8. Discussion
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Bronchial asthma is a chronic inflammatory disease, characterized by both bronchoconstriction and airway inflammation which leads to bronchial hyper-responsiveness to various stimuli, in which many cell types play a role, more important being mast cells, eosinophils and T-lymphocytes (Bousquet et al., 2000).

The present study was aimed to evaluate the antiasthmatic activity of chloroform, ethanol and aqueous extracts of roots, stem and leaves of Clerodendrum serratum Linn.

Morphological examination of drug refers to evaluation by colour, odour, taste, size, shape and special features, like touch, texture etc. It is a technique of qualitative evaluation based on the study of morphological and sensory profiles of whole drugs. Organoleptic evaluation means conclusions drawn from studies resulted due to impression on organs of senses (Kokate et al., 2003). All these parameters were recorded for the plant in order to assess the quality of raw materials used for the study.

Microscopical techniques provide detailed information about the crude drug. Microscopical inspection of crude drugs from plant origin is essential for the identification of the grounded or powdered materials. Though microscopy alone cannot provide complete evaluation profile of a herbal drug, still it can provide supporting evidence, which when combined with other analytical parameters can be used to obtain the full evidence for standardization & evaluation of herbal drugs (Mukherjee, 2002). The important histological findings in case of leaves suggests that the ground tissue is parenchymatous, angular, thin walled and compact. There are five discrete vascular bundles of which three are adaxial bundles and two are abaxial bundles. The mesophyll tissue consists of distinct palisade zone and spongy parenchyma. Stem has a continuous intact epidermis, narrow cortex, cortical sclerenchyma, phloem, xylem and pith. Along the inner boundary of cortex, there are discrete, thick masses of fibres which are lignified. The pith is wide parenchymatous with thin angular compact cells. In the root secondary xylem is unique in the distribution pattern of the vessels. There are central cluster of vessels which are thin walled, wide, angular and solitary. Calcium oxalate sand crystals are abundant in cortical parenchyma along with starch grains.

Controlled incineration of crude drugs results in an ash residue consisting of inorganic materials (metallic salts and silica). This value varies within fairly wide limits
and is therefore an important parameter for the purpose of evaluation of crude drugs. Moreover direct contamination, such as by sand or earth, is immediately detected by the ash value. Sometimes, inorganic variables like calcium oxalate, silica, carbonate content of crude drug affects “total ash” values, such variables are then removed by treating with acid (as they are soluble in hydrochloric acid) and then acid-insoluble ash value is determined (Mukherjee, 2002). The total ash, acid-insoluble ash and water soluble ash were found to be within range.

The separated, cleaned and powdered plant parts i.e Roots, Leaves and Stems were subjected to successive extraction using chloroform, ethanol and water.

Qualitative chemical tests revealed the presence of major phytoconstituents such as flavonoids, saponins, alkaloids and sterols.

The anti asthmatic activity was evaluated by using various *in-vivo* and *in-vitro* animal models like isolated goat tracheal chain preparation, isolated guinea pig ileum preparation, milk-induced leucocytosis, milk-induced eosinophilia, bronchial hyperactivity in guinea pig, passive paw anaphylaxis, clonidine-induced catalepsy in mice and mast cell degranulation and broncohalveolar lavage in rats.

Acute toxicity studies were aimed at establishing the therapeutic index i.e. the ratio between the pharmacologically effective dose and the lethal dose, and also to perform the primary screening. After toxicity studies the doses of the extract were fixed at 50, 100 and 200mg/kg p.o for pharmacological screening.

Histamine is an important mediator of immediate allergic (type-1) and inflammatory reactions. It causes bronchoconstriction by activating H1-receptors. The trachea was used for the experimental purpose rather than the bronchi since it is easier to dissect and has the same reactions to spasmogenic and spasmolytic drugs. Although, the method is known for its suitability in the study of antispasmodic drugs in general, emphasis is given on its use in the testing of bronchodilators. This is because of the close anatomical and physiological association, which exists between tracheal and bronchial musculature (Castillo et al., 1947).

