5. Discussion

Inflammation usually occurs when infectious microorganisms such as bacteria, viruses or fungi invade the body, reside in particular tissues and/or circulate in the blood (Artis and Spits, 2015; Isailovic et al, 2015; Pedraza-Alva et al, 2015). Inflammation may also happen in response to various processes such as tissue injury, cell death, cancer, ischemia and degeneration (Lucas et al, 2009; Rock et al, 2011; Fernandes et al, 2015; Heppner et al, 2015; Loane et al, 2016; Waisman et al, 2015). Generally, both the immune responses such as innate and adaptive are involved in the development of inflammation (Artis and Spits, 2015, Rock et al, 2011, Waisman et al, 2015). The innate immune system is the foremost defensive mechanism against invading microorganisms and cancer cells, macrophages, mast cells and dendritic cells. The adaptive immune systems involves the activity of more specialized cells such as T and B cells that are responsible for eradicating the invading pathogens and cancer cells by producing specific receptors and antibodies. Abundant of inflammatory mediators are secreted during an inflammatory process. Inflammatory substances are usually divided into two main categories: pro-inflammatory and anti-inflammatory mediators. Nevertheless, some mediators such as interleukin-12 possess both pro- inflammatory and anti-inflammatory properties (Vignali and Kuchroo, 2012). Among the inflammatory mediators and cellular pathways that have been extensively studied in association with human pathological conditions are cytokines (e.g., interferons, interleukins and tumor necrosis factor α), chemokines (e.g., monocyte chemoattractant protein 1), eicosanoids (e.g., prostaglandins and leukotrienes) and the potent inflammation modulating transcription factor such as nuclear factor κB (Vignali and Kuchroo, 2012).

The management of inflammatory diseases is very difficult due to the chronic and prolonged treatment of disease that may result in to drug resistance. The glucocorticoid plays main role in the variety of fundamental processes such as cell proliferation, inflammation, immune responses, metabolic homeostasis, development and reproduction (Beato and Klug, 2006). Topical GCs are used for the treatment of inflammatory diseases such as dermatitis whereas; inhaled GCs are used for the treatment of asthma and chronic obstructive pulmonary disease (COPD) (Newton et al, 2010; Barnes, 2013). But, the long term use of GCs shows severe and permanent side effects such as osteoporosis, tissue wasting, cataracts, peptic ulcer, diabetes mellitus, Cushing’s syndrome, hypertension, skin atrophy, osteoporosis, psychosis and hypothalamic–pituitary–adrenal axis suppression (McDonough et al, 2008).
Glucocorticoids at higher dose binds with the DNA recognition sites for activation of transcription by increased histone acetylation of anti-inflammatory genes and transcription of several genes linked to side effects (trans-activation). The severe asthmatic patient sometimes shows decreased responsiveness to glucocorticoid treatment. GCs also shows post-transcriptional effects and decrease stability of pro-inflammatory mRNAs. Many mechanisms of GC resistance have been identified such as, phosphorylation; post-translational modifications of GR, reduced histone deacetylase-2 expression, increased P-glycoprotein-mediated drug efflux, cytokine activation of mitogen activated protein kinase pathways, excessive activation of transcription factor activator protein and increased levels of the macrophage migration inhibitory factor (Barnes and Adcock, 2009).

Thus, the above mentioned treatments are ineffective and accompanied by a series of undesirable systemic side effects (Mease and Menter, 2006). Therefore, currently research has been thrown on searching effective anti-inflammatory treatments that interfere with the inflammatory cascade such as inhibition of nuclear factor kappa B (NF-κB). NF-κB transcription factor regulates the transcription of essential pro-inflammatory genes, such as genes for cytokines, chemokines, adhesion molecules, nitric oxide synthase, cyclo-oxygenase-2 (COX-2) and others. Thus, inhibition of NF-κB transcription factor results in the decreased release of these mediators that ultimately causes a decline of the inflammatory process (O’Neill, 2006). A large number of plant derived natural products have established anti-inflammatory activity such as; flavonoids, steroids, tannins and triterpenes that interfere with an inflammatory cascade (Rios et al, 2009). Many of the above mentioned compounds act by non-specific mechanism (e.g. antioxidants), but may also act via specific mechanisms such as the inhibition of inflammatory mediators (Calou et al, 2008). In order to overcome these undesirable side-effects, the research work was focused on to identify novel bioactive phytoconstituents with therapeutic potential with no or minimum side effects.

Thus, the present study demonstrates the anti-inflammatory property of HG and HG + FC combination on various inflammatory models. The major objective of current research work was to curtail the transactivation mediated side effects and to produce the transrepression mediated anti-inflammatory effect of synthetic glucocorticoid Fluticasone (FC) by combining with plant steroid at the low dose.

Numerous medicinal plants present in the plant flora exhibits anti-inflammatory activity. Currently the research is carried out on steroidal compounds present in the medicinal plants.

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Anti-inflammatory plants containing steroidal structure includes *Agave sisalana* (Hecogenin), *Trigonella foenum graecum* (Diosgenin), *Solanum xanthocarpum* (Solasodine), *Boswellia serrata* (Boswellic acid), *Glycyrrhiza glabra* (Glycyrrhizin), *Commiphora mukul* (Guggulsterone), *Withania sominifera* (Withaferin-A) (Paik et al, 2005; Shishodia and Aggarwal, 2006; Govindana et al, 2004; Chrubasik et al, 2007; Akamatsu et al, 1991; Gebhard et al, 2009; Kazutoshi et al, 1999). In the present study selection of plant steroid was done by studying an effect of various plant steroids like Hecogenin, Diosgenin, Solasodine, Glycyrrhizin, Boswellic acid, Guggulsterone, Sarsasapogenin or Withaferin-A on Croton oil induced ear edema in mice. All plant steroids have exhibited significant anti-inflammatory activity by virtue of inhibition of ear edema in mice. The HG showed maximum inhibition of ear edema at 50 µg/mice dose as compared to other plant steroids which was found comparable to FC. Therefore, HG was selected for further anti-inflammatory activity in various animal models.

