6. DISCUSSION

Inflammatory bowel disease is a chronic relapsing inflammatory disorder involving the gastrointestinal tract. In IBD, the bowel i.e intestine becomes inflamed, leading to abdominal cramps and diarrhea. Incidences have been gradually increasing in India due to adaptation to western living style. The pathogenesis of IBD is the result of combination of environmental, genetic, and immunological factors. T-Cell Responses are similarly activated in all forms of IBD to cause intestinal inflammation by the release of various pro-inflammatory cytokines. Also, due to various targets involved in the pathophysiology of IBD, very few drugs are available which can targets those sites. The available treatments have serious adverse effects, compliance problems and increased rates of relapse. Surgical options add to the cost of therapy. Therefore, filtrating high effects and low toxicity of natural anti-inflammatory medications are desired for the treatment of patients with IBD.

Hence the search has been extended to herbal drugs which apart from being safe may afford better protection and decrease the incidence of relapse. Majority of available herbal formulations are multiple combinations of plants. Most of the polyherbal formulations face problems in standardization and quality control. Also, it is very difficult to say exactly which plant is responsible for the efficacy or which plant is responsible for the side effect seen. If any clinically proven monoherbal treatment is available, with evidences for its action on the sites involved in the pathogenesis of the disease, coupled with elucidation of the exact mechanism of action responsible for therapeutic action in preclinical studies, it would be a relatively affordable alternative treatment with more acceptability for the patients.

In the pathogenesis of IBD, oxidative stress plays a major role. The combination of excessive generation of reactive oxygen species (ROS) and decreased antioxidant defenses is a major pathogenetic mechanism contributing to the initiation and progression of IBD. Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against inflammatory and degenerative disorders. Hence, antioxidants can be used as therapeutic agents. The medicinal plants chosen for the study were selected on the basis of the active chemical constituents present and activities reported. Phytoconstituents like natural steroids, phenolic
Discussion

Compounds (Amarowicz et al., 2007), flavonoids (Defeudis et al., 2003), saponins (Yoshikawa et al., 2003), terpenoids (Takeoka and Dao, 2003) and alkaloids (Shaheen et al., 2005) are well known for their antioxidant and antiinflammatory activities. Preliminary phytochemical screening of both the plants by successive solvent extraction revealed the presence of various phytoconstituents like alkaloids, carbohydrates, tannins, fats, oils, steroids, saponins and flavanoids in both the medicinal plants. Kurchicin and conessine are principle active alkaloids of Holarrhena antidysenterica is highly effective against causative micro-organisms of diarrhea, dysentery i.e. especially amoebic type (Bhutani et al., 1988). The essential oil from Cyperus rotundus contains at least 27 components comprising sesquiterpene hydrocarbons, epoxides, ketones, monoterpenes and aliphatic alcohols and triterpenes like β-sitosterol and linoleic acid. The tuber and rhizome are used to treat chronic diarrhea with mucus and other abdominal problems. Holarrhena antidysenterica and Cyperus rotundus are being used for their anti diarrhoeal effects; their possible modulatory role in inflammatory bowel disease with precise mechanism of action is yet to be scientifically verified.

The study was planned in three parts viz. firstly; phytochemical tests and preliminary efficacy study with whole hydromethanolic plant extracts was conducted to see the effect in experimental IBD. In the next study, we obtained different extracts of both plants using various solvents with increasing polarity successively, performed phytochemical tests and then checked which extract showed maximum effect. Finally, main efficacy studies was done using optimum dose levels and most efficacious extracts of both the plants.

In the present days of modernization, herbs when used as medication have to face new challenges regarding the identity, quality and efficacy. Queries prevail as to how the plants are collected, processed, preserved and used. To meet this, standardization of herbal drugs is mandatory. Authenticity is the important aspect of the standardization and quality control. In order to assure a consistent and acceptable quality herbal product, care should be taken right from the identification and authentication of herbal raw materials to the verification process of final product. Researchers are trying to evaluate many plant drugs used in traditional system of medicine. The pharmacognostical study is one of the major criteria for identification of plant drugs. In our study, the pharmacognostic investigation authenticated the quality of stem bark
of *Holarrhena antidysenterica* and tubers of *Cyperus rotundus* and thus has justified that the drug used under study.

