Discussion
DISCUSSION

The Triphala churna is popular traditional Ayurvedic herbal compound formulation, consisting of fine powder prepared by mixing equal proportion of dried pericarp of Haritaki (*Terminalia chebula* Retz.), Bibhitaka (*Terminalia belerica* (Gaertn) Roxb.) and Amalaki (*Emblica officinalis* Gaertn) fruits (Anonymous, 2003). However, there are no clear cut guidelines with regards to the proportion of the three ingredients. Some use equal proportions and some authors prepare Triphala by mixing one parts of Haritaki, two parts of Bibhitaka and four parts of Amalaki (Sarma and Tripathi, 1966; Vaidya, 1968).

Triphala is the one of the magic remedy of Ayurveda with multiple mechanisms of protective actions. According to ancient text, Triphala categorized as Tridoshik rasayana and antioxidant rich herbal formulation (Vaidya, 1968; Jeena and Kuttan, 1995; Sharma and Das, 1996; Jagetia et al., 2002). Rasayanas are nontoxic Ayurvedic complex herbal formulations or individual herbs used to rejuvenate or attain the complete potential of an individual in order to prevent diseases and degenerative changes that leads to diseases and promote longevity by providing strength and immunity (Sharma, 1994; Vayalil et al., 2002).

Triphala is traditionally been used as laxative in chronic constipation, colon cleansing, digestion problems and poor food assimilation. It also been used in cardiovascular diseases, all diseases of eyes, diabetes, poor liver function, large intestine inflammation and ulcerative colitis (Anonymous, 1952; Murthy, 1998; Mukherjee et al., 2006). Clinical report showed Triphala powder has been effective as laxative and used in management of hyperacidity and other gastric problems (Mukherjee et al., 2006). Modern scientific evaluations of herbal drugs is mainly of concern with validating the traditional uses of herbal medicines and unexplored wonders and phenomena which lay in the depth of traditional systems are to be studied and integrated with exiting system. Therefore in light of above discussion, we have standardized the Triphala formulations by pharmacognostic and phytochemical studies and evaluated for their gastrointestinal cytoprotective effects.
1. PHARMACOGNOSTICAL STUDY

In pharmacognosy, microscopic examination of the powdered drug is an inevitable procedure among the study of the transverse section, which aids in the identification and authentification of any material of plant origin. When a polyherbal powder is studied, establishment of powder characters of each ingredient is highly essential. Diagnostic characters from the ingredients can be taken as parameter for the establishment of identity of the formulation, particularly churnas (Anonymous, 1998). The powdered ingredients of plant origin such as fruits of *Emblica officinalis*, *Terminalia belerica* and *Terminalia chebula*; stem of *Tinospora cordifolia*; stem bark of *Azadirachta indica*, and leaves of *Trichosanthes dioica* studied as per the standard protocol for the microscopic examination and their powder characters were established.

2. PHYTOCHEMICAL STUDIES

After authentification of suitable plant materials, next step is to do a routine quality control tests for the assessment of the quality of the formulations. Physicochemical parameters including loss on drying, ash value, acid insoluble ash and extractive value of three Triphala formulations carried out to ensure the quality of formulations. The results showed all formulations have values in almost similar fashion.

It has been mentioned that the study of a crude drug of natural origin is useful only to the extent that it contains some active principles, which are pharmacologically significant, and therefore, unless these principles definitely identified, the real value of a crude material cannot be justified. Phytochemical screening of Triphala formulations reveals the presence of tannins, flavanoids, sugar, phenolic compounds, sterol, and carboxylic acid as major components.

The test of heavy metals is designed to determine the content of metallic impurities. Contamination of medicinal plant materials with heavy metals can be attributed to many causes including environment pollution and traces of pesticides.
Permitted levels of heavy metals in drugs, food and food supplements and herbal preparations as per standard WHO guidelines (Anonymous, 1998) and Ayurvedic Pharmacopoeia of India, Part II (Anonymous, 2007) lead is 100 ppm, arsenic 3 ppm, cadmium 0.3 ppm and mercury 1 ppm. As could be observed from the study trace metals do not seem to be present in significant quantities in Triphala equal, Triphala unequal and Chinnodbhavadi kwath.

HPTLC-finger printing is considered as the most important pre-requisite for undertaking any studies involving medicinal plants. Chromatographic fingerprinting should be done with emphasis on identification of specific chemical marker compound representative of specific herb. This is necessary to ensure reproducibility of the results obtained and to trace the differences in biological activity to the chemical profile when required. Triphala equal, Triphala unequal and Chinnodbhavadi kwath are standardized using gallic acid as a marker compound. Gallic acid was observed at 0.52 Rf value, when scanned at 254 nm. The formulations show the almost same Rf values as observed for gallic acid.

3. PHARMACOLOGICAL STUDIES

3.1. GASTRO-CYTOPROTECTIVE ACTIVITY

Gastric acid hyper secretion and peptic ulcer are very common human suffering today and represent a major health problem, both in terms of morbidity and mortality. Stress related gastric mucosal damage (Miller, 1987; Cook et al., 1991), non steroidal anti-inflammatory drug induced gastric lesion (Ivey, 1988; Langman et al., 1991) and Helicobacter pylori mediated gastroduodenal ulcers (Goodwin et al., 1986; Konturek and Konturek, 1994) are amongst the most frequently observed stomach related problems.

The gastric mucosal layer provided by the epithelial cells is postulated to act as the 'First line of defense' against mucosal injury. This layer of mucus together with the 'Second line of defense' a healthy epithelial cells themselves, have been thought to play significant role in defense of the mucosa against injury and ulceration (Lipkin, 1971). For many years physiological properties within the body that lead to or prevent ulceration of gastric mucosa have been thought of as being 'aggressive' or
“defensive” in nature. Peptic ulcer was assumed to develop when the summation of aggressive factors (like acid, pepsin and *Helicobacter pylori* infection) is greater than defensive factors (like secretion of bicarbonate, mucus and prostaglandins) (Jain *et al.*, 2007).

Research advances during the last few years have offered new insight into the pathophysiology of different gastric disorders resulting in evolution of better therapy and preventive measures to treat gastro duodenal ulceration. These measures are directed at strengthening the mucosal defense system rather than by attenuating the aggressive acid-pepsin factors responsible for the induction of ulcer (Goel and Bhattacharya, 1991). Although few drugs like sucralfate and prostaglandin analogue i.e. misoprostol increase the defensive factors without affecting the offensive acid-pepsin secretion. They are termed as cytoprotective drugs and are proved useful in ulcer therapy (Vergn and Kori-Lindner, 1990). Several natural drugs have been reported to possess anti-ulcerogenic effect by virtue of their predominant effect on mucosal defensive factors (Goel *et al.*, 1987; Rao *et al.*, 2000; Sairam *et al.*, 2001).

Triphala formulation is one the renowned formulation used alone or along with other ingredients in Ayurvedic practice for the treatment of gastric problems. Hence the present study was undertaken to elucidate the probable mechanism underlying anti-ulcer action observed with this combination. Rats have been used as experimental animals in both ulcerogenic procedures mainly because the lower three-fifth glandular secretory portion of its stomach is analogous to the body of the stomach in man both anatomically and functionally (Shay *et al.*, 1945) and rats being omnivorous resemble man nutritionally.

