6.

Discussion
Plants and other natural products have been in use since ages for health and maintenance of life. Standardization and development of reliable quality protocols for herbal formulation is one of such important issues. The objective of quality protocol development activities should be to ensure a 'minimum therapeutic guarantee' to the user. Such objective can be accomplished by ensuring a fairly consistent quality in the raw materials, a validated process control system and adequate checks on the finished product.

Asthma is a disease which claims many lives of people every year. The popularity of shared care in the management of asthma means that general Practitioner, Nurses and Pharmacy Staff are working together to get the best outcome for the patient.

The pathological changes occur in asthma is like airway obstruction due to combination of various factors include spam of airway smooth muscle, edema of airway mucosa, increased mucus secretion, cellular infiltration of the airway walls and injury and desquamation of the airway epithelium. Development of asthma involves both genetic and environmental factors. Exposure to the antigens triggers release of inflammatory mediators includes histamine and products of arachidonic acid metabolism like leukotrienes and thromboxanes which increase airway responsiveness. The cysteinyll leukotrienes LTC$_4$ and LTD$_4$ are potent bronchoconstrictors. Activation of T-cell and their secretion products (Cytokines-CD4 Th2) promote growth and differentiation of inflammatory cells. The principal cytokines involved include interleukin (IL-4), which is necessary for IgE production, IL-5 which is a chemoattractant for eosinophils and granulocyte-macrophage colony stimulating factors which is similar to IL-4 but less potent. Release of
Neuropeptides like substance P, neurotkenin A, and calcitonin gene related peptide cause vascular permeability, mucus secretion, bronchoconstriction and bronchovasodilation. Cholinergic reflex, bronchoconstriction due to inhalation of irritant substances.

Thus the treatment goal of asthma is to prevent chronic symptoms, maintain pulmonary function as near normal as possible, maintain normal activity levels, prevent exacerbations, minimize hospitalization and avoid adverse effect of treatment. The asthma therapies are thus divided into two categories (i) Symptomatic relief which include the drugs like β agonists, theophylline and anticholinergics. They mainly serve as bronchodilators. (ii) Long term control includes anti-inflammatory agents like corticosteroids, mast cell stabilizers and leukotriene modifiers.

Currently available inhaled bronchodilators and anti-inflammatory drugs are effective in most asthmatics, but this palliative therapy required long term daily administration and is associated with serious toxicities (particularly with the steroidal drugs). As a result there is high prevalence of usage of complementary and alternative medicines for treatment of asthma.

Three anti-asthmatic drugs are chosen for development of standardization parameters, evaluation of anti asthmatic activity and their synergistic effect is also checked.

*Albizzia lebbeck* is commonly known as “Shirisa” belonging to family Mimosaceae, used mainly in diseases like leucoderma, bronchitis, piles etc. Tree is mainly distributed in deciduous and semideciduous forest in Asia.

*Euphorbia hirta* is commonly known as “Dudheli” belonging to family Euphorbiaceae, used mainly in diseases like asthma, as galactogogue, to
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treat diabetes etc. Herb is distributed throughout the world most widely in tropical and subtropical countries.

*Sphaeranthus indicus* is commonly known as “Gorakhmundi” belonging to family Asteraceae, used mainly in insanity, asthma, spleen diseases, elephantiasis, anemia, leucoderma etc. plant grows well in rice fields and dry waste places and cultivated in India, Sri Lanka, Africa and Australia from sea level to 1200m altitude.

**Pharmacognostical study:**

*Albizia lebbeck:*

Morphologically bark was thick with varying length, outer surface was buff colored and rough due to fissures and cracks. Inner surface was striated and dark reddish brown. It was having characteristic sternutatory and astringent taste.

Histology of bark revealed important diagnostic features like outgrowth, wide rhytidoma and phloem fibers in group of 20-22 with crystal sheath.

Powder of bark was coarse, granular, brownish, sternutatory odor and astringent and slightly bitter taste. Microscopy of powder revealed important identifying characters like fungal spores, phloem fibers with prisms, tangential and radially cut medullary rays, sclereids and brownish matters.

