V. DISCUSSION

The present study was carried out to assess the pharmacological activities and to evaluate the safety of cow urine in laboratory animals. The results obtained under various pharmacological activities viz., analgesic, anti-inflammatory, wound healing, hepatoprotective and antidiabetic activities and safety evaluation done under sub-acute and sub-chronic toxicity studies using pregnant and non-pregnant Deoni cows’ urine are discussed here.

5.1 Examination of cow urine for physical, chemical and microscopic characteristics

Immediately after collection, the urine was subjected to physical, chemical and microscopic examinations. The average pH ranged from 8.2 to 8.47 and the average specific gravity ranged from 1.020 to 1.030. The clear urine free from deposits, casts and microbes was used for the experiments.

5.2 Fractionation of cow urinary proteins by SDS-PAGE

In the present study, when pregnant cow urine was subjected to PAGE, 7 protein bands were found. Where as, in non-pregnant cow urine 3 protein bands were found with molecular weights of 67.57, 62.27 and 55.04 KDa. The increase in the number of bands in pregnant cow urine could be due the excretion of some of the protein hormones through urine.

A similar study was carried out in non-pregnant cow urine by Amita (2003), where she found bands of 69, 63, 50, 38, 34, 25 and 13 KDa.

5.3 Evaluation of cow urine for antimicrobial activity in vitro

A clear zone of inhibition was seen from 10 to 60 µl with an ascending order of width of the clearance zone. This shows that cow urine has antimicrobial activity against \textit{E. coli} or inhibits the growth of \textit{E. coli} at the administered concentrations. Amita (2003) evaluated the cow urine for antimicrobial activity (in vitro) against \textit{E. coli} serotype 026 by disc diffusion method and noticed that bacteria were sensitive to cow urine.
5.4 Evaluation of pharmacological activities of cow urine

5.4.1 Evaluation of anti-inflammatory activity

5.4.1.1 Acute inflammation study

5.4.1.1.1 Formalin-induced paw edema

Formalin-induced edema is considered as one of the most suitable test procedures to screen anti-inflammatory agents as it closely resembles human arthritis (Greenwald, 1991).

The nociceptive effect of formalin is biphasic, an early neurogenic component followed by a tissue-mediated response (Aceto and Cowan, 1991). The first phase is attributed to the release of histamine, 5-HT and kinins occurs within an hour of injection and is partly due to the trauma of injection, while the second phase is related mainly to prostaglandins measured around 3 hours (Vane and Booting, 1987). However, NSAIDs inhibit cyclo-oxygenase in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors (Fields, 1987).

In the present study, there was no significant decrease in paw thickness observed in cow urine treated animals when compared to control. However, aspirin treated group (reference control) showed a significant decrease in the paw thickness. Similarly, Jagadeesh (2007) did not find any anti-inflammatory activity of cross bred HF cow urine in rats.

5.4.1.2 Sub-acute inflammation study

5.4.1.2.1 Cotton pellet granuloma in rats

The cotton pellet granuloma method is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The moist weight of the cotton pellet correlates with the transudate and the dry weight of the pellet correlates with the amount of the granulomatous tissue (Olajide et al., 2000).
In the present study, there was no significant decrease in the weight of cotton pellets in cow urine treated animals when compared to control. However, aspirin treated group (reference control) showed a significant decrease in the cotton pellet weight.

5.4.2 Evaluation of cow urine for analgesic activity

5.4.2.1 Paw licking time

Cow urine treated animals did not show any significant decrease in paw licking time when compared to the control group. Group II animals treated with aspirin showed a significant decrease in paw licking time both in early and late phases when compared to the control group.

5.4.2.2 Hot plate reaction

The hot plate test has been found to be suitable for the evaluation of centrally acting analgesics. The validity of this test has been shown even in the presence of substantial impairment of motor performance (Plummer et al., 1996).

In the present study, cow urine treated animals did not show any significant difference in reaction time when compared to the control group. Group II animals treated with aspirin showed a significant increase in hot plate reaction time when compared to the control group. Jagadeesh (2007) also evaluated cross bred HF cow urine for analgesic activity by hot plate method and did not find significant results.

5.4.2.3 Acetic acid induced writhing

In acetic acid-induced abdominal writhing which is the visceral pain model, the processor releases arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis plays a role in the nociceptive mechanism (Franzotti et al., 2002).

