6. Discussion

In the present study, seven bioactive components were isolated i.e. Achillicin and chamazulene from *Achillea millefolium*, rubiadin and mollugin from *Rubia cordifolia*, costunolide, dehydrocostus lactone and cynaropicrin from *Saussurea lappa*. The characterization and identification of these compounds has done by IR, NMR (\(^1\)H & \(^{13}\)C), MASS and HPTLC.

Mass spectrum of achillicin showed molecular ion peak at \((m/z)\) 306, acetoxy functional group has shown fragment peak at \((m/z)\) 246.14 - \(C_{15}H_{18}O_3^+\) and O-H group has shown fragment peak \((m/z)\) 228.13 - \(C_{15}H_{16}O_2^+\). IR spectrum has shown characteristic peak of O-H group at 3453 cm\(^{-1}\), saturated \(\gamma\)-lactone C=O stretching was observed at 1773 cm\(^{-1}\) and C=O stretching of acetoxy group was observed at 1727 cm\(^{-1}\). PMR spectrum of achillicin has shown characteristic peaks of methyl groups Me-15, 13, 17 and 14 at \(\delta\) 1.29, 1.61, 2.08 and 2.18, respectively whereas O-H proton has showed peak at \(\delta\) 1.72. Form the above spectral data suggesting molecular formula of achillicin was found to be \(C_{17}H_{22}O_5\).

Further, achillicin was treated with 5\% NaOH and heated to afford chamazulene carboxylic acid \((C_{15}H_{16}O_2)\), which was confirmed by mass \((m/z)\) 229. IR spectrum of chamazulene carboxylic acid has shown characteristic peaks at 3359 (O-H str.), 1700 (C=O str.) and at 2975 cm\(^{-1}\) (CH stretching). PMR spectrum of chamazulene carboxylic acid showed characteristic peak of three methyl protons at \(\delta\) 1.63-1.59 (Me-13), 2.65 (Me-15) and 2.83 (Me-14). The O-H proton has showed broad peak at \(\delta\) 11.96 due to high deshielding effect of carboxylic group.

The IR spectrum of rubiadin showed characteristic peaks at 3417 (OH str.), 2919 (alkyl neat CH str.), 2881, 1670 (unchelated C=O) and 1631 cm\(^{-1}\) (chelated C=O). PMR spectrum of rubiadin has shown signals of \((O-H_1)\) showed peak at 12.98, aromatic protons \((Ar-H_{(4,5,6,7,8)})\) showed peaks at \(\delta\) 7.39, 8.16, 7.92, 7.93 and 8.26, respectively. Aliphatic group such as methyl \((CH_{3(2)})\) proton showed peak at \(\delta\) 2.18. Mass spectrum of rubiadin showed molecular ion peak of aglycone at \((m/z)\) 254-C\(_{15}H_{10}O_4\).

Mollugin showed characteristic peak of O-H showed peak at 3165, C=O stretching at 1645 and methyl neat stretching peak at 2972 cm\(^{-1}\) in its IR spectrum. PMR spectrum of mollugin has shown characteristic peaks hydroxyl proton \((OH_{(1)})\) at \(\delta\) 12.13. Aromatic protons \((Ar-H_{(1',6,7,8,5)})\) showed peaks at \(\delta\) 7.05, 7.44-7.58, 8.16 and 8.34 respectively. Methyl protons \((CH_{3(3',7)})\) showed peak at \(\delta\) 1.49 and peak of
methoxy (OCH$_3$) protons was observed at δ 3.96. Mass spectrum of mollugin showed a molecular ion peak at (m/z) 284.3-C$_{17}$H$_{16}$O$_4$.

IR spectrum of costunolide showed characteristic bands at 2945 (C-H str.), 2870 (Methyl C-H str.) and 1762 cm$^{-1}$ (C=O str.). PMR spectrum of costunolide showed characteristic peak of methyl (OCH$_3$) protons at δ 1.71. The methylene (CH$_2$) protons showed peaks at δ 5.58-6.05. The other aliphatic (C$_4$,C$_5$,C$_8$) protons showed peaks at δ 1.48, 1.97, 2.02 and 1.99, respectively. Mass spectrum of costunolide showed molecular ion peak at (m/z) 232.15-C$_{15}$H$_{20}$O$_2$.