*Euphorbia hirta:*

Herb was erect with copious crisped hairs with characteristic leaf anatomy like oblong to lanceolate shape, serrulate margin with obliquely cordate base.
Histology of root showed typical structure of dicot root in which 4-5 layers of parenchymatous cortex, narrow phloem and wide wood with uniseriate medullary rays were present. Starch was present throughout the section except cork region. Stem in transverse section showed single layered epidermis with simple multicellular hairs. It was followed by 2-3 layers of collenchyma, 10-15 layers of parenchymatous cortex embedded with pericyclic fibers in group of 10-15. Phloem was narrow with wide wood region with all the four elements and was traversed by uniseriate medullary rays. In the centre, parenchymatous pith was embedded with starch grains. Transverse section of leaf was dorsiventral and transcurrent. It showed characteristic 2-9 celled trichomes and anomocytic stomata. Latex glands were present in mesophyll region which is important characteristic feature of Euphorbiaceae family. In the micrib 3-5 sets of vascular bundles were present.

Powder of herb was light green and fibrous. Microscopy of powder showed simple starch, latex cells, anther head and petal in surface view as identifying characters. Besides this it also showed anomocytic stomata, 2-9 celled trichomes, annular and pitted wood vessels.

Stomatal number of upper epidermis was lower 213 than lower surface 286. Stomatal index was also higher 17.04 for lower surface than upper surface 16.421. Vein islet no and vein termination number were 24.44 and 41.77 respectively. Palisade ratio was 7-8.
Sphaeranthus indicus:

Plant was easily identified with sessile, decurrent obovate to oblong leaves. Pink colored heads were characteristic diagnostic feature to identify the plant.

Microscopy of root showed characteristic brownish tissue called metaderm. It showed pericyclic fibers, stone cells alternating with radially arranged secretory canals in the secondary cortex. Secondary phloem and secondary xylem were traversed by pitted lignified bi-pentaseriate medullary rays. Stem in transverse section showed papillose cuticle covered with 3-5 celled trichomes. In the cortex region it showed discontinuous ring of lignified pericyclic fibers in group of 20-25. Stelar region showed well developed ring of bicollateral vascular bundle surrounding the pith and traversed by lignified, pitted and uniseriate medullary rays. Characteristic feature of leaf histology was abundant trichomes of varying types on both the epidermis. Simple covering 3-4 celled, collapsed cell, knee shaped trichomes which measuring 130.8-145.2µ in length and 29.0-43.5µ in width. Club and clavate shaped glandular trichomes were measuring 43.5-72.5µ in length and 30.2-43.5µ in width. Both the epidermis showed anisocytic stomata. Midrib showed 3-4 collateral vascular bundles associated with group sclerenchymatous cells on either side.

Powder was light greenish brown with aromatic odor and bitter or slightly pungent taste. It showed above mentioned trichomes, pollen grains in pollen sac, balloon shaped trichomes on the calyx and bordered pitted vessels with other wood elements.
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Stomatal number in lower surface 47-54 while for upper surface 16-21, stomatal index in lower surface 30-39 while for upper surface 18-31. Vein islet number is 23-27.

Physicochemical evaluation:

Various type of ash values (Total, acid insoluble and water soluble) were found to be highest in *S. indicus* and lowest in *A. lebbeck* while comparing all drugs chosen for study. These revealed the presence of inorganic materials such as carbonates, silicates and oxalates etc. as heating lost organic materials in the form of CO$_2$ left behind the inorganic compounds.

Water soluble extractive value of two drugs (*E. hirta* and *S. indicus*) was higher than their respective alcohol soluble extractive value which revealed that presence of more water soluble compounds in these drugs. However, alcohol soluble components are more than water soluble components in *A. lebbeck* as evident from its respective extractive value.

Moisture content (loss on drying) of these drugs was found to be in following descending order *E. hirta* > *S. indicus* > *A. lebbeck*. All above variations may be due to different portions of drugs chosen (Table 11). All these parameters of *A. lebbeck* and *S. indicus* were found to be within limits mentioned in Ayurvedic pharmacopoeia$^{103,300}$. No report of these parameters of *E. hirta* available in pharmacopoeia.

Heavy metals analysis of all drugs showed presence of cadmium, lead and arsenic below their respective detection limits thus these could be carried free from heavy metals contamination for further use. (Table 12)
Extractive values for successive extract were varying for each plant according to the presence of non polar and polar components. (Table 13)

Extractive value of successive solvent extraction of Albizzia lebbeck were petroleum ether 0.5 %w/w, benzene 0.7 %w/w, chloroform 1.2%w/w, methanol 8.1 %w/w and water 11.9 %w/w.

