma is a chronic respiratory disorder associated with airway hyperresponsiveness, bronchoconstriction and inflammatory changes in the airways. The present study was undertaken to evaluate the in vitro and in vivo anti-asthmatic activity of Eugenia jambolana bark extracts. The bark was extracted using aqueous and alcoholic solvents to obtain aqueous extract of Eugenia jambolana (AQEEJ) and methanolic extract of Eugenia jambolana (MEEJ). The percentage yields of the aqueous and alcoholic extracts were reported as 20.48% w/w and 11.45% w/w, respectively. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids/terpenoids, carbohydrates, resins and other phytoconstituents.*

*In the guinea pig ileum preparation, both extracts inhibited acetylcholine-induced contraction, with AQEEJ producing comparatively greater inhibition than MEEJ. The acute toxicity observations reported in the study showed no significant behavioural or morphological toxic manifestations at the investigated doses. The observed inhibitory effects against histamine- and acetylcholine-induced smooth-muscle responses indicate that Eugenia jambolana bark extracts possess promising preliminary anti-asthmatic activity. The activity may be associated with the bioactive phytoconstituents present in the extracts. Further well-controlled in vivo studies and mechanistic investigations are required to establish the efficacy, safety and therapeutic potential of Eugenia jambolana bark extracts in asthma.*

***Keywords:*** *Eugenia jambolana; Bark extract; Anti-asthmatic activity; Histamine; Acetylcholine; Guinea pig ileum; Bronchoconstriction; Phytochemical screening.*

**Corresponding author:**

**Chichula Gouthami**,
*Department of Pharmacology,*
*KGR Institute Of Technology And Management,*
*Secunderabad, Telangana.*
*Mobile:*
***Email Id:*** *patelgouthami703@gmail.com*

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## 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-induced asthma[6]. Table 1.1 shows the main features of these disorders.

### Historical background

The modern school of thought regarding asthma as a disease is so powerful that it is difficult to imagine asthma, as it was once conceptualized. During the seventeenth century, Once English physicians Thomas Willis and Sir John Floyer began arguing that asthma was different from other breathing disorders and is the same from person to person. They mentioned that asthma, as a specific form of disordered breathing, must be treated differently from other forms of breathlessness cases. By the late nineteenth century, physicians believed that asthma was a disease which had a specific set of causes, clinical consequences, and requirements for treatment, despite the diversity of individual experiences as shown in Table 1.1. Because of the spectrum of severity within asthma, some people with asthma only rarely experience symptoms, usually in response to triggers, where as other more severe cases may have marked airflow obstruction at all times [1].

**Table 1.1. Classification of asthma** [1]

| Severity | Symptom Frequency | Symptoms at night | Peak expiratory flow rate or $FEV_1$ of predicted | Variability of peak expiratory flow rate or $FEV_1$ |
|---|---|---|---|---|
| Intermittent | <once a week | ≤ twice per month | ≥80% predicted | <20% |
| Mild Persistant | >once per week but<once per day | >twice per month | ≥80% predicted | 20-30% |
| Moderate persistent | Daily | >once per week | 60-80% predicted | >30% |
| Severe | Daily | Frequent | <60% predicted | >30% |

Asthma exists in two states: the steady-state of chronic asthma, and the acute state of an asthma exacerbation. The symptoms are different depending on what state the patient is in. Common

symptoms of asthma in a steady-state include: nighttime coughing, shortness of breath with exertion but no dyspnea at rest, a chronic 'throat-clearing' type cough, and complaints of a tight feeling in the chest. Severity often correlates to an increase in intensity of symptoms. Symptoms can worsen gradually and rather insidiously, up to the point of an acute exacerbation of asthma. It is a common misconception that wheezing is common in patients with asthma-some never wheeze, and their disease may be confused with another Chronic Obstructive Pulmonary Disease (COPD) such as emphysema or chronic bronchitis. An acute exacerbation of asthma is commonly referred to as an asthma attack. The cardinal symptoms of an attack are shortness of breath (dyspnea), wheezing, and chest tightness[7]. Some patients present primarily with coughing, and in the late stages of an attack, air motion may be so impaired that no wheezing may be heard. When present the cough may sometimes produce clear sputum. The onset may be sudden, with a sense of constriction in the chest, breathing becomes difficult, and wheezing occurs (primarily upon expiration, but can be in both respiratory phases). Asthma is classified according to the frequency of symptoms, Forced Expiratory Volume (FEV1) and peak expiratory flow rate (PEFR) [8]. Though symptoms may be very severe during an acute exacerbation, between attacks a patient may show few or even no signs of the disease[9].