3.1.1. Gastric ulcers- induced by aspirin plus pyloric ligation in albino rats.

In pylorus ligation model, it has been proposed that the digestive effect of accumulated gastric juice and interference with gastric blood circulation are responsible for the induction of gastric ulcer (Brodie, 1966; Patel *et al.*, 2000). Enhanced secretion of acid-pepsin leading to auto digestion of gastric mucosa and break down of mucosal barrier is also responsible for gastric ulcers (Goel *et al.*, 1991). Stimulation of pepsin secretion, with or without secretion of acid, is the major factor in the development of gastric ulcers (Anoop and Jagadeesan, 2003).
Aspirin causes mucosal damage by inhibiting prostaglandins synthesis and interferes with protective mechanisms, such as mucus and bicarbonate secretion, surface epithelial hydrophobicity and mucosal blood flow (Langman et al., 1991; Rao et al., 2000). These changes promote diffusion of acid through the breached surface to destroy cells, capillaries and vein causing haemmorhagic ulcer (Ajaikumar et al., 2005). Aspirin was administered to pyloric ligated rats; in this procedure it is reported that aspirin further aggravates the acidity and attenuates the resistance of the gastric mucosa by causing extensive damage to the glandular region of the stomach (Sanmugapriya and Venkataraman, 2007).

Triphala unequal and Chinnodbhavadi kwath pretreatment showed significant gastric ulcer protective effect as evident from decreased intensity of gastric mucosal ulceration against aspirin treated pyloric ligated rats. The antiulcer activity in this model is evident from significant reduction in free acidity and total acidity along with significant increase in mucin activity. Hypersecretion of acid is due to uncontrolled secretion of hydrochloric acid from the parietal cells of the gastric mucosa through the proton pumping H\(^+\)-K\(^+\) ATPase (Harsey and Sachs, 1995). Blockade of this offensive factor resulted in high healing of gastric and duodenal ulcer in many experimental and clinical gastric ulcer models (Al-Rehaily et al., 2002). Thus the ability of all three Triphala formulations to reduce the acidity may be due to direct action on the acid producing parietal cells or through inhibition of proton pumping H\(^+\)-K\(^+\) ATPase. Earlier study showed significant inhibition of gastric H\(^+\)-K\(^+\) ATPase and acid secretion by ellagic acid (Murakami et al., 1991). Plant glycosides are also known to inhibit chloride transport in the stomach (Machen and Forte, 1979). The ellagic acid is the major chemical constituents present in the ingredients of Triphala formulations (Rastogi and Mehrotra, 1990; Row and Murthy, 1968; Saleem et al., 2002). Glycoside is the major constituents of *Tinospora cordifolia* (Gangan et al., 1994) and *Terminalia belerica* (Awasthy and Nath, 1968), hence the observed effect may be due to the presence of glycosides and ellagic acid in Triphala formulations and their contribution towards antacid effect.

The entire surface of the gastric mucosa is covered by a continuous layer of mucus gel. Mucus is secreted from mucus neck cells and serves as 'first line of defense' against ulcerogens. The defense is mostly due to active secretion of
bicarbonate and improving the buffering of acid in gastric juice and by acting as an effective barrier to back diffusion of hydrogen ions (Williams and Tumberg, 1980; Venables, 1986). Secreting mucus cover the gastric mucosa, that would strengthen the healing process in the stomach by providing a physical barrier and a stable unstirred layer between the apical surface of the epithelial cells and lumen. All these can reduce the aggressive action of acid on the epithelium and at the ulcer site to provide a better environment for wound repair (Allen et al., 1986).

A copious amount of gastric mucus is secreted during superficial mucosal damage and provides favorable microenvironment for repair by restitution (Goel et al., 1985). Mucin is viscous glycoprotein and makes up the major part of the mucus (Zalewsky and Moody, 1979). Hence the estimation of mucin secretion is valuable for the study of mucosal defensive mechanisms against ulcerogens. Triphala unequal and Chinnodbhavadi kwath significantly increased the TC: TP ratio, which reflects the functional integrity of mucosal barrier and could be taken as reliable index for mucin secretion (Goel et al., 1985). Increase in mucin was due to increase in individual mucopolysaccharide like total hexose, fucose and hexosamine leading to significant increase in total carbohydrate in both unequal and kwath treated groups. Triphala equal also increased the level of total carbohydrate and mucin activity (p>0.05). All the three Triphala formulations have little or no effect on total protein content of gastric juice. Hence, the augmentation of the mucosal barrier by all the three Triphala formulations was due to increase in secretion of dissolved mucus in the gastric secretion.

Rapid proliferation of gastric mucosa plays an important role in mucosal protection during normal state following mucosal damage. Following extensive damage of the surface epithelial cells, repair occurs within few hours through a process called restitution and which could be followed by cell proliferation (Prabha et al., 2003). The DNA and RNA content is theoretically similar in all diploid cells of the same animal and therefore biochemical determination of DNA content of the tissue provides an estimate of the total number of cells in tissue (Manonmani et al., 1995). Increase or decrease in life span of mucosal cells can be expressed as amount of DNA and RNA in the gastric wall mucosa. The increase in DNA content of gastric wall mucosa in all three drug treated groups indicate decreased cell shedding and
increased life span of cells (Mukhopadhyay et al., 1987). A recent study suggested that phenolic compound (Suresh kumar et al., 2006), ascorbic acid and flavonoids (Sarma and Keshavan, 1993; Shimoi et al., 1997) in Triphala formulations are responsible for the protection of DNA. Hence the major mechanism of Triphala formulations to maintain integrity of gastric mucosa could involve reduction in DNA and RNA fragmentation of the cells and induction of the process of cell proliferation.

Histopathological studies showed that, aspirin plus pyloric ligation procedure produced severe epithelial destruction and cell infiltration in muscular layer in stomach tissue from control animals. Triphala equal pretreatment produced severe epithelial destruction in rats. Triphala unequal and Chinnodbhavadi kwath showed almost normal cytoarchitecture.

From the present study, it is concluded that Triphala formulations possess significant anti-ulcer activity. This activity depends mainly on inhibition of acid secretion, increase in mucin secretion, which enhances the stability of gastric mucosal barrier and gastric cytoprotection against aspirin plus pyloric ligation- induced gastric ulceration in rats and thus can be classified among cytoprotective drugs (Robert, 1979). Among the three formulations studied, Chinnodbhavadi kwath showed more pronounced effect followed by Triphala unequal and Triphala equal.

3.1.2 Stress - induced gastric ulcer and hypothermia.

Various physical and psychological stresses cause gastric ulceration in humans and experimental animals (Miller, 1987). The precise biochemical changes during ulcer generation are not clear yet, although various hypothesis have been proposed from time to time. Stress-induced ulcers are probably caused by the release of histamine, by enhanced acid secretion and reduction in mucus production (Pal and Nagchaudhury, 1991), increased gastric motility (Garrick et al., 1986), vagal over activity (Cho et al., 1976), reduction in mucosal blood flow and peripheral neural influences (Gaton et al., 1993), mast cell degranulation (Cho and Ogle, 1979), and decreased prostaglandins level (Miller, 1987).