Currently, toxicology encompasses mainly activities to determine the potential for adverse effects from chemicals (both natural and synthetic), with the objective of assessing hazard and risk to humans and animals. After preliminary positive results of efficacy studies, in order to establish the safety of a new drug, *in vivo* toxicological studies are very essential experiment carried out in animals. We all believe that natural therapy using herbs is safe. But it is also very important to assess scientifically the potential beneficial or adverse effects of all medicinal plants extensively used by us. In order to establish the safety of a new drug, various guidelines are established for preclinical studies. The toxic effects are seen for single dose as well as repeated dose so that their acute effects and chronic effects can be established especially if they are to be used in varying doses for a longer period of time. Hence acute and sub-acute toxicity studies are always invaluable in evaluating the safety profile of phytomedicines. In acute toxicity studies, the maximum tolerated dose for hydromethanolic extract of *Holarrhena antidysenterica* is up to 1500mg/kg (Sharma et al, 2005) while that for *Cyperus rotundus* is up to 2000mg/kg (Sharma et al, 2005).

The sub acute toxicity study allows the establishment of the existence or not of adverse effects, and later for the identification and characterization of the affected organs. Generally, the reduction in body weight gain and internal organ weights is a simple and sensitive index of toxicity after exposure to potentially toxic substances (Teo et al, 2002). In addition, the diet if well accepted does not cause any alterations in carbohydrate, protein or fat metabolism in these experimental animals. It should not adversely interfere with the nutritional benefits (e.g. weight gain, stability of appetite) expected of animals that are continually supplied with food and water *ad libitum*. Subacute toxicity study in our studies revealed that extracts of both the plants at various doses did not appear to retard growth or affect food consumption and utilization.

The haematopoietic system is very sensitive to toxic compounds and serves as an important index of the physiological and pathological status for both animals and humans (Adeneye et al, 2006). The levels of SGOT and SGPT are good indicators of
liver functions. Nephrotoxicity of the drug can be estimated by renal function markers like creatinine and BUN. There were no treatment related changes in the haematological indices. No significant changes were observed due to treatment which indicates that the drug did not interfere with the renal and hepatic functions. The treatment did not show any significant changes in lipid parameters and glucose levels.

The macroscopic appearance and weight of the major organs like liver, heart, kidney and lung and histopathological studies of their sections did not reveal any evidence of treatment related degenerative structural changes or necrosis at any dose levels. The study did not report any mortality and was not associated with any adverse effects suggesting that the doses were well tolerated by both male as well as female rats, and is not toxic. According to these results, the no-observed-adverse-effect level (NOAEL) for MEHA and CHCR extracts are 1350mg/kg body weight/day and 1800mg/kg body weight/day respectively, administered orally for 28 days in rats under the conditions of this study (Copplestone, 1988; WHO, 1987). This may be an important point in assessing the suitability of MEHA and CHCR for repeated administration for therapeutic use.

Having established the safety profile of MEHA and CHCR, efficacy studies were undertaken to evaluate the effect of the plants on experimentally induced animal model of IBD. Since IBD like conditions do not prevail in animals, different types of exogenous irritants are used to induce inflammatory reactions of the gut. DNBS induced colitis is a well characterized transmural T\textsubscript{H}-1 driven inflammation of the colon & may be considered a model of IBD. DNBS is a nitroaryl oxidizing acid with extreme oxidizing properties. For preliminary pharmacological studies, DNBS dissolved in 50% alcohol was used to induce colitis. After disruption of mucosa integrity by ethanol, hapten DNBS is bound to colon tissue proteins, & changed into a modified protein compound, which is recognized by macrophages as an abnormal antigen & presented quickly to the sensitizer T - Lymphocytes. So a series of immune responsiveness & severe colon inflammation are initiated subsequently. The colon injury correlates with a significant rise of apoptosis which is associated with a significant increased expression of proapoptotic Bax and decreased colon content of antiapoptotic Bcl-2. (Emanuela et al, 2006). This inflammatory response is also related to activation of NF-κB and phosphorylation of c-Jun as well as FAS ligand expression.
in the colon (Emanuela et al, 2006). In our study, DNBS (120 mg/kg in 50% ethanol) administration caused significant colon inflammation as revealed by increased macroscopic score over normal control animals. Intrarectal administration of DNBS resulted in an inflammatory response characterized by extensive mucosal disruption, linear and deep ulcers, hemorrhage and submucosal edema. There was macroscopic evidence of extensive colonic mucosal injury along the 1-3 cm segment at the site of instillation of DNBS. Inflammation leads to infiltration of polymorphonuclear leukocytes, macrophages, eosinophils and lymphocytes inside the cells. While in normal control animals goblet cells were large in number along with intact mucosal lining.

