Discussion

The present study revealed the anti-asthmatic activity of hydroalcoholic extract of *Onosoma bracteatum* at 100 and 200 mg/kg using citric acid induced cough and ovalbumin induced asthma in Guinea pigs. The synthesized flavones also showed potent bronchodilator activity as observed by a number of in vitro and in vivo experimental models viz relaxant effect of synthesized flavones on tracheal chain contracted by histamine or antigen and protection against bronchoconstriction induced by histamine aerosol and was evaluated using citric acid model induced cough and ovalbumin induced asthma in guinea pigs to evaluate the anti-asthmatic potential of flavonoids in details along with the docking score of most effective synthesized flavones.

In present study, acute toxicity study of as per (OECD 423) guidelines was carried out at doses 2000 and 5000 mg/kg and plant was found to be safe. Hence, 100 and 200 mg/kg dose of *O. bracteatum* was selected for the evaluation of anti-asthmatic potential of the *O. bracteatum*. The synthesized flavones in two doses levels 1000 & 3000 mg/kg were found to be safe causing no significant change in behavioral signs, toxic manifestations and along with zero mortality.

In qualitative phytochemical screening, the hydroalcoholic of *O. bracteatum* showed the presence of flavonoids, alkaloids, glycosides, and saponins. The leaves of *O. bracteatum* were subjected to successive solvent extraction. The successive solvent extraction of the drug with solvents of increasing polarity generally results in the separation of the constituents according to their polarity. The non-polar constituents are extracted in solvents like petroleum ether (2.94 % w/w) being non-polar, semi polar constituents are extracted in chloroform (3.69 % w/w) and ethyl acetate (3.20 % w/w ), while the polar and highly polar are found in ethanol (3.02 % w/w), while the polar constitutes are extracted in chloroform (3.69%) and ethyl acetate (3.20 % w/w),while the polar and highly polar constituents are found in ethanol (3.02 % w/w) and water (4.76 % w/w).Thus, the values of successive solvent extraction provide an idea regarding the presence of various non-polar, semi-polar and polar constituents (Kokate et al., 2005). The proteins and fats were found to be absent. These results revealed that plant contains flavonoids which may be responsible for the protective effect of *O. bracteatum* against asthma.
Total phenolic content in the hydroalcoholic extract of *O. bracteatum* as calculated to be 193.6 ± 0.611 mg GAE/g crude extract. The hydroalcoholic extract of *O. bracteatum* possessed high content of phenolic compounds. Total flavonoid content in the hydroalcoholic extract of *O. Bracteatum* was calculated to be 137.6 ± 2.645 mg RE/g crude extract. Total alkaloid content present in the hydroalcoholic extract of *O. Bracteatum* was calculated to be 93.6 ± 2.081 mg AE/g crude extract. Total saponin content present in the hydroalcoholic extract of *O. bracteatum* was calculated to be 85.5 ± 0.680 mg DE/gram crude extract. Hydroalcoholic extract resulted in 54.27% DPPH radical scavenging activity at highest concentration, which was significantly lower than that observed for ascorbic acid (75.27%) at same concentration. Also the hydroalcoholic extract of *O. bracteatum* showed hydroxyl radical scavenging activity 59.60 % at highest concentration which was significant as observed from ascorbic acid 61.14%

Asthma is characterized by bronchoconstriction and bronchial inflammation which are associated with airway obstruction and mucus production that leads to increased airway hyper-responsiveness resulting to recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night or in the early morning upon exposure of allergens, chemicals, drugs, bacteria and viruses. The factors like goblet cell hyperplasia, increased area of sub mucosal glands result in hyper secretion of mucus with decreased mucociliary clearance contribute to chronic airway inflammation, airway obstruction, and asthma exacerbation (Woolcock 1988; Takeyama, Fahy et al., 2001). The chronic airway inflammation of asthma is unique in which the airway wall is infiltrated by T-lymphocytes (TH-2 phenotype), eosinophils, neutrophils, macrophages/monocytes and mast cells which further results in some detrimental effects on airway function, including airway smooth muscle contraction, induction of airway hyper responsiveness, mucus hypersecretion, epithelial shedding and vascular exudation (Gavett, O'hearn *et al*., 1995; Johnson and Knox 1997; Cohn, Elias *et al*., 2004). Excessive production of cytokine and chemokine induces oxidative stress sensitive transcription of nuclear factor-kB in bronchial epithelial cells (Roebuck 1999). Therefore, the use of bronchodilatory agents is recommended as immediate therapy with the use of anti-inflammatory agents in most asthmatic syndrome (Li *et al*., 2008). This palliative therapy requires long term daily administration and is associated
with major limitations owing to low efficacy, associated adverse events and compliance issues. As a result there is high prevalence of usage of herbal medicine for treatment of the asthma (Detroja et al., 2011)