Extractive value of successive solvent extraction of Euphorbia hirta were petroleum ether 0.77 %w/w, benzene 0.9 %w/w, chloroform 1.8 %w/w, methanol 9.2 %w/w and water 18.8 %w/w.

Extractive value of successive solvent extraction of Sphaeranthus indicus were petroleum ether 1.5 %w/w, benzene 2.41 %w/w, chloroform 0.95 %w/w, methanol 7.99 %w/w and water 9.9 %w/w.

Albizzia lebbeck and Euphorbia hirta showed more water extractive value indicated presence of more polar components than non-polar components while successive extractive value of Sphaeranthus indicus indicated that the presence of significant amount of non-polar and polar components.

**Phytochemical Studies:**

*Albizzia lebbeck* showed presence of flavanoids, saponins, carbohydrates, tannins, phenolics, phytosterols and triterpenoids.

*Euphorbia hirta* showed presence of flavanoid, carbohydrates, tannins, coumarins, phytosterols and triterpenoids.

*Sphaeranthus indicus* indicated presence of alkaloids, carbohydrates, phenolics, volatile oils, phytosterols and triterpenoids.

TLC studies (Tables 19, 20 & 21) of successive solvent extracts of all drugs were carried out using suitable mobile phase and spraying agent on precoated silica gel G plate. Petroleum ether and benzene extracts of *A. lebbeck* showed the presence of five and three components of different $R_f$
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Extractive values for successive extract were varying for each plant according to the presence of non polar and polar components. (Table 13) Extractive value of successive solvent extraction of *Albizia lebbeck* were petroleum ether 0.5 %w/w, benzene 0.7 %w/w, chloroform 1.2%w/w, methanol 8.1 %w/w and water 11.9 %w/w.

Extractive value of successive solvent extraction of *Euphorbia hirta* were petroleum ether 0.77 %w/w, benzene 0.9 %w/w, chloroform 1.8 %w/w, methanol 9.2 %w/w and water 18.8 %w/w.

Extractive value of successive solvent extraction of *Sphaeranthus indicus* were petroleum ether 1.5 %w/w, benzene 2.41 %w/w, chloroform 0.95 %w/w, methanol 7.99 %w/w and water 9.9 %w/w.

*Albizia lebbeck* and *Euphorbia hirta* showed more water extractive value indicated presence of more polar components than non-polar components while successive extractive value of *Sphaeranthus indicus* indicated that the presence of significant amount of non-polar and polar components.

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TLC studies (Tables 19, 20 & 21) of successive solvent extracts of all drugs were carried out using suitable mobile phase and spraying agent on precoated silica gel G plate. Petroleum ether and benzene extracts of *A. lebbeck* showed the presence of five and three components of different $R_f$
values respectively, same extracts of *E. hirta* showed presence of single component in both. All spots were developed on spraying the plates with Anisaldehyde sulphuric acid reagent further confirmed the presence of triterpenoids and phytosterols. TLC studies of chloroform extract of *A. lebbeck* and *E. hirta* did not respond any color spot on spraying the plate with Dragendorff's reagent further indicating devoid of alkaloids, while same extract of *S. indicus* showed single spot with Dragendorff's reagent confirming the presence of alkaloid component in it. Methanol extract of all drugs showed variable number of components when visualized the plates in UV light, iodine vapours and finally sprayed with ferric chloride reagents indicated presence of coumarins, tannins, phenolic compounds and flavanoids etc. Water extract of these drugs also showed the presence 2-4 components when sprayed the plates with anisaldehyde sulphuric acid reagent revealing the presence of highly polar phytoconstituents like proteins, carbohydrates etc.

