Chapter 6

Discussion and Conclusion
In the present study the anti-inflammatory, analgesic and hemotoxic activities of the extracts of leaves of *Lawsonia inermis* were evaluated. An attempt was also made to standardize the plant leaf extract and identify the principle(s) responsible for the pharmacological action.

The effect of *Lawsonia inermis* extracts was examined on the manifestation of cardinal signs of inflammation. For screening and evaluation of anti-inflammatory agents, one of the most commonly employed method is based on the ability of the agent to inhibit edema produced in the hind paw of rats by injection of various phlogistic agents. It has been reported that edemogenic activity of kaolin diminishes when the substance is suspended in sterile media; dextran induced edema is inhibited by chlorpromazine, anti-histaminics and by some adrenergic agents, but not by cortisone and phenylbutazone (Winter et al., 1962). Formaline induced edema is not as sensitive to inhibition by phenylbutazone and other related drugs, as are edemas induced by other irritants. Moreover indomethacin and flufenamic acid are ineffective in this test at doses known to produce significant inhibition in other assays. Antihistaminic agents are also effective in this method (Winter 1965). Yeast-induced edema appears to be relatively insensitive to standard anti-inflammatory drugs (Winter, 1965). In dextran and albumin-induced edema also, phenylbutazone appears to be ineffective unless given at toxic doses. Edema induced by dextran could also be influenced non-specifically (Winter 1966; Garrattini et al., 1965).

On the contrary, carrageenin-induced edema in rat paw is used extensively for its reproducibility and specificity to anti-inflammatory drugs and being least affected by non-specific factors and variations in strain, sex or body weight (Swingle, 1970). Standard anti-inflammatory drugs are also effective in this model and yield linear and parallel dose response curves (Winter, 1965; Swingle et al., 1971). Accordingly the effect of the extracts of Lawsonia on carrageenin-induced paw edema was examined. The alcoholic extract in doses of 0.75 g and 1.5g / kg p.o. significantly inhibited the carrageenin induced paw edema. It may be mentioned here that the extract did not produce any mortality upto a dose of 2 g/kg . The LD$_{50}$
of the extract is reported to be above 2 g/kg i.p (Anand et al., 1992; Ali et al., 1995; Iyer et al., 1998). The potency of anti-inflammatory effect produced by Lawsonia inermis was found to be less than that of phenylbutazone. Thus from the observations it may be stated that Lawsonia inermis alcoholic extract effectively inhibited the edema which is one of the cardinal signs of inflammation. However no parallel inhibitory action was observed by the aqueous and chloroform extracts.

In order to correlate the anti-inflammatory activity with biochemical changes, the effect of Lawsonia extracts on transaminases activity was studied. It has been proposed that the anti-inflammatory drugs inhibit certain enzyme system like transaminases (Steegle et al., 1961). Experimental results revealed that the levels of serum Glutamic oxaloacetic acid transaminases (SGOT) and serum Glutamic pyruvic acid transaminase (SGPT) increased in the control (carrageenin treated) rats. Lawsonia partially prevented the rise in the enzymatic activity, which were associated with the inflammatory reactions.

Carrageenin induced inflammation in rats was accompanied by a significant increase in lipid peroxides by the liver. Carrageenin triggers some as yet uncharacterized metabolic reaction in the liver. As a result of this metabolic disturbance, the output of lipid peroxides in the liver was increased. Since damage of lysosomes is implied in the increased output of liver peroxides, it is likely that carrageenin elicits some local reaction in the paw which is transmitted to the liver, which, in turn causes activation of lysosomes as a defensive measure (Robert and Wai, 1979). In present study it was found that γ GTP levels was raised in serum of carrageenin treated group. The raise in the γ GTP in serum indicate that carrageenin could cause damage to liver cell membrane even, if it is administered at paw region. The damage might be caused either due to carrageenin or due to release of autocoids.