**Epidemiology**

Asthma has no standard definition. Attempts to define asthma have generally resulted in descriptive statements invoking notions of variable airflow obstruction over short periods of time, sometimes in association with markers of airway hyper-responsiveness and cellular pathology of the airway[10].

Asthma is diagnosed clinically on the basis of symptoms of wheeze, dyspnea, and cough, and by objective evidence of variable airflow obstruction. Allergic asthma can present for the first time at any age, but incidence is highest in childhood[11]. Asthma in childhood frequently remits during adolescence but can recur in adult life[12]. Asthma in adults tends to persist for life and is probably associated with an accelerated decline in lung function[13]. Deaths from asthma are now uncommon, especially in children and young adults; in the past they have occurred in epidemics thought to have been caused by use of high dose formulations of relatively unselective β-agonist drugs.[14]

Asthma is an airway disease that can be classified physiologically as a variable and partially reversible obstruction to air flow, and pathologically with overdeveloped mucus glands, airway thickening due to scarring and inflammation, and bronchoconstriction, the narrowing of the airways in the lungs due to the tightening of surrounding smooth muscle. Bronchial inflammation also causes narrowing due to edema and swelling caused by an immune response to allergens[1].

**1.3 Bronchoconstriction**

During an asthma episode, inflamed airways react to environmental triggers such as smoke, dust, or pollen. The airways narrow and produce excess mucus, making it difficult to breath. In essence, asthma is the result of an immune response in the bronchial airways. The airways of asthma patients are "hypersensitive" to certain triggers, also known as ‘stimuli’ (see below). (It is usually classified as type I hypersensitivity). In response to exposure to these triggers, the bronchi (large airways) contract into spasm (an "asthma attack"). Inflammation soon follows, leading to a further narrowing of the airways and excessive mucus production, which leads to coughing and other breathing difficulties. Bronchospasm may resolve spontaneously in 1–2 hours, or in about 50% of subjects, may become part of a 'late' response, where this initial insult is followed 3–12 hours later with further bronchoconstriction and inflammation. The normal caliber of the bronchus is maintained by a balanced functioning of these systems, which both operate reflexively. The parasympathetic reflex loop consists of afferent nerve endings which originate under the inner lining of the bronchus. Whenever these afferent nerve endings are stimulated (for example, by dust, cold air or fumes) impulses travel to the brain-stem vagal center, then down the vagal efferent pathway to again reach the bronchial small airways. Acetylcholine is released from the efferent nerve endings. This acetylcholine results in the excessive formation of inositol 1, 4, 5-trisphosphate (IP3) in bronchial smooth muscle cells which leads to muscle shortening and this initiates bronchoconstriction[3].

**1.4 Triggers of asthma** (Stimulus that aggravate asthma response)

- Allergens from nature typically inhaled, which include waste from common household pests, the house dust mite and cockroach, as well as grass pollen, mold spores, and pet epithelial cells[15].
- Indoor air pollution from volatile organic compounds, including perfumes and perfumed products. Examples include soap, dishwashing liquid, laundry detergent, fabric softener, paper tissues, paper towels, toilet paper, shampoo, hairspray, hair gel, cosmetics, facial cream, sun cream, deodorant, cologne,

shaving cream, aftershave lotion, air freshener and candles, and products such as oilbased paint [15]