Oxidative damage is considered to be common factor in the pathogenesis of ulcers by different experimental and clinical models. The increase in free radicals
generation apart from acid pepsin factors are also held responsible for induction of ulcers (Miller, 1987). Stress causes both the sympathetic and parasympathetic stimulation of the stomach, which induces an increased gastric motility and muscular contraction leading to vascular compression and mucosal ischemia, which in turn causes free radical generation (Das and Banerjee, 1993). Currently, a concept is emerging that proposes that gastric mucosal lesions caused by various factors are due to increased cell death by apoptosis with simultaneous block of cell proliferation process. Stress-induced gastric lesions have been associated with lowering of proliferating cell nuclear antigen labeling index and also with an imbalance between Bcl-2 family of anti-apoptotic protein and Bax protein, which promotes apoptosis (Konturek et al., 1999). In this connection, it is anticipated that any agents which can promote healing process through decrease of apoptosis and increase of cell proliferation together with the attenuation of free radicals generation and inflammation at the ulcer crater, would be useful to ameliorate the complication of stress in the stomach (Cho et al., 2003). It would be interesting to study the effect of the test formulation for their influence on the process of apoptosis.

The similarity in etiology, histology and clinical management between experimental stress ulcers and clinical stress ulcers, enables the use of animal stress ulcer models as an experimental tools for stress-induced gastric bleeding and ulceration (Brodie and Hanson, 1960). Based on this assumption and the fact that they are already in use for the treatment of human gastric disorders, it can suggested that the test formulations especially Chinnodbhavadi kwath and Triphala of unequal proportion could be very useful in the treatment of stress linked gastric ulceration in clinical settings.

In stress-induced hypothermia procedure, reduction in rectal temperature (hypothermia) was observed in rats subjected to forced swimming stress for 15 mins. It is reported that rasayana or adaptogenic drugs have been proved to produce complete potential in order to prevent diseases and degenerative changes that leads to diseases and promote longevity by providing strength and immunity (Sharma, 1994; Vayalil et al., 2002). All the three Triphala formulations showed significant reduction in hypothermia almost in similar fashion in rats subjected to stress. However, this effect was found more pronounced in Triphala unequal group which could be due to
higher concentration of *Emblica officinalis* in this formulation. The efficacy in kwath treated group may be due to presence of *Tinospora cordifolia* along with other ingredients.

Effect of stress on RBC and related parameters showed that stress significantly reduces the haemoglobin level compared to normal control group. Triphala unequal showed significant increase in haemoglobin level, increase in MCH and MCHC indicating that Triphala may prove to be useful remedy for stress-induced anaemia as per previous report (El-Mekkawy et al., 1995).

Serum alkaline phosphatase and transaminases are cytoplasmic in location and are released in to circulation after cellular damage (Sallie et al., 1991). Stress significantly increases the level of these enzymes in serum indicating increased cellular damage. The increased values of alkaline phosphatase and SGPT were significantly attenuated by the pre-treatment with Triphala unequal compared to stress control group confirming the cytoprotective effect of drug against stress.

Increase in generation of reactive oxygen species (ROS) during stress conditions led to oxidative damage to gastric mucosa resulting gastric ulceration (Cochrane et al., 1983; Miller 1987). Superoxide, hydrogen peroxide and hydroxyl radical are important reactive oxygen species causing oxidative damage. Among all ROS's, especially hydroxyl radical plays a major role in damage to gastric mucosa in almost all types of gastric ulcers (Bandyopadhyay et al., 2002; Das and Banerjee, 1993). Lipid peroxide level is an indicator for the generation of ROS in the tissue. Our data have also shown significant increase in hydroxyl radical and LPO in stress ulcerated stomach homogenate revealing generation of ROS during stress.

Cell membrane lipid is very susceptible to hydroxyl radical attack and initiates the formation of LPO. Lipid peroxide involves the formation and propagation of lipid radicals, the uptake of oxygen and rearrangement of double bonds in unsaturated lipids, which eventually results in destruction of membrane lipids. Biological membranes are often rich in unsaturated fatty acids and bathed in oxygen rich metal containing fluid. Therefore, membrane lipids are susceptible for peroxidative attack (Cheesman, 1993). Theses induces cell degradation, impaired ion transports, increase
in membrane fluidity, permeability and loss of structural and functional integrity of cell membrane (Freeman and Crapo, 1982).

Preventive antioxidants, such as superoxide dismutase (SOD) and catalase enzymes are the first line of defense and glutathione is the second line of defense against ROS. SOD is the most important enzymes that acts by quenching of superoxide anion, which is active oxygen radical produced in different aerobic metabolism (Pryor, 1986). In addition to removing a highly reactive species that might directly contribute to tissue necrosis, the dismutation of superoxide anion by SOD may be protective by extending the half life of endothelium derived relaxing factor, nitric oxide (Villegas et al, 2001). Earlier studies showed that superoxide anions produced in various models of gastric ulcer in rats, which is catalyzed or dismutated by SOD provide protection to gastric mucosa (Tondon et al., 2004; Bafna and Balaraman, 2005). Catalase is a tetrameric enzyme, present in most of the cells and act by catalyzing the decomposition of peroxyl radical, $\text{H}_2\text{O}_2$ to water and oxygen (Pryor, 1986). Accumulation of $\text{H}_2\text{O}_2$ occurs in the mitochondria and cytosol, if not scavenged by catalase, can itself cause lipid peroxidation by increasing the generation of hydroxyl radical (Das et al., 1997). Hence decrease in catalase level in present study in stress condition led to increase in accumulation of these reactive products and thus, has caused increased LPO and tissue damage. The effect is further aggravated by decreased activity of gastric peroxidase during stress (Boyd et al., 1981).

Glutathione (GSH) is an important constituent of intracellular protective mechanisms against oxidative stress. Additionally GSH scavenges superoxide anion and protect protein thiol group from oxidation (Villegas et al., 2001). Depletion of GSH is known to result in enhanced lipid peroxidation and excessive LPO can cause increased GSH consumption and increase the susceptibility of gastric mucosal cells to oxygen metabolites. A significant depletion of gastric glutathione has been reported in gastric ulceration in rats (Boyd et al., 1981; Anandan et al., 1999).

In the present study, we have observed that water immersion stress- induced severe hemorrhagic lesions in stomach of rats. The rats pretreated with all the Triphala formulations and omeprazole reduces the severity of ulcer lesions as well as ulcer index in rats subjected to stress. Further treatment Triphala unequal and Kwath

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significantly reversed the stress-induced generation of hydroxyl radical and lipid peroxide. This may be due to restoration of free radicals scavenging enzymes viz; SOD, catalase and glutathione in gastric mucosa, effectively counter acting the free radicals generated by cascade of reaction as described above. Earlier study also revealed that biological activities of Triphala is due to its ability to scavenge free radicals (Sabu and Kuttan, 2002; Naik et al., 2005).