IR spectrum of dehydrocostus lactone showed characteristic peaks at 3100 (C=CH$_2$ str.), 1800 (C=O str. of γ lactone), 1641 (C=CH$_2$ str. of γ lactone), 1644 (heptacyclic CH$_2$) and 902 cm$^{-1}$ (C=CH$_2$ str.). PMR spectrum of dehydrocostus lactone showed characteristic peaks of methylene at δ (2CH$_2$) and 5.48 (CH$_2$). Mass spectrum of dehydrocostus lactone showed molecular ion peak at (m/z) 230.13-C$_{15}$H$_{18}$O$_2$.

IR spectrum of cynaropicrin showed characteristic peaks at 3300 (O-H str.), 1762 (C=O str. of γ-lactone) and 1670 cm$^{-1}$ (C=CH str.). PMR spectrum of cynaropicrin showed characteristic signals at δ 1.71 (CH$_2$), 2.01 (O-H$_{(3,4')}$), 2.19 (CH$_{2(9)}$), 4.37 (CH$_{2(4')}$), 5.03 (CH$_{2(13)}$, s, 2H), 5.38 (2CH$_{2(14,15)}$) and 5.98 (CH$_{2(3')}$, s, 2H). Mass spectrum of cynaropicrin showed molecular ion peak at (m/z) 330.15-C$_{19}$H$_{22}$O$_5$.

Isolated compounds were further identified by high performance thin layer chromatography (HPTLC). Thus, obtained isolated compounds were further evaluated by in-vitro and in-vivo studies.

In-vitro studies were carried out to evaluate their potential spasmolytic activity in isolated tracheal strips of Wistar albino rats. pD$_2$ values are indicative of the potency of individual isolated compound, higher the pD$_2$ values-higher is the potency. Furthermore, maximal relaxation obtained was indicative of the efficacy of the individual isolated compounds. Among the seven individual isolated components, the order of potency was found to be costunolide (5.90) ≥ mollugin (5.90) > chamazulene (5.41) > rubiadin (5.25) > cynaropicrin (5.11) > dehydrocostus lactone (4.91) > achillicin (4.45). Isoprenaline was found to be more potent pD$_2$ value (7.2) compared to all isolated compounds. Extracts of these plants were earlier studied for anti-inflammatory properties and since all isolated compounds were able to relax the tracheal strips preparations, it can be interpreted that all the isolated compounds were
efficacious in spasmolytic study. Calcium is mainly responsible for calmodulin (CaM: calcium-modulated protein) modulated smooth muscle contraction. Any attempt to block this intracellular availability of calcium may produce smooth muscle relaxation. Since sesquiterpenes lactones are known to have sequestering action on calcium ions either through blockade of voltage-dependent Ca$^{2+}$ channels (Pinho-da-Silva et al., 2012) or by inhibition of the Ca$^{2+}$-ATPase (Wictome et al., 1993), costunolide, a sesquiterpenes lactone, produced smooth muscle relaxation and showed maximum relaxation out of all isolated compounds. Other compounds such as mollugin and rubiadin (anthraquinones) may possess their smooth muscle relaxing property either due to blockade of voltage gated calcium channels (Cavalcante et al., 2008) or through NO production. However, by comparing the pD2 value, sesquiterpenes exhibited a stronger smooth muscle relaxant activity compared to anthraquinone and naphthoquinones due to secondary mechanisms such as by inhibition of phospholipase A$_2$ activity similar to glucocorticoids (de Alvarenga et al., 2011), one of the biological activity exhibited by sesquiterpenes or due to their higher cell permeability owing to their lipoidal nature.

Among the seven isolated compounds, achillicin, chamazulene, rubiadin, mollugin, costunolide, dehydrocostus lactone and cyanaropicrin, the compounds showed better activity in in-vitro study such as chamazulene, mollugin and costunolide, were selected for further in-vivo study. These compounds were found to be safe in earlier reports (Wang et al., 2012; Eliza et al., 2009). Sensitisation of Wistar albino rats, results in production of anti-bodies IgE and subsequent exposure of antigen will precipitate antigen and IgE reaction culminating to bronchospasm and asthma like symptoms. Evaluations were carried out using wet/dry lung weight ratio; haematological markers (TLC & DLC) in blood and BALF; biochemical markers (PAF, IL-6, IL-8, EPO, SOD, TNF-α, NO) in lung tissue homogenate and BALF; Lung lavage protein concentration and airway immunohistochemistry (H&E).