- Association between some medications, including aspirin[16], ß-adrenergic antagonists (ß-blockers), and food allergies such as milk, peanuts, and eggs with asthma have been shown. However, not all people with food or other allergies have asthma [15]
- Use of fossil fuel related allergenic air pollution, such as ozone, nitrogen dioxide, and sulfur dioxide, which is thought to be one of the major reasons for the high prevalence of asthma in urban areas[7].
- Various industrial compounds and other chemicals, notably sulfites; chlorinated swimming pools generate chloramines-monochloramine, dichloramine and trichloramine in the air around them, which are known to induce asthma[17].
- Early childhood infections, especially viral upper respiratory tract infections. Children who suffer from frequent respiratory infections prior to the age of six are at higher risk of developing asthma, particularly if they have a parent with the condition. However, persons of any age can have asthma triggered by colds and other respiratory infections even though their normal stimuli might be from another category (e.g. pollen) and absent at the time of infection. In many cases, significant asthma may not even occur until the respiratory infection is in its waning stage, and the person is seemingly improving[7]. In children, the most common triggers are viral illnesses such as those that cause the common cold.
- Exercise or intense use of respiratory system in one or other way can also stimulate asthma attack. These are thought to be primarily in response to the exposure of the airway epithelium to cold, dry air.
- Hormonal changes in adolescent girls and adult women associated with their menstrual cycle can lead to a worsening of asthma. Some women also experience a worsening of their asthma during pregnancy, whereas others find no significant changes, and in other women their asthma diminished during their pregnancy[7].
- There is growing evidence that psychological stress is a trigger. It can modulate the immune system, causing an increased inflammatory response to allergens and pollutants. Cold weather can make it harder for patients to breathe. Whether high altitude helps or worsens asthma is debatable and may vary from person to person[18].

### 1.5 Bronchial inflammation

The mechanisms behind allergic asthma- i.e., asthma resulting from an immune response to inhaled allergens - are the best understood of the causal factors. In both people with asthma and people who are free of the disease, inhaled allergens that find their way to the inner airways are ingested by a type of cell known as antigen-presenting cells (APCs). APCs then "present" pieces of the allergen to other immune system cells. In most people, these other immune cells (T0 cells) "check" and usually ignore the allergen molecules. In asthma patients, however, these cells transform into a different type of cell (Th2), for reasons that are not well understood. The resultant Th2 cells activate an important arm of the immune system, known as the humoral immune system. The humoral immune system produces antibodies against the inhaled allergen. Later, when a patient inhales the same allergen, these antibodies "recognize" it and activate a humoral response. Inflammation results: chemicals are produced that cause the wall of the airway to thicken, cells which produce scarring to proliferate and contribute to further 'airway remodeling', causes mucus producing cells to grow larger and produce more and thicker mucus, and the cell-mediated arm of the immune system is activated. Inflamed airways are more hyper-reactive, and will be more prone to bronchospasm. The "hygiene hypothesis" postulates that an imbalance in the regulation of these Th2 cell types in early life leads to a long-term domination of the cells involved in allergic responses over those involved in fighting infection. The suggestion is that for a child being exposed to microbes early in life, taking fewer antibiotics, living in a large family, and growing up in the country stimulate the Th1 response and reduce the odds of developing asthma[19].

## MATERIALS AND METHODS:

### Drugs and Chemicals

Drugs and Chemicals Compound disodium chromoglycate (DSCG), histamine dihydrochloride, acetylcholine chloride, atropine sulfate, mepyramine meleate, and toluidine blue were purchased from CAL Laboratories, Supplied by Kasliwal Brothers, Indore. Histamine solution was freshly prepared in normal saline (NaCl, 8.5 g/l). All the other chemicals were of analytical grade.

**Extraction**



**Figure 7.1 Soxhlet apparatus for the extraction of different plant Extracts**

**Collection, identification and authentication of plant materials**

The bark of *Eugenia Jambolana* was collected. The collected plant samples were deposited in the Department of Pharmacology, Sura labs, Dilsukhnagar, India for future reference.

**Preparation of the test extracts**

The polyherbal formulation was suspended in 1% SCMC in distilled water and administered orally or intraperitonially. The control animals were given an equivalent volume of SCMC vehicle. The standard group received various standard anti-asthmatic Drugs.

**Preparation of plant extracts**

**Collection of plant materials:**

The bark of *Eugenia Jambolana Bark* was collected from the outfield of Nimar Region, MP, India in February-March 2013. The plant materials were identified and authenticated by Dr. Rao, HOD, Botany and Plant Anatomy, Khargone, India.