Determination of membrane associated enzyme activities like ATPase indicates changes in membrane under physiological conditions. ATPases (Na\(^{+}-K^{+}\), Mg\(^{2+}\) and Ca\(^{2+}\) ATPase) are lipid dependent membrane bound enzymes, involved in active transport process (Devi et al., 2004). Enhanced level of lipid peroxidation observed in the present study, might have led to loss of protein thiol and there by changing membrane functions (Adhirai and Selvam, 1997). Depletion of GSH during stress also decreases membrane bound ATPase, as they require –SH group to maintain their structure and functions (Garner and Garner, 1983). Thiol groups are main components of the intracellular non-protein sulfahydryl groups and they participate in many cellular functions including drug metabolism and detoxification of free radicals (Lai et al., 1991). Triphala unequal and Kwath reversed the decreased level of membrane bound ATPase in stress condition. The effect may be due to significant decrease in LPO and restoration of the level of GSH and there by protecting the protein thiol and sulfahydryl groups which are essential for structural integrity and function of ATPase. It is pertinent here to refer to a previous report which suggested that *Emblica officinalis* extract (Al-Rehaily et al., 2002) and *Azadirachta indica* (Bandyopadhyay et al., 2002) significantly prevented depletion of non-protein sulfahydryl groups, which might have contributed to the protective effect of Triphala unequal and Kwath.

Recent studies indicate that gastro-duodenal ulceration is due to apoptotic cell death of the gastric mucosa. DNA fragmentation of the cells is indicative of apoptotic cells death in stress-induced gastric damage in rats (Bandyopadhyay et al., 2004) and thus by decreasing the life span of gastric mucosal cells (Mukhopadhyay et al., 1987). Stress significantly reduce the strength of gastric mucosa and there by reduces the resistance towards ulcers as evident by significant decrease in the level of DNA and RNA. The increased level of DNA and RNA in rats pretreated with Triphala unequal
and Kwath indicated that the drugs reduce the apoptotic cell death and erosion of gastric mucosa to some extent in stress condition.

Histopathological studies showed that, stress produced severe epithelial destruction, submucosal oedema and hemorrhage in stomach tissue from stress control animals. Triphala equal produced submucosal oedema with few haemorrhage patches. Triphala unequal and Chinnodbhavadi kwath showed mild to moderate epithelial disruption, moderate oedema in submucosa and muscular layer.

From the present study, it can be concluded that Triphala formulations possess significant anti-ulcer activity. This may be due to their rasayanic (rejuvenatives) activity and partly due to its antioxidants and cytoprotective activity. It is conceivable that the cytoprotective activity of Triphala formulations may relate primarily to the inhibition of the oxidative modification of certain proteins whose free radical mediated structural modification and loss of functions is particularly relevant to the development of cell injury.

Triphala equal powder is prepared by mixing equal proportion of dried pericarp of *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*, while Triphala unequal prepared by mixing three ingredients in unequal proportion (1:2:4). Chinnodbhavadi kwath (Decoction) prepared by mixing equal proportion *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, *Tinospora cordifolia*, *Azadirachta indica* and *Trichosanthes dioica* (Sarma et al., 1966; Anonymous 2003). Previous report suggested that among all these ingredients, *E. officinalis*, *T. cordifolia* and *A. indica* produced significant anti-ulcer activity. *A. indica* has been reported to produce anti-ulcer activity through inhibition of acid secretion through anti-histaminergic mechanism (Raj et al., 2004), block oxidative damage by preventing LPO, hydroxyl radical and thiols depletion and DNA damage (Bandyopadhyay et al., 2002). The major anti-ulcer compound isolated from the aqueous extract of Neem bark characterized to be phenolic glycoside in nature (Bandyopadhyay et al., 1998).

*Emblica officinalis* is reported to have produced anti-ulcer activity through significant decrease in acid-pepsin secretion and increase in mucosal protective factors like mucus secretion, cellular mucus and life span of mucosal cells (Rao et al.,
2001; Sairam et al., 2002), by scavenging free radicals and restoring the antioxidant level, ATPase activity and thiol groups (Rajeshkumar et al., 2001; Al-Rehaily et al., 2002, Sairam et al., 2002). The antioxidant activity of *E. officinalis* could be due to presence of Emblicanin A and B, which improve the efficacy of vitamin-C in reducing dehydroascorbic acid to ascorbic acid, that is also reported to be antioxidant in nature (Ghosal et al., 1996; Bhattacharya et al., 2000). Additionally saponins and tannins are known to affect the integrity of mucus membrane (Oliver, 1960). Tannins with their protein precipitating and vasoconstriction effects could be advantageous in preventing ulcer development (Aguwa and Nwako, 1988). Tannins also being astringent may have precipitated micro proteins on the site of ulcer there by forming an impervious protective pellicle over the lining to prevent toxic substances and resist the attack of proteolytic enzymes (John and Onabanzo, 1990; Nwafor et al., 2000). *Terminalia belerica* and *Emblica officinalis* are well established major antioxidants and their free radical scavenging effect is due to the presence of ellagic acid and gallic acid compared to *Terminalia chebula* (Sabu and Kuttan, 2002).

Emblica officinalis and *Tinospora cordifolia* are categorized as ‘Rasayanas’ (rejuvenatives). Rasayanas are non-toxic Ayurvedic complex herbal preparations or individual herbs used to rejuvenate or attain the complete potential of an individual in order to prevent diseases and degenerative changes that lead to disease condition. The emerging data suggest that the possible mechanisms may be by immunostimulation, quenching free radicals, enhancing cellular detoxification mechanisms, repair damaged non-proliferating cells, inducing cell proliferation and self-renewal of damaged proliferating tissues, and replenishing them by eliminating damaged or mutated cells with fresh cells (Vayalil et al., 2002; Bafna and Balaraman, 2005).

At the dose level studied, the Chinnodbhavadi kwath and Triphala unequal provides significant protection in different models of gastric ulcer as compared to Triphala equal. The reason for the observed efficacy of this preparation is the increased quantity of two of its ingredients namely *Terminalia belerica* and *Emblica officinalis* in Triphala unequal. Of the two, *Emblica officinalis* is well-known for its cytoprotective effect. Chinnodbhavadi kwath produced anti-ulcer effect due to the presence of Triphala along with *Tinospora cordifolia* and *Azadirachta indica*. In the light of above discussion, it can be suggested that Triphala unequal formulation and
Chinnodbhavadi kwath are better suited for the treatment of gastric ulceration due to stress or other related conditions in comparison to Triphala equal formulation in clinical settings.

3.2. INTESTINAL CYTOPROTECTIVE ACTIVITY

Methotrexate-induced small intestine injury

It is well known that many oral chemotherapeutic treatments can induce intestinal mucosal injuries. Such observations have been reported, for examples, after treatment using cyclophosphamide and Mytomycm-C (Mizuno et al., 1987), 5-flurouracil (Yamazaki et al., 2004) or methotrexate (Sener et al., 2006). Methotrexate (MTX) is a widely used drug in anti-metabolite cancer therapy and also used as anti-arthritic, anti-inflammatory and immunosuppressive agent. In recent years, the high doses of MTX therapy has extensively been tried for patients with osteosarcoma, acute leukemia and sever psoriasis. The occurrence of severe unpredictable toxicity associated with this therapy is a serious clinical problem. Since the cytotoxic effect of methotrexate is not selective for the cancer cells, it also affects the normal tissue that have a high rate of proliferation, including the actively dividing cells of gut mucosa and the haemopoietic cells of the bone marrow. Methotrexate inhibits the dihydrofolate reductase and there by affects synthesis of DNA, RNA and protein, which can cause an acute injury to the intestinal epithelium (Rampton, 2001; Sener et al., 2006; Li et al., 2005).