The dose of HG (50 µg/animal) and in combination HG + FC (25 µg/animal each) was selected on the basis of pilot study. Initially the dose finding study was performed by selecting various doses of HG such as 50, 75 and 100 µg/animal and all doses exhibits significant anti-inflammatory activity in mice. Hence, smallest dose of drug was selected for further anti-inflammatory activity in various animal models (50 µg/animal). We have demonstrated the anti-inflammatory activity of the HG and its combination with FC in various acute and chronic anti-inflammatory animal models such as Croton oil induced ear edema in mice, Cotton pellet induced granuloma in rats, TNBS induced colitis in rats (Ingawale and Patel, 2016), DNFB induced dermatitis in mice, OVA induced lung edema in mice and CFA induced arthritis in rats.

The distinctive symptoms of acute inflammation are redness, heat, edema and pain. Therefore measurement of edema is important tool for evaluation of anti-inflammatory activity of drug. It is also useful for the measurement of skin inflammation stimulated by phlogistic agents such as croton oil. Mice ear edema is one of the most common *in vivo* inflammatory models that have been extensively used in the exploration of anti-inflammatory activity (Phanse et al, 2012). The model not only allows us the identification of the potential allergenic agent on the basis of challenge induced increases in ear thickness in sensitized animals, but also examines the subsequent study of potential inhibitory agents (Kimber et al, 1999). Numerous drug classes such as bradykinin antagonists, histamine antagonist and serotonin antagonist, vanilloid antagonists, steroidal and non-steroidal anti-inflammatory agents can modify the ear edema.
edema response depending on the nature of edema producing substance. The croton oil induced inflammation is the very common model for study of an exudative type of acute inflammatory conditions (Tonussi and Ferreir, 1994). Croton oil induced ear edema is a widely used model for studying the skin inflammatory process and for pinpointing the anti-inflammatory agents that could be beneficial for the skin disorders treatment (Young, 1989; Gabor, 2000). Intense vasodilatations, inflammatory cell infiltration, edematous changes of skin are the distinctive signs of acute inflammation are observed on croton oil topical application (Ighodaro et al, 2010).

Croton oil is a phlogistic agent extracted from *Croton tiglium* belonging to the family Euphorbiaceae. It shows an irritant and vesiculant effect on the skin. It consists of a mixture of lipids that contains 12-O-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as chief irritant agents. The topical application of croton oil or TPA to the skin stimulates an acute inflammatory reaction characterized by proliferation, vasodilatation, edema formation, polymorphonuclear leukocyte infiltration to the tissue and activation of nuclear oncogenes (Garg et al, 2008). TPA is also stimulated protein kinase C that further activates other enzymatic cascades, such as mitogen activated protein kinases and phospholipase A2 with the release of platelet activation factor and arachidonic acid. The mediators involved in this inflammatory cascade endorse vasodilation, vascular permeability, the release of histamine and serotonin, leukocytes migration, the synthesis of prostaglandins and leukotrienes. Corticosteroids, leukotriene B4 antagonists, cyclooxygenase inhibitors and 5-lipoxygenase inhibitors had previously shown the topical anti-inflammatory activity in croton oil induced skin inflammation (Murakawa et al, 2006).

In this model, topical application of HG and Combination applied to the mice ear treated with 2.5 % v/v croton oil showed the significant decrease in the dermal thickness of croton oil treated mice. The data is further supported by histological analysis of ear tissue and measurement of serum pro-inflammatory cytokines levels. It has been reported that croton oil application showed marked increase in the ear thickness with clear evidence of edema, epidermal hyperplasia and substantial inflammatory cell infiltration in the dermis with accompanying connective tissue disruption (Boukhatem et al, 2014). In the present study the croton oil treated mice showed increase in the dermis thickness, characterized by connective tissue loosening, disorganization of extracellular matrix fibers and epidermal hyperplasia which was significantly inhibited by treatment of mice with HG and Combination indicates combination has beneficial effect in acute inflammatory conditions.
Cellular infiltration denotes an imperative characteristic of skin inflammation and neutrophils are the major type of cells that participates in the cellular infiltration around the inflammatory area. Neutrophils play a vital role in the cutaneous inflammation. Accumulation of leukocytes in the skin is essential for the advancement of inflammatory process as well as for increasing the expression of certain inflammatory enzymes such as cyclooxygenase-2 (Sanchez and Moreno, 1999; Schaefer et al, 2004). MPO is an enzyme found in the primary granules of polymorphonuclear neutrophils and used as an index of inflammation severity (Masoodi et al, 2011). MPO activity is directly related to the amount of leukocyte infiltration at the site of inflammation. This parameter reveals the degree of cell damage and level of cell injury in the mice ear tissue (Krawisz et al, 1984). MPO (biomarker of inflammation) activities were significantly increased in the mice ear tissue treated with croton oil (2.5% v/v) as compared with normal control mice only. Topical treatments with HG and its combination were able to inhibit MPO activity significantly, indicating that these compounds may influence migration of inflammatory cells in the inflammatory process (Petrovski et al, 2008).

TNF-α is a pleiotropic pro-inflammatory cytokine that elicits a wide spectrum of physiologic and pathogenic events such as proliferation, differentiation, cell death, modulation of gene transcription, and inflammation (Qidwai and Khan, 2011). It plays a key role in the inflammatory and autoimmune disorders that is produced mainly by macrophages and T cells in response to a variety of stimulations (Jash et al, 2011). IL-12 is an important pro-inflammatory cytokine that plays a main role in the acute inflammation (Choi et al, 2010). These cytokines (TNF-α and IL-12) are important components of innate and adaptive immunity that arbitrate the activation and trafficking of immune cells in response to inflammation. The increased level of TNF-α and IL-12 was observed in mice treated with croton oil as compared to the normal control group. While, treatment of mice with HG, FC and combination showed significant reduction in the TNF-α and IL-12 levels. Hence, from this experiment it was observed that the main anti-inflammatory effect of HG and combination could be due to suppressive effect on the pro-inflammatory cytokines.