The guinea pig tracheal chain is a classical preparation, but requires some skill to prepare and is not very sensitive for many agonists (Nagchauduri et al., 1974).
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The goat tracheal chain is easier to handle and to prepare; it is also much more sensitive than guinea pig tracheal chain. Therefore, the dose relative contractile responses of different agonists like Acetylcholine, Histamine, 5-Hydroxytryptamine and Bradykinin can be observed in isolated goat trachea. (Nagchauduri et al., 1974). With these agonists, the concentration necessary to produce contraction is generally less with goat tracheal chain than with guinea pig tracheal chain.

It is also reported that isolated goat trachea contracts in response to acetylcholine (0.1-12.8 μg), histamine (0.1-102.4 μg), and barium chloride (0.1-51.2 μg) in a dose dependent manner and to 5-HT in a narrow dose range. Pheniramine maleate (H1-receptor antagonist) blocks contractions to histamine while Cimetidine (H2-receptor antagonist) potentiated the contraction. These observations suggest the presence of both H1-excitatory and H2-inhibitory receptors for histamine on the isolated goat trachea (Kulshrestha, 1983).

In the present study, ethanolic extract of roots of Clerodendrum serratum (concentration of 30 μg/ml perfused in P.S.S.) significantly inhibited (p<0.001) the histamine (10 μg/ml) induced contractions on isolated goat tracheal chain preparation. The inhibition of ethanolic extract of roots was more significant than the others i.e chloroform and aqueous extracts of leaves and stem.

Histamine is one of the major inflammatory mediators in the immediate phase of asthma, causing airway hyper responsiveness and bronchial airway inflammation. The study regarding involvement of H1 and H2 receptors has been done in experimental asthma in guinea pig using respiratory smooth muscles. (Yamatake et al., 1977) and it was confirmed that there is prominent involvement of H1 receptors as compared to H2 receptors especially in asthma (Jena et al., 1994). In the present study ethanolic extract of roots of Clerodendrum serratum inhibited histamine-induced contractions of isolated guinea pig ileum at a concentration 30μg/ml. Thus the possible mechanism through which Clerodendrum serratum inhibits histamine induced contractions of guinea pig ileum may be by blockade of H1 receptors leading to inability of smooth muscle to respond to histamine induced spasm leading to inhibition of bronchoconstriction.

Thus the significant inhibition of histamine-induced contractions produced by ethanolic extract of roots of Clerodendrum serratum on isolated goat tracheal chain
preparation and guinea pig ileum preparation indicates that the plant has antihistaminic (H1-receptor antagonist) action.

Histamine when inhaled has been shown to induce bronchoconstriction by direct H1-receptor activation and also by a neurally mediated bronchoconstrictor effect via vagal reflexes. Histamine has been shown to activate action potentials in intrapulmonary vagal afferents. Thus, the broncho-protective effect of a test drug could be due to its H1-blocking effect or due to a direct bronchodilator effect. (Gokhale and Saraf, 2000).

In the present study, ethanolic extract of roots at the dose of 200mg/kg significantly protected the guinea pigs against histamine-induced broncho-spasm. The guinea pigs exposed to histamine aerosol showed signs of progressive dyspnoea leading to convulsions. The root extract significantly prolonged the latent period of convulsions as compared to control following exposure to histamine aerosol. The standard antihistaminic drug Chlorpheniramine maleate (2 mg/kg, i.p.) used in the study produced a significant increase in the latent period of convulsion at the 1st, 4th and 24th hour. Therefore, the result of present study indicates the utility of the plant in the treatment of asthma and bronchitis by virtue of its broncho-dilating activity.