The inhibition of the increase of carrageenin induced paw edema in the Alc Ext D1 & D2 treated group shows anti-inflammatory effect of Alc Ext D1 & D2. The increase in serum GSH in the Alc Ext treated group might be due to the presence of flavonoids in the extract. The inhibition in the rise of γ GTP levels also indicates
the membrane stabilizing effect of Alc Ext. The combined anti-oxidant and membrane stabilizing effect might be responsible for inhibiting the rise of SGOT and SGPT levels in the Alc Ext treated group.

The histopathological examination of rat liver sections treated with carrageenin showed completely normal sections. Though carrageenin treatment significantly elevated the enzyme levels in the liver, but the microscopical examination does not reveal any morphological changes. However, further studies using electron microscopy would reveal the minute changes if any.

The constituent, Lawsone, of Lawsonia is known to possess anti-inflammatory activity (Tripathi et al., 1980). It also contains flavonoids, which have anti-oxidant and anti-inflammatory properties. Present study suggests that Lawsonia has significant effect on acute phase of inflammation caused by carrageenin. Anti-inflammatory, antioxidant and membrane stabilizing action of Lawsonia might be responsible for this action.

Carrageenin induced paw edema is reported to involve three distinct phases of mediator release. The early phase is attributed to the release of histamine and serotonin, and the intermediate phase is mediated by kinin like substances and the next phase is due to the release of prostaglandin like substances (Di Rosa and Willoughby, 1971; Di Rosa et al., 1971a). To ascertain the inhibitory effect of Lawsonia extracts on the mediators, anti-inflammatory activity of the extracts was evaluated against histamine, serotonin, bradykinin and prostaglandin E2 induced paw edema. The alcoholic extract at dose 1.5 g/kg p.o. significantly inhibited the edema formation induced by PG E2 (31% inhibition at 3rd hr). But the inhibition was non-significant in the edemogenic effect of histamine, serotonin and bradykinin. Hyalluronidase is known to act by polymerizing the hyaluronic acid in capillary endothelium and thereby producing an increase in the vascular permeability (Ghosh et al., 1963). Lawsonia extracts showed no inhibition in the hyalluronidase-induced paw edema in rats. The non-significant inhibition of edema
by these phologistic agents is also marked by non-significant inhibition of the biochemical markers, SGOT and SGPT.

Lipoxygenase inhibitors also possess significant anti-inflammatory effect against carrageenin-induced paw edema (Chawla et al., 1987). So inhibition of carrageenin-induced paw edema by Lawsonia extracts could also be due to its inhibitory effect on lipoxygenase (the enzyme involved in lipoxygenase pathway of inflammation). \( \gamma \) GTP is involved in regulation of tissue glutathion, its activity and serum levels are known to be elevated in inflammation (Meister, 1983; Hangin et al., 1994). The significant inhibition in carrageenin induced rise of \( \gamma \) GTP in alcoholic extract treated group is suggestive of its anti-inflammatory effect.

The metabolites of arachidonic acid formed via Cyclooxygenase and lipoxygenase pathways represent two important classes of inflammatory mediators. Prostaglandins (products formed via Cyclooxygenase pathway) PG \( E_2 \) in particular is known to cause or enhance the cardinal signs of inflammation. Similarly, leukotriene (LTB4) is a mediator of leukocyte activation in inflammation and inhibitors of lipoxygenase may have potential therapeutic value. The description of the phologistic activities of leukotrienes (O'Flaherty, 1982) has directed the search for agents that inhibit both the Cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (Griswold et al., 1987). Arachidonic acid induced paw edema in rats is an in vivo model to distinguish between Cyclooxygenase and lipoxygenase inhibitors (Di Martino et al., 1987). Chlorpheniramine maleate (antihistaminic) and cyproheptadine (antihistaminic and antiserotonin agent) also had inhibitory effect on edema formation which appears to be due to intervention of the action of histamine released from mast cell, suggesting that mast cell mediator release may contribute at least in part to arachidonic acid induced paw edema.

It has been observed earlier that alcoholic extract of Lawsonia inhibited carrageenin-induced edema, which involves release of histamine and serotonin.
Pain is one of the cardinal signs of inflammation. Hence, it is necessary to evaluate whether any new drug modifies the inflammatory pain, which appears to be the most relevant test because this type of pain is present in most of the conditions for which the anti-inflammatory drugs are used. For testing analgesic activity of Lawsonia extracts, tail immersion and Eddy's hot plate methods were used. At 1.5 g/kg the aqueous and alcoholic extracts showed significant analgesic action. A rise in the central threshold of pain may be involved in this action.