Macroscopic and histological indices like Disease Activity Index (DAI), Colon Mucosal Damage Index (CMDI), Degree of Necrosis, Degree of Inflammation and Degree of Fibrosis are usually applied to evaluate the severity of DNBS induced injuries (Wang et al, 2001; Zhang et al, 2010). DAI and CMDI scores reveal edema, hyperplasia due to fibrosis, hemorrhage, epithelial exfoliation and necrosis, presence and intensity of ulcers on epithelium and loss of epithelium and entire crypts. Colitis induced in disease control rats showed marked increase in these scores (Wei-Guo et al, 2003). The histopathological study showed diffuse inflammation in mucosal and submucosal layers, distortion in crypt architecture, granuloma formation and presence of fissures or aphthous ulcers. These observations found in our study are in line with endoscopic reports which highlight colonic inflammation, erosions and ulcers in IBD patients. Thus DNBS could mimic the colon damage occurring in human due to disease progression. Regression of the disease pathogenesis following treatment with the 5-ASA and plant extracts significantly declined the scores of all inflammatory indices. Thus histopathological assessment supplemented the protective activity of MEHA and CHCR.

DNBS administration caused significant reduction in water intake, food intake, and body weight. Treatment with 5-ASA, MEHA and CHCR resulted in significantly less percentage reduction in body weight and did not change food intake and water intake when compared to model control. Reduction in body weight is the sign of the generation of IBD. Food and water consumption are also reduced in IBD patients due to inflammation which further results in decreased body weight (Hendrikson et al,
This is thus an indirect evidence of reduction in inflammation of the colon with MEHA 600 mg/kg, CHCR 800 mg/kg and 5-ASA 100 mg/kg drug treatments.

DNBS administration resulted in increased Stool Consistency Score which was attenuated by 5-ASA, hydromethanolic whole extracts of *Holarrhena antidysenterica* (200 mg/kg, 400 mg/kg, 600 mg/kg) and *Cyperus rotundus* (300 mg/kg, 500 mg/kg, 800 mg/kg) pretreatment. It has been suggested that increased intestinal permeability is a primary etiologic factor in both IBD (Stein *et al*, 1998) and DNBS induced colitis (Cuzzocrea *et al*, 2004; Wallace *et al*, 1995). Furthermore, alterations in active electrolyte transport by the diseased epithelium would result in altered water flux and could thereby contribute to the secretory diarrhea (Haiyan Zhou *et al*, 2000). IBD patients often complain of frequent and watery diarrhea with blood and mucus.

Wet colon length was significantly decreased in model control group as compared to normal control animals. Due to severe inflammation and necrosis, average wet colon weight and wet colon weight/length ratio on intracolonically administration of DNBS was significantly increased in model control group as compared to normal control animals. Colon weight increases and colon length decreases due to hyperplasia, edema, ulcers, inflammation and necrosis of colon tissue in animal models. As colitis progresses, the length of the colon becomes shorter and it becomes thicker in circumference as the muscles of the colon contract. 5-ASA, hydromethanolic whole extracts of *Holarrhena antidysenterica* (200 mg/kg, 400 mg/kg, 600 mg/kg) and *Cyperus rotundus* (300 mg/kg, 500 mg/kg, 800 mg/kg) treatment significantly prevented the alteration in colon weight, and colon length as compared to DNBS Control animals due to decreased hyperplasia, edema, inflammation and necrosis of mucosal tissue.

Efficacy studies using hydromethanolic (70% methanol) whole extracts of both the plants reversed the development of experimental colitis. This solvent mixture, being highly polar, extracts the polar constituents like alkaloids and flavonoids. All water soluble as well as insoluble alkaloids and flavonoids were extracted in hydromethanolic solvent. Extracts obtained using different solvents of increasing polarity were also used in our study. Phytoconstituents viz. steroidal alkaloids like conessine, holarrhenine and holarricine and flavonoids were present in
Discussion

Hydromethanolic extract of *Holarrhena antidysenterica* while chloroform extract of *Cyperus rotundus* showed the presence of compounds containing steroidal nucleus like sesquiterpene hydrocarbons, monoterpenes, aliphatic alcohols and triterpenes. Phytoconstituents like natural steroids, flavonoids (Defeudiv *et al.*, 2003), terpenoids (Takeoka and Dao, 2003) and alkaloids (Shaheen *et al.*, 2005) are well known for their antioxidant and antiinflammatory activities. These active constituents alone or in combination may be responsible for the pharmacological actions of these extracts. MEHA (600 mg/kg) and CHCR (800 mg/kg) treatments significantly decreased the wet colon wt/l, CMDI and stool consistency scores as compared to DNBS Control animals.