Cow urine treated animals did not show any significant difference in the number of writhings when compared to the control group. Group II animals treated with aspirin showed a significant decrease in the number of writhings when compared to control group.
5.4.3 Evaluation of cow urine for anti-pyretic activity

Fever may be a result of infection or one of the sequelae of tissue damage, inflammation, graft rejection, or other disease states. Antipyretics are drugs, which reduce the elevated body temperature. Regulation of body temperature requires a delicate balance between production and loss of heat, and the hypothalamus regulates the set point at which body temperature is maintained. In fever this set point elevates and a drug like paracetamol does not influence body temperature when it is elevated by the factors such as exercise or increase in ambient temperature (Goodman and Gilman, 1996).

In the present study, cow urine treated animals did not show any significant decrease in temperature when compared to the control group. Group II animals treated with aspirin showed a significant decrease in temperature when compared to control group. A similar study was conducted by Jagadeesh (2007) using cross bred HF cow urine where in the author did not observe anti-pyretic activity.

5.4.4 Evaluation of cow urine for wound healing activity

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. It occurs in 3 phases; 1) inflammatory phase characterized by hemostasis and inflammation, 2) proliferative phase characterized by epithelization, angiogenesis and collagen deposition and 3) maturational phase, where the wound undergoes contraction resulting in a smaller amount of apparent scar tissue (Nayak and Pereira, 2006).

Healing does not normally require much help as it is a hormonal, physiological response to injury, but still wounds cause discomfort and prone to infection resulting in other complications. Therefore, agents expediting healing process are indicated to reduce the complications (Shukla et al., 1999).

In the present study, the selection of cow urine was based on its traditional medicinal use. There is ample experimental and clinical evidence available supporting the use of cow urine in the treatment of wounds. The study was designed to investigate the influence of cow urine on different parameters of wound healing namely wound contraction, epithelisation, tensile strength, protein content, hydroxyproline content and histological changes at the wound site.
5.4.4.1 Wound area reduction and healing time

Healing and contraction was fast in cow urine-treated wounds when compared to control and povidone iodine-treated wounds. Maheshwari et al. (2005) have reported decrease in the healing time with less infection by urine application to wounds in dogs when compared to antiseptic cream application. Jagadeesh (2007) also looked for the wound healing activity of cross bred HF cow urine and got satisfactory results.

The faster wound contraction rate in the treatment groups could be due to the stimulation of interleukin-8, an inflammatory α-chemokine, which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes. It increases the junctional intracellular communication in fibroblasts and induces a rapid maturation of granulation tissues (Moyer et al., 2002). This statement was supported by the histological findings, where the inflammatory cells, fibroblasts and keratinocytes were more in cow urine-treated group when compared to the control group.

5.4.4.2 Tensile strength

Povidone iodine and cow urine-treated wounds showed a significant increase in the tensile strength when compared to control group animals. Increase in tensile strength of treated wounds may be due to the increase in collagen concentration and stabilization of fibers (Udupa et al., 1995).

Freifelder (1987) reported that tensile strength depends on the Vander walls interaction among the hydrogen ion bonds of the triple helix collagen leading to twisting of the collagen fibers, more the twisting, greater the tensile strength, hence better the healing.

5.4.4.3 Hydroxyproline content

According to Kumar et al. (2006), collagen is the major component which strengthens and supports extra cellular tissue is composed of the amino acid, hydroxyproline, which is used as a
biochemical marker for tissue collagen. In the present study, cow urine-treated wounds demonstrated a significant increase in the hydroxyproline content of the granulation tissue indicating increased collagen turnover. These findings were well correlated with the histological findings of abundant proliferation of fibroblasts and collagen bundles in the healing tissue of cow urine-treated animals when compared to control groups. Amita (2003) created surgical wounds in rats and treated them systemically along with topical application of cow urine. She found early development of granulation tissue and regeneration of epidermis with fibroplasias in cow urine-treated animals when compared to the control group.

5.4.4 Total protein content

Cow urine and povidone iodine-treated animals showed a significant increase in the total protein content when compared to the control animals. According to Spanheimer and Peterkofsky (1985), protein synthesis indirectly depicts collagen synthesis and increased wound tensile strength. Similarly, Haydock and Hill (1986) have reported significant lower healing rates in decreased protein synthesis. Thus, increased protein content in the treatment groups could be one of the reasons for increased tensile strength and collagen content found.

5.4.5 Histological examination

On day 4, povidone iodine applied wounds were showing exudates, inflammatory cell infiltration and proliferation of surface epithelium just below the exudate. Cow urine-treated wounds revealed comparatively less exudate formation, mild hemorrhage and more inflammatory cell infiltration. Neovascularization and epithelial cell proliferation was marked in cow urine-treated wounds when compared to control and reference groups.