In ovalbumin induced model, intraperitoneal injection of ovalbumin cause allergic reactions in guinea pigs. This model is widely used for the evaluation of anti-asthmatic activity. Histamine, prostaglandin D2, tryptase, the cysteinyl leukotrienes LTC4, LTD4 and LTE4 were all identified mast cell products with proinflammatory effects. Mast cells are also sensitive to stimulation of asthma. Cytokines produced by T-helper type 2 lymphocytes, specially granulocyte macrophages colony stimulating factor and interleukins IL-4, -5, -9, and -13, attract the active eosinophils and stimulate IgE production by B lymphocytes, are responsible for more sustained bronchoconstricition, cellular infiltration of the airway mucosa, and mucus hypersecretion of the late asthmatic reaction. These chronic inflammatory disorders of the airways lead to tissue injury and structural changes (Rickel et al., 2008)

In present study, citric acid control animals showed significant increase in decrease in latency time and increase in cough frequency as compared to normal treated animals which signifies significant cough response in the form of sneefing and sneezing. However on the treatment with the pet ether and hydroalcoholic extract of O. bracteatum at doses 100 and 200mg/kg produced reversible response as induced by the citric acid, as evidence by significant decrease in cough frequency and increase in latency time. The effect of hydroalcoholic extract may be due to the correction of activation of C fibres and Afibres. The pet ether extract obtained from the parent O. bracteatum extract showed significant increase in cough frequency and decrease in latency time.

In ovalbumin model, administration of ovalbumin cause significant increase in coughs frequency, decrease in latency time and produced oxidative stress However treatment with hydroalcoholic O.B. extract at doses 100 and 200 mg/kg produced significant protection from asthma as evidenced by decrease in frequency and increase in latency time.
Oxidative stress is one of the key roles in asthmatic pathogenesis and very well-known pathway exhibiting the tissue damage (Barnes, 2008). Oxidative stress can have many detrimental effects on airways function, including airway smooth muscle contraction, induction of airway hyperresponsiveness (Weiss, 1986; Alder, 1990), epithelial shedding malondialdehyde, are end-product of cell membrane lipid peroxidation by reactive oxygen species and are considered reliable markers of oxidative tissue injury (Aruoma et al., 1989). In the present study it was found that TBARS content in BAL fluid and lung tissue homogenates of the sensitized-challenged animals was significantly increased, as compared to normal control. On comparing SC with standard drug treated group AMN reduced the TBARS content in both BAL and lung tissue samples. BAL and lung tissue samples from the groups treated with O. bracteatum showed reduction in TBARS level dose dependently, as compared to SC and the highest dose i.e. 400 mg/kg of both the plant pet ether extracts were able to produce reduction in TBARS level and was insignificant to AMN.

Lipid peroxidation is the oxidation process of polyunsaturated fatty acids present in the cell membrane which yields peroxide radical-lipid hydroperoxides and aldehyde products such as Malondialdehyde (MDA) (Sharma et al., 2003). Present study showed the high level of LPO in ovalbumin control group whereas O. bracteatum extract treated groups showed increase in LPO level in lung tissue and BAL fluid. TBARS was found to be increased in control group while plant showed the significant increase in TBARS levels in lung tissue and BAL fluid in treated groups. These products in turn enhance the vascular permeability and leucocytes chemotaxis and alter the prostaglandin synthesis and histamine release thus perpetuating inflammation. LPO and degradation products formed by free radicals are an important measures of an ongoing oxidative damage. Serum MDA peroxidation by measuring serum MDA during an acute attack and in symptoms free period of bronchial asthma.