Chronic inflammation is the long term reaction arises when the acute response is inadequate to remove the inflammatory agents. The chronic inflammatory process involves infiltration of neutrophils and proliferation of fibroblasts with exudation of fluid at the site of inflammation. Such a process happens by the development of proliferative cells that can either spread or form granuloma (Gupta et al, 2003).
The Cotton pellet induced granuloma model is famous for screening the chronic phase anti-inflammatory activity that is characterized by fibroblast proliferation, monocyte infiltration, exudation and angiogenesis (Majno, 1998; Vogel, 2002). The transudate phase of chronic inflammation causes increase in the wet weight of cotton pellet while presenting inflammatory response to the sub-cutaneously implanted cotton pellet shows the formation of granuloma. Therefore, increase in the dry weight of cotton pellet is considered as proliferative phase of inflammation (Cuman et al, 2001; Swingle and Shideman, 1972; Kumar et al, 2005). The implanted cotton pellets absorbs the surrounding tissue fluid increases the wet weight of the granuloma tissue and dry weight correlates well with the granulomatous tissue formed surrounds the cotton pellet (Raju et al, 2005; Zhu et al, 2011; Olajide et al, 1999; 2000). In this study, HG and combination decreased the weight of dry granuloma tissue significantly as compared to DC group rats. This may be due to the ability of HG in reducing the number of fibroblasts, the synthesis of collagen, mucopolysaccharide and prevention of an angiogenesis process (Babu et al, 2009).

Mucopolysaccharide is a naturally occurring antithrombotic and anti-inflammatory agent used for the treatment of thrombophlebitis, osteoarthritis and thromboembolism (Heimendinger et al, 1953). Cerqueira et al., 2012 already have reported the anti-inflammatory activity of HG in rats by evaluating various biomarkers of inflammation such as oxidative stress, lipid peroxidation and myeloperoxidase. The inflammatory process leads to production of various reactive oxygen species (ROS) and fibroblast is one of the important endogenous sources of production of oxidants (ROS) (Dalle-Donne et al, 2005). Hence, it is concluded that HG and its combination HG + FC is effective in preventing cotton pellet induced granuloma by reducing the number of fibroblasts, the synthesis of collagen, mucopolysaccharide and prevention of an angiogenesis process. This may be due to the ability of HG in reducing the number of fibroblasts, the synthesis of collagen, mucopolysaccharide and prevention of angiogenesis process (Babu et al, 2009).

TNF-α and IL-12 are two important pro-inflammatory mediators that have been released by various cell (including mast cells, macrophages, neutrophils, eosinophils, and epithelial cells) in response to inflammatory reactions. The elevated levels of both the cytokines were significantly decreased by rats treated with HG and combination. The inhibition of release of inflammatory cytokines such as TNF-α and IL-12 may be responsible for the anti-inflammatory action (Yadav et al, 2011).
The adrenal gland, spleen and thymus gland are an imperative organ serves as a reservoir for the cells and antibody formation that participates in the immune responses (Ismail et al, 2008; Pedernera et al, 2008). The adrenal and thymus gland indices of rat treated with acetonised cotton pellets were significantly decreased as compared to normal control rats. The index of both the gland was significantly increased in rats treated with HG and combination as compared to FC treated rats indicating significantly less deterioration related to immune cell apoptosis by HG and its combination.

The Ulcerative colitis (UC) and Crohn's disease (CD) are two major types of Inflammatory Bowel Disease (IBD). These are characterized by unrelieved and relapsing inflammation of the gastrointestinal tract (Cho, 2008). The pathogenesis of IBD is complex and a variety of factors responsible for IBD are participation of environmental triggers, microbial agents, genetic predisposition and immune dysfunction (te Velde et al, 2006). TNBS induces diffuse colonic inflammation, characterized by increased leukocyte infiltration, edema and ulceration (Isik et al, 2011). In fact, TNBS induced colitis is a hapten induced colitis model in which Th1-mediated immune response involving various cytokines including TNF-α and IL-12 serving as effector cytokine that result in transmural infiltration and inflammation (Ikeda et al, 2008; Takagi et al, 2010). The characteristic features of TNBS induced colitis inflammation of the rat resemble to those of human IBD by numerous clinical and histopathological features (Ford et al, 2011). The first choice of treatment for IBD is 5-aminosalicylic acid but, these drugs are not helpful and resulted into remission of the disease. This condition of IBD is further treated with corticosteroids and the patients may become either resistant or dependent on the drugs (Lima et al, 2010).

The present study confirmed, that treatment of rats with HG and combination was able to reduce the severity and extent of the acute colonic damage induced by TNBS. The presence of adhesions between the colon and adjacent organs, which results from transmural inflammation, is a common feature of TNBS induced colitis (Morris et al, 1989). The decrease in the extent and severity of colitis induced by hapten was accompanied by a lower incidence of diarrhoea, adhesions and minimum macroscopic score. In the present study effect of HG and combination on lesions score, diarrhea score and adhesion score was evaluated. The trans-rectal instillation of TNBS in rats shows the presence of diarrhoea as an indicator of the colon inflammation. The rats also suffered from a marked colonic mucosal damage, edema, deep ulcerations and hemorrhage. The trans-rectal administration of HG and combination
significantly elevates the severity and extent of colonic injury and diarrhea. The data was supported by histopathological analysis of rat colon tissue.

Several chemically induced methods of colitis have shown proliferation of inflammatory cells, increase in crypt length, moderate to marked hyperplasia, edema and inflammation (Ulrike et al, 2014). In our study the severe disruption of normal architecture of colon with extensive ulceration and inflammation with necrosis, edema and presence of inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and eosinophils) infiltration in the mucosa and distortion of crypts architecture were significantly reduced by treatment of rats with HG and combination.