**Separation and analysis of phytomarkers:**

*A. lebbeck*

Chromatographically (preparative TLC), pure betulinic acid was isolated in bark of *A. lebbeck* and identified by determining m.p; m.m.p.; co-TLC and overlain of IR spectra with standard betulinic acid (*Fig. 17*). Betulinic acid is reported as anti-anaphylactic biomarker; therefore it was preferred to estimate its content in plant drug and developed HPTLC method. HPTLC data and chromatogram scanned at 523 nm revealed the presence of betulinic acid (*fig 19, 20*) and its content in bark was found to be 0.02 %w/w (*Fig 18, Table 25*)
Method was validated for its validation parameters like linearity 0.9937; precision (%C.V.) i.e. repeatability of measurement 0.382-0.82, repeatability of application 0.42, interday 0.422-0.81 and intraday 0.61-0.85; Limit of detection 1.66 ng/spot; Limit of quantitation 5.54 ng/spot; accuracy 99.88-100.20 %. (Table 30) Developed method is precise, specific and selective.

E. hirta:
Quercitrin was isolated by fractionation and crystallization followed by purification with preparative thin layer chromatography. Isolated compound was confirmed by m. p. obtained between 178°-181° C. Hydrolysis of isolated quercitrin gave aglycone quercetin which was confirmed by co-TLC with standard quercetin at R_f 0.72. Co-TLC of aqueous phase and standard rhamnose gave spot at same R_f 0.3.
Quercitrin is reported as a marker for antioxidant and anti-inflammatory activities so preferred to estimate its content in plant by HPTLC method. HPTLC data and chromatogram scanned at 254 nm revealed the presence of quercitrin (Fig. 24 & 25) and its content in aerial parts was found to be 3.03 %w/w (Fig. 23 and Table 32)
Method was validated for its validation parameter like linearity (0.9922); precision (%C.V.) i.e. repeatability of measurement. 0.26-4.49, repeatability of application 1.19, interday 0.33-4.49 and intraday 0.21-3.51; Limit of detection 1085.28 ng/spot; limit of quantitation 3617.6 ng/spot; accuracy 99.66-100.56 %. (Table 37) Developed method is precise, specific and selective.

S. indicus:
The volatile oil was isolated from aerial parts of *S. indicus*, yield of which was found to be 0.3 % v/w (Clavanger’s method). Isolated volatile oil was yellowish brown having characteristic aroma. It was analyzed by GLC combined to mass spectroscopy (GCMS) technique. The chromatogram showed peaks at retention time (RT) 9.03, 14.06, 14.31, 14.59, 16.39, 17.69, 19.81, 21.41, 22.45 and 24.69 (Fig. 26). As per literature, the peak at 14.59 RT revealed presence of β-Caryophyllene. It was also confirmed by its mass fragmentation exhibiting peaks at m/e 27, 41, 55, 69, 93, 105, 120, 133, 161, 175, 189 and 204 with standard β-Caryophyllene (Fig. 27 & 28).

β-Caryophyllene was isolated from the oil and was identified by boiling point at 254-257°C and Co-TLC with standard (Sigma) using petroleum ether: CCl₄:: 75:25 as mobile phase and sprayed with anisaldehyde sulphuric acid reagent showed blue spot at Rf 0.3 (Fig. 29). Isolated β-Caryophyllene was further confirmed by obtaining overlain gas chromatogram and exactly matching with standard β-Caryophyllene chromatogram. (Fig. 30)

Literature survey revealed anti asthmatic activity of β-Caryophyllene, therefore its quantity was determined in the volatile oil by GLC technique. The quantity of β-Caryophyllene in volatile oil was found to be 1.27 % v/v as per data obtained from chromatogram. (Table 39)

Method was validated for its validation parameters like linearity(0.99); precision(% C.V.); repeatability of measurement 0.32-0.71, repeatability of application 0.17, interday 0.65-0.73 and intraday 0.13-0.52; limit of detection 5.75ppm; limit of quantitation 19.18ppm; accuracy 99.60-100.25 %. Developed method is precise, specific and selective.
Pharmacological activity:
The dried barks of *Albizia lebbeck* are used as anti-inflammatory agent also have bronchodilatory, mast cell stabilizer and anti allergic activities. The dried leaves and stem of *Euphorbia hirta* are used as antispasmodic as an antiasthmatic, as broncholytic, as a sedative, carminative depurative. The dried entire plant of *Sphaeranthus indicus* has been used as laxative, anthelmintic, as fish poison, in leucoderma, asthma, dysentery, bronchitis, tuberculosis. In present study, we evaluated the alcoholic extract of bark of *Albizia lebbeck*, aerial parts of *Euphorbia hirta* and *Sphaeranthus indicus* and developed a formulation combining these drugs for their anti asthmatic activity.