Glutathione is intercellular thiol antioxidant present in the lung tissue. Low level of GSH in lungs cause inflammation and hyperresponsiveness (Aggarwal et al., 2008). Present study showed the low level of GSH in ovalbumin control group whereas O. bracteatum extract treated groups showed increase in GSH level in lung tissue and BAL fluid. TBARS was found to be increased in control group while plant showed the significant decrease in
TBARS levels in lung tissue and BAL fluid in treated groups. Lung tissue is also known to produce and release NO in allergic asthma (Franova et al., 2011). The anti-asthmatic activity of hydroalcoholic extract was demonstrated by the inhibition of the inflammatory mediators, macrophages, interferons, leukotrienes and interleukins etc in ovalbumin model. Cellular infiltration is assessed for airway inflammation, which is characterized by increased level of leukocytes and eosinophils as evident is asthmatic syndrome and can be estimated by total leukocyte count and eosinophil count in bronchoalveolar lavage.

(BAL) fluid (Bousquet et al., 1990; Kirby et al., 1987; Adelroth et al., 1990; DeMonchy et al., 1985; Foresi et al., 1990). Ovalbumin induced a significant cellular infiltration into the BAL fluid which was recovered from sensitized animals (SC) than normal control (NC) group. Eosinophils in allergic conditions have a definite role in inflammation and airway hyper-responsiveness (Dunn et al., 1988; Ishida et al., 1989). Eosinophil constitutes of granule basic protein , such as major basic protein (MBP), eosinophil peroxide and eosinophil cation protein (ECP) which triggers airway remodeling. Eosinophil synthesized eicosanoids (PGI$_2$) and cysteinylleukotrienes and release a range of cytokines and chemokines (Kariyawasam and Robinson, 2006). Increased eosinophil numbers found in peripheral blood, sputum and lung tissue of asthmatic patients (Kay, 2005) which can be correlated with greater asthma severity. T lymphocytes with other airway resident cells, can also determine the development and degree of airway remodeling. Elevated level of Th2-lymphocytes have been observed in the bronchial mucous of biopsy samples, bronchoalveolar lavage and sputum from patient with asthma. Th2- cytokines resulted in the overproduction of IgE, eosinophilic inflammation and airway hyper-responsiveness (Larche et al., 2003). Eosinophilic infiltration into lungs and BAL fluid has been considered the hallmark of inflammation. There is a correlation between infiltration of eosinophils and airway inflammation, thus plays a significant role in the pathogenesis of asthma (Petres-Golden, 2004). Treatment with hydroalcoholic extract of O. bracteatum showed significant reduction in cellular infiltration and activation of eosinophils in comparison to sensitized animals and the effect produced due to the highest dose i.e. 400 mg/kg of the pet ether extract was comparable to aminophylline. The standard drug treated group aminophylline
exhibited reduced cellular infiltration and recruitment of eosinophils among other groups except normal control group.

The TLC of ethanolic extract was done and calculated $R_f$ value. The solvent system used for TLC was Toluene, chloroform, ethyl acetate and n-butanol fractions of powdered *O. bracteatum*. On the basis of phytochemical screening results, the flavonoids were present in n- butanol fraction. Column chromatography was performed with n-butanol fraction in chloroform: methanol (9:1) as solvent system. Fractions (1- 100) were collected and TLC was observed, three different single spots were found in range of 40-50, 55-60 and 85-100 test tube respectively. The identical TLC pattern ones were pooled. Three isolated compounds were obtained, one was yellow rosette shaped crystals, another one was yellow needle shaped and last one was amorphous in nature.

*Onosma bracteatum* belongs to family: Boraginaceae commonly called as Gaozaban and Gojihva. It is found in western Himalayas, from Kashmir to kumaon between 3600-4500m elevations. In Himachal Pradesh, it is reported from districts of Kangra, Pangi-Bharmour, Kinnaur, Lahaul and Spiti. It is an average sized perennial herb and the leaves are lanceolate with conspicuous hairy pallid bases. Some species of Onosoma genus e.g. *O. arenaria* contains 1, 2-unsaturated pyrrolizidine alkaloid (Shu, 2003), *O. argentatum* Hub.-Mor. contain deoxyshikonin, acetyl shikonin, shikonin (Reidl, 1978) whereas *O. bracteosum* Hausskn and *Onosma thracicum* Velen. consist of oleic and $\alpha$-linolenic acids. The phytochemical investigations done on *O. bracteatum* found to be merely showing naphthaquinone, alkaloid, phenolic compounds, fatty acids and tocopherol (El-shazlyet al., 2003). The plant is used as a tonic, demulcent, diuretic and spasmylytic (Patel et al., 2011). Its flowers are useful in rheumatism, syphilis, leprosy and act as stimulant and cardiac tonic. It is recognized as therapeutic plant traditionally in Ayurveda for dealing of asthma as well bronchitis (Ahuja, Gulati et al., 2009). The plant parts are used as chief adjuvant in marketed preparations e.g. Joshanda and Zeal for treating cough and bronchitis (Patel et al., 2011).