The TNBS treated rats showed significant decrease in the adrenal, thymus and spleen gland indices due to colonic inflammation and that can be significantly increased by the treatment of rats with HG (50 µg/rat) and Combination (25 µg/rat, each). The decrease in body weight during inflammation is due to reduced absorption of nutrients through the intestine. Therefore, the restoration of the body weight in rats by HG may involve the improvement in the absorption of the nutrients through the intestine of rats. The elevated blood sugar levels were also significantly reduced in rats treated with HG and combination treated rats.

The level of MPO enzyme was increased in TNBS treated group (Lima de et al, 2010; Dutra et al, 2011; De-Faria et al, 2012). On the contrary, HG and combination treatment led to reduced MPO activity in inflamed colon, pointing out an inhibitory effect on granulocyte infiltration. The dysregulation of either immunity or intestinal barrier function allows the initiation of IBD resulting in chronic active inflammation with the production of pro-inflammatory cytokines such as TNF-α and IL-12 (Lee and Fedorak, 2010; Hai et al, 2011; Kavanaugh et al, 2011). Elevated levels of TNF-α have been observed in the experimental colitis patients (Lee and Fedorak, 2010; da Silva et al, 2010). In this experiment, the levels of TNF-α were significantly increased in the colon at 48 h after TNBS instillation. In contrast, the HG and combination treated rats showed a significant decline in the TNF-α level (Huang et al, 2010).

Another cytokine released during an infiltration process is IL-12, which is well known as a strong immunosuppressive, mainly through its negative effects on antigen presentation. During IBD process, we observed that IL-12 levels were elevated in colonic tissues, when compared with the normal control group (Lindsay et al, 2002; Zhou et al, 2010). On the other
hand, treatment of rats with HG and combination was able to prevent IL-12 elevation, suggesting that this results in a better modulation of inflammatory process and protection of gut mucosa.

TNF-α and MPO results indicate that HG and combination treatment may be effective in protecting the gut mucosa by preventing immune cell infiltration, and consequently, preventing MPO activation and pro-inflammatory cytokine release. The results of colitis are supported by histopathological analysis of rat’s colon tissue. The severe disruption of normal architecture of colon with extensive ulceration and inflammation with necrosis, edema and diffuse inflammatory cells (polymorphonuclear leukocytes, lymphocytes, and eosinophils) infiltration in the mucosa and distortion of crypts architecture were significantly reduced by treatment of rats with HG and its combination with FC.

The etiological treatment of Atopic Dermatitis (AD) involves avoidance of the offending agent. In certain situations, removal of the allergenic agent is not possible and the therapy involves assuaging the inflammatory reaction (Saint-Mezard et al., 2004). AD is a common skin disease characterized by edema, erythema, excoriation and scaling of skin (Leung and Bieber, 2003; Yang, 2012). The well-established topical treatment for AD involves the use of corticosteroids and phototherapy but, these treatments may be ineffective because of their risks or unresponsiveness. Topical steroidal therapy is very successful for the treatment of AD but, because of its potential adverse effects it cannot be administered for long term use (e.g. skin atrophy and hyperpigmentation of skin) (Cohen and Heidary, 2004). Therefore, it is necessary to minimize the corticosteroid dosage schedule. Owing to the low cost and safety, herbal medicines can be a newer drug therapy for treatment of AD in patients. Newly developed anti-inflammatory and immune-modulators drugs may be better options for the treatment of AD (Saint-Mezard et al., 2004). Therefore, we have confirmed the anti-inflammatory effects of HG and combination on AD like skin lesions in Balb/c mice.

The skin inflammatory reaction by painting mice ear tissue with hapten (DNFB) follows three different steps. First, activation of skin innate immunity; Second, production of IFN-γ by activated T cells and cytotoxicity that shows activation of resident cells in skin and production of new inflammatory mediators. Third, leucocytes are recruited at the site of inflammation and induce the morphological changes such as edema, epidermal spongiosis and infiltration of inflammatory cells (Saint-Mezard et al11., 2004). The hallmark of Th1 skewing reaction of T cells is IFN-γ that shows elevated production of various cytokines and chemokines such as
IL-1, TNF-α, GM-CSF and macrophage inflammatory protein-2, resulting in immense accumulation of leukocytes (Kobayashi, 2008).

In our animal model, repeated application of DNFB causes an increase in ear thickness, ear weight and induced hyperplasia, edema and spongiosis in AD mice (Jeonghyeon et al, 2013). These results imply that increased ear thickness and weight were due to inflammatory reactions including hyperplasia, spongiosis, edema and immune cell infiltration which are believed to mimic the human AD like condition. The treatment of mice with HG and combination effectively prevented the enlargement of ear thickness and ear weight, as well as hyperplasia, edema and spongiosis in inflamed tissues. These results indicate that HG can effectively prevent these inflammatory reactions, leading to inhibition of ear thickness and weight increases in inflamed tissues. So, the results of present study showed that combination could replace the usage of corticosteroids for the treatment of allergic AD.

The measurement of MPO activity reveals the degree of cell damage and level of cell injury in the mice ear tissue (Krawisz et al, 1984). MPO level was significantly increased in the mice ear tissue treated with DNFB challenge when compared to normal control mice. Topical treatments of Balb/c mice with HG and combination were significantly inhibited the MPO activity, indicating that these compounds may influence migration of various inflammatory cells in the inflammatory process (Pietrovski et al, 2008).