Dysfunctional immunoregulation of the gut is believed to be the main culprit behind pathogenesis of IBD. Amongst the immunoregulatory factors, reactive oxygen species are produced in abnormally high levels in IBD. Their destructive effects may contribute to the initiation and/or propagation of the disease (Hendrickson *et al.*, 2002). Myeloperoxidase (MPO), Malondialdehyde (MDA), Nitric oxide (NO) and Superoxide Dismutase (SOD) are the enzymes playing important role in oxidative stress. Changes in these enzymes serve as the foremost markers of oxidative stress in IBD (Rezaie *et al.*, 2007).

Colonic injury by DNBS administration was characterized by an increase in MPO activity compared with normal control, indicative of neutrophil infiltration in inflamed tissue confirming the enhanced leucocyte infiltration seen at histological inspection. In this study, the extent of MPO activity closely paralleled the increase of tissue MDA along with decline in SOD activity in model control compared with normal control. These observations reiterated that oxidative stress played an important role in the occurrence of colitis. Oxidative stress induces production of reactive metabolites of oxygen (ROS) and nitrogen (NO) which in turn lead to the pathological aggravation of a series of free radicals chain reactions contributing to the pathophysiology of IBD. Monocytes from patients with Crohn’s disease (Kitahora *et al.*, 1998) and polymorphonuclear neutrophils from patients with ulcerative colitis (Shiratora *et al.*, 1993) have an increased capacity to generate free oxygen radicals. These free radical chain reactions strongly attack DNA, proteins, enzymes, biological membranes as well as disrupt the integrity and function of intestinal mucosa barrier,
hindering mucosal recovery, especially in case of impaired endogenous defence system and ultimately leading to activation of inflammatory mediators (Kirsner et al., 1982; Jewell et al., 1986; Buffinton and Doe, 1995).

The enzymatic antioxidant defense system is the nature protector against lipid peroxidation. SOD enzyme is important scavenger of superoxide ion and hydrogen peroxide. These enzymes prevent generation of hydroxyl radical and protect the cellular constituents from oxidative damage. SOD and CAT enzymes usually act in a synergetic manner. SOD catalyzes dismutation of the $O_2$ into $H_2O_2$. $H_2O_2$ can then be transformed into $H_2O$ and $O_2$ by CAT. Inflammation of mucosa produced by inflammatory mediators causes impairment of the SOD and makes tissue more susceptible to oxidative damage (Buffinton and Doe, 1995; Fantone et al., 1982). In turn, superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, secreted by neutrophils and phagocytes accumulating in the inflammatory lesion, cause impairment of cellular membrane stability and cell death by leading lipid peroxidation. Immense reduction in these enzymes level was reported in IBD (Misra and Irwin, 1972; Rezaie et al., 2007).

MPO is released by activated neutrophils into the extracellular milieu, where it uses $H_2O_2$ and chloride anion (Cl$^-$), forming hypochlorous acid which is a powerful oxidant and reacts with amines to form chloramines. Thus, it promotes oxidative stress and additionally it induces neutrophil infiltration on mucosal area causing further damage to the tissue. This fact is documented in both animal models, and patients with IBD (Megan et al., 2012; Benjamin et al., 2013). Lipid peroxidation is a free radical mediated process, which has been implicated in a variety of disease states like UC. MDA is final product of oxidative stress and is good indicator for extent of oxidative stress (Forbes et al., 2006). Colonic biopsy from specimens of patients with active IBD had enhanced levels of lipid peroxidation products like MDA and MPO activity. These findings suggest that chronic gut inflammation promotes an imbalance between pro-oxidant and antioxidant mechanisms, leading to the net accumulation of oxidatively modified proteins and lipids (Reifen et al., 2004). Antioxidants show beneficial effects on experimental colitis (Deng et al., 1994; Wei-Guo et al., 2003).
However, MEHA 600mg/kg and CHCR 800 mg/kg pretreatment significantly reversed the pathogenesis of IBD by preventing neutrophil infiltration produced by DNBS, as seen by decrease in MPO activity and also prevented the increased accumulation of MDA. Administration of MEHA 600 mg/kg and CHCR 800 mg/kg significantly prevented the reduction in activities of SOD (10.19±1.03, 9.23±1.06 U/gm) respectively when compared to model control (1.77±0.2 U/gm) animals. These results were found comparable to conventionally prescribed drug 5-ASA. Similar changes are observed with respect to the alterations in these enzyme levels in colitis patients, the beneficial antioxidant effect of the plant extracts can be extrapolated for human use.

