5. DISCUSSION

Inflammation is a protective process that is essential for the preservation of integrity of organism in the event of physical, chemical, and infectious damage. Often, it is found that inflammatory response to severe lesions erroneously damages normal tissue (Lunardelli et al., 2006). The harmful stimuli such as pathogens, damaged cells or irritants lead to the complex biological response of vascular tissues characterized by redness, joint pain, swollen joint that is warm to touch, its stiffness and loss of joint function. This response may be acute or chronic. Under chronic state it becomes a causative factor in the pathogenesis. Being a self-defense reaction in its first phase, inflammation is regarded as the main therapeutic target and a preferred choice for the treatment of disease and alleviating the symptoms.

In the current study two medicinal plants - Artemisia amygdalina and Gentiana kurroo belonging to two different plant families were investigated for anti-inflammatory activity. Analysis of the results indicated that both A. amygdalina and G. kurroo have strong anti-inflammatory potential. In this study, whole plant material from both plants was used and fractionated with different solvents. The different fractions obtained were screened for anti-inflammatory activity using carrageenin induced paw edema model. This animal model is most widely used for screening of novel anti-inflammatory drugs and one of the most well established models in association with edema formation (Handy and Moore, 1998). Of these different fractions, methanolic fraction in both plants was found to cause maximum decrease in edema formation. The methanolic fractions from both plants were again tested in different doses and were found to decrease the edema in dose dependent manner. This may be because of the presence of more bioactive agent/s in this fraction. Most of the bioactive agents present in the medicinal herbs belong to secondary metabolites. These may be terpenoids or
flavonoids in nature. As many monoterpenoids like camphene, borneol, and β-pinene are known to possess anti-inflammatory property (Lin et al., 2008; Tung et al., 2008), flavonoids like 6-methoxytricin have been seen to show anti-inflammatory and analgesic activities (Yin et al., 2008). The increased activity with increased doses may be because of the higher concentration of bioactive agent/s in these extracts. Similar type of study has been carried out on Xeromphis spinosa. The bark of this plant was extracted with petroleum ether, ethyl acetate and methanol in equal proportions. These extracts were used orally at doses of 200 and 400 mg/ kg body weight in carrageenin induced paw edema model and were found to exhibit anti-inflammatory activity by inhibiting the edema formation significantly as compared to control (Biswa et al., 2009). In other study on Albizia lebbeck Benth. the petroleum ether, ethyl acetate and methanol extracts of the bark of this plant were found to inhibit the paw edema volume after 4 h of carrageenin injection by 36.68% at 400 mg/kg dose level (Achinto and Muniruddin, 2009).

Our study also reported higher potential in the methanolic extract of G. kurroo than that of A. amygdalina. Similar results were reported when the anti-inflammatory potential of two plants namely- Moringa oleifera and Ocimum gratissimum was investigated by carrageenin induced hind paw edema method. For this study leaves of both plants were taken and extracts were prepared separately in methanol and water. The extracts of Moringa oleifera showed maximum reduction in edema formation at oral doses of 500 mg/kg bw with methanolic extract showing higher activity than the aqueous extract. Similar results were reported in Ocimum gratissimum, where again the methanolic extract had higher effect than aqueous extract at oral administration of 100 mg/kg bw. The overall study concluded that Ocimum gratissimum had higher anti-inflammatory potential than Moringa oleifera, as the anti-inflammatory effect produced
by the latter plant at the dose of 500mg/kg bw was produced by former plant at only
100mg/kg bw (Yadav and Shah, 2014).

With these methanolic extracts this experiment was further extended for the
study of chronic inflammation using mycobacterium induced adjuvant arthritic model.
The extracts were given orally to arthritic rats of CFA model for 14 days in different
doses. The extracts decreased the paw edema in a gradual manner recorded on 4, 7, 11
and 14 day. During this study it was also found that the development of arthritis was
less in extract treated animals which was obvious from inflammation seen in uninjected
paw. In control group the uninjected paw was found to have high inflammation after 14
days, while in extract treated group and standard group the inflammation found was
very much less. Again these effects were higher in animals treated with methanolic
extract of G. kurroo than those treated with methanolic extract of A. amygdalina. In
adjuvant arthritis, the cellular immune response to mycobacterial antigens has been
detected and is probably involved in the development of arthritis (Whitehouse, 1982).
Identification of the 65 kDa protein as the main target of T-cell mediated responses
(Gaston et al., 1989a; Gaston et al., 1989b) and its cross-reactivity to purified
cartilage proteoglycans (Eden et al., 1985) included in rheumatoid arthritis (Quayle et
al., 1992) has emphasized its importance in arthritis during mycobacterial infections.