*O. bracteatum* has been used to treat inflammation, analgesia, memory retention, rheumatic pain and tachycardia. Our study revealed significant response in all the parameters for the asthma as decrease in cough frequency, increase in latency time and reduction in TBARS,
NO level and elevation in GSH level which prevent asthma. Thus conclusively we found that the plant *O. bracteatum* has constituents which may be anti-tussive and anti-asthmatic and may be useful for the prevention of asthma.

Flavonoids are a group of chemical moiety of the compounds whose structure is based on C₆-C₃-C₆ i.e. two phenyl rings are attached through a propane bridge. Flavonoids are a family of plant compounds with a similar flavone backbone composed of two aromatic rings and an oxygen heterocycle attached (Yao *et al.*, 2004) Flavonoids can be classified into various classes *i.e.* Flavonols (Quercetin, Kaempferol), Flavones (Luteolin, Apigenin), Flavanones (Hesperetin), Flavonoid Glycosides (Rutin), Flavanolignans (silibinin), Flavans (Catechin, Epicatechin), Isoflavones (Genistein, Daidzein), Anthocyanidins (Cyanidin, Delphinidin), Aurones (Leptosidin, Aureusidin), Leucoanthocyanidins (Teracacidin), Neoflavonoids (Coutareagenin, Dalbergin), Chalcones (Harborne and Williams, 2000). All classes of flavonoids exhibits variety of biological activities, but among them, the flavones have been considerably explored. In recent years, substituted synthetic flavonoids have been dragging continuous attention due to their broad range of biological activities. Flavonoids are very well known and documented to possess antioxidant effects, antiviral, and leishmanicidal activity, ovipositor stimulant of phytoalexins, anti-HIV, and vasodilator, bactericidal, DNA cleavage, anti-inflammatory, anti-mutagenic, anti-asthmatic and anticancer activities. Especially, flavones (2-phenylchromones) exhibit a wide variety of activities (Singh *et al.*, 2014; Gaspar *et al.*, 2014; Xiao *et al.*, 2011; Evans and Miller, 1998). Therefore, they exhibit diverse type of properties that are beneficial for human health via interacting with a number of cellular targets involved in critical cell signaling pathways in the body. Especially, flavone derivatives have been reported to possess anti-asthmatic activity via targeting FceRI receptors as well as exhibiting leukotriene antagonism (Tamura *et al.*, 2010) Histamine is implicated as a mediator of some of the symptoms of allergic rhinitis and other allergic diseases, but its importance in asthma is much less well understood. However, some studies also suggests the potential value of H₁ antihistamines in the treatment of asthma and number of groups have been reported compounds of potential therapeutic benefit that were claimed to possess the combined properties of both histamine...
H₁ antagonism and mast cell stabilization, likely, BM 15100 (Blank et al., 1989; Nugroho et al., 2011).

Substituted flavones are also known to show potential pharmacological profile but no natural flavonoids have been reported with halogens as substituent. Substitution of the B ring is known to enhance antibacterial activity, with 3'-chloro, 4'-chloro and 4'-bromo analogues each being approximately twice as effective as their parent compound against S. aureus, and four times more active against Enterococcus faecalis. Also, the 2',4'-dichloro derivative exhibited a 4 - 8 folds improvement in activity against S. aureus (Guz et al., 2001). However, a little work has been carried out on iminoflavones revealing anti-asthmatic and antimicrobial activities. Additionally, we were also interested to evaluate the anti-asthmatic and antimicrobial activity of flavones derivatives possessing nitrogen atom at 4-position. Therefore, in order to search for new compounds that have potential activity for asthma and microbial infections, this paper describes the synthesis of variably substituted 4-iminoflavones. Moreover, to gain much understanding, the docking studies were performed on specific targets in order to examine the binding affinity of respective molecules within the pocket.