Proliferation of small intestinal epithelial cells occurs in the crypt cells. Crypt cells which rapidly regenerate and migrate to the villus tip lead to replacement of intestinal epithelium. This process is completed within two days in rats and three days in humans (Yuncu et al., 2004). The anti-mitotic effect of MTX is known to give rise to the malabsorption syndrome. This syndrome is accompanied by histopathological changes in the intestine, such as shortened microvilli which possibly results from the depressed generation of intestinal epithelial cells following damage to crypt cells by MTX. Their depressed generation consequently lead to decreased absorption surface area and in turn to lesser absorption of nutrient and drugs (Tsurui et al., 1990).
The chemical constituents of intestine have been found to change under the condition of the malabsorption syndrome induced by the anti-tumour drug administration (Takeuchi et al., 1989; Tsurui et al., 1990). Protein, phospholipid and total lipid are the major constituents of cell membrane (Arrieta et al., 2006) appeared to have decreased in the homogenate of the small intestine of MTX treated rats. The MTX treatment leads to drastic changes in membrane fluidity as a result of phospholipid degradation. Fluidity of membrane is responsible for the functioning of membrane bound enzymes and various other membrane transport molecules (Molenaar and Hulsteart, 1980). These findings adequately reflect the MTX-induced histological changes in the intestine, which are characterized by rapid protein breakdown, lipid degradation, abnormal intestinal morphology, break in epithelial barrier and mucosal permeability (Fromont et al., 1999). The decrease in membrane protein and lipids (total lipid and phospholipid) of the small intestine has been prevented by Triphala equal and Triphala unequal co-administration with MTX. This effect of both the formulations on protein content was more evident in the BBMV of the small intestine. Our results suggested that Triphala equal and unequal pretreatment stabilizes the lipid bilayer of cell membrane which is further supported by the low level of LPO in the Triphala treated groups.

The brush border membrane lining the enterocytes is highly specialized to perform varieties of function mainly digestion and absorption of nutrients. The activity of the digestive enzymes is important in the digestion and absorption of the food. For this purpose, the enzymes particularly located in the brush border section of the intestine are very important since they are responsible for the final stage of degradation and assimilation food (Hakim et al., 2006). Intestinal alkaline phosphatase, γ-glutamyl transpeptidase (GGT) and disaccharidase (sucrase and lactase) are located in the brush border on the apical surface of the mature villous enterocytes in the small intestine. (Jung et al., 2006). GGT together with disaccharidase constitutes ectoenzymes which are localized at the external surface of membrane while alkaline phosphatase is located in the hydrophobic core of the brush border membrane (Shayiq et al., 1986).

The disaccharidase is responsible for the digestion of the dietary disaccharides that are absorbed only after hydrolysis (Henning et al., 1994). Sucrase-isomaltase
complex is a membrane bound dimeric enzyme. Apart from having a hydrolytic role, it has trans-glucosidase activity. It also acts as carrier for delivering the products of sucrose hydrolysis across the brush border membrane in the enterocytes (Storelli et al., 1972). Lactase hydrolyses the β- galactosidase, one of its substrate; β-lactose is hydrolyzed to β-D galactose and β-D glucose and also acts as carrier for delivering the products of lactose hydrolysis (Tauber, 1950). γ-Glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl residue from glutathione and other γ-glutamyl compounds to an amino acid or peptide as acceptors. The GGT activity seems to be involved in the active transport of some amino acids from apical to basolateral membrane or in hydrolysis of γ-glutamyl substances (Sobiech, 1981; Harpaz and Uni, 1999). Alkaline phosphatase is responsible for the hydrolyzing phosphoric ester bond of organic compounds and most likely plays a role in fat absorption (Mahmood et al., 1994). It is also considered to be involved in the intestinal transport of calcium (Gracey et al., 1975).

Mucosal enzymes can serve as markers for small intestinal damage. The striking loss of brush border membrane enzyme activities in the present study could therefore be a result of changes in membrane fluidity, disintegration and subsequent release of the enzymes caused by MTX concentration in the lumen. Reduction in enzymes activities would result in accumulation of undigested materials within small intestine causing mal-digestive diarrhea (Jung et al., 2006). The decrease in alkaline phosphatase activity may be due to the inactivation of enzymes by free radical injury because metal binding sites of this enzyme are more susceptible to direct oxidation (Sisley et al., 1999). The decrease in alkaline phosphatase, GGT and disaccharidase activities in the intestine followed by MTX treatment are consistent with the result reported previously in rats treated with MTX (Taminiau et al., 1980).

Methotrexate co-treatment with Triphala unequal inhibited the depletion of sucrase, lactase and alkaline phosphatase and kwath enhanced the level of sucrase. The level of GGT also got enhanced by Triphala unequal and kwath as compared to MTX control group. The protective effect could be ascribed to epithelial cell integrity or to a stabilizing effect on tight epithelial junction of small intestine due to direct effect on membrane lipid as shown above. Restored level of alkaline phosphatase also may be due to antioxidant properties of Triphala unequal formulation.
Methotrexate is known to depress the metabolic activity and active transport capacity of the intestinal mucosa (Robinson et al., 1966; Capel et al., 1979). Intestinal ATPase plays a crucial role in the cellular physiology of nutrient absorption and electrolyte homeostasis. The Na\(^{+}\)-K\(^{+}\) ATPase is the main enzyme responsible for the maintenance of transcellular Na\(^{+}\)/K\(^{+}\) gradients, which play a vital role in the absorption of nutrients and electrolytes in the intestinal tract. The Na\(^{+}\)-K\(^{+}\) ATPase, is an ubiquitous nucleotidase located at the basolateral membrane of virtually all polarized cell types, including intestinal epithelial cells, where it supports the function of numerous transport processes. In addition to Na\(^{+}\)-K\(^{+}\) ATPase activity, intestinal mucosa also contain residue ATPase activity, which becomes evident in the absence of K\(^{+}\), which probably resembles the activity of different Mg\(^{2+}\) dependent ATPase (Diaz et al., 1998). Mg\(^{2+}\) ATPase is involved in energy requiring process in cells (Devi et al., 1998) and Ca\(^{2+}\) ATPase is involved in the maintenances of Ca\(^{2+}\) homeostasis by calcium transport system (Thor et al., 1985).

Disruption of activity of these enzymes may have occurred by oxidation of vital protein thiol (Adhirai and Selvam, 1997), which is a major constituent of non-protein sulphydryl groups. The marked loss of ATPase activity in MTX treated group may be due to the loss of protein thiol and sulphydryl groups, a consequence of depletion of glutathione and lipid peroxidative damage that is observed in MTX treated group. Co-treatment with Triphala unequal reversed the loss of ATPase activity almost near to normal control group values, probably by its ability to maintain levels of GSH and LPO in methotrexate treated rats.