Our results are in concordant with the results obtained during study on ethanolic
leaf extract of C. caudate. This extract was administered orally at doses of 200 and 400
mg/kg bw to arthritic rats of CFA model for 28 days. The extract was reported to show
gradual decrease in paw volumes and change in body weight recorded on 7, 14, 21 and
28 day (Pashikanti et al., 2014). Different studies are reported on the use of these two
acute and chronic inflammatory models for the study of anti-inflammatory potential of
medicinal plants. In one such study the n-butanol extract of I. stolonifera (BE-IS
significantly decreased edema formation and reduced the carrageenin-induced exudates and cellular migration in carrageenin model. Further in CFA model improvement was shown because of treatment of these animals by BE-IS. The preliminary mechanistic work also demonstrated decrease in the levels of myeloperoxidase (MPO) and malondialdehyde (MDA), increased activity of anti-oxidant enzyme superoxide dismutase (SOD) in the BE-IS treated animals in vivo (Cai et al., 2014). Again *Euphorbia prostrata* (EPA) was investigated for anti-inflammatory and anti-arthritic potential employing these two in vivo models - carrageenin induced inflammation and *Mycobacterium* adjuvant induced chronic inflammation respectively. Results of in vivo studies revealed it to possess highly significant anti-inflammatory/anti-arthritic activity by decrease in edema formation and also with the γ-Glutamyl transpeptidase (γ-GT) concentration significantly reduced at the site of inflammation (Singh et al., 2011). 

During inflammation leukocytes and serum proteins migrate to areas of tissue injury. Recruitment of cells to inflammatory sites is dependent on release of vasoactive and chemotactic factors that increase regional blood flow and microvascular permeability and promote migration of leukocytes from the intravascular space into the tissues (Suffredini et al., 1999). It is well established that activated immunocytes are involved in inflammation process, particularly macrophages, which play a crucial role in specific and nonspecific immune responses during inflammation (Romeo et al., 2012). So these plant fractions were used to demonstrate the effect on the immune system using HA titre and DTH Balb/C models for such study. HA titre was carried out for determining the effect of these fractions on the humoral immune response, while DTH method was used for checking their effect in cell mediated immune response. All the fractions from *A. amygdalina* were observed to have an immunosuppressive effect by inhibiting antibody formation and the cellular immune response. Methanolic fraction
was again found to have more inhibitory effect on the immune response. So, only methanolic extract was used in different doses for evaluating dose dependent effect against the immune response. Cyclophosphamide was used as the standard immunosuppressant drug. The %age suppression in specific and non-specific immune responses by methanolic extracts at 750mg/kg bw was found almost similar as that by cyclophosphamide at a dose of 50mg/kg bw. Similar results were observed in *G. kurroo*, with methanolic fraction showing higher potential; however the activity observed was higher in methanolic extract of *G. kurroo* than *A. amygdalina*. The inhibition in immune response was observed to increase with the increase in dose of the methanolic extract. Further the inhibition was more in secondary humoral response than the primary response. Also the inhibition in cellular mediated response increased from 24 to 48 h. The immunosuppressive effect of these extracts is consistent with their anti-inflammatory activity. The capacity of these plants to inhibit humoral and cellular immune responses can have useful applications in some immune-mediated inflammatory disorders including autoimmune diseases (*Mirshafiey et al., 2004*). Our results followed pattern of the study of *Cleome gynandra* Linn. The ethanolic extract of aerial parts of this plant showed immunosuppressant effect in dose dependent manner in haemagglutination antibody titre and DTH method on Wistar albino rats (*Gaur et al., 2009*). Till now, the results determined the anti-inflammatory and immunosuppressive potential of *G. kurroo* and *A. amygdalina*.