Clonidine, a α2-adrenoreceptor agonist induces dose dependent catalepsy in mice, which is inhibited by histamine (H1) receptor antagonists but not by H2 receptor antagonist (Jadhav et al., 1983). Histamine acts as a modulator of pre-synaptic catecholamine processes in the CNS by causing depletion of the transmitter stores in the nerve terminals. Muley et al., (1979) showed that intracerebroventricular injection of histamine in conscious mice induced catalepsy, which was inhibited by H1 antagonists but not by H2 antagonist. It is known that Clonidine releases histamine from mast cells (Lakdawala et al., 1980). Schwartz (1977). Brain histamine does play a definite role in the production of the extra pyramidal motor it has been suggested that the cataleptic effect of Clonidine in the mouse be mediated by histamine (via H1 receptors) which is released from brain mast cells in response to stimulation of α2 adrenoreceptors by Clonidine (Balsara, 1983). In the present study, Clonidine produced maximum catalepsy after 120 min. of administration in the vehicle treated group. While, in the groups treated with ethanolic extract of roots (200mg/kg), there was significant inhibition of catalepsy.
The standard drug used, Chlorpheniramine maleate (10 mg/kg, i.p.) has also significantly inhibited the Clonidine induced catalepsy.

Mast cells are widely distributed in the connective tissue, with a preferential localization adjacent to small blood vessels. The mast cells contain basophil granules literally loaded with active substances which, if allowed to escape themselves or via enzymatically formed products, causes vascular and other tissue reactions similar to those characteristic of inflammatory process (Uvnas, 1969). In the rat mast cell granules the histamine concentration has been calculated to be around 0.3M. Uvnas(1969) studied the mast cell degranulation and its correlation with the release of histamine after the administration of compound 48/80, the mast cell degranulating agent. Both clonidine and compound 48/80 act through the dynamic expulsion of granules without causing any damage to the cell wall (Stanworth, 1973). Lakadawala et al., (1980) have shown that clonidine releases histamine from the mast cells in the similar manner to a selective liberator like compound 48/80. It is known that disodium cromoglycate, a standard mast cell stabilizer prevents degranulation of mast cell by raising the cyclic adenosine mono phosphate (Geetha et al. 1981) It has been known that al pharmaceutical agents that increase the cAMP relax airway smooth muscle and inhibit the release of autacoids from the tissue and basophils (Bertelli et al.1973). In the present study, ethanolic extract of roots at the dose of 200mg/kg significantly significantly inhibited the mast cell degranulation and percent protection was found to be 60.51%

Ayurveda provides number of herbs for the treatment of asthma and herbal formulations, which include some anti-stress herbs to enable adoption to stress since excessive stress may aggravate symptoms of asthma (Ryland et al., 2000).

Adaptogens are the medicinal substances that are meant to put an organism into a state of non-specific heightened resistance in order to better resist stress and adaptation to external challenges (Lazarev, 1958). An important feature of the adaptogens is their capacity to increase organism’s resistance to various adverse effects of a physical, chemical and biological nature (Brehman and Dardymov, 1969). An adaptogen may produce normalization that reveals itself irrespective of the direction of previous pathologic shift. After parenteral administration of milk there is increase in TLC, and this
stress full condition can be normalized by administration of an adaptogenic drug. It was demonstrated that there is increase in leukocyte count after parenteral administration of milk (4 ml/kg, s.c.) (Bhargava and Singh, 1981).

In the present study the vehicle treated group of mice, after parenteral administration of milk showed significant increase in leukocyte count, where as the groups in which ethanolic extract of roots(200mg/kg) was administered, have shown normalization of leukocyte count. This indicates the adaptogenic activity of the plant.

Most allergic and non-allergic asthmatics, including those with mild asthma, have bronchial eosinophilia and there is a significant association between eosinophil activation and asthma severity as well as bronchial hyper-responsiveness. The involvement of eosinophils into bronchial mucosa in which allergic inflammation occurs is a critical contributor to the late asthmatic reaction of congestion and mucus hypersecretion. When these cells arrive, they degranulate and perpetuate underlying airway inflammation.