**Phytochemical screening of aqueous and ethanolic extracts of *Eugenia Jambolana Bark***

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 were 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:

**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.

**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% CuSO4 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.

**Acute Toxicity Test (Determination of LD50)**
The acute toxicity test (LD50) was determined according to the procedure described by Kabore Adama, Albino mice (20–25 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 intraperitoneally (i.p.), 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 250, 500, 1000 mg/kg were chosen and administered i.p. respectively to three groups of one mouse 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 polyherbal formulations the polyherbal formulations was composed of ethanolic extracts of Solanum xanthocarpum (50 mg), Aegle marmelos (50 mg), Caesalpinia bonduc(100 mg), and Murraya koenigii(100 mg).

**Experimental Animals**

**Mast Cell Degranulation Studies**

Albino rats of either sex were divided into 6 groups of six animals each and sacrificed by cervical dislocation. The animals were immediately injected with 15 ml of prewarmed (37°C) buffered salt solution (NaCl 137 mM; KCl 2.7 mM; $MgCl_2$; 1 mM; $CaCl_2$ 0.5 mM; $NaH_2PO_4$ 0.4 mM; Glucose 5.6 mM; HEPES 10 mM) into the peritoneal cavity, and massaged gently in this region for 90 s, to facilitate cell recovery. A midline incision was made and the peritoneum was exposed. The pale fluid was aspirated using a blunted plastic Pasteur pipette, and collected in a plastic centrifuge tube. The fluid was then centrifuged at 1000 rpm for 5 min, and the supernatant was discarded to reveal a pale cell pellet. The cell pellets were resuspended in fresh buffer and recentrifuged.

The peritoneal cell suspension divided in six parts viz. – ve control, +ve control, reference standard (Ketotifen 10 µg/ml) and ethanolic extract of *Eugenia jambolana* at different concentration i.e., 600, 800, 1000 µg/ml, each containing 0.1 ml of cell suspension and incubated at constant temperature 37°C in water bath for 15 min. Then 0.1 ml of compound 48/80 was added in all samples except in – ve control group and suspensions were further incubated for 10 min at 37°C. The cells were then stained with 10% of toludine blue solution and observed under the higher magnification by microscope. The percent granulated and degranulated mast cells were counted in each group.

**Antihistaminic and anticholinergic studies on guinea pigs**

Antihistaminic and anticholinergic studies were conducted on guinea pigs (350-500 g) of either sex. For mast cell stabilization paradigm, adult Wistar albino rats weighing 140–160 g of either sex were used. All the Experimental animals were fed on commercial pellet diet (Poshak Ahar Pvt.ltd. India). They were group housed under standard conditions of temperature (22 ± $2^0$C), relative humidity (60 ± 5%) and 12:12 light/dark cycle, where lights on at 0700 and off at 1900 h). They were divided in groups of ten animals each.

Group 1. The saline fed group served as control

Group 2. Treated with a standard drug. Before experimentation, the animals were kept on fast for 24h but water was given ab de libitum.

During experiments, animals were also observed for any alteration in their general behavior.

All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC).

***In-vitro* Anti-asthmatic Evaluation:**

**Isolated Goat Tracheal Chain Preparation:** The goat trachea was collected from the slaughterhouse from freshly slaughtered goat and was immediately transferred to a thermostat flask containing cold Krebs's Hansleit solution (4°C). The trachea was kept in Krebs's Hansleit solution at 4 °C in the refrigerator until used on the next day. The Goat trachea was cut transversely between cartilage segments to give several rings of tracheal muscle. The bioassays of goat tracheal preparation in 10 µg/ml of histamine in Krebs's Hansleit solution and presence of 100 µg/ml of plant extract were carried out to obtain the differences in the dose-response curve. The percentage maximum contractile response was plotted to generate the dose-response curve of histamine in the absence and presence of the plant extract.

**Isolated Guinea Pig Ileum Preparation:**

Overnight fasted guinea pigs of either sex weighing 400-600 g were sacrificed using the head blow stunning method. The animal was quickly dissected, and the ileum was taken out, mounted in an organ bath containing 10 ml Tyrode solution under the basal load of 500 mg. The solution was continuously aerated with 6-8 bubbles/minute at 37.5 °C temperature. The responses of the ileum to the drug were recorded on smoked kymograph paper on Sherrington rotating drum. The tissue was allowed to equilibrate for 30 min, during which the bathing solution was changed every 10 min. The bioassays of ileum preparation in 10 µg/ml of histamine in Tyrode solution and 100 µg/ml of plant extract were carried out to obtain the differences in the dose-response curve. The contractile responses of the ileum to agonist acetylcholine were also recorded in the presence and absence of plant extract.