On day 8, povidone iodine-treated wounds revealed epithelial cell proliferation and angiogenesis with mild keratinization. Cow urine-treated wounds showed complete epithelisation with keratinization, proliferation of fibroblasts with bundles of collagen and more blood vessel formation in the area when compared to the control and standard groups.
Angiogenesis is an important component in wound healing and increased vessel growth can facilitate both the extent and direction of fibroplasias (Shukla et al., 1999). Angiogenesis was predominant in cow urine-treated groups when compared to control and reference animals, which may be contributing to their wound healing property. Angiogenesis plays an important role in wound healing and newly formed blood vessels comprise 60 per cent of the repair tissue, which helps hypoxic wounds to attain the normoxic conditions. The increased vessel growth can facilitate both the extent and direction of fibroplasia (Ehrlich et al., 1972). Improved angiogenesis, therefore, might be responsible for better wound healing activity of cow urine treated wounds.

According to Getie et al. (2002), any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis. Thus, cow urine may act by inhibiting lipid peroxidation to assist in wound healing.

Several local growth factors like basic fibroblast growth factor (bFGF) involve in the process of wound healing and target endothelial cells, fibroblasts, myoblasts etc. (Schweigerer, 1988). It is presumed that the cow urine may possess growth factor like activity or may enhance the expression of growth factors as in the case of wound healing process.

### 5.4.5 Evaluation of cow urine for antidiabetic activity

In the present study, the cow urine (group IV, V, VI and VII) was evaluated for the antidiabetic activity, which was compared with the normal animals (group I), diabetic animals (group II) and glibenclamide, a standard oral hypoglycemic drug treated animals (group III). Glibenclamide, a second generation sulphonyl urea derivative, essentially stimulates the insulin secretion through ATP dependent channel (K\(_{\text{ATP}}\) channel). Hence glibenclamide requires functionally active β-cells to stimulate insulin secretion (Melander et al., 1987). Single dose of glibenclamide provokes a brisk release of insulin from pancreas. It acts on cell membrane leading to enhanced calcium flux across it, hence degranulation. After chronic administration the insulinaemic action of glibenclamide declines, but improvement in glucose
tolerance is maintained. Thus, it is an oral antidiabetic preparation with an efficient hypoglycemic action (Prasad et al., 2009).

### 5.4.5.1 Clinical signs

Streptozotocin effectively induced diabetes in rats characterized by polydipsia, polyuria, weight loss, decreased physical activities and hyperglycemia, which is in agreement with earlier findings (Shenoy and Ramesh, 2002).

### 5.4.5.2 Body weight

Diabetic control and cow urine treated animals showed a gradual and significant decline in the body weight when compared to normal animals throughout the experiment. The decrease in body weight is attributed to lack of insulin resulting in wasting due to enhanced catabolism of proteins and lipids with subsequent weight loss. However, glibenclamide treated animals showed a significant increase in the body weight when compared to normal control diabetic control animals. The probable reason for the increase in body weight in this group could be due to either enhanced secretion of insulin (Persuad et al., 1999) or by rejuvenation of β-cells of pancreas (Latha and Pari, 2003).

### 5.4.5.3 Blood glucose

In the present investigation, streptozotocin was administered at the dose of 45 mg/kg body weight in Wistar albino rats which caused a significant diabetogenic response when compared to the normal control group. The diabetic animals attained a blood glucose level of more than 300 mg/dl, 96 hrs post STZ injection. This is because streptozotocin action in β cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, the hyperglycemia is observed with a concomitant drop in blood insulin. About six hours later, hypoglycemia occurs with high levels of blood insulin. Finally, hyperglycemia develops and blood insulin levels decrease (West et al., 1996).
When compared with the normal control animals, diabetic animals revealed a significant increase in blood glucose levels. Glibenclamide-treated animals (group III) showed a significant decrease in the glucose levels when compared to the diabetic group. Cow urine treated animals did not show significant decrease in blood glucose levels when compared to diabetic control group. These changes in blood glucose and insulin concentrations reflect abnormalities in B cell function. STZ impairs glucose oxidation (Bedoya et al., 1996) and decreases insulin biosynthesis and secretion (Nukatsuka et al., 1990).

5.4.5.4 Serum biochemistry

5.4.5.4.1 Serum cholesterol and Triglycerides

There was a significant increase in the levels of serum cholesterol and triglyceride levels in diabetic animals when compared to the normal animals. Van Horn (1996) stated that plasma cholesterol and triglyceride levels are strongly related to the degree of diabetes. The increased total cholesterol and triglyceride levels observed in diabetic rats may be the result of impaired liver function caused by the damage done by streptozotocin, which acts either directly or indirectly by enhancing the plasma glucose level.