The contact of hapten with skin causes the release of TNF- α and IL-12 during the DNFB sensitization phase (Saint-Mezard et al, 2004). Therefore, TNF-α and IL-12 are the important mediators of cutaneous inflammatory response (Kock et al, 1990; Piguet et al, 1991). The previous research reports suggested that the inhibition of pro-inflammatory cytokines such as TNF-α and IL-12 might be useful for the treatment of AD (Griffiths et al, 2005; Groves et al, 1995; Watanabe et al, 2007). Furthermore, TNF-α also exerts a stimulatory effect on skin resident cells, resulting in leukocytes enrollment during contact hypersensitivity responses (Grabbe and Schwarz, 1988). In the present study, the levels of TNF-α and IL-12 were elevated by DNFB challenge in inflamed ear tissues. Moreover, HG and combination treatment in mice effectively reduced the production of TNF-α and IL-12 in inflammatory tissues. These data indicates that HG is an anti-inflammatory agent by its Th 1 skewing reaction, resulting in the diminution of inflammatory reactions (hyperplasia and spongiosis) and immune cell infiltration.
It has been previously established that, DNFB application results in inflammatory skin which leads to dermal thickening along with skin lesions involving edema, erythema, hemorrhage and hyperkeratosis (Kim et al, 2015). Following DNFB application, secretion of inflammatory cytokines and chemokines leads to infiltration of inflammatory cells and increasing epidermal keratinocytes promotes accumulation of collagen, dermal thickening and tissue damage (Purwar et al, 2008). In our study DNFB induced AD model in mice, the treatment with HG and combination effectively prevented the skin atrophy characterized by decrease in ear thickness and ear weight. These results indicate that combination can effectively prevent these inflammatory reactions; leading to inhibition of skin atrophy in inflamed tissues indicates its beneficial role in dermatitis.

Previously reported study by Bergot et al., 2015 showed that DNFB induced mouse develop characteristic pathological features of AD similar to those described patients of dermatitis. Pathological features of ear skin include diffuse epidermal hyperplasia with hyperkeratosis, light spongiosis within the stratum spinosum, and dermal thickening (Bergot el, 2015). Histopathological studies revealed that DNFB challenge increased the dermal thickening, hyperplasia, presence of an inflammatory cells, increase in the size and number of mast cells in DNFB treated mice. Inflammatory hyperplasia, increase in the size and number of inflammatory cells were decreased in the histopathological section of mice treated with HG and combination effectively.

Asthma is one of the serious, worldwide public health problems that affect all age group. It is an airway inflammatory disease that can be exacerbated by numerous extrinsic factors, such as allergens (Agarwal and Gupta, 2011). The infiltration of cells into the lung tissues, the alveolar fluids, in the blood and alterations in lung histology are features largely associated with asthma (Pynn et al, 2012). However, the pathophysiological mechanism of asthma is unclear despite the increasing prevalence of this disease. Moreover, the current therapy for asthma fails to provide an adequate curative solution. At the present conditions, corticosteroids are the drugs most commonly used drugs to control the bronchial airway inflammation. However, the corticosteroid therapy has important adverse effects on their long term use and some patients shows complete resistance to corticosteroids therapy or fail to show clinical improvement after the high dose of glucocorticoids treatment. Therefore, the development of safer and more effective anti-asthmatic drugs is the need of today’s life (Ribeiro-Filho et al, 2013).
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Balb/c mice are sensitive responders to OVA and are a well-established airway inflammatory disease model that represents human asthma (Inman et al, 1999; Corry and Kheradmand, 2009). In OVA induced lung edema model, OVA serves as a source of allergenic agent for the initiation of asthma. After sensitization and aerosol challenge with OVA in a murine model, type 1 immune response is triggered. The Th2-skewed response is characterized by release of Th2 cytokines such as IL-4, IL-6, and IL-12 mediate production of ovalbumin-specific IgE immunoglobulin by B cells and recruitment of eosinophils, mast cells, and mucus-secreting goblet cell hyperplasia and other inflammatory cells (Camateros et al, 2007). Pro-inflammatory cytokine participation mainly by IL-1, IL-6 and TNF-α further intensifies the inflammatory response (Wills-Karp, 2004). The early response to allergen is as a result of IgE dependent type I hypersensitivity reaction driven by mast cell-derived mediators, whereas the late response develops as a result of activation of antigen-specific T cells. The secretion of pro-inflammatory cytokines by activated mast cells, T-lymphocytes and injured bronchial cells were responsible for the pathogenesis of OVA induced bronchial asthma in mice (Kumar et al, 2008). In this search, the HG and combination were investigated in the present study for their anti-asthmatic activity on OVA induced lung edema in Balb/c mice.

Since, the migration of inflammatory cells into the lungs is inevitable in allergic and asthmatic disorders; it has been found that a significant increase in the number of total cell and differential cell count in BALF of OVA challenged mice. The total cell count is an important parameter in asthma. The results revealed that HG and combination shows significant reduction in the total cell count as compared to OVA challenged mice. In the differential cell count the increased number of eosinophils and neutrophils has been observed. HG has the potential of attenuating the proliferation and transmigration of eosinophils and neutrophils in the lung that plays an important role in allergic asthma (Drazen et al, 1996; Thomas et al, 1995). The increased number of eosinophils shows the phenomenon of eosinophilic infiltration and the increased number of neutrophils leads to activation of IL-8 which induces sputum during allergic asthmatic condition. The result revealed that combination proved to be more potent in reducing the eosinophil and neutrophil count as compared to HG alone. From, the results it could be suggested that HG and combination dampens the asthmatic inflammation in the lung by its action on inflammatory cells (eosinophils).

The adrenal, thymus and spleen glands indices are important organs of an immune system whose weight were significantly decreases in the OVA sensitized mice and that can be significantly increased by the treatment of mice with HG (50 µg/rat) and combination (25
The body weight of OVA sensitized mice also get decreases due to reduced absorption of nutrients through the intestine. Therefore, the body weights of mice were found to be improved by the treatment with HG and combination. The elevated blood sugar levels were also significantly reduced in mice treated with HG and combination.

The important parameter estimated with the lung homogenate of Balb/c mice is MPO. Eosinophils and neutrophils play a major role in the presence of MPO, which is considered as the marker for inflammation. MPO is hemeprotein catalyzes the formation of HOCl from H₂O₂. HOCl contributes directly to tissue dysfunction and destruction of protein along with other free radicals. The elevated level of MPO has been observed in lung tissue of OVA sensitized mice which was significantly decreased by HG and combination. The combination of HG with FC showed most significant reduction in the elevated MPO level that indicates the cellular damage leading to the altered cellular function within the inflamed lung (Kirkham and Rahman, 2006).