Our earlier observations indicate that alcoholic extract of Lawsonia possess significant anti-PG activity. Hence, it is natural that the extract would inhibit the inflammatory response involving this mediator and free radicals.

Studies on Lawsonia extracts discussed so far indicate anti-oxidant and anti-inflammatory activity of the alcoholic extract. Since most of the non-steroidal anti-inflammatory drugs are ulcerogenic, effects of Lawsonia extracts on induction of gastric ulceration in animals were evaluated in its toxicity studies. No ulcers developed at the dose of 2 g/kg p.o.

The LD$_{50}$ of the extracts has been reported to be above 2 g/ kg i.p. (Anand et al., 1992; Ali et al., 1995; Iyer et al., 1998). The results of studies on sub acute toxicity following administration for a longer period also did not reveal any untoward effect of the extracts on behavioral response, body weight, normal reflexes and visceral appearance. The extract did neither produce any ulcerogenic effect nor any histopathological changes in the kidney and liver tissues.

In the course of investigation, general Pharmacodynamics of Lawsonia extracts were also studied. The extracts produced no significant change in the behaviour pattern but reduced the movements of animals. The plant extract is reported to potentiate the pentobarbitone induced sleeping time (Anand et al., 1991). Also its use as stuffed pillows for sleep induction has been reported (Abdul Hannan, 1997).
The alcoholic extracts of Lawsonia non-significantly prolonged blood-clotting time (P>0.05). The effect appears to be due to PG inhibitory effect of the extract.

Lawsonia extract is reported to possess potent antibacterial activity against *Nocardia asteroides*, *Candida albicans*, *C. krusei*, *Cryptococcus neoformans* and mould spp (Goncalves et al, 1972). Henna is strongly active against *Trichophyton rubrum*, *T. mentagrophytes*, *T. tonsurans*, *T. violaceum*, *T. verrucosum*, *T. schoenleinii*, *Epidermophyton floccosum*, *Micobacterium pyogenes*, *Streptococcus pyogenes*, *D. pneumoniae*, *B. subtiles*, *E. coli*, *Salmonella typhi*, *Vibrio comma*, *Shigella dysentriae*, *Microsporum ferrugineum*, *M. gypseum*, *M. canis* and *Sporotrichum [Sporothrix] schenckii* (Itani, 1973). Ethanolic extracts of henna leaf have fungitoxic property (Dube and Tripathi, 1987; Natrajan and Lalitha Kumari, 1987). While aqueous solution of lawsone has clear fungistatic property (Millet at al, 1989). Bark extract of *Lawsonia inermis* is active against ringworm fungi (Singh and Pandey, 1989) and also against several fungi implicated in dermatitis (Datta et al, 1989). Henna bark has fungistatic activity against *Microsporum gypseum* and *Trichophyton mentagrophytes* (Singh and Pandey, 1989). A herbal combination which included *Lawsonia alba* powder inhibits the growth of *Trichophyton* and *Microsporum* species isolated from clinical cases of sheep ringworm (Kader et al, 1995). Henna based herbal drug combinations have been tested for antileprosy activity (Asthana et al, 2001). Leaf extracts are effective against *Helminthosporium sativum* (Pandey et al, 2002) and several bacteria (Radhika et al, 2002; Ahmed et al, 2003).

The antibacterial activity of the extracts combined with its anti-inflammatory and analgesic properties would definitely increase its therapeutic potential because existence of all the three activities mentioned in a single test substance is really unique.