Regarding oxidative stress, the results of the present study is consistent with the hypothesis that the increase in reactive oxygen species production and impaired antioxidant defense mechanism are postulated to be causative factors of mucosal damage in inflammatory diseases (Han and Meydani, 2000). Quantitatively the main free radicals in tissue are superoxide that can be produced by both endothelial cell through xanthine oxidase (XO) and by activated neutrophils via myeloperoxidase. Gastrointestinal mucosa is particularly rich in xanthine oxidase. It has been postulated that xanthine oxidase would be capable of extensive damage to vascular endothelium and responsible for multiple tissue dysfunction (Park, 1989; Villegas et al., 2001).
The severity of intestinal damage induced by MTX was related to a significant increase of xanthine oxidase activity. The increase of xanthine oxidase in MTX control rats indicated that the free radicals derived via action of xanthine oxidase were in part responsible for the oxidative tissue damage of intestinal mucosa. These finding are consistent with those from other studies which showed that XO activity increased after administration of anti-inflammatory drugs (Tanaka and Yuda, 1996; Villegas et al., 2001). All three triphala co-treated groups showed significant decrease in XO activity in the present study suggesting that xanthine activity reverted towards normal in radiation induced oxidative damage in intestine of rats (Sandhya et al., 2006b). The observed activity may relate to antioxidant activity of Triphala formulations through which, it may protect the oxidation of essential sulfahydral groups and thus maintain the XOR predominantly in XDH state.

In addition to direct damaging effect on tissue, free radicals and inflammation seems to trigger the accumulation of leucocytes in the involved tissue and thus aggravate the tissue injury indirectly through activated neutrophils (Vaziri, 2004). It is well known that phagocytic leucocytes, when suitably stimulated, increased their oxygen consumption, which is called as respiratory or oxidative burst of neutrophils. It has been shown that activated neutrophils secret enzymes such as myeloperoxidase (MPO) from their azurophillic granules (Bell et al., 1995; Sullivan et al., 2000). In turn, MPO play fundamental role in triggering of free radicals release which may exerts toxic effect on fatty acid residues in membrane lipids (Han and Meydani, 2000). Myeloperoxidase activity is considered as quantitative measure of neutrophils inflammatory response in variety of clinical and experimental studies (Krawisz et al., 1984). Previous study showed that significant elevation of MPO level observed in the ileum of the rats treated with Methotrexate is due to oxidative organ injury (Sener et al., 2006). The elevated level of MPO in small intestine in the present study indicates that the neutrophils accumulation contributes to MTX- induced injury. Co-treatment with Triphala equal and Triphala unequal has a preventive effect against methotrexate -induced damage through inhibition of neutrophils infiltration via its anti-inflammatory activity (Rasool and Sabina, 2007) and depressed oxidative burst function of the neutrophils via its antioxidant activity (Sabu and Kuttan, 2002; Naik et al., 2005).
Lipid peroxidation, mediated by oxygen free radicals is believed to be an important cause of destruction and damage to cell membranes and has been suggested to be a contributing factor to the development of MTX mediated tissue damage (Sener et al., 2006) as confirmed by the elevated level of MDA in the present study. The increased lipid peroxidation is responsible for the formation of the lipid hydroperoxides in membrane and would result in damage of membrane bound enzymes (Fridovich and Portar, 1981). The accumulation of lipid peroxides introduces hydrophilic moieties in to hydrophobic phase and thus alters membrane permeability and its functions. (David et al., 1991).

Methotrexate is stored inside the cell in polyglutamated form. Long term administration can cause accumulation of methotrexate polyglutamates and decrease folate level (Galivan et al., 1983). On the other hand, MTX could decrease the availability of NADPH in cells leading to depletion of glutathione. Under normal condition NADPH is used by glutathione reductase to maintain the reduced state of cell glutathione (Caetano et al., 1997). As reported by Ross, cell injury and enhanced cell susceptibility to toxic chemicals are related to insufficient intracellular GSH content (Ross, 1988). Considering the relationship between GSH and the deleterious effects of methotrexate, it seems likely that GSH may play a critical role in limiting the propagation of free-radical reactions, which would otherwise result in extensive lipid peroxidation. In the present study, the significant reduction in GSH levels, promoted by methotrexate, led to a reduction of effectiveness of the antioxidant enzyme defense system, sensitizing the cells to reactive oxygen species (Babiak et al., 1998). However, due to its antioxidant activity, Triphala equal and unequal formulations reduced the methotrexate induced oxidative injury and restored the GSH levels significantly. The restored level of GSH in Triphala equal and unequal groups prevent the propagation of free radical reactions and thus inhibit the methotrexate-induced lipid peroxidation.

Two major transport routes are thought to be active in the intestinal absorption of nutrients and drugs. One is the transcellular pathway and the other is the paracellular pathway (Powel, 1981). Intestinal absorption in MTX-induced malabsorption has so far been studied in terms of absorption through the transcellular pathway, since nutrients are mostly absorbed via this pathway, and absorption through
the paracellular pathway has been little studied. In this study the effect of MTX on intestinal absorption through the paracellular pathway has been examined by employing phenol red (PR) permeation test in everted segments of damaged small intestine of rats treated orally with MTX.

Phenol red is generally used as a non-absorbable marker in *in-vitro, in-situ* and *in-vivo* absorption experiments. This non-absorbable marker permeated the small intestine very slightly in the small intestine of normal untreated rats in the *in-vitro* everted sac experiment. This suggests that phenol red permeation assay can be used to assess MTX-induced damage to the small intestine, since it may permeate the damaged intestine more easily because of the disordered barrier function of the intestinal epithelium (Nakamaru *et al.*, 1998; Horie *et al.*, 1999). The MTX administration to rats in present study indicated a high permeation of phenol red through the small intestine as shown in the *in-vitro* everted small intestine technique (Fig.-4), which was in conformity with the result obtained from mice (Nakamaru *et al.*, 1998). Interestingly, the permeation was depressed by Triphala equal and unequal co-treatment with MTX in rats. The data have been shown that Triphala equal and unequal protect the small intestine from MTX - induced intestinal damage. The observed effect may be due to decrease in LPO level and thus preventing the alteration in membrane permeability and its functions (David *et al.*, 1991).

In addition to above, histopathological studies have shown that methotrexate produced marked loss of cytoarchitecture, epithelial destruction and necrosis in colon of rats. Triphala equal showed mild epithelial damage and cell infiltration. Triphala unequal showed moderate cell infiltration while Chinnodbhavadi kwath showed fatty changes in intestine of rats.

The observed effect in this model may be attributed to antioxidants activity of flavanoids, ascorbic acid and phenolic compounds present in Triphala formulations. The Triphala unequal contains higher proportion of such antioxidants which would be responsible for its pronounced effect against MTX followed by Triphala equal and kwath. Thus the data generated during the present study indicate presence of very good intestinal protective activity in the Triphala unequal formulation, moderate activity in Triphala equal formulation and mild activity in the Chinnodbhavadi kwath.
The exact reaction for this quantitative difference remains to be identified. This may be due to pharmacodynamic and possible pharmacokinetic differences.