TNF-α and IL-12 are two important pro-inflammatory mediators that have been released by various cell (including mast cells, macrophages, neutrophils, eosinophils and epithelial cells) in response to allergic inflammation. This pro-inflammatory mediator played a vital role in immune-regulation to induce lung edema in asthmatic condition (Thomas et al, 1995). Moreover, it caused the release of neutrophils, myofibroblasts as well as eosinophils activation and alteration in vascular permeability (Kandhare et al, 2012; 2015). Significantly elevated levels of TNF-α and IL-12 also have been documented in the airways of asthmatics patients (Broide et al, 1992). The increased level of TNF-α and IL-12 in the serum sample of Balb/c mice reflects the activation of pro-inflammatory response whereas the administration of HG and combination significantly inhibits these levels may be virtue of its anti-inflammatory potential.

IL-6 is a multifunctional cytokine produced by various hematopoietic and non-hematopoietic cells in response to different stimuli (Kishimoto, 2005). It is responsible for activation of insulin-like growth factor 1 (IGF-1), erythroid differentiation factor (EDF) and platelet derived growth factor (PGDF) that produces activation of fibroblast and smooth muscle hyperplasia (Gupta et al, 2014). The increased level of IL-6 was observed in the serum samples of OVA sensitized mice. This increase in the IL-6 level was significantly decreased by HG and combination demonstrated uniform inhibition of IL-6 level.
TXA$_2$ is a prostanoid lipid mediator and act as a potent airway constrictor. It is synthesized by oxidation of arachidonic acid into PGH$_2$ under the influence of either cyclooxygenase-1 or cyclooxygenase-2. The enzyme thromboxane synthase catalyzes the isomerization of PGH$_2$ to TXA$_2$, which has a very short half-life and is rapidly hydrolyzed to the stable, inactive metabolite TXB$_2$ (FitzGerald et al, 1985). In this OVA induced asthma model, the elevated level of TXB$_2$ increases intracellular Ca$^{2+}$ ions level that is often accompanied by contraction of bronchial smooth muscles (Dorn, 1993; Janssen and Daniel, 1991).

This anti-asthmatic activity has been further strengthened by histopathological results obtained from OVA sensitized model. After pre-sensitization of Balb/c mice by intraperitoneal (i.p.) injection of OVA (50 μg) and alum (1 mg) solution on 0 and 7$^{th}$ day they were challenged with OVA aerosol 2.5% (w/v) through a nebulizer (inhalation) on 14$^{th}$ and 21$^{st}$ day. This has resulted in structural changes in the asthmatic bronchi airways which included goblet cell hyperplasia and metaplasia, epithelial fragility, enlarged mucus glands, matrix deposition in the airway wall, increased airway smooth muscle mass, thickening and elastin abnormalities in the bronchial wall. The slides stained with H & E were analyzed for change in lung architecture such as thickness of the epithelium, sub epithelial smooth muscle layers and small airways. The slides stained with Giemsa were analyzed for goblet cell arrangement feature including goblet cell hyperplasia, hypertrophy and metaplastic condition of the bronchi. Both the histopathological studies were correlates with previous studies ((Bai and Knight, 2005; Olmez et al, 2009). The histology of lung tissue stained with H & E and Giemsa reveals that HG and Combination were effective in restoration of bronchial changes in Balb/c mice.

In the laboratory animal’s arthritis is primarily induced by chemical (formaldehyde) or biological (Mycobacterium) agents used for screening of a range of anti-arthritic agents. On the other hand, it is also said arthritis is not only a disease of joints, but also associated with immune, hepatic, renal and other organ systems damage that directly or indirectly affects on the joints. Hence, it is needed to determine the pathological and biochemical aspects of arthritis that are necessary in evaluating the activity of drugs (Rainsford, 1982). Osteoarthritis and rheumatoid arthritis are two mainly common types of arthritis characterized by joint inflammation, immune cell infiltration, synovial hyperplasia, joint pain and swelling that result in the destruction of joint integrity and function disability (Neugebauer et al, 2007).
Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting about 1% of the population in developed countries (Amresh et al, 2007). The clinical findings of human arthritis such as proliferative synovitis, swelling of limbs, inflammatory cell infiltration, bone erosion and destruction of cartilage are common to adjuvant induced arthritis in rat. RA is a symmetric polyarticular arthritis that mainly affects the small diarthrodial joints of the hands and feet. In addition to inflammation of the synovium (joint lining), the pannus (front of tissue) invades and destroys the local articular structures (Noguchi et al, 2005). The synovium is a cellular structure with a delicate lining of the joint. The hyperplasia of delicate lining results from a noticeable increase in fibroblast like and macrophage like synoviocytes. Locally expressed degradative enzymes, including metalloproteinases, serine proteases, and aggrecanases, digest the extracellular matrix and destroy the articular structures (Noguchi et al, 2005).

CFA induced arthritis is well a recognized model and has been commonly used for evaluation of anti-inflammatory and anti-arthritic potential of various agents (Costa et al, 2004; Walz et al, 1971). In the CFA induced arthritis model, rats were selected for induction of arthritis because rats develop a chronic swelling in the multiple joints with infiltration of inflammatory cells, destruction of joint cartilage and bone that shows close similarities with human RA (Sing and Majumdar, 1996). CFA is composed of an inactivated and dried *Mycobacteria tuberculosis*, that contain different pathogen-associated molecular patterns including toll-like receptor 2, 4, and 9 agonists and responsible for stimulation of a cell mediated immunity that increases the synthesis of certain immunoglobulins (Akira et al, 2006). The injection of CFA to rats shows reactivity to heat shock proteins, cartilage proteoglycans, and interactions with intestinal flora (van Vollenhoven et al, 1998; van de Langenijt et al, 1994). The CFA induced arthritis follows a biphasic response to acute local inflammatory reaction that subsides after 3-4 days and a chronic systemic reaction that shows a relapsing-remitting course after initial two weeks and can persist for several months (Neugebauer et al, 2007). Further, release of diverse inflammatory mediators such as, cytokines (TNF-α, IL-6, IL-12 and TXB₂), lysosomal enzymes, hydrolytic enzymes, and prostaglandins (PGs) are known to participate in the pathogenesis of RA (Eric and Lawrence, 1996; Sandoval et al, 2000; Pandey et al, 2005; Naik and Wala, 2014; Billiau and Matthys, 2001).