DNBS administration in model control animals led to elevation of colonic tissue NO levels (297.3±14.91 nmol/gm), which was significantly inhibited by 5-ASA 100 mg/kg, MEHA 600 mg/kg and CHCR 800 mg/kg (202.5±12.95, 176.5±9.47, 210.8±14.75 nmol/gm) respectively. Besides direct and severe impairment of the function of intestinal barrier by ROS, excessive NO also participates in complicated system between inflammatory cells and immunocytes in IBD. Activated macrophages produce NO via the inflammatory activation of inducible nitric oxide synthase (iNOS). Furthermore, advanced stages of bowel inflammation in humans (Cameron et al, 2002; Boughton-Smith et al, 1993; Nathan et al, 1994; Lundberg et al, 1994) and animals (Morris et al, 1989; Ikeda et al, 1997; Aiko and Grisham, 1995; Ribbons et al, 1995; Mourelle et al, 1996) are associated with an enhanced local formation of NO by iNOS. NO produced in abundance by iNOS, directly reacts with O2− to form peroxynitrite (ONOO−) and peroxynitrous acid (ONOOH). Alternatively, nitrite (NO2−), a major product derived from NO, may be oxidized by MPO to nitrogen dioxide (NO2) (Mehmet et al, 2013). An in vivo study gives direct evidence on NO-induced injury on gut epithelial cells supporting the detrimental role of excessive NO in colitis (Bostoglu and Dimistros, 1994; Avdagic et al, 2013) Increased production of NO, and the presence of iNOS protein and iNOS mRNA have been demonstrated in affected areas of gut in patients suffering from UC or CD (Rachmilewitz et al, 1995; Singer et al, 1996; Kimura et al, 1997). Inhibition of physiological NOS has been reported to enhance intestinal lesions in inflammation (Laszlo and Whittle, 1999; Pfeiffer and Qiu, 1995). Prolonged production of high amounts of NO by iNOS on the other hand is proinflammatory and inhibition of iNOS seems to ameliorate the inflammatory
response and tissue injury in experimental colitis (Hogaboam et al, 1995; McCafferty et al, 1994). Thus, the probable mechanism behind efficacy of MEHA and CHCR in IBD could be through this pathway as well.

An increase in serum Cortisol level in model control animals was observed. Treatment with 5-ASA, MEHA and CHCR reversed the increase in serum Cortisol level induced by DNBS suggesting, a possible protective role against stress induced IBD. Endogenous corticotrophin releasing factor (CRF) mediates the stress-induced inhibition of the upper GI tract and the stimulation of colonic motility (Tadakazu et al, 2007). CRF is thought to have the potential to change the production of several cytokines including lymphocytes and NK cells thus contributing to the pathophysiology of human UC. CRF levels are increased in lamina propria mononuclear cells from patients with active UC. Chronic stressful events and continuous inflammation result in high endogenous serum levels of ACTH (Michie et al, 1988) and Cortisol. ACTH and Cortisol were found to be elevated, for example, after intravenous administration of IL-1 (Sapolsky et al, 1987) or IL-6 (Perlstein et al, 1991; Mastorakos et al, 1993). In the larger group of CD patients, a positive association of Cortisol serum levels and high humoral inflammatory activity was confirmed (Rainer et al, 1998).

From these exploratory studies for efficacy and probable mechanism of action, we found that MEHA (450mg/kg, 600mg/kg) and CHCR (600 mg/kg and 800 mg/kg) showed beneficial effect in DNBS induced IBD in rats. Traditionally *Holarrhena antidysenterica* is useful in amoebic dysentery and diarrhea. Various fractions of *H.antidysenterica* showed promising activity against experimental amoebiasis in rats and hamsters (Dutta and Iyer, 1968). The fruit extract (50% ethanolic) showed antiprotozoal effect against *Ent. histolytica* strain STA, *Trypanosoma evansi* (Dhar et al, 1968). *Cyperus rotundus* has been reported to have antispasmodic effect (Shamkuwar et al, 2012). Based on these reports and our studies, the possible mechanism of protective effect might be attributed to anti-oxidant and anti-inflammatory effects of alkaloids, flavonoids and triterpenes present through maintaining enzymatic defense activities against free radicals and scavenging the free radicals as well as increased endogenous NO. These cumulatively inhibit the damage to the mucosa.
Food and bacteria damages the mucosal barrier and exposes the sub-mucosa. Innate and acquired immune responses are engaged to produce large amounts of cytokines from intestinal epithelium. Prolonged or inadequate activation of the intestinal immune system participates in the pathological events of chronic mucosal inflammation (Sartor, 1997). Pathological induction of NF-κβ plays a pivotal role in the pathogenesis of IBD and most likely mediated by its ability to activate genes that encode proteins such as cytokines, chemokines, adhesion molecules, and enzymes involved in mediator synthesis and the further amplification and perpetuation of the inflammatory response. Neurath et al described increase p65, a subunit of NF-κβ’s activity in isolated mononuclear cells from the lamina propria patients with crohn’s disease (Neurath et al, 1998). Th1 responses are characterized by secretion of IL- 1, IL- 2, IL-6, IL-12, IL-18, TNF- α and IFN- γ. Ulcerative colitis exhibits additional response of defective Th2 responses characterized by secretion of IL- 4, IL-5 and IL-10 (Manuela Neuman, 2004). Although the initial purpose of inflammatory response is to provide protection against the invasion of foreign antigens, in IBD the immune response becomes dysregulated, resulting in damage to host intestinal tissues. Increased levels of IL-4, IL-6, IL-12 and IFN-gamma found in intestinal tissue and peripheral blood of Inflammatory Bowel Disease patients (Manuela Neuman, 2004).