A significant protection of rat mesentric mast cells from disruption caused by compound 48/80 was observed in animals treated with alcoholic extract of bark of *Albizia lebbeck*, aerial parts of *Euphorbia hirta* and *Sphaeranthus indicus* in our study. (Table 44, Fig. 34)

It is well known that mast cells are extensively involved in the pathophysiology of bronchial asthma. Mast cell disruption is mediated by activation of IgE antibodies. Sodium cromoglycate, Nedocromil are important agents for the drug therapy of bronchial asthma and stabilization of mast cell membrane is the major mechanism responsible for their effectiveness.²²,²³

In mice anaphylaxis induced mortality or anaphylactic hypotension and passive cutaneous anaphylaxis are used as indicators of the level of anaphylaxis. There are two types of PCA technique: homologous PCA and heterologous PCA. In the present study, we used these models to evaluate the effectiveness of the drug on immediate hypersensitivity.
Pretreatment with antiovalbumin antisera is reported to produce inflammation and wheals. Mediators like leukotrienes, prostaglandins, platelet activating factors and cytokines are reported to be responsible for such inflammatory response. Later, a delayed localized inflammatory response can be observed due to enhanced vascular permeability and leukocyte infiltration at sites of allergen challenge. In our study antiovalbumin antisera obtained from rats was injected intradermally to the mice, forty eight hours later egg albumin was injected intravenously along with Evan’s blue dye. The penetration of the dye in to the skin area of mice, where antiovalbumin antisera were injected, was an indicator of amount of inflammatory response produced. The amount of dye penetration in control animals was significantly higher, suggesting the significant extent of inflammation development taken place due to antigen antibody reaction. The leakage of dye was significantly less in the animals treated with alcoholic extract of bark of Albizzia lebbeck, aerial parts of Euphorbia hirta and Sphaerantus indicus at the dose of 250mg/k, P. O. each drug. (Fig. 35) This can partly be due to inhibition of leukotriene synthesis. There was no effect of PEF on leakage of dye.

Asthma generates CO$_2$ in the serum and sensitized animal showed in increase in pCO$_2$ level while treatment with ADF reduced pCO$_2$ level. (Table 45, Fig. 36) In asthma oxygen level is decreased and hence sensitized animal showed decreased level of oxygen which could be in animal treated animals. (Table 46, Fig. 37)

Intravenous administration of egg-albumin in guinea pigs showed significantly higher serum bicarbonate level. (Table 47, Fig. 38) This is mainly due to the higher carbon dioxide tension in blood which is...
transported as bicarbonates. Developed formulation treated animals showed significantly lesser serum bicarbonate level. There was also raised resistant to air flow so air flow rate was sudden decreased. Due to airway obstruction, there may be low volume of air inspired or expired per breath which indicates there was sudden decreased in tidal volume. Developed formulation treated animals showed significantly higher tidal volume and air flow rate. (Table 49; Fig. 40; Table 50; Fig. 41)

The presence of eosinophils in the airways of asthma patients was recognized over years ago in histological preparation of lung tissue. Intravenous administration of egg-albumin to guinea pigs after 14 days of antigen challenge is result in selective pulmonary eosinophilia, a response that has been associated with airway hyperreactivity. The evidence of eosinophil involvement in inflammatory reactions within the asthmatic lung has been provided by demonstration of an increased bronchial eosinophilia in patients exhibiting a late asthmatic response. Eosinophils cause epithelial damage by releasing major basic protein, which may lead to increased airway reactivity, either by exposing sensory nerve endings or by removing the protective effects of an epithelial derived relaxant factor.25

In our study, we used sensitized guinea pigs to demonstrate the effect of alcoholic extract of developed formulation on eosinophil accumulation following antigen challenge. The guinea pig was well suited for such studies since airway hyper reactivity and eosinophilia readily demonstrated in this species. As compared with unsensitized animals, significantly higher level of eosinophils was observed in untreated sensitized animals challenged with egg albumin. Animals treated with alcoholic extract of developed
formulation (ADF) showed significantly lower level of eosinophils as compared to untreated sensitized animals. (Table 51, Fig. 42-46) Eosinophilia which is a common phenomenon during inflammatory process was also observed in our study due to antigen challenge. Egg albumin sensitized alveolar septa is markedly congested, whole of the intestinal tissue was infiltrated by chronic inflammatory cells consisted of lymphocytes mainly with few neutrophils and plasma cells compared to normal alveoli. While, treated animals showed normal structure of alveoli compared to sensitized animals. (Fig. 47)