Paw swelling is an index of measurement of anti-arthritic activity of various drugs (Rajendran and Krishnakumar, 2010). The measurement of paw swelling is simple, quick and sensitive procedure for evaluating the intensity of inflammation (Begum and Sadique, 1988). The RA
shows the presence of edema of periarticular tissues such as ligaments and joint capsules. The intensity of ligaments swelling and joint capsule swelling increases in the initial phase of inflammation and becomes constant in further two weeks of arthritis. These changes in the paw volume are associated with increase in the concentration of granulocytes and monocytes cells in the joint tissue (Kweifio-Okai and Carroll, 1993). In chronic inflammatory state activation of macrophages results in the production of several cytokines such as IL-6 and TNF-α that have been associated in immune arthritis (Arend and Dayer, 1990; Thorbecke et al, 1992).

In the present study, oral treatment of HG and combination to rats showed anti-arthritic effect in all the inflammatory parameters. The significant increase in a paw thickness after the subplantar administration of CFA is reflecting the status of arthritis. The treatment of rats with HG and combination showed the significant decrease in the paw thickness by inhibiting the release of inflammatory mediators, indicating its anti-inflammatory. In this model, arthritis score is an index of the joint inflammation after CFA immunization (Yu et al, 2006). A selective reduction in the arthritis score distinguishes the immunosuppressive effects of HG from its anti-inflammatory effects. So, the present study revealed that paw volume, arthritic score and joint diameter were significantly increased in CFA challenged rats as compared to NC rats. The oral treatment of rats with HG and combination significantly decreased the paw volume, arthritic score and joint diameter as compared to CFA treated rats.

The spleen is an imperative organ and serves as a reservoir for the cells and antibody formation involved in immune responses. The decrease in spleen weight and increase in thymus weight are correlated to a stimulatory effect on the immune system (Pedernera et al, 2006). The subplantar administration of CFA significantly decreased the spleen and thymus gland index that can be significantly increased by administration of HG and combination as compared to CFA treated rats. The significant increase in the spleen and thymus weight might be observed due to immune-stimulatory effect of HG (Ismail et al, 2008; Pedernera et al, 2008).

Anemic condition is frequently associated with chronic arthritis patients because of gastrointestinal blood loss due to arthritic medications and structural changes in the bone marrow which prevents the release of iron for incorporation into red blood cells (Glenn et al, 1977; Mowat, 1971; Allard et al, 1992). In CFA induced arthritis, the decreased levels of RBCs and Hb in CFA treated rats is associated with the reduced levels of erythropoietin
hormone that may be caused due to decreased bone marrow response to erythropoietin and destruction of premature RBCs (William, 1996). The treatment of rats of CFA treated rats with HG and combination significantly elevated the decreased level of RBC and Hb.

The elevated level of IL-1β inflammatory response results in an increase in granulocyte and macrophages colony stimulating factors that results in to the elevation of WBC level in CFA treated rats (Eric and Lawrence, 1996). Treatment of rats with HG and combination might play important role in inhibition of IL-1β inflammatory mediator release that may significantly decrease the WBC level in CFA treated rats.

In the present study, challenge of rats with 0.1 ml CFA significantly elevated the serum AST, ALT and ALP levels. Liver impairment is a typical feature in rheumatoid arthritis. Assessment of liver injury is done by determining the biomarkers levels such as AST, ALT and ALP. Elevated serum levels of these enzymes indicate damage to hepatic architecture resulting in leaching of these enzymes into the systemic circulation. The estimation of serum parameters provides valuable information about liver and kidney impairment indices that is also considered as an important feature of adjuvant arthritis. They play a critical role in the formation of biologically active chemical mediators such as bradykinins in inflammatory process (Mythilypriya et al, 2008). So, in the present study significant rise in the aminotransferase level was observed in CFA treated animals suggests that it might be released from the damaged liver cells (Banji et al, 2011). The treatment of rats with HG and Combination significantly decreased the level of AST, ALT and ALP that confirms the treatment have protective effects on liver and kidney function.

The accumulation of neutrophilic infiltration at the inflammation site is an important feature of MPO assay. It produces demolition of lysosomes by dent of increase in array of reactive oxygen species (ROS) that up regulates hydroxy and peroxide radicals leading to tissue membrane damage (Halliwell et al, 1988). HG is a potent antioxidant and possesses free radical scavenging property (Cerqueira et al, 2012). Treatment with HG and combination significantly reduces the elevated MPO level in CFA treated rats. In our earlier studies, HG and combination inhibited MPO in granular tissue of TNBS induced colitis in rat model (Ingawale and Patel, 2016).

In a human body the network of cytokines such as pro-inflammatory and anti-inflammatory mediators available in the balanced state which is disturbed by a variety of factors such as
infectious agent and environmental exposure in RA (Sweeney and Firestein, 2004; Firestein, 2005). These cytokines plays an essential role both in the initiation and continuation of localized and systemic inflammatory changes (Miossec, 2004; Hackett et al., 2008). These cytokines contribute to numerous features of rheumatoid arthritis, such as inflammation of synovial tissue, the proliferation of synovial tissue, cartilage tissue damage and bone damage. The levels of inflammatory cytokines (TNF-α and IL-12) were found to be elevated in the early phase of arthritis (Ferraccioli et al, 2010).