Asthma was induced by administration of ovalbumin in rats resulted hypersensitivity reaction and alteration in immunity. Results of cell count analysis exhibit that there is an infiltration of inflammatory scavenging cells (macrophages and eosinophils) from blood to BALF in challenged rats, previously sensitized by ovalbumin. Significant reduction was seen in this infiltration of eosinophils and macrophages after treatment with isolated chamazulene, mollugin and costunolide, showed percentage inhibition of eosinophils and macrophages 41.67 & 18.2%, 48.67
Furthermore, their combination (76.39 & 62.73%) was found to be more effective in reducing this infiltration compared to individual compounds and standard, dexamethasone (52.78 & 45.45%). Reduction in eosinophils infiltration was more significant in BALF as compared to blood, may be due to decreased vascular permeability exhibited by the isolated compounds. The increased infiltration of macrophagic cells, after the challenge, also lead to increased lung weight, due to the macrophagic nature of these cells they caused inflammation in airways, as a result oedema was established and subsequently lung W/D ratio increased. The increased lung W/D ratio, due to challenge, was mitigated by combination and individual isolated compounds. Furthermore, eosinophil peroxidase (EPO) a key enzyme mainly found in eosinophils, was found to increase in challenge rats, previously sensitised by ovalbumin. Increase in EPO is in direct co-relation with increase in eosinophils at airways inflammation site. Degranulation of eosinophils lead to release of EPO along with eosinophil granule proteins such as EPX and major basic protein (MBP), which turns damages the protective layer of respiratory epithelial cells, and also produced bronchoconstriction, mucus hyper secretion and vasodilation.

Results in the present study indicate that challenge with ovalbumin in previously sensitised rats lead to significant increase in IL-6 levels. Further treatment of these rats with isolated compounds their combination and standard drug (dexamethasone) lead to a significant decrease in IL-6 levels (30.43%). However, combination of these compounds was found to me most effective followed by costunolide and then mollugin, with percentage inhibitions of (44.8%), (37.57%) and (32.99%), respectively. Decrease in neutrophil/eosinophil infiltration leading to decreased lung epithelial damage could be the possible causes for reduction in IL-6 levels. Degranulation of mast cells and neutrophils results in the release of IL-6 and IL-8, moreover IL-6 gene is constitutively expressed in lung epithelial tissue and is activated by allergens. An increased level of IL-6 leads to release of various cytokines and activate JAK/STAT pathway (Pernis and Rothman, 2002) which triggers certain genes responsible in remodelling of airway smooth muscles. However there are evidences of protective behaviour of IL-6, as reported, elevation of eosinophils is seen in IL-6 deficient mice.

Our isolated compounds mollugin, costunolide and their combination has been significantly (P<0.001) found to reduce the elevated levels of platelet activating factor
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PAF (PAF) compared to challenge, with percentage inhibition of (50.0%), (63.64%) and (73.23%), respectively. PAF is a key mediator responsible for bronchoconstriction and airway hyper-responsiveness. It is stored in mast cells and eosinophils. Stimulation of Th2 cells due to allergen induced sensitization followed by ovalbumin challenge leads to activation of B cells which in turn increases eosinophils count in blood. Since eosinophils are the only source of PAF in blood, sensitization induced eosinophilia is the primary cause of increased PAF in blood. Furthermore, increase in PAF levels in BALF is either due to eosinophil infiltration or due to mast cell degranulation. The released PAF in the airway further leads to chemotaxis, mucus production, eosinophil recruitment and finally asthma. Any reduction in PAF at the site of inflammation will inhibit the airway hyper-reactivity. This reduction in PAF levels could either be due to impairment in its production via JAK/STAT induced MAP kinase pathway (Ferby et al., 1994) or due to impairment in its release which needs to be further investigated.

In the present study, our isolated compounds mollugin, costunolide and combination showed significant (P<0.001) inhibition of the elevated levels of TNFα when compared to asthmatic group, with percentage inhibition of (66.86%), (73.12%) and (79.67%), respectively. Isolated compounds were found to exhibit a response in inhibiting TNFα equivalent to dexamethasone. Tumor necrosis factor alpha (TNFα) is the most widely studied pleiotropic cytokine of the TNF superfamily. In pathophysiological conditions, generation of TNFα at high levels leads to the development of inflammatory responses that are hallmarks of many diseases. Implication of TNFα has been reported earlier in asthma. In addition to its underlying role in the inflammatory events, there is increasing evidence for involvement of TNFα in the cytotoxicity. TNFα depletes cellular antioxidant enzymes levels. Both in vitro and in vivo studies showed that TNFα stimulates the high level reactive oxygen species (ROS) generation from endothelial cells and neutrophils. Of the different cell types, ROS derived from neutrophils, have a very important role in TNFα-dependent alteration of pulmonary vasoreactivity.