Since the process of inflammation not only involves the immune cells but the pro-inflammatory cytokines also play a major role. One of the major pro-inflammatory cytokine is TNF-α which is produced by different immune cells and its rate of production and hence the concentration in blood demonstrates the extent of inflammatory response (*Beutler and Cerami, 1989*). Blood samples were taken from
treated and untreated groups of animals used in HA titre and DTH models. The serum from blood samples was analysed for the concentration of TNF-α. The results indicated a marked difference in the concentration of TNF-α between the treated and untreated groups. The animals treated with methanolic extracts had low serum TNF-α concentrations as compared to untreated control animals. Further the serum TNF-α concentration in serum of treated animals showed decrease with increase in dose of the methanolic extract and hence dose dependent effect. Results of the study carried out on ethanolic extract of *Artemisia morrisonensis* Hayata was also reported to decrease the TNF-α levels in the serum of extract treated animals as compared to untreated animals (Chou et al., 2012).

Cytokines have been observed to play an important role at the site of inflammation in edema formation by induction of vasodilatation and increased vascular permeability (Dur’an et al., 2010). Recent studies have shown that carrageenin in particular induces peripheral release of NO and PGE2 (Omot et al., 2001) and also the release of TNF-α, which then promotes IL-1 and IL-6 production in the tissue (Cunha et al., 1992). These pro-inflammatory cytokines mediate and regulate immunity and inflammation. In case of organ injury induced by endotoxin such as LPS, TNF-α is regarded as principal mediator in stimulating secretion of other cytokines along with IL-1β and IL-6 that are mainly synthesized by immune cells and play a pivotal role in the process of inflammation (Roshak et al., 1996). Lipopolysaccharide (LPS)-stimulated macrophages using Raw264.7 cells is a well-established *in vitro* inflammation model (Kim et al., 2008; Yang et al., 2013). This model was used for investigating the suppressive effect of methanolic extract of *A. amygdalina* on the release of inflammatory mediators-NO, TNF-α and IL-6 by LPS activated macrophages. The extract was taken in a dose range of 25-200μg/ml and inhibited the production of these
mediators in a dose dependent manner from LPS stimulated macrophages. The extract showed the similar inhibitory effect on these inflammatory mediators. This observation was made in the supernatant obtained from the macrophage culture. Similar results were observed with *G. kurroo* also but the suppression of these mediators was more as compared to the *A.amygdalina*. During the progression of inflammation, the most known important mediators produced by macrophages include NO, prostanoids, TNF-α, and Interleukins (*Watson et al., 1999; Kubes and McCafferty, 2000*). Among these inflammatory cytokines, TNF-α, IL-1β, and IL-6 are considered most crucial mediators for the inflammatory process, mediating immunity and activating macrophages (*Yang et al., 2013, Dai et al., 2009*). Further among the signalling molecules which are associated with the production of these inflammatory mediators are NO and PGs produced by the activity of the most crucial enzymes that is iNOS and COX-2 (*Reddy and Herschman, 1994; Dur´an et al., 2010*). Importantly, the macrophages isolated from iNOS knock-out mice and cultured in presence of LPS/IFNγ were unable to show the acute inflammatory response, thus implying the importance of NO in inflammation (*Coffey et al., 2004*). From this information it can be inferred that the contribution of methanolic extracts in anti-inflammatory activity might be by the inhibition in production of these pro-inflammatory mediators from the immune cells.

Many studies on other medicinal plants are reported where this model has been used for the analysis of inflammatory response under *in vitro* conditions. In one such study the n-butanol extract of *I. stolonifera* used in LPS-activated RAW264.7 macrophages *in vitro* reduced the production of nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in them (*Cai et al., 2014*). The anti-inflammatory effects were reported in citrus peel extracts against LPS-induced macrophage cells. The cells treated with these extracts showed slightly
decreased expression of iNOS, COX-2 and about 40% inhibition in NO production. However the extract had not shown any concentration dependent change in these effects. Further the extracts had also inhibited the production of pro-inflammatory cytokines with IL-6 mostly inhibited (44%) followed by TNF-α (13%) and no inhibition in IL-1 production (Kang, 2014). The in vitro anti-inflammatory studies were carried out on ethanolic extract of Siegesbeckia orientalis. In cell culture experiments, LPS stimulated RAW264.7 cells pre-treated with ethanol extract showed reduction in the production of NO, IL-6, and TNF-α (Hong et al., 2014).