3.3. COLON CYTOPROTECTIVE ACTIVITY

**Acetic acid-induced experimental Colitis in albino rats.**

Induction of colitis by acetic acid in rats is one of the standardized and well established commonly used method to produce an experimental model of inflammatory bowel disease with some resembles to human acute intestinal inflammation. Several major causative factors in the initiation of human colitis such as enhanced vasopermeability, prolonged neutrophils infiltration, increased production of inflammatory mediators and excessive oxygen derived free radicals release by inflamed mucosa are involved in the colitis by acetic acid in rats (Sharon and Stenson, 1985; Elson et al., 1995; Millar et al., 1996). The initial injury in this model of colitis is a relatively bland epithelial necrosis and oedema that variably extended in to the lamina propria, submucosa and external muscular layer. Mucosal and submucosal inflammation that follows the initial epithelial cell injury is associated with the activation of arachidonic acid pathway. This phase of the inflammatory response is inhibited by leukotriene blockade, phospholipase A2 inhibitors, glucocorticoids, prevention of neutrophil recruitment, interleukin 1 receptor antagonist, platelet activating factor inhibitors, mast cell stabilizers, and reactive oxygen metabolite scavengers (Yamada et al., 1991; Elson et al., 1995).

The acetic acid-induced colitis in the present study was associated with the macroscopic, microscopic, functional and biochemical changes. The acetic acid produced inflammation and ulceration appears to involve the entry of protonated form of the acid in to epithelium, where it dissociates to liberate protons within intracellular milieu leading to acidification that most likely accounts for the epithelial injury and ulceration (Yamada et al., 1991).

The severity of colonic damage, increased wet weight and shortening of inflamed colon tissue and impairment of fluid absorption are considered as reliable and sensitive indicators of severity and degree of acute inflammatory response after instillation of acetic acid in to colon of rats (Galvez et al., 1997; Stegmund et al.,
Pre-treatment with Triphala unequal and sulfasalazine affords protection to rat colonic mucosa as shown by the significant attenuation of the extent and severity of macroscopic mucosal inflammation, ulceration, haemorrhagic lesion in the colon of rats as assessed by significant decrease in colonic damage score. The results produced are in agreement with the previous finding of Sharad (2003), who showed Triphala unequal significantly reduced the damage score in colon of colitic rats.

The increase in wet weight and shortening of inflamed colon resulted in to increased wet weight/length (mg/cm) ratio, indicating the occurrence of severe oedema in ulcerative colitic rats (Gotteland *et al.*, 1997). Triphala unequal showed significant reduction in weight/length ratio which was comparable with sulfasalazine (*p* > 0.05).

One of the classical symptoms of colonic mucosal inflammation is diarrhoea. Several authors have directly examined the impact of experimental colitis on hydroelectnc transport. Thus acetic acid colitis is accompanied by a significant decrease in colonic basal net sodium and chloride absorption, as a consequence of colonic barrier disruption and increased permeability. Disturbance in permeability has been suggested to be involved in the pathogenesis of inflammatory bowel disease (Fedorak *et al.*, 1990; Fedorak *et al.*, 1995). Therefore, the impairment of fluid absorption in colitic rats in our study primarily reflect the loss of viable epithelial surface in the early stage of colitis as reported in earlier studies (Galvez *et al.*, 1997; Gotteland *et al.*, 1997). Pre-treatment with Triphala equal, Triphala unequal and sulfasalazine to colitic rats, reversed the net secretion in to net absorption, although to a limited degree compared to normal fluid absorption (normal control). The observed reduction in damaged area by Triphala unequal and Sulfasalazine and amelioration in the impairment of colonic fluid transport may be partly ascribed to mucosal protection from inflammation. Number of flavanoid compounds have been reported to inhibit intestinal motility and secretion (Rao *et al.*, 1997) and one of the most common flavonoid occurring in nature quercetin is reported to be helpful in mucosal recovery from lactose-induced chronic diarrhoea in rats (Galvez *et al.*, 1995). Therefore, the effect of Triphala equal and unequal can be ascribed partly to their flavanoid content in their ingredients. Nevertheless, there must be other mechanism involved, since
Ulcerative colitis is a chronic recurrent inflammatory bowel disease of unknown origin. Oxidative stress has been implicated in the pathogenesis of ulcerative colitis in experimental animals (Keshavarzian et al., 1990) and in humans (Kitahora et al., 1998). In the present study, we observed levated level of myeloperoxidase, nitric oxide and lipid peroxidation in colonic mucosa that was parallel with the depleted glutathione content and superoxide dismutase, which is indicative of oxidative stress. Excess production of reactive oxygen metabolites e.g., superoxide, hydroxyl radical, hydrogen peroxide, hypochlorous acid and oxidant derivatives, such as N-chloramines, are detected in the inflamed mucosa and may be pathogenic in inflammatory bowel disease (Keshavarzian et al., 1992). Sustained production of reactive oxygen metabolites during colonic inflammation may overwhelm the endogenous antioxidant defense system that regulates their production leading to oxidative injury (Blau et al., 1999). Decreased endogenous antioxidant levels in patients with ulcerative colitis have also been reported (D'Odorico et al., 2001). The main sources of reactive oxygen metabolites in the inflamed mucosa are activated phagocytic leukocytes, capable of producing superoxide and a cascade of various species leading to a very reactive hydroxyl radical and peroxide. The xanthine oxidase pathway in colonocytes also produces superoxide anion by conversion of xanthine/hypoxanthine to uric acid. A third possible source is the oxidation of arachidonic acid either through the lipooxygenase reaction, producing leukotrienes, or the prostaglandin generating cyclooxygenase reaction (Loguercio et al., 1996).

Neutrophils play a crucial role in the development and full manifestation of gastrointestinal inflammation. Activated neutrophils are recruited into inflamed mucosa and submucosa of the large intestine during acute inflammation by local production of cytokines and can then contribute to tissue destruction by the production of reactive oxygen metabolites, granule enzymes, and lipid mediators that can further amplify the inflammatory response by their effects on macrophages and lymphocytes (Martin et al., 2004; Paola et al., 2005). The principal free radical in tissues is superoxide anion ($\mathrm{O}_2^-$) which is converted to the secondary oxidant $\mathrm{H}_2\mathrm{O}_2$ by superoxide dismutase. $\mathrm{O}_2^-$ can be produced by both endothelial cells through xanthine
oxidase and activated neutrophils through NADPH oxidase, which reduces molecular oxygen to the $O_2^-$ radical, and through the enzyme myeloperoxidase (MPO). This enzyme catalyzes the formation of such potent cytotoxic oxidants as hypochlorous acid from $H_2O_2$ and chloride ions and N-chloramines (Hagar et al., 2007; Martin et al., 2004). Measurement of MPO activity has been used as an indicator of neutrophils influx in to inflamed gastrointestinal tissue (Mustafa et al., 2006) and inflamed mucosa of rats in acetic acid-induced ulcerative colitis (Hagar et al., 2007).