According to Bopanna et al. (1997), in streptozotocin-induced diabetes there is excess of fatty acids in the serum, which are converted into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed at the same time in liver may be discharged into blood in the form of lipoproteins. The abnormal high concentration of serum lipids in the diabetic subject is due mainly to increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the hormone sensitive lipase.

In glibenclamide-treated animals, there was a significant decrease in the levels of serum TGs and cholesterol when compared to the diabetic control group. However, cow urine-treated animals did not show significant changes when compared to diabetic control group. On the contrary, Vadivelan (2007) found decrease in the levels of cholesterol and TGs in cow urine-treated Wistar albino rats.

5.4.5.4.2 BUN and creatinine
There was a significant increase in the levels of BUN and serum creatinine in diabetic control group when compared to normal animals. Both BUN and creatinine are the better indicators of glomerular filtration rate (Finco, 1997). This shows renal dysfunction, which might be due to the direct toxic effects of streptozotocin on kidney combined with the glucosuric effect that causes renal tubular destruction. These results were well supported by histological findings of tubular and glomerular degeneration in the kidney sections.

Glibenclamide-treated animals revealed a significant decrease in the levels of BUN and creatinine when compared to diabetic control group, where as cow urine treated animals showed no significant alterations when compared to diabetic control animals. These findings were well correlated with histological findings.

5.4.5.5 Histopathology

**Pancreas:** The pancreas of normal control (group I) was normal in appearance of acini and islets of Langerhans. The Pancreas of diabetic control (group II) and cow urine-treated animals (group IV, V, VI and VII) showed destruction of β-cells, degeneration and necrosis of β-cells. The decrease in cellularity within islets of Langerhan’s observed in the present study reflects the cytotoxicity of streptozotocin where cells undergo destruction by necrosis (Szkudelski, 2001). The pancreas of glibenclamide-treated group (group III) showed mild congestion and regeneration of β-cells.

**Liver:** In group I animals, the hepatic architecture was apparently normal. Where as in diabetic control group, there was vacuolar degeneration of hepatocytes. Glibenclamide-treated animals showed mild congestion with few hepatocytes showing vacuolar degeneration. Cow urine-treated animals showed dilated sinusoidal spaces; hepatocytes were swollen and degenerating. Few hepatocytes revealed karyomegaly with prominent nucleoli.

**Kidney:** The histological preparations of kidney of normal animals showed normal appearance of kidney tubules with abundant glomeruli. Diabetic control animals showed tubular and glomerular degeneration with homogenous eosinophilic material in the tubular lumen. Glibenclamide-treated animals
showed apparently normal tubular epithelium with less eosinophilic material in the tubular lumen. In cow urine-treated animals there was tubular epithelium degeneration, vacuolar cytoplasm and homogenous eosinophilic material in the tubular lumen similar to diabetic control group.

The present study revealed that cow urine has no antidiabetic property, which is in contrast to the findings of Vadivelan (2007).
5.4.6 Evaluation of cow urine for antihepatotoxicity/hepatoprotectant activity

Hepatotoxicity is frequently encountered either as primary or secondary to other diseases resulting in significant alterations in metabolism. Biochemically it manifests with elevations in marker enzymes and alterations in antioxidant defense mechanism (Shahjahan et al., 2005). CCl₄ is biotransformed by cytochrome P450 system to produce trichloromethyl free radicals which again react with oxygen to form trichloromethylperoxy radicals, which attack lipids on the membrane of endoplasmic reticulum to elicit lipid peroxidation, thus resulting in cell necrosis and consequently cell death (Shyamal et al., 2006).

Liver injury induced by CCl₄ is the distinct symptom of xenobiotic-induced hepatotoxicity and commonly used model for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs. CCl₄ is known to cause hepatic damage with a marked elevation in serum levels of LDH and aminotransferase enzymes (AST and ALT) because these enzymes are cytoplasmic in location and are released into the blood after cellular damage (Recknagel et al., 1991).

In the present study, an attempt was made to see the hepatoprotectant/antihepatotoxic effect of cow urine in Wistar albino rats. Acute hepatotoxicity was induced by intraperitoneal administration of CCl₄. Silymarin was used as the reference with which the cow urine treatments were compared to check for the anti-hepatotoxic activity. Direct evidence of this anti-hepatotoxicity was noted in the occurrence of alterations in various hepatic parameters (AST, ALT, LDH, ALP etc.).