***In-vivo* Anti-asthmatic Evaluation**

**Pharmacological Screening (Antiasthmatic paradigms)**

**Histamine-induced Bronchospasm in guinea pigs**

The guinea pigs fasted for 24 h were exposed to an atomized 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). 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 (PCD). By

observation experience was gained so that the preconvulsion 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 ten animals each. Mepyramine (8 mg/kg) and polyherbal formulation (300 mg/kg) were administered intraperitonially 30 min prior to exposure. Animals, which did not develop typical asthma within 6 minutes were taken as protected.

**Acetylcholine-induced bronchospasm in guinea pigs**

Procedure was similar to the histamine aerosol study except that animals were exposed to aerosol of 0.5% acetylcholine in another set of animals (n = 10). Atropine sulphate (2 mg/kg) was used as a standard drug. Mast cell degranulation by compound 48/80 this was carried out as per the method described by Kaley and Weiner (1971) with little modification. Male albino rats were sacrificed by cervical dislocation. The animals were immediately injected with 15 ml of prewarmed (37°C) buffered salt solution (NaCl 137 mM; KCl 2.7 mM; MgCl 2; 1 mM; CaCl20.5 mM; NaH2PO40.4 mM; Glucose 5.6 mM; HEPES 10 mM) into the peritoneal cavity, and massaged gently in this region for 90 s, to facilitate cell recovery. A midline incision was made and the peritoneum was exposed. The pale fluid was aspirated using a blunted plastic Pasteur pipette, and collected in a plastic centrifuge tube. The fluid was then centrifuged at 1000 rpm for 5 min, and the supernatant discarded to reveal a pale cell pellet. The cell pellets were re-suspended in fresh buffer and re-centrifuged. Aliquots of the cell suspension were incubated with the test compounds or disodium cromoglycate, before challenge with compound 48/80. The aliquots were carefully spread over glass slides and the mast cells were stained with 1% toluidine blue and counterstained with 0.1% light green. The slides were dried in air and the mast cells counted from randomly selected high power objective fields (X450). The effect of polyherbal formulation on mast cells was studied by incubating the mast cells for 10 min with the above formulation in a concentration of 1, 10 or 100 µg/ml. In another set of experiments the mast cells which were pre-incubated with polyherbal formulations were exposed to the mast cell degranulator, compound 48:80 (10 µg/ml) and the incubation continued for a further 10 min. Then, the mast cells were carefully spread over glass slides. The percent degranulation of the mast cells was calculated. Disodium cromoglycate (DSCG) (20 µg/ml) was included in one of the study groups for comparison.

**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 $P < 0.05$.

## 8. RESULTS AND DISCUSSION:

**Extraction**

Dried leaf powder of *Tamarindus indica Seed* (500 gm) were extracted with water and alcohol (95% w/v) to get concentrated aqueous and alcoholic extract. The yields of extracts were as follows: AQEEJ (20.48% w/w), MEEJ (11.45% w/w).

**Phytochemical screening of different plant extracts**

Table 8.1: depicts results of screening of different plant extracts for various phytochemical constituents. AQEEJ showed the presence of alkaloids, proteins, anthraquinone glycosides, flavonoids, saponins, sterols, tannins, and carbohydrates. MEEJ was found to contain alkaloids, flavonoids, saponins, steroids, and tannins.

**Table 8.1: Phytochemical screening of different plant extracts**

| 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

**Acute toxicity test (Determination of LD50)**

The LD50 of the AQEEJ 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.