A reduction in the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity and antioxidant activity (Veljaka et al., 1995; Mustafa et al., 2006). The reduction in colonic MPO activity following treatment with Triphala equal and Triphala unequal could be attributed to reduced neutrophils infiltration in inflamed colonic tissue via its anti-inflammatory effect and such inhibitory activity of Triphala on neutrophils can result in reduced free radical generation in inflamed colon via its antioxidants effect as evidenced by attenuation in the increased level of malondialdehyde level associated with acetic acid colitis. Increased lipid peroxidation that occurs in colonic tissue can initiate a vicious cycle that generates more and more reactive metabolites, which exhausts cellular antioxidants and favors the consequent development of further inflammation (Paiva et al., 2004). It is therefore, reasonable to assume that Triphala equal and Triphala unequal treatment improve colonic oxidative balance in animals in ulcerative colitis because they were able to reduce the level of malondialdehyde, a good indicator of lipid peroxidation (Ohkawa et al., 1979). Sabu and his coworkers (2002) have reported the presence of the \textit{in-vitro} antioxidant activity of Triphala by scavenging oxygen radicals together with the inhibition of lipid peroxidation. Thus, our findings of the present study are parallel with these observations.

Nitric oxide (NO), a reactive free radical gas, is generated enzymatically in a variety of cells from the L-arginine pathway by three isoforms of NO synthetase (Yue et al., 2001). In the gastrointestinal tract, NO can be either protective or damaging to tissues, depending on what type of NOS is involved in the pathological condition. Constitutive NOS (cNOS) is cytoprotective by directly acting as an inducer of defense response in the GI tract, while NO derived from iNOS, (iNOS) is an important inflammatory mediator together with other free radicals, contribute significantly to the
inflammatory response in the colon. The mechanism of this inflammatory response is likely to be the interaction of NO with superoxide to produce peroxynitrite, which is a strong oxidizing agent, associated with colonic epithelial cell death that initiates lipid peroxidation. High concentrations of NO are known to exhibit toxic effects through the formation of nitroso derivatives (Dijkstra et al., 1998; Rachmilewitz et al., 1995; Yue et al., 2001). Inhibition iNOS seems to ameliorate the inflammatory response and tissue injury in experimental colitis (Hogaboam et al., 1995). The reduction in NOS activity, which was observed in the present study, following treatment with either Triphala equal or unequal corroborates the previously reported findings of Jagetia et al. (2004). Similarly Vitamin-C as present in Emblica officinalis has also been reported to inhibit or mitigate the adverse effect of nitric oxide (Fraga et al., 1991). This reduction in NO activity under the study may be attributed mainly to the anti-inflammatory and to, some extent to the antioxidant effect of test formulations.

The tripeptide glutathione is the most important intracellular antioxidant which play key role in controlling redox state of cell. GSH deficiency signifies an excessive production of reactive oxygen species in acetic acid control group (Sido et al., 1998). Other investigators (Galvez et al., 1997; Millar et al., 1996) had shown reduction in glutathione levels following use of acetic acid-induced experimental colitis. They suggested that the levels of GSH were reduced in tissues when the antioxidant was neutralized by the liberated oxygen-derived free radicals. Treatment of rats with Triphala unequal and kwath significantly counteracted the depletion of total glutathione level in colonic mucosa.

Superoxide dismutase (SOD) is an intracellular metalloenzyme that catalyses the dismutation of the superoxide radicals with subsequent oxidative damage. A role of super oxide dismutase in the colitis is supported by protective and beneficial effect of orgotem, a native super oxide dismutase enzyme on patients of IBD (Niwa et al., 1985) and protective effect of SOD mimetic enzymes in rodent model of colitis (Cuzzocrea et al., 2001). Triphala unequal increased the SOD level in colonic mucosa. Therefore, it may be suggested that the elevated level of SOD and glutathione might be attributed to the free radical scavenging capacity of Triphala unequal formulation. This scavenging capacity of formulation results in prevention of the vicious cycle that generate more and more reactive metabolites and thus maintain
cellular antioxidant level and reduce the inflammation produced by free radicals (Paiva et al., 2004).

Lysosomal enzymes are the main factors playing a vital role in disrupting the cell membranes, tissue injury and repair, phagocytosis and also participate in pathological processes such as inflammation and degenerative processes (Gallin, 1988). It was further postulated that lysosomal enzymes are released in inflammatory diseases to stimulate the synthesis of prostaglandins (Gupta et al., 1992). In the present study, the activity of Cathepsin-D was elevated in mucosa of colitic rats compared to normal control group. The reduction of the release of lysosomal enzymes proves beneficial and indirectly confirms the protective effect of drugs but Triphala formulations have not shown any remarkable effect on the level of Cathepsin-D in mucosa of ulcerated colon.

Further, the histopathological studies have shown acetic acid-induced necrosis and disruption of epithelial, loss of cytoarchitecture, hemorrhagic spot with cell infiltration. Triphala equal produced moderate disruption of epithelial layer while Triphala unequal produced mild cell infiltration with patches of haemorrhage. Chinnodbhavadi kwath produced oedema in muscular layer and moderate epithelial erosion.

At the dose level studied, the Triphala unequal provided more significant protection than Triphala equal against acetic acid-induced ulcerative colitis than the latter while kwath has no effect. It is important to note that the efficacy of Triphala unequal in the down regulation of the activated inflammatory response that was comparable with sulphasalazine. Flavanoids may be useful in inflammatory bowel diseases because of their multilevel anti-inflammatory action and radical scavenging activity. Previous reports showed that quercetin is helpful in trinitrobenzene-sulfonic acid-induced colitis in rats (Sanchez de Medina et al., 1996). Quercetin is one of the flavonoids present in Triphala formulation along with tannin, phenolics and ascorbic acid leading to increased anti-oxidant and cytoprotective effect to colon in rats. Thus Triphala equal and unequal has antioxidant with anti-inflammatory effects constituting an interesting approach in the down regulation of this inflammatory condition.
Our results have shown significant protective effects of Triphala unequal formulation against different kinds of experimental injuries induced in stomach, small intestine and colon of rats. Triphala equal formulation exhibited moderate cytoprotection in all the three models. The reason for the observed efficacy of Triphala unequal preparation is the increased quantity of two of its ingredients namely *Terminalia belerica* and *Emblica officinalis* in Triphala unequal. They are well-established rasayana drugs, major antioxidants and their free radical scavenging effect is due to the presence of ellagic acid and gallic acid. Of the two, *Emblica officinalis* is well known for its cytoprotective effect due to its powerful anti-oxidant active constituents. Increased proportion of this ingredient would have lead to increase in the tannin, ascorbic acid, phenolic compounds and flavanoid content of the preparation which might result in to increased anti-oxidant and cytoprotective effect.

Thus for general GI tract cytoprotective effect Triphala unequal should be the preferred one in therapeutics. However, the Chinnodbhavadi kwath produced better effect as cytoprotective against gastric injury produced by aspirin plus pyloric ligation and water immersion stress - induced gastric ulcers. This may be due to additive effect of guduchi present in the kwath. The data generated during the study clearly shows that formulation factors are important for the expression of pharmacological activities.