Phytochemicals are potential candidates in an emerging novel and cost effective approach to block the expression NF-κβ for the treatment of many diseases including IBD. Inhibition of NF-κB, MAPK and related cytokines by whole pomegranate fruit extract occurred in vivo in mouse skin exposed to 12-O tetradecanoylphorbol-13-acetate (TPA) (Afaq et al, 2005). In our laboratory also, expression of IL-1β, TNF- α, IL-18 and NF- κβ were found to be upregulated in experimental model of DNBS induced IBD and pretreatment with 5-ASA, Punica juice, Punicalgin and Aegle marmelos significantly reversed these gene expression changes.

In our studies, analysis of inflammatory cytokine production by colon tissue culture and real time RT-PCR in present study detected high amount of IL-4 mRNA (1.00) in disease control group, treatment with 5-ASA , MEHA and CHCR significantly lowered mRNA expression for cytokine IL-4 (0.255, 0.535, 0.591) respectively. Oxidative stress upregulates the transcription of IL-4 (Zhognlin Wu et al, 2000). The treatments reduced the oxidative stress due to its antioxidant effect and downregulated
the transcription of IL-4 mRNA and re-affirmed the reported findings. Increased production of IL-4 in IBD may contribute to disease pathogenesis and its suppression reverses the condition.

We evaluated the mRNA expression of cytokine IL-6 which is another well known proinflammatory cytokine and found increased amount of IL-6 genes in colon samples of DNBS treated rats. Cytokine expression after the treatment with standard drug and investigational herbal extracts decreased when compared to the rats treated with DNBS alone. In IBD, T\textsubscript{H}1 lymphocytes increase production of IL-6 and initiate cytotoxic, apoptotic and acute phase responses. IL-6 and soluble IL-6 receptor have been implicated in the inflammation of IBD (Manuela Neuman, 2004). IL-6 plays important role in SAMP1/Yit murine model of Crohn’s like ileitis (Pizzaro et al, 2006). From these data it is apparent that IL-6 may contribute to enhanced susceptibility to injury. Increased IL-6 levels in the colonic tissue support their role in the immunopathogenesis of this disorder. Thus treatments showed cytoprotective, antiapoptotic and anti-inflammatory effects in experimentally induced IBD model.

DNBS led to enhanced expression of mRNA of IL-12 (1.00) and aggravated colon injury. The expression significantly decreased after 5-ASA, MEHA and CHCR administration (0.23, 0.509, 0.424). Considerable data point to a prominent role for IL-12 produced from activated macrophages in the development of T\textsubscript{H}1 CD4+ T cells in the intestinal mucosa. In several rodent models of IBD, treatment with anti-IL-12 antibody prevents the development of colitis. Further, when intercrossed with IL-12 knockout mice, the TNF ARE knockout mice do not develop intestinal inflammation, emphasizing the importance of IL-12 in the initiation of the mucosal inflammation in this mouse model (Barbara et al, 2002). Administration of monoclonal antibodies blocking the IL-12/p40 subunit can be useful to induce and maintain clinical remission in CD patients (Peluso et al, 2006). Our study strongly indicates that IL-12 induces intestinal mucosal inflammation and the treatments prevented specifically this target to modulate the progression of colitis.

IFN-\textgamma mRNA expression levels were significantly upregulated in DNBS model group (1.00) and the same was downregulated in 5-ASA 100mg/kg, MEHA 600mg/kg and CHCR 800 mg/kg pretreated groups (0.23, 0.517, 0.591). IFN-\textgamma plays a role in
mediating the inflammation in animal models of IBD (Barbara et al, 2002). Increased synthesis of IFN-γ has been correlated with severity of disease in IBD patients which is supported by the reduction in the IFN-γ producing lamina propria mononuclear cells in colonic biopsies from anti-TNF-α treated patients (Manuela Neuman, 2004). Thus, our treatment had suppressive effects on the actions of mononuclear cells. Thus, in DNBS-induced colitis model, there is direct stimulation of immune cells leading to direct TH1-cell response which can be controlled only by direct abrogation of IFN-γ production with exogenous administration of MEHA and CHCR extracts.