However, IL-12 is associated with enhanced levels of inflammatory cells infiltration and cartilage destruction, whereas TNF-α stimulates the production of other inflammatory cytokines such as IL-1 and IL-6 and responsible for regulating the joint swelling (Kuiper et al, 1998; Van den Berg et al, 1999; Brennan and McInnes, 2008; Zuo et al, 2014). In addition, TNF-α is mainly expressed in the synovial membrane lining at the beginning of arthritis, whereas IL-12 is detected at the later stage of arthritis (Feldmann et al, 1996). In the present study, levels of TNF-α and IL-12 were significantly increased in the serum samples of CFA arthritic rats. In agreement with this, our results demonstrate that HG and Combination exerts an anti-arthritic effect by suppressing the production of TNF-α and IL-12 induced by the administration of CFA in rats. Among ILs, TNF-α and IL-12 are found to be the most active mediators that participates in the pathogenesis and progression of arthritis, by promoting the production of matrix metalloproteinase and PGE₂, through increasing COX-2 expression (Sakaki et al, 2004; Duque et al, 2006).

IL-6 is immune-modulatory cytokines considered to be of great importance in the pathogenesis of RA in humans (Jacques et al., 2006). This cytokine plays a crucial role in the initiation and perpetuation of localized and systemic inflammatory changes (Hackett et al., 2008). They resemble with the variety of arthritic inflammation features, such as synovial proliferation, synovial tissue inflammation, cartilage and bone damage. This cytokine cascade increases the level of IL-6 and stimulates cartilage matrix degradation and increased production of matrix degrading enzymes such as matrix metalloproteinase (Smolen and Steiner, 2003). In CFA induced arthritis model, the significant increase in IL-6 level can be effectively prevented by HG and its Combination in rats.

TXB₂ is the key inflammatory mediator that is derived from AA through the COX pathway in cell membranes of mammals and over expressed in inflammatory conditions such as RA and cancers (Khanapure et al, 2007, Huang and Huang, 2014; Huang et al, 2013). It shows many
pro-inflammatory effects such as vasodilation, increased blood flow, increasing vascular permeability, pyrexia and potentiation of pain. It also promotes the production of some MMPs and stimulates bone resorption (Lewis et al, 1990). So, the elevated level of TXB₂ in CFA induced arthritis model can be significantly prevented by HG (50 µg/rat) and its combination (25 µg/rat, each) in rats.

Bone destruction is a common feature of rheumatoid arthritis that was examined by radiological analysis of ankle joints (Patel et al, 2012). X-ray examination of rat paws showed that treatment with HG and Combination inhibited the arthritis associated with joint alterations. The histology of ankle joints from CFA immunized rats showed severe proliferation of synovium, with significant infiltration of inflammatory cells, cartilage damage and bone erosion. From the studies, it was evident that the inflammation of connective tissue, cartilage damage and bone erosion was controlled by the treatment of rats with HG and its Combination.

Chronic inflammation involves the release of a numeral inflammatory mediators that are responsible for the pain, bone and cartilage destruction that leads to severe disability (Eric and Lawrence, 1996). Prostaglandins are formed in the inflammation process under the influence of two enzymes, COX-1 and COX-2. The enzyme COX-1 is accountable for the maintenance of gastric mucosa integrity, renal function and homeostasis. The expression of COX-2 enzyme was upregulated in inflamed tissue. These upregulated expressions of COX-2 may be responsible for increased production of PGs (Griswold and Adams, 1996). The activity of COX-2 was significantly decreased in HG and Combination treated rats at the dose of 50 and 25 µg/rat, each.

The above research studies have provided new insights into the Hecogenin mechanisms of action acts by GR-mediated effects through inhibition of COX enzyme and pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and TXB₂. The study has led down to the hypothesis that therapeutic effects are mediated via transrepression process, whereas side effects are predominantly mediated by transactivation (Reichardt et al, 2001).

Hecogenin and combination induces significantly fewer transactivation mediated side effects, such as increased blood glucose, body weight and skin atrophy in mice and rats. Therefore, we believe that the risk of diabetes induction, body weight gain and skin atrophy with Hecogenin should be clearly lower than that for Fluticasone alone. The reduction of lymphoid
organ weights such as spleen, adrenal and thymus gland weight was also diminished after topical administration of Hecogenin and its combination.

In the acute oral toxicity study, the dose of HG 2000 mg/kg was found to be safe in mice and no mortality was observed. In light of these toxicity findings study, it was concluded that HG is not toxic up to the dose of 2000 mg/kg and not showing any signs of toxicity or mortality.

In the safety pharmacological study, the mice in NC and HG treated groups were monitored daily for fourteen day for any toxic signs and mortality. During the period of 14 days there were no observable symptoms of toxicity or deaths. All of the mice gained weight and displayed no significant changes in behavior. Apart from that, physical appearance features such as skin, fur and eyes were found to be normal and whilst the body weight of the mice showed as increase. Organ weight is also an important index of physiological and pathological status in animals. The heart, liver, kidney, spleen and lungs are the primary organs affected by metabolic reaction caused by toxicant (Dybing et al, 2002). The histological analysis was done to further confirm the alterations in cell structure of the organs. The histological examination is the golden standard for evaluating the treatment related pathological changes in tissues and organs (OECD Guidelines, 2001).

In the present study, histopathological analysis of an oral administration of HG did not adversely affect the morphology of mice organs and there was no structural damage to the organs such as heart, liver, kidney, lungs, and spleen of the mice. The results obtained from biochemical analysis, body weight, organ weight and histopathology analysis of HG drugs indicates that the drugs were well tolerated by mice for 14 days. The hematopoietic system is very sensitive to noxious compounds and serves as an imperative index of the pathological and physiological status in animals (Adeneye et al, 2006). After 14 days of treatment with HG there were no changes in the hematological parameters such as Albumin, globulin and their ratio. This indicates that there were no significant changes of serum levels of ALP, ALT and AST. So, from the safety pharmacological study it was concluded that, HG did not show any toxicity or produce any notable histopathological signs or alterations in serum enzymes.