Kandil et al., (1996) and Hazara, (2002) have reported certain toxic effects attributed to the use of Lawsonia, of which, multiple reports on hemotoxicity appear in the literature and could be the major limitation with its use. In the toxicity evaluation of Lawsonia extracts at a higher level of therapeutic dose of 2 g/ kg po
no significant changes were evident in the haematological parameters. It is suggestive of the fact that percutaneous absorption (Kandil et al., 1996) of large quantity of cytotoxic naphthoquinone (Ali and Grover, 1998) or use of irrational dose could be responsible for the hemotoxicity, which was not observed even at the higher therapeutic dose. The extracts also did not show any significant alteration in the markers of cell damage; \( \gamma \) GTP, SGOT, SGPT \((P>0.05)\). Serum GSH levels were also not altered significantly.

Next efforts were made to identify the active principle(s) responsible for the anti-inflammatory activity of leaves.

In view of the pharmacological activity of alcoholic extract of henna leaves, it was considered worthwhile to undertake phytochemical standardization of this important medicinal plant.

Preliminary phytochemical screening by the method of Zafar et al, 2004 indicated the presence of alkaloids, amino acids, carbohydrates, flavonoids, phenolics, proteins, saponins and steroids in the leaves.

Flavonoids in henna leaves have been reported by Agarwal et al., 1959 and Mahmoud et al., 1980. Flavone glycosides in henna leaves have been reported by El-Negoumy, 1991. Flavonoids are a diverse group of phytoconstituents divided into several categories. Flavonols, flavones and flavonones constitute the three major flavonoid subtypes (Evans, 2003). As the Shinoda / Pew test for flavonoids was positive, the UV- characterization was thought necessary to identify the subtype of flavonoids. The alcoholic extract of leaves had principal maxima at 272 nm indicating the presence of flavonols and absence of flavones. This preliminary analysis offered the possibility of presence of rutin – a typical flavonol- in the alcoholic extract. Hence, qualitative HPLC of alcoholic extract was undertaken with respect to rutin by the modified method of Dubber and Kanfer, 2004. The retention time of standard Rutin \((90\% \text{ purity})\) was found to be 9.4 min \((\text{water: acetonitrile 65: 35, 0.5 ml/min, 350 nm})\) comparable with the retention time of 8.944 min
(Dubber and Kanfer, 2004). The absence of a peak at 9.4 min, indicated the absence of rutin in alcoholic extract of leaves. In view of the absence of rutin among flavonols, it was considered necessary to quantify total flavonols.

Standard rutin had an UV absorption maxima at 268 and 322 nm which was comparable to 265 nm maxima of rutin reported in the literature (Therapeutic goods administration, 1995). The total flavonol content in henna leaves was quantified by modified TGA method (Therapeutic goods administration, 1995) at 268 nm. A standard plot of rutin (absorbance versus concentration) was made at 268 nm. The experiment had a correlation coefficient $r = 0.9973$ ($p<0.002$, very significant) indicating the suitability of this method. The linear regression equation for rutin concentrations 0-10 $\mu$g/ml was found to be $y = 0.0006x - 0.003$.

The total flavonol content in alcoholic extract (dried leaves extractive value 33 %) was found to be 8.06 $\mu$g/ml which corresponds to 2.6598 % total flavonols in dried henna leaves.

The content of individual constituents were however, found to be varying from the previous work which could be attributed to geographical and collection time variations. The anti-inflammatory and analgesic activities of extracts were compared. The results show that percentage inhibition in paw edema were on higher side with alcoholic extract treatment, which indicates that pharmacological activities are due to flavonoids in the extract.

Thus it may be inferred that flavonoids present in alcoholic extract of *Lawsonia inermis* could be responsible for its anti-inflammatory activity, which would be synergised in the samples containing lawsone. The extract has potential to inhibit both the Cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (dual inhibition property).

From the present investigation we may conclude that *Lawsonia inermis* extracts possess potent anti-inflammatory activity due to dual inhibition of arachidonate
metabolism. The extracts also possess significant analgesic activity. Most of the non-steroidal anti-inflammatory drugs are ulcerogenic but the extracts do not have ulcerogenic property. The extract also possesses antimicrobial property and is reported to be effective against several organisms. Since Lawsonia possesses potent anti-inflammatory, analgesic and antimicrobial activities without any noticeable toxicity, there appears to be good prospect of this plant for commercial exploitation. However, further studies are required for the same.