The effect of methanolic extracts on the proliferation of B-cells and T-cells in response to LPS and ConA respectively, was investigated using MTT assay. The assay was performed by taking the single-cell suspension of spleen isolated from Balb/C mice. The methanolic extracts were found to significantly inhibit the proliferation of both cells in a dose dependent manner. The extract indicated more inhibition of T-cell than the B-cell; however the difference was not so high. Here again the G. kurroo showed more potential than A. amygdalina. These results are consistent with our earlier results where the methanolic extract was found to inhibit the humoral and cell mediated immune response. It is well known that B-cells produce anti-bodies in response to the antigens while T-cells help in framing the cellular response against the antigens. Hence the decrease in humoral and DTH response can be referred to the inhibition of the B and T-cell proliferation respectively. Similar type of study was carried out on Lawsonone and was found to show immuno suppressive effect by inhibiting the splenocyte proliferation (Ali et al., 2013).

The inflammatory response is transduced by a variety of signalling pathways. Nuclear factor kappa B (NF-κB) is one of the essential signalling molecules among them which increase the production of mediators and effector molecules resulting in
acceleration of the inflammatory process (Caamano and Hunter, 2002). A variety of adaptor molecules-MyD88, TIRAP/Mal etc. are recruited during the binding of LPS to the Toll-like receptor-4 for the activation of NF-κB pathway for the induction of pro-inflammatory genes (Barton and Medzhitov, 2003; Miller et al., 2005). NF-κB is normally present in the cytoplasm bound to I-κBα. After phosphorylation and subsequent degradation of I-κBα, NF-κB is released from the NF-κB/I-κBα complex. It then gets translocated into the nucleus where it causes induction of pro-inflammatory genes by binding to their promoter region. Because NF-κB acts as transcription factor and thus causes induction of iNOS, TNF-α, and IL-6 genes by binding to their promoter regions (Chen et al., 1995; Roshak et al., 1996; Liu et al., 2000; Vila-del Sol and Fresno, 2005; Yoshimura, 2006). So it becomes essential to investigate its expression while the production of cytokines mentioned above is studied. The expression of NF-κB (p65) was evaluated in mice peritoneal macrophages stimulated with LPS in presence of different concentrations of methanolic extract of A. amygdalina. In presence of LPS (stimulated) increased concentrations of NF-κB (p65) were registered in protein extracts of peritoneal macrophages after 24 hours as compared to vehicle (unstimulated). While the results indicated the reduced expression of NF-κB (p65) in presence of methanolic extract. Similar effect was observed in presence of the methanolic extract of Gentiana kurroo but the NF-κB (p65) expression was less (0.23 fold) as compared to methanolic extract of Artemisia amygdalina (0.41 fold) at a dose of 100μg/ml. Further this activity shown by both plants was dose-dependent. So the reduced production of the pro-inflammatory mediators observed above may be attributed to the inhibition in the expression of NF-κB (p65) and hence the effect against inflammation. This type of study is also reported by different researchers using other medicinal plants. The in vitro studies were carried out on leaf ethanolic extract prepared from Dictamnus dasycarpus
using LPS-stimulated RAW 264.7 cells. The extract was used at doses of 0.5 and 1 mg/mL and production of cytokines, transcription factor NF-κB and different enzymes involved in the promotion of inflammation were compared between the treated and untreated groups in LPS activated macrophages. The results showed suppression in NO production, decrease in pro-inflammatory cytokines viz. IL-1β and TNF-α, down-regulation in the expression of transcription factor NF-κB and decreased synthesis of pro-inflammatory enzymes, iNOS and COX-2 due to their attenuation in treated groups as compared to untreated groups in LPS activated macrophages. Further these effects were shown to be dose dependent (Ghosh et al., 2014). Furthermore, the ethanolic extract of *Siegesbeckia orientalis* was reported to inhibit LPS-induced NF-κB activation by blocking the degradation of IκB-α. Also the *in vivo* studies demonstrated decreased edema formation and less concentration of IL-6 in serum of the treated mice (Hong et al., 2014).

