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Evaluation of Cow urine Piper betel extract for antiulcer activity in rats

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ABSTRACT

The antiulcer effect of cow urine extract of Piper betel (family Piperaceae) was evaluated in pyloric ligation induced ulceration model in Wistar rats. The antiulcer activity was evaluated by measuring ulcer index and percentage of ulcer healing. The extract (500mg/kg b.w) showed significant antiulcer activity as evidenced from the data obtained. Histopathological findings also confirm the antiulcer activity of Cow urine Piper betel extract.

Keywords: Piper betel, antiulcer activity, pylorus ligation.

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INTRODUCTION

Peptic ulcer disease is a group of disorders characterized by the presence of ulcers in any portion of gastrointestinal tract (GIT) exposed to acid in sufficient concentration and duration. Although these ulcerations are commonly affects stomach (gastric ulcer), or small intestine (duodenal ulcer)[1].

Peptic ulcers are relapsing lesions that are most often diagnosed in middle aged to elder adults, but they may first become evident in young adults life. They often appear without precipitating conditions and may then after a period of weeks to months of active disease which may heal with or without therapy. Even after healing, the tendency to develop peptic ulcer remains, due to recurrent infections with H.*pylori.* Although it is difficult to estimate the prevalence of active disease, autopsy studies and population survey indicates a prevalence of 6% to 14% for men and 2%-6% for women. The male to female ratio of duodenal ulcers is about 3:1, and for gastric ulcers about 1.5 to 2.1[2].

Ulcers are deep lesions penetrating through the entire thickness of the gastrointestinal tract (g.i.t) mucosa and muscularis mucosa. The etiology of peptic ulcer was fiercely debated. It is believed that peptic ulcers develop due to an imbalance between aggressive factors (Helicobacter pylori, NSAIDs, gastric acid) and protective factors (mucin, bicarbonate, and prostaglandins), leading to an interruption in the mucosal integrity.

In recent years, focus on plant research has increased worldwide and several studies had showed immense potential of medicinal plants[3]. Herbal medicines derived from plant extract are increasingly being recognized in treating various clinical diseases, with little knowledge of their modes of action [4].

One of such is leaves of *Piper betel* (family Piperaceae) commonly known as Betel vine in English. The Betel plant is an evergreen perennial creeper, with glossy heart-shaped leaves. The Betel plant was originated in South and South East Asia [5]. It needs a compatible tree or a long pole for support. It requires well drained fertile soil. Leaves are yellowish green to dark green in color with glossy upper surface. They have characteristic and pleasant odour. They are aromatic with varied taste, ranging from sweet to pungent due to the presence of essential oil. Traditionally the plant is reported for its antiulcer activity. In the present study an effort has been made to establish the scientific validity to the antiulcer property of the leaf extract with cow urine of Piper betel in pylorus ligation induced ulcer model in Wistar rats.

MATERIALS AND METHOD

The betel vine was collected from nearby plantations and authenticated by Dr. Muralidhar, Prof and HOD, Department of biotechnology, Dayananda Sagar Institutions. Later leaves were shade dried and powdered. Ranitidine (50mg/kg) was used as standard drug.

Preparation of extract:

200gm of powdered leaf was extracted with fresh cow urine by maceration process for 4 days then filtered and subjected for evaporation to remove moisture content in rotary vaccum evaporator and the crude extract was collected and kept in dessicator for further pharmacological studies.

Qualitative phytochemical investigation

The cow urine betel vine extract was then subjected to phtochemical investigation for the presence of various secondary metabolites such as tannins, amino acids, alkaloids, flavonoids, saponins and proteins using standard methods [6, 7, 8].

Experimental animals

Wistar rats weighing between 200-250gms were procured from the animal house of Dayananda Sagar College of Pharmacy, Bangalore. They were maintained at standard housing condition at the room temperature of 22^0C ($\pm 3^0$C) and relative humidity of 44-56% with light and dark cycles of 10-14hrs respectively [9,10]. The rats were provided with standard rodent pellet diet and the water was given *ad libitium* 11. The study protocol was duly approved by Institutional Animal Ethics Committee (CPCSEA, Reg no: DSCP/M.Pharm/ IAEC/106/Pharmacology).

Acute toxicity studies.