5.4.6.1 Serum biochemistry

There was a significant increase in the levels of AST, ALT, ALP, LDH, and bilirubin in CCl₄ treated (group II) animals when compared to the normal animals which is due to the hepatocytes damage by CCl₄. Damage to the structural integrity of the liver by CCl₄ is reflected by an increase in the activities of AST, ALT, ALP, and LDH, which are released into the circulation after cellular damage (Wolf, 1999).

There was a significant decrease in the levels of TSP in CCl₄ treated (group II) animals when compared to the normal animals. A decrease in the level of TSP may be associated with a decrease in the
number of hepatocytes, which are responsible for protein synthesis (Shahjahan et al., 2005). However, in silymarin and cow urine treated animals there was a significant increase in TSP levels when compared to CCl₄ treated animals (group II). This could be due to the recovery of hepatocytes, which was confirmed through histopathology.

5.4.6.2 Lipid peroxidation

Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA) and is used as an indicator of tissue damage involving a series of chain reactions (Vaca et al., 1988). It reacts with thiobarbituric acid, producing red-coloured products. Lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity, osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity. It has been hypothesized that one of the principal causes of CCl₄-induced hepatotoxicity is lipid peroxidation of hepatocyte membranes by free radical derivatives of CCl₄ (Recknagel et al., 1991). The observation of elevated levels of hepatic MDA in group II rats (administered CCl₄ alone) in the present study is consistent with this hypothesis. Thus, the maintenance of near normal levels of hepatic MDA in silymarin and cow urine-treated animals suggests the protective nature of silymarin and cow urine from lipid peroxidation.

5.4.6.3 Histopathology

The histopathological observations supported the results obtained from biochemical observations. In the group II animals, the normal hepatic architecture was lost with vacuolar degeneration of the hepatocytes. Silymarin and cow urine treated animals showed recovery from the CCL₄-induced liver damage as evident from normal hepatic architecture.

Hepatoprotective drug reduces the injurious effects or preserves the normal hepatic physiological mechanisms, which have been disturbed by a hepatotoxin (Yadav and Dixit, 2003). In the present study, lowered activities of marker enzymes and maintenance of TSP after treatment along with the histopathological findings confirms the hepatoprotective nature of both pregnant and non-pregnant cow urine.
Antioxidative action plays an important role in protection against \( \text{CCl}_4 \)-induced liver injury (Ko and Lim, 2006). According to Chauhan and Singhal (2006), cow urine has antioxidant properties and thus it neutralizes the oxidative stress produced in body through action on free radicals. Thus, the hepatoprotective action observed in the present study can be attributed to its antioxidant property.

### 5.5 Safety evaluation

In this study, cow urine was evaluated for the safer use by conducting sub-acute and sub-chronic oral toxicity studies in rats. This study provides information on the health effects and hazards likely to arise from repeated daily consumption of cow urine over a period of time.

In both the studies there were no significant alterations found in the body weight, hematological, biochemical parameters, gross and histopathological observations when compared to the control group. These findings are in contrast to the observations of Amita (2003), who found a significant increase in the body weight, Hb, PCV TLC and TSP in urine treated animals when compared to the control animals. However, Jagadeesh (2007) also noticed normal range of values in his study in cow urine-treated rats.

Thus, pregnant and non-pregnant cow urine was declared to be safe for daily consumption at the doses studied in rats.

The following conclusions were drawn from the present study.

1. The average pH of pregnant Deoni cow urine is 8.2 to 8.45 and that of non-pregnant urine is 8.20-8.47. The average specific gravity is 1.020 to 1.030 in both pregnant and non-pregnant cows’ urine.

2. In pregnant Deoni cow urine 7 proteins are excreted with molecular weights of 67.11, 61.40, 58.86, 37.01, 25.45, 17.68 and 11.06 KDa. Where as, in non-pregnant cow urine 3 proteins were found with molecular weights of 67.57, 62.27 and 55.04 KDa. In pregnant cow urine, nitrogen content was \(22.2 \pm 1.2\) mg/ml, P was \(0.80 \pm 0.4\) mg/ml, K was \(1.2 \pm 0.02\)mg/ml and Ca was \(0.002\)
mEq/L. In non-pregnant cow urine the mean levels were 26.5 ± 0.9, 0.95 ± 0.1, 1.0 ± 0.01, 0.002 mEq/L, respectively.

3. Pregnant and non-pregnant cow urine does not have analgesic, anti-pyretic, anti-inflammatory activities at the administered doses.

4. Both pregnant and non-pregnant cow urine showed wound healing property following topical application.

5. Pregnant and non-pregnant cow urine does not have antidiabetic property but possess hepatoprotectant action.

6. Cow urine was found to be safe in both 28 day and 90 day oral toxicity studies.