The late asthmatic phase reaction to an allergen often coincides with an increased number of eosinophils in the airway. Eosinophils have also been regarded as one of the major effector cells in this disease, because eosinophils can influence airway function by producing effects on airway remodeling through release of cytotoxic proteins, lipid mediators, oxygen free radicals, cytokines and transforming growth factor-β. Though eosinophils can increase in number in body fluids and tissues, emphasis is placed on the number of eosinophils in blood. It was suggested that increase in total eosinophil count reflects asthmatic activity and used for early detection of exacerbations (Horn et al., 1975). Eosinophil degranulation is an important immunologic mechanism leading to allergic inflammation in cutaneously manifested cow's milk allergy.

In the present study it was observed that, parenteral administration of milk (4 ml/kg, s.c.) to the vehicle treated group significantly increased the eosinophil count after 24 hour, where as, in the groups treated with ethanolic extract of roots (200mg/kg, p.o.) there was significant inhibition of milk induced eosinophilia in mice. Thus the present study shows that Clerodendrum serratum can prevent the release of inflammatory mediators by decreasing the elevated eosionophil count in the asthma patients.
Allergic asthma is a chronic inflammatory process occurring due to exposure of allergen resulting in the activation of T-lymphocytes with subsequent release of inflammatory mediators. Immunomodulating agents are useful in the treatment of asthma by virtue of inhibiting the antigen-antibody (AG: AB) reaction thereby inhibiting release of inflammatory mediators.

Administration of egg albumin (s.c.) to rat raises the antiserum to egg albumin in the plasma and sub plantar injection of plasma containing these antibodies, then challenged with egg albumin leads to passive paw anaphylaxis in rats (Pungle et al., 2003). The present study revealed that in the animals pretreated with ethanolic extract of roots, there was significant reduction (p<0.01) in the paw volume at all the time intervals. The beneficial effect of Clerodendrum serratum could be due to either inhibition AG: AB or anti-histaminic activity.

Allergic inflammation associated with airway hyper reactivity is the main feature of allergic asthma. The inflammatory response is characterized by an increase in the numbers of eosinophils and mast cells (Wardlaw, 1988) mucus hyper secretion and activation of T cells (Lukacs, 2001). Several studies have shown that T-helper type (Th2) cells play a major role in the initiation and maintenance of allergic airway inflammation and asthma through their increased production of Th2-type cytokines (IL-4, IL-5, and IL-13) (Elias, 2003). These inflammatory cytokines, also produced in the bronchial tissue by mast cells, alveolar macrophages and epithelial cells, play a significant role in the pathogenesis of airway inflammation (Busse, 2001). This role has been highlighted in several studies using gene knockout and cytokine ablation approaches (Taube, 2002; Kopf, 1993; Foster, 1996; Herrick, 2003). Th2 cytokines mediate a series of events in the inflammatory cascade leading to the development of allergic asthma. Such events include B cell maturation and IgE isotype switching, activation and regulation of mast cell, eosinophil and neutrophil function, and regulation of chemokine and adhesion molecules and mucus production (Zhu, 1999; Andrew, 1998; de Vries, 1998; Shim, 2001). Ovaalbumin increases the neutrophiles, eosinophils, macrophages, monocytes, leucocytes, lymphocytes epithelial cells and mucus etc. in Bronchoalveolar lavage fluid. The root ethanolic extract of Clerodendrum serratum at dose of 200mg/kg showed
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significant inhibition in TLC and neutrophiles, lymphocytes and monocytes, differential leucocytes count as compared to others.

Thus, the present study demonstrates that the roots of *Clerodendrum serratum* has potential to inhibit allergic reaction induced by immunological as well as chemical stimuli. The plant has also shown adaptogenic and anti allergic activity. This suggests its potential in the treatment and prophylaxis of various allergic conditions including asthma and justifies its use in the treatment of asthma.