***Effect of ethanolic extract of Elephantopus scaber on induced mast cell degranulation in albino rats***

| ***Treatment*** | ***Conc. (µg/ml)*** | ***Mast cells*** | |
|---|---|---|---|
| | | ***Granulated %*** | ***Degranulated %*** |
| -ve control | - | 86.10 ± 2.10 | 10.63 ± 1.60 |
| +ve control | - | 22.23 ± 2.63 | 77.30 ± 1.12 |
| Ketotifen | 10 | 75.13 ± 1.15* | 23.45 ± 3.54 |
| MEEJ | 600 | 35.76 ± 3.43# | 60.10 ± 2.36 |
| MEEJ | 800 | 52.63 ± 1.60* | 46.12 ± 2.11 |
| MEEJ | 1000 | 60.14 ± 1.92* | 39.33 ± 2.42 |

**MEEJ = *Ethanolic extract of Eugenia jambolana, values are expressed as Mean ± SEM, n = 6, * P<0.001, # P<0.01 as compared to positive control group.***

Mast cell degranulation is important in the initiation of immediate responses following exposure to allergens. An attempt was made to find out whether Methanolic extract of *Eugenia jambolana* has any effect on the rate of disruption of mast cells following exposure to compound 48/80, an agent which causes histamine release. The present study, *Eugenia jambolana* offered significant protection against Compound 48/80 induced mast cell degranulation by stabilizing it, which is responsible for the decreasing airway inflammation by preventing release of various inflammatory mediators.

The relaxant effects of ethanolic extract of *Eugenia jambolana* on tracheal chains of guinea pigs might be produced by different mechanisms including stimulation of b-adrenergic receptors, inhibition of histamine H1 receptors, or an anticholinergic property of this plant. The relaxant effects of all concentrations of the extract of *Eugenia jambolana* obtained were significantly lower than control group. These findings suggest probable b-adrenergic stimulatory, muscarinic and/or histamine H1 blocking properties of the plant extract.

## ***IN VITRO* EVALUATION OF ANTI-ASTHMATIC ACTIVITY**

**Evaluation of Anti-Asthmatic Activity Using Isolated Guinea Pig Ileum Preparation:**

The MEEJ and AQEEJ are subjected to a dose-response curve using isolated guinea pig ileum preparation. The percentage decrease in response was more in AQEEJ as compared to MEEJ at a

dose of 100 µgmL-1 as shown in Table 8.3 and Fig. 8.2.

**Table 8.3: showing responses of various extracts of bark of *Eugenia Jambolana* on isolated guinea pig ileum preparation in presence of acetylcholine**

| Groups | Drug (Dose) | % Response | | | | |
|---|---|---|---|---|---|---|
| | | 0.1 ml | 0.2 ml | 0.4 ml | 0.8 ml | 1.6 ml |
| Control | Acetylcholine (10µg/ ml) | 80.20 | 95.36 | 98.63 | 96.25 | 97.30 |
| MEEJ | Acetylcholine (10µg/ml) + MEEJ (100µg/ ml) | 60.14 | 69.36 | 75.96 | 70.10 | 68.01 |
| AQEEJ | Acetylcholine (10µg/ml) + AQEEJ (100µg/ml) | 56.69 | 60.25 | 50.33 | 32.95 | 30.51 |