In view of the importance of pharmacological active flavones, 4-iminoflavones were aimed to synthesize. The replacement of oxygen atom of the keto group was carried out with an amino group containing phenyl hydrazine. Our synthetic approach was initiated with the synthesis of various intermediate chalcones3(a-j) which was achieved through Claisen-Schmidt condensation. Targets compounds were synthesized via initial preparation of chalcones 3(a-j) using easily accessible starting materials, 2-hydroxy-4-methoxyacetophenone and substituted aldehydes in presence of 10% KOH and ethanol (Scheme 1). Thereafter the chalcones were then oxidative cyclized in the presence of iodine to furnish the flavone derivatives 4(a-j) in good yields. Notably, the reaction was conducted under 1,4-dioxane solvent system. It has been reported that the method followed for preparation of flavones corresponds to final products with satisfactory yields with both electron withdrawing and electron donating moieties. The plausible mechanism for the formation of flavone ring system 4(a-j), using iodine mediated oxidative cyclization is presented in Scheme 1. The mechanism involves; isomerization of the initial adduct
followed by intramolecular cyclization resulting in its hemiacetal species (a) which gets converted to more reactive flavylum ion, (b) furthermore, water molecules attach on the more reactive position to form adduct and finally, (c) on oxidation in presence of iodine yields final products \(4(a-j)\). The final product series \(5(a-j)\), 4-imino flavones was synthesized by refluxing the flavone \(4(a-j)\) with phenylhydrazine in presence of concentrated \(H_2SO_4\) in ethanol absolute. All the compounds were fully and satisfactory characterized through spectroscopic techniques.

Most employed and popular software was used in the present study in order to visualize and optimize the binding affinity of synthesized new molecules on specific drug targets. The significant result of anti-asthmatic and antimicrobial activity prompted is to investigate the docking of molecules on well-known targets. Active site residues of human histamine H1 receptor according to co-crystal structure with inhibitors; Trp 428(A), Phe 432(A), Asp 107(A), Tyr 108(A), Ser 111(A), Tyr 431(A), Trp 158(A), Tyr 458(A), Asn 198(A), Thr 112(A), Thr 194(A), Phe 424(A), Phe 435(A) were taken to define binding pocket within target protein, whereas, active site residues of glucosamine-6-phosphate synthase according to co-crystal structure with inhibitors (Cys 300, Gly 301, Thr 302, Ser 303, Ser 347, Gln 348, Ser 349, Thr 352, Val 399, Ser 401, Ala 602 and Lys 603) were taken to define binding pocket within target protein.

Protein ligand analysis also showed strong interactions with target protein and had four hydrogen bond interaction with different residues (Tyr 108, Ser 111, Tyr 431 and Tyr 458) in H1 receptor site, whereas, six hydrogen bond interaction with different residues (Ser 303, Ser 347, Thr 352, Thr 355 and Glu 488) in active site of antimicrobial and antifungal activity.

The compound at optimized doses showed significant relaxation. Interestingly, the relaxation was found to increase with increment in dose, whilst, control (at different dose) were found to show contraction due to induction of histamine. This revealed that \(5(f)\) considerably inhibited the contractile effect of histamine thus produces bronchodilation. Therefore, compound \(5(f)\) was found active in all the experiments, supporting each other.
In this present study, among the synthesized compounds, 5(f) was found to be the most promising against asthmatic models, whilst, 5(h) was found active against all the microbial strains. Anti-asthmatic activity was correlated and evaluated with various models, likely, citric acid induced cough model, OVA induced asthma model (Biochemical estimation for cell infiltrations, estimation of lipid peroxidation and glutathione and estimation of TNF-α and IL-6 and histamine induced response).

Compound 5(f) was found to show decrease in number of cough whilst in comparison to codeine compound 5(f) produced considerable reduction in neutrophils lymphocytes, eosinophils, and leukocytes.

The synthesized compounds 5(a-j) were tested (30 mg/kg) in order to analyze their impact over LPO and GSH. Compounds 5(f), 5(j), 5(h), 5(c) and 5(i) showed substantial and considerable effects over LPO as well as GSH. Among these, 5(f) having methyl substitution at para position was found to be the most active one. The present experiment showed that

The antimicrobial activity was determined in terms of minimum inhibitory concentration (MIC) of the synthesized compounds 5(a-j). The activity was determined against the gram-positive Bacillus subtilis, Stapylocococcus aureus, the gram-negative Escherichia coli, Salmonella typhi and the fungi Candida albicans. The obtained data revealed that compounds inhibited the growth of the selected microorganisms in vitro showing MIC values between 3.1 to 100 µg/ml. Interestingly, compound having halogen substitution were found to show better inhibition against C. albicans in comparison to other compounds. However, very few of the synthesized compounds were found inactive against specific strains.