Reactive oxygen species (ROS) such as $O_2$, $H_2O_2$ AND $OH$ are highly toxic to the cells. Severe oxidative stress can produce major disturbances on the cell metabolism that includes, DNA, and RNA damage. Intracellular freee ions, other proteins and lipid peroxidation. The lungs are always exposed to higher levels of oxygen than most other tissues that leads to DNA damage by free radical related mechanism. Most other active metabolites in human lungs are superoxide radical, hydroxide peroxide and hydroxyl radical which are generated by multiple enzymatic reactions. Medicinal plants contain a wide range of chemical compounds that could serve as “Leads” in the development of anti asthamatic agents (D.Srinivasarao *et al.*, 2006). In the present study the roots of *Clerodendrum serratum* demonstrated significant antioxidant activity through the DPPH free radical scavenging activity, reducing power assay and scavenging of hydrogen peroxide (Bhujbal *et al.*, 2009) which supports its role in the treatment of asthma.

As the ethanolic extract of the roots demonstrated significant anti asthamatic activity as compared to chloroform, ethanol and aqueous extracts of stems and leaves, it was further subjected to isolation and characterization studies to find out possible chemical constituents responsible for its anti asthamatic action.

Chromatographic studies of the ethanolic extract of roots were performed on Camag HPTLC instrument which enabled to establish the finger printing profile for Apigenin, one of the important phytoconstituent isolated from the roots of the plant.

4. Toluene : ethylacetate (1:4)

Research has demonstrated that flavonoids and saponins are being used in the treatment of asthma and as the phytochemical screening also revealed the presence of these constituents, an attempt was made to isolate the flavonoids and saponins from the roots. The isolation and characterization studies ($^1H$-NMR, FAB-MS and DART-MS)
confirmed the presence of a flavonoid glycoside, Apigenin-7-glucoside\(\text{C}_{21}\text{H}_{20}\text{O}_{10}\) and a new saponin Icosahydropenic acid\(\text{C}_{51}\text{H}_{80}\text{O}_{19}\). Apigenin is known to reduce allergic airway inflammation due to alteration of TH1/TH2 polarization via the suppression of GATA-3 (Jun-Rim Choi et al., 2009, 918-924). Thus the presence of apigenin in the roots of the plant is attributable for its anti asthamatic activity.

Newly isolated saponin Icosahydropenic acid, was also evaluated for anti asthmatic activity by employing isolated goat tracheal chain preparation, isolated guineapig ileum preparation, mast cell degranulation studies in the rats and clonidine induced catalepsy in mice. From the results of acute toxicity studies the isolated saponin at the dose of 100mg/kg was used for the animal studies. In the isolated goat tracheal chain and guinea pig preparation the isolated saponin (concentration of 15 \(\mu\)g/ml perfused in P.S.S.) significantly inhibited \(p<0.001\) the histamine (30 \(\mu\)g/ml) indicating the anti asthmatic action. In the mast cell degranulation studies in the rats the isolated saponin at the dose of 100mg/kg significantly inhibited the mast cell degranulation and percent protection was found to be 59.22\% as compared to standard sod.cromoglycate (50mg/kg) where percent protection was found to be 64.48\%. In the clonidine induced catalepsy studies in mice the clonidine produced maximum catalepsy after 60 min. of administration in the vehicle treated group. While, in the groups treated with isolated saponin (100mg/kg), there was significant inhibition of catalepsy. The standard drug used, Chlorpheniramine maleate (10 mg/kg, i.p.) has also significantly inhibited the clonidine induced catalepsy.

The results of the animal studies confirmed that roots, stems and leaves of the Clerodendrum serratum Linn demonstrated significant anti asthmatic activity in various experimental animal models. This also justifies the traditional claim of the roots in the treatment of asthma. The study thus confirms that the activity exerted by the roots is attributable due to the presence of phytoconstituents like Apigenin, newly isolated pentacyclic triterpenoid saponin, Icosahydropenic acid, alkaloids and various plant sterols.