Results found in our study were in accordance with various studies and lend support to the immune theories behind IBD. Treatment with investigational extracts leads to modulation of cytokine IL-4, IL-6, IL-12 and IFN-γ mRNA expression. Thus protective effects are due to downregulation of genes of proinflammatory cytokines affecting the immune system and inflammatory processes. The positive results reinforce the crucial role of the extracts in modulating the immune mechanisms involved in IBD, addressing various pathogenetic mechanisms of IBD and thus rarefying the progression of the symptoms of colitis. The protective effects of investigational herbal drugs could be due to the presence of individual or the combined effects of active principles like alkaloids, flavonoids and triterpenes present in Holarrhena antidysenterica and Cyperus rotundus. These findings can contribute to the development of novel targets in medical therapies for the treatment of IBD.

In context to above results, having established the toxicity profile, pharmacological efficacy in IBD and probable mechanism of actions, the next objective was to conduct clinical study using randomized and parallel group design. The results from this study may offer great potential for the development of novel, effective and satisfactory herbal formulation. Holarrhena antidysenterica extract was selected to evaluate its efficacy and safety in patients with chronic ulcerative colitis. At present, polyherbal formulations are available containing several plants or their extracts for the treatment of IBD patients. Monoherbal formulation with single ingredient would have better therapeutic effects, safe compared to modern allopathic formulations and cheaper than surgical procedures. The monoherbal formulation can be marketed as a scientifically proved and tested formulation for people suffering from IBD. This can help in reducing the need of steroids and other surgical methods and thereafter their untoward
side effects and complications. Allopathic drugs have been shown to produce side effects; hence if we can see the effects of monoherbal formulation with Allopathic drug, we can reduce the dose of Allopathic drug and thus reduce the risk of side effects. We wanted to develop one monoherbal formulation that could go to the market. Both the plants showed similar efficacy but we chose only one plant for clinical studies. Hence, the purpose of clinical study was to compare the efficacy of Monoherbal formulation containing *Holarrhena antidysenterica* alone as well as in combination with Modern (Allopathic) treatment in patients with chronic ulcerative colitis.

In our patient population, more prevalence was observed in 31-40 and 51-60 years of age and 60% patients out of total 30 patients having UC were males. The epidemiological data and theories behind the pathophysiology of IBD reported were found to be correlating with our clinical studies. Kelvin Thia in 2008 and Cosnes Jacques in 2011 reported that the peak age for UC is 30–40 years with slight male predominance. 30% of patients enrolled for the study were having habit of smoking. The treatment with test drug reduced the high scores for signs and symptoms for ulcerative colitis in all smokers as well as nonsmokers. The strongest environmental risk factor for IBD is tobacco smoking but in 1998, Zijistra reported that current smoking is protective against UC.

In our study 80% patients inspite of leading active lifestyle were suffering from UC which probably could be due to their private jobs (66.67% patients) which imbibes a sense of insecurity, tremendous workload, financial stress and lackadaisical attitude towards personal health. People leading active life with outdoor activities have not been found to suffer from UC (Hanauer, 2006). In our studies, 70% patients were experiencing stress due to financial problems, overwork and poor health. Higher mortality from IBD has been reported in managerial, clerical, and sales positions, which typically involve sedentary and indoor work. All the treatments in the present study reduced mucus in stools. Stress increases the risk of developing IBD. Stress causes neuroendocrine response which decreases the mucus secretion and thereby weakens the mucosal barrier & increases the permeability. An impaired colonic mucosal barrier leading to increased intestinal permeability has been demonstrated in patients with UC. Local leaks due to apoptosis of colonic epithelium and highly
permeable ulcerous lesions comprise the primary lesion in mild UC (Gitter et al, 2001).

All the patients of our study, irrespective of vegetarian or non-vegetarian were regular consumers of street food. They had complaints of intermittent diarrhea and constipation along with flatulence. They expelled mucus and blood in their feces and suffered with gastrointestinal infections. These symptoms were reduced after the treatment with test drug. The scores were significantly different before and after the treatment. IBD is most prevalent in developed regions. It is postulated that this is the result of “westernization” of lifestyle, such as changes in diet, smoking and variances in exposure to sunlight, pollution, and industrial chemicals (Lakatos et al, 2007). Higher intake of fatty acids, sucrose, fast food increases the risk for IBD.