The methanolic extract of *A. amygdalina* was analysed for the identification of bioactive compounds by liquid chromatography-mass spectrometry (LC-MS). The compounds identified in the ESI⁺ mode [M + Na]⁺ are dihydroartemisinic acid, artemisinin, arteannunin B, dihydroartemisinic aldehyde. The compounds dihydroartemisinic alcohol and artemisinic acid were identified in the ESI⁺ [M + H]⁺ mass spectra mode. While in ESI⁻ [M – H]⁻ mass spectra mode, dihydroartemisinin, eupatilin and artemisitene were identified. The compounds identified were secondary metabolites and terpenoids in nature except for the eupatilin which is a flavonoid. Artemisinin is one of major drugs used for the treatment of malaria and has been isolated from *Artemisia annua* in 1972 by Chinese scientists (Liu et al., 1979; Klayman, 1985; Luo and Shen, 1987). The peak obtained in our LCMS report corresponds to artemisinin but does not necessarily authenticate its presence unless the
results are not compared with the standard artemisinin evaluated under the same conditions in LCMS, and finally its isolation is carried out. A number of compounds isolated from *Artemisia* species belonging to flavonoids, terpenoids, acetylenes, caffeoylquinic acids, coumarins and sterol have been shown to possess antiviral, anticancer, anti-angiogenesis, antifungal, and immunosuppressive activities. (Lee, 2007; Tan et al., 1998). Eupatilin have been reported to be present in *Artemisia umbelliformis* Lam. and *Artemisia genipi* Weber and also to possess the topical anti-inflammatory activity (Giangaspero et al., 2009). The anti-inflammatory activity of the *A. amygdalina* may be because of eupatilin or it may be some other compound/s. In order to determine the actual bioactive compound/s responsible for these activities, compound isolation and extensive study is necessary.

The analysis of liquid chromatography-mass spectrometry (LC-MS) report of the methanolic extract of *G. kurroo* revealed the presence of secondary compounds belonging to iridoids, alkaloids and flavonoids. The identified compounds in the ESI+ mode [M + Na]+ were loganic acid, swertiamarin, gentiopicroside, gentisin. In ESI+ [M + K]+ mass spectra mode single compound gentianine was identified. The compounds sweroside and norswertianolin were identified in the ESI+ [M + H]+ mass spectra mode, while in ESI− [M − H]− mass spectra mode 4″-O-β-D-glucosyl-6′-O-(4-O-β-D-glucosyl caffeoyl) linearoside, swertisin, gentioside and isogentisin were identified. The plant secondary metabolites - Iridoids and flavonoids are reported to have anti-inflammatory potential (Aquila et al., 2009; Ocampo et al., 2013). Among the various compounds identified in the methanolic extract, loganic acid, swertiamarin and gentianine are reported to possess the anti-inflammatory potential (Kwak et al., 2005; Wei et al., 2013; Saravanan et al., 2014). The anti-inflammatory potential of methanolic extract may be attributed to these compounds. Our results are in
concordance with the results obtained from the LCMS analysis of root methanolic extract of *G. kurroo*. The report also shows the presence of same compounds in the root methanolic extract except for the gentianine (Wani et al., 2013). Hence our results validate the presence of these compounds in this medicinal herb. Some of these compounds have also been reported in different species of the same genus such as *Gentiana affinis*, *Gentiana algida*, *Gentiana alpina*, *Gentiana asclepiadea*, *Gentiana atuntsiensis* and *Gentiana bulgarica* (Jensen et al., 1975). Lastly, this work was a trial based study; the detailed research is still to be carried out for the authentication of the actual bioactive compound/s responsible for the anti-inflammatory activity.