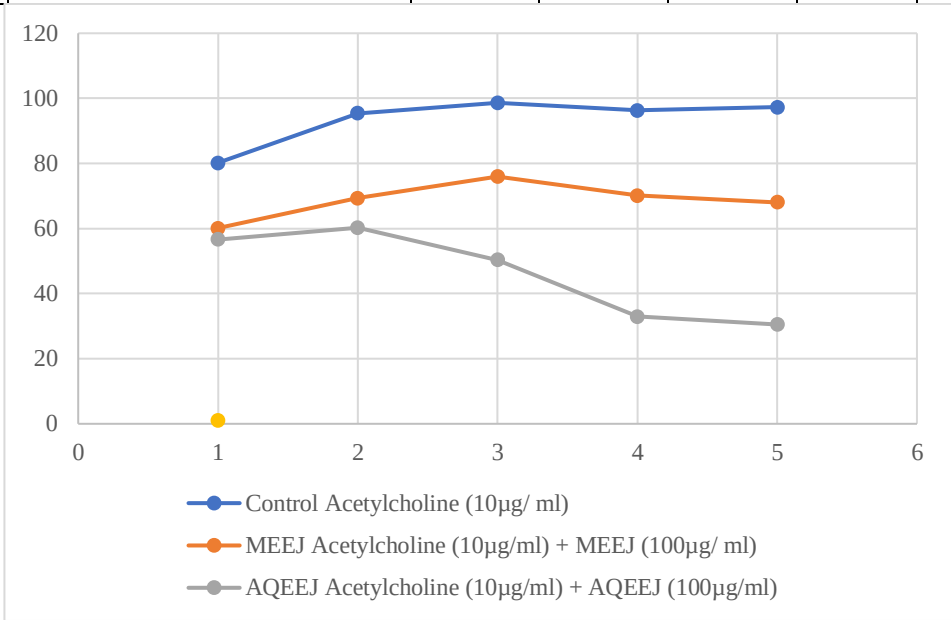



**FIG. 8.2: Showing The Log-Dose Response Curve of Acetylcholine in the Presence and Absence of Guinea Pig Ileum**

## *IN VIVO* EVALUATION OF ANTI-ASTHMATIC ACTIVITY

**Table 8.4: Effect of polyherbal formulation on histamine-aerosol in guinea pigs**

| Treatment | Dose mg/kg,ip | Preconvulsion time | Protection |
|---|---|---|---|
| Control | Saline | 130.2±1.42 | - |
| Mepyramine | 8 | 462±1.24 | - |
| Polyherbal formulation | 300 | 380±2.13 | 52.01 |

Values are mean ± S.E.M. (n = 10); **P< 0.01 vs. control; Dunnett's t-test after way ANOVA

Table 8.5: Effect of polyherbal formulation on acetylcholine induced bronchospasm in guinea pigs

| Treatment | Dose mg/kg,ip | Preconvulsion time | Protection |
|---|---|---|---|
| Control | Saline | 125.1±1.41 | - |
| Atropine Sulphate | 2 | 390±2.15 | - |
| Polyherbal formulation | 300 | 262±1.52 | 59.12 |

Values are mean ± S.E.M. (n = 10); **P< 0.01 vs. control; Dunnett's t-test after way ANOVA.

**Table 8.6: Effect of polyherbal formulation on mast cell**

| Treatment | Dose mg/kg,ip | Weight of granuloma (mg) | Inhibition % |
|---|---|---|---|
| Control | Saline | 120.2±2.41 | - |
| Prednosoline | 10 | 69.1±1.47 | 40.14 |
| Polyherbal formulation | 300 | 62±1.22 | 48.38 |

Values are mean ± S.E.M. (n = 10); **P< 0.01 vs. control; Dunnett's t-test after way ANOVA.

Acetylcholine-aerosol provoked bronchoconstriction in all animals of control group. The polyherbal formulation (300 mg/kg, i.p.) significantly protected animals (P< 0.01) from acetylcholine-induced bronchoconstriction. Mepyramine (a standard antimuscarinic drug, 2 mg/kg, i.p.) significantly prolonged pre-convulsion time to 462 sec (P< 0.01) and protected animals from asphyxia (Table 3). The LD50 of the polyherbal formulation was calculated to be 850.7 mg/kg, i.p. In the present study, we have used histamine and acetylcholine as spasmogens in the form of aerosols to cause immediate bronco constriction in guinea pigs. Mepyramine (8 mg/kg) were used as reference standard against histamine and acetylcholine induced bronchospasm respectively (Kumar A, Ramu P). The bronchodilatory effect of polyherbal formulation was found comparable to the protection offered by both the reference standard drug Mepyramine.

## DISCUSSION:

The present study was undertaken to evaluate the potential anti-asthmatic activity of bark extracts of *Eugenia jambolana* using suitable ***in vitro*** **experimental models**. Both methanolic extract of *Eugenia jambolana* (MEEJ) and aqueous extract of *Eugenia jambolana* (AQEEJ) were investigated and compared with appropriate standard treatments.

**Extraction and Phytochemical Screening**

The extraction process produced aqueous and alcoholic extracts with reported yields of 20.48% w/w and 11.45% w/w, respectively. The preliminary phytochemical screening indicated the presence of several classes of secondary metabolites. The aqueous extract was reported to contain alkaloids, flavonoids, saponins, sterols, tannins, carbohydrates, proteins, resins and related constituents, whereas the alcoholic extract showed alkaloids, flavonoids, saponins, steroids, tannins, carbohydrates and resins.