Due to the presence of a less developed antioxidant defense mechanism, lung is particularly vulnerable to injury by reactive oxygen species. Considerable efforts have been made on using antioxidants to protect the lung against the egg-albumin toxicity. Therapeutic strategies, designed to augment cellular endogenous defense systems have been identified as a promising approach to combat oxidative stress-associated disease conditions.

Lipid peroxidation is often the first parameter to prove the involvement of free radicals in cell damage. The reason for this are, (1) Lipid peroxidation is an extremely likely consequence if a reactive free radical is formed in a biological tissue where polyunsaturated fatty acids are generally abundant. (2) Lipid peroxidation is a very important process in free radical pathology as it is damaging to cells. The thiobarbituric acid (TBA) assay is the most popular and earliest method used as an indicator of lipid peroxidation.

In the present study it was observed that there was significantly higher MDA level in the control group when compared to the normal group. Significantly
lesser level of MDA was observed in animal treated with alcoholic extract of developed formulation suggest inhibition of lipid peroxidation. (Table 52, Fig. 48)

An extensive range of antioxidant defenses, both endogenous and exogenous, are present to protect cellular components from free radical induced damage, which is attributed to antioxidant enzyme like SOD, catalase, reduced glutathione (GSH) etc. In the present study, activity of SOD and catalase and level of GSH were measured.

The most abundant reactive oxygen species (ROS) generated in living cells is superoxide anion and its derivatives, particularly highly reactive and damaging hydroxyl radical, which induces peroxidation of cell membrane lipid. Lipid peroxidation is known to induce cellular damage and is responsible for ROS induced organ damage. In this respect, any increase in SOD activity of the organ appears to be beneficial in the event of increased free radical generation. However, it has been reported that a rise in SOD activity, without a concomitant rise in the activity of catalase might be detrimental. It is due to the fact that SOD generates hydrogen peroxide as a metabolite, which is cytotoxic and has to be scavenged by catalase. Thus a simultaneous increase in catalase activity is essential for an overall beneficial effect of increase in SOD activity.

There was significantly lesser catalase activity in the control group when compared to the normal group. Significantly higher in the catalase activity was observed in animals treated with alcoholic extract of developed formulation as compared to the untreated animals. (Table 53, Fig. 49)
higher in catalase activity suggests inhibition of egg-albumin induced oxidative stress \(^{301,302,303}\)

In the present study, egg-albumin intraperitonially resulted into a significantly lower reduced glutathione levels. Treatment with alcoholic extract of developed formulation showed significantly higher reduced glutathione levels as compared to untreated animals. (Table 55, Fig. 51) Murakami et al. in 1996 suggested that GSH levels were suppressed during oxidative stress. Hence, the lower GSH level in control group can be attributed to oxidative stress. So higher levels of GSH in test drug treated animals suggest inhibition of oxidative stress.

In case of Dexamethasone treated animals there was a significantly lower level of MDA as compared to untreated animals. Significant increase in catalase activity, significantly higher levels of GSH were observed in standard group in comparison to the control group. This suggests that Dexamethasone also inhibits the oxidative stress induced by egg-albumin.

It was demonstrated that alcoholic extract of developed formulation may mediate its antioxidant effects by modulation of lipid peroxidation and enhancing antioxidant enzymes.

The results of this study showed that the alcoholic extract of bark of *Albizia lebbeck*, aerial parts of *Euphorbia hirta* and *Sphaerantus indicus* and developed formulation were found to be effective in various experimental models of asthma. Stabilization of mast cells, inhibitory effects on
immediate hypersensitivity reactions, antieosinophilic activity and anti-oxidant activity appear to be involved in its mode of action.

Our study provide scientific evidence for anti asthmatic activity, supports the anti-oxidant properties as demonstrated earlier and suggests the possible anti asthmatic effect of selected plant drugs. From these studies it can be considered that the selected plant drugs are potential source for anti asthmatic active substance of which few potential substances were identified.