Most of the patients complained of loose motions or watery diarrhea. All the patients showed positive result for occult blood test done in stool samples which reversed significantly in all the treatment groups. Rise in hemoglobin levels of the patients after the trial treatments could be due to decreased blood loss through feces. The clinical studies conducted in 30 patients of ulcerative colitis, irrespective of their etiology showed a marked reduction in the frequency and consistency of the stools after the test drug treatment. Patients suffering from several years with chronic diarrhoea responded positively to the trial treatments. Patients treated with monoherbal tablets alone showed maximal reduction in abdominal pain, diarrhoea, and bowel frequency per day and stool consistency scores and the results were better than Mesalamine treated patients. The treatment reduced the colonic inflammation as evident from ESR value. Patients with UC have also demonstrated decreased colonic mucin. An in-vitro study demonstrated a possible interaction between bacterial peptides and the mucosa in UC, resulting in depletion of mucus secretion by goblet cells (Leiper et al, 2001) and excessive mucus expulsion in stool after colonic damage. Microbes are considered as an antigen which leads to activation of intestinal immune system & epithelial cells. Release of various inflammatory mediators cause local mucosal damage (Blumberg et al, 2001). Blood is almost always present in stools of IBD patients. Medical therapy leading to remission improves gut barrier integrity. Thus, the results were similar to our pre-clinical studies in which the MEHA showed beneficial effects and reduced stool consistency scores.
In our trial, patients were found to have stool infection on the day of enrollment for study. Treatment with Monoherbal tablet alone and in combination with modern medicine Mesalamine (Mesalazine) significantly reduced the stool infection while Mesalamine alone could not resolve the infection. This property of controlling infection in monoherbal treatment will be additional benefit over Mesalamine alone. Gastrointestinal infections like amoebic dysentery is the major and most frequent problem found in IBD patients. As traditionally *Holarrhena antidysenterica* is useful in amoebic dysentery and diarrhea. *Holrrhena antidysenterica* showed promising activity against experimental amoebiasis in rats and hamsters (Dutta and Iyer, 1968), The fruit extract (50% ethanolic) showed antiprotozoal effect against *Enta. histolytica* strain STA, *Trypanosoma evansi* (Dhar et al, 1968). Clinical tests with connessine, which is the important alkaloid content of *Holrrhena antidysenterica* showed anti amoebic activity with intestinal and hepatic amoebiasis patients (Signier et al, 1949). All the treatments significantly reduced the cumulative scores for abdominal pain, diarrhea, stool consistency and bowel frequency/day in all the patients irrespective of their smoking habit, type of stress, age, food habits or chronicity of symptoms. In context to these reports and the observations obtained in our studies, the monoherbal test formulation alone and in combination with Mesalamine showed significant improvement in combating the clinical symptomatology of IBD.

Patients treated with monoherbal therapy and combination therapy did not report any side effects, relapse or complications while 50% patients treated with Mesalamine exhibited the relapse of the symptoms like diarrhea and flatulence after drug withdrawal. The most common problem existing with Aminosalicylates class of drug prescribed quite often for the treatment of IBD is relapse on stoppage of treatment even after the regression of the symptoms. Any therapy which provides relief from symptoms and treats the disease at basic level and improves the quality of life of patients suffering from distressing disease like IBD would be easily acceptable. Thus, being monoherbal preparation, the overall cost of treatment was minimum along with benefits of similar efficacy, safety and less chances of relapse when compared to available conventional treatments like salicylates, steroids, antidiarrhoels etc. Thus, the efficacy observed in ulcerative colitis patients treated with monoherbal formulation containing extracts of *Holarrhena antidysenterica* and the group treated
with both herbal and Mesalamine was found to be better than Mesalamine alone treatment.

In conclusion, treatment with extracts leads to reduction in MDA, MPO activity, NO and cortisol levels, increase in SOD activity and downregulation of expression of genes for proinflammatory cytokines like IL-4, IL-6, IL-12 and IFN-γ in colon tissues of rats. Clinical reports of our studies further support the efficacy of investigational drug extracts in resolving the symptoms of IBD. *Holarrhena antidysenterica* and *Cyperus rotundus* contain phytoconstituents like alkaloids, flavonoids and terpenes, which are the key factors in the medicinal value of these plants. Thus, the possible mechanism of protective effect might be attributed to these active principles exhibiting anti-oxidant and anti-inflammatory via downregulation of proinflammatory cytokines.

The formulation containing extracts of *Holarrhena antidysenterica* can be used as a novel, safe, effective and more economical alternative in case of ulcerative colitis compared to modern system of medicine and polyherbal formulations available in the market. The results obtained in the present study can be used to conduct Phase II and III clinical trials with larger sample size and determine the problems associated with the management of IBD.