The presence of flavonoids, tannins, alkaloids, saponins and other phytoconstituents may be pharmacologically relevant because these classes of compounds are commonly associated with biological activities involving smooth-muscle responses, inflammation and oxidative processes. However, the present phytochemical screening is qualitative and therefore does not establish which individual constituent is responsible for the observed anti-asthmatic activity.

**Acute Toxicity Study**

The acute toxicity study reported no significant changes in the normal behaviour of the experimental animals and no apparent toxic symptoms at the doses investigated. The reported toxicity findings were subsequently used as a basis for dose selection for the pharmacological studies.

These observations indicate that the tested extracts were tolerated under the conditions of the reported acute study. Nevertheless, acute toxicity findings alone cannot establish long-term safety, and further subacute or chronic toxicity studies would be required for a comprehensive safety assessment.

***In Vitro*** **Anti-Asthmatic Activity using Isolated Guinea Pig Ileum**

The isolated guinea pig ileum preparation was employed to investigate the effect of the extracts against acetylcholine-induced contraction. Acetylcholine produced a marked contractile response in the control preparation, with responses ranging from 80.20% to 98.63% across the tested concentrations.

Both MEEJ and AQEEJ reduced the acetylcholine-induced response. MEEJ produced responses of 60.14%, 69.36%, 75.96%, 70.10% and 68.01%, whereas AQEEJ produced lower responses of 56.69%, 60.25%, 50.33%, 32.95% and 30.51% at the corresponding concentrations.

The greater reduction in acetylcholine-induced response observed with AQEEJ indicates that the aqueous extract showed stronger inhibitory activity than MEEJ in the isolated guinea pig ileum model. This finding differs from the goat tracheal chain experiment, in which MEEJ showed greater inhibition of the histamine-induced response. Such differences may be related to differences in the phytochemical composition of the extracts and the pharmacological pathways involved in histamine- and acetylcholine-mediated smooth-muscle contraction.

**Overall Interpretation**

Taken together, the in vitro findings demonstrate that both MEEJ and AQEEJ possess inhibitory effects against mediator-induced smooth-muscle responses. MEEJ showed comparatively greater activity against histamine-induced contraction in the goat tracheal chain preparation, whereas AQEEJ demonstrated greater inhibition of acetylcholine-induced contraction in the guinea pig ileum preparation. The differential activity of the two extracts suggests that *Eugenia jambolana* bark may contain multiple phytoconstituents capable of influencing different pathways associated with bronchoconstriction. The observed activity provides preliminary experimental support for further investigation of the bark extracts as potential sources of anti-asthmatic agents.

The results also contain separate tables describing histamine- and acetylcholine-induced bronchoconstriction and mast-cell-related in vivo experiments involving a “polyherbal formulation.” Those results should not be directly attributed to

*Eugenia jambolana* bark unless the experimental records confirm that the formulation consisted exclusively of the *Eugenia jambolana* extracts.

Therefore, based on the clearly identified *Eugenia jambolana* results, the study demonstrates promising ***in vitro*** **anti-asthmatic activity**, particularly through inhibition of histamine- and acetylcholine-induced smooth-muscle responses. Further well-controlled *in vivo* studies are required to establish the anti-asthmatic efficacy and mechanism of action of the extracts.

## CONCLUSION:

The present study was undertaken to evaluate the in vitro and in vivo anti-asthmatic activity of *Eugenia jambolana* bark extracts. The aqueous and methanolic extracts showed the presence of several phytoconstituents, including alkaloids, flavonoids, saponins, tannins, steroids/terpenoids, carbohydrates and resins, which may contribute to the observed pharmacological activity.

The acute toxicity observations indicated that the extracts did not produce significant behavioural or morphological toxic effects at the investigated doses.

Thus, the findings of the present investigation provide preliminary experimental evidence for the anti-asthmatic potential of *Eugenia jambolana* bark extracts, particularly against mediator-induced smooth-muscle contraction. The observed activity may be associated with the phytoconstituents present in the extracts. However, further well-designed in vivo investigations, mechanistic studies, dose-response studies, and detailed safety evaluation are necessary to establish the therapeutic potential of *Eugenia jambolana* bark as an anti-asthmatic agent.

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