Unlike the cytokines mediators (IL-6, IL-8, TNFα, PAF) nitric oxide (NO) is a secondary messenger usually synthesized due to nitric oxide synthase (NOS). NOS have three isoform iNOS, nNOS and cNOS. Among all these iNOS being the inducible enzyme releases the burst of nitric oxide at the site of inflammation. Since inflammation plays a pivotal role in setting up of asthma, nitric oxide produced by
iNOS could be a major contributor of asthma. Furthermore it is well established from clinical data that NO levels were found to be significantly increased in exhaled air in asthmatic patients. As evident in the present study, previously sensitized challenged rats a significant increase in NO levels. The increase was subsided significantly with combination, costunolide and mollugin, with percentage inhibition of (56.73%), (50.21%) and (42.0%). Nitric oxide reacts with superoxide radicle and gets converted into more harmful free radical peroxynitrite. Further it dilates airway blood vessels which results in infiltration of inflammatory cells and precipitation of asthma. Our isolated compounds either inhibit NOS or may act as free radical scavenger which needs to be investigated. Furthermore SOD levels concomitantly found to increase with these drugs which may support their antioxidant potential. Since glucocorticoids are known to inhibit NOS and thereby prevents asthma or inflammation, a structural similarities of our isolated sesquiterpenes might produce there action through glucocorticoids based NOS inhibition.

Histopathology of lungs, after staining with haematoxylin and eosin (H&E), in experimental induced asthmatic rats model (Ovalbumin challenged groups) showed marked increase in the inflammatory cells (eosinophils & macrophages), perfused from blood through blood capillaries, and thickening of basement membrane in the lining epithelium (Br. Ep.) of the terminal bronchiole (BL) when compared to control group. Subsequent treatment with our isolated compounds showed marked reduction in infiltrated cells and as well as in thickening of basement membrane. This results suggests that our isolated compounds particularly, costunolide was significantly able to reduce inflammatory responses occurred due to allergen exposure. Histopathological results suggest that costunolide showed more significant results when compared to dexamethasone by comparing severity of infiltration and thickening of basement membrane. The order of activity of isolated compounds was found to be costunolide > mollugin > chamazulene. This clearly shows significance of our isolated compounds in the treatment of experimentally induced asthma, particularly costunolide whereas mollugin showed similar results when compared to dexamethasone. However, chamazulene was not able to subsidise the inflammatory response upto the marked level as compared to dexamethasone.

In present study, effect of the three isolated moiety's i.e. costunolide, chamazulene, mollugin are due to sesquiterpene and naphthoquinone nature, sesquiterpene (costunolide and chamazulene) is earlier found to inhibit MAPK
pathway and has been shown to inhibit IL-6 and IL-8 inhibitor (Hoffmann and Schmidt, 2010; Lindenmeyer et al., 2006), further in neuronal phagocytic cells, costunolide is known to inhibit iNOS by downregulating its mRNA in RAW 264.7 cell lines (Kang et al., 2004). It is also known to inhibit NF-κB and thereby inhibits MAP kinases which are integral part of JAK/STAT pathway (Koo et al., 2001). Whereas chamazulene (also known as naturally occurring profen) due to its structural similarity with profens (flurbiprofen, ibuprofen) is known to inhibit cyclooxygenase and has a marked anti-inflammatory effect – a hallmark for inflammation (Oehler and Imming, 2007). Further, chamazulene is also known to inhibit leukotriene-B4 and elicit its anti-asthmatic potential (Safayhi et al., 1994). Mollugin is known to upregulate heme-oxygenase-1 in hippocampal microglial cells (Jeong et al., 2011) and act as neuroprotective by preventing the inflammation through inhibition of iNOS, TNF-alpha and COX-2 in RAW 264.7 cell lines LPS induced inflammation was markedly reduced in Mollugin through inhibition of JAK/STAT pathway via inhibition of JAK 2 (Zhu et al., 2013; Kim et al., 2009).

Moreover, biological activity displayed by majority of sesquiterpene lactones is due to the presence of α-methylene-γ-lactones and α,β-unsaturated cyclopentenone ring (Chaturvedi, 2011). β-sitosterol, a sesquiterpene is also known to act via L type calcium channels and found to increase the uptake of calcium and thereby activating the neutrophils. As a result this will result in the burst of neutrophils as reported in our experimentally induced asthma (Liz et al., 2013). Furthermore, activation of neutrophils due to calcium uptake is another reason for inhibition of interleukins and TNF-α.