Acute toxicity was carried out as per the CPCSEA and OECD guidelines 425.

Group of 6 healthy mice weighing 20-25gms were selected and kept for 3-4hrs fasting with free access to drinking water. Doses were calculated according to the body weight.The test extract were dissolved in cow urine and administered orally at the starting dose of2000mg/kg and were kept for observation for any mortality and behavioral changes for 24hrs, and further dose was increased upto 5000mg/kg b.w. It was observed that no mortality was observed even at the higher dose of extract and no change in their behavior. As per the OECD guidelines $1/10^{th}$ and $1/5^{th}$ of the maximum tolerated dose was selected. Therefore effective therapeutic doses were 250 and 500mg/kg b.w.

Pylorus ligation ulcer induce model [11]:

The animals were separated in 5 groups each containing 6 rats. The groups were as follows:

Group I: Negative control (saline treated).

Group II: Positive control (cow urine treated).

Group III: standard (Ranitidine 50mg/kg).

Group IV: Test extract 1 (250mg/kg).

Group V: Test extract 2 (500mg/kg).

Negative control received normal saline 1ml/kg orally. Positive control received 1ml/kg cow urine orally; standard group received Ranitidine 50mg/kg orally. The remaining two groups received test extract at the doses of 250 and 500mg/kg orally for 14 days on the 15th day under light ether anesthesia, the abdomen was opened by midline incision below the xiphoid process. The pylorus portion of the stomach was slightly lifted out and ligated, avoiding damage to the blood supply. The stomach was placed back carefully and the abdomen wall was closed with sutures. The animals were deprived of food and water during the postoperative period and the animals were sacrificed six hours after pyloric ligation by over dose of ether anesthesia. The stomach was isolated and the contents of the stomach were collected and centrifuged. The gastric juice collected was centrifuged at 1000rpm for 10 minutes and the volume was measured and further used for the estimation of total and free acidity.

Parameters measured:

Ulcer index and % protection was measured. The volume and pH of the gastric juice was measured and later was used for estimation of free and total acidity.

The ulcers scores were given based on their intensity as follows.

0- no ulcer

1- Superficial ulcer

2- Deep ulcers

3- Perforations.

Evaluation of ulcer index:

An ulcer index was calculated as:

$UI=UN+US+UP\times10^{-1}$

- UN= average number of ulcers per animal
- UI= ulcer index
- US=average of severity scores
- UP=percentage of animals with ulcers

% Protection= UI (CONTROL) - UI (TREATED)/ UI (CONTROL)

Determination of total and free acidity[12]:

To 1ml of gastric juice 3 drops of topfers reagent was added in conical flask and titrated against 0.01N NaoH until the development of orange color, which indicates the free acidity. Titration was continued till the solution turns to pink colour which indicates total acidity. The result was expressed as µEq/ml for acid concentration and output respectively.

Total acidity= volume of NaOH × Normality of NaOH×100 /0.1

Statistical analysis:

The statistical significance was assessed using one way of variance (ANOVA) followed by Dunnet comparison test. The values are expressed as Mean ±SEM.

Result and Discussion:

Phytochemical analysis:

The leaf extract contains brown residue. The preliminary phytochemical studies of Cow urine Betel vine extract of the leaves confirms the presence of flavonoids, Alkaloids, Phenolic compounds, phytosterols and saponins. Further the extract was taken for pharmacological studies. The results of phytochemical studies were as shown in **Table 1.**

Table 1: Phyto-chemical investigation of Cow urine Betel vine extract.

Sl.no	Type of test	Cow rine extract
1	**Test for CARBOHYDRATES**	
	▪ Molisch's test	+
	▪ Fehling's test	+
	▪ Barfoed's test	+
2	**Test for STARCH**	-
3	**Test for PROTEINS and FREE AMINO ACIDS**	
	▪ Million's test	+
	▪ Biuret's test	+
	▪ Ninhydrin's test	+
4	**Test for PHYTOSTEROLS**	+
5	**Test for FIXED OILS and FATS**	
	▪ Spot test	+
	▪ Saponification	+
6	**Test for ALKALOIDS**	
	▪ Mayer's test	+
	▪ Dragendroff's test	+
	▪ Wagner's test	+
	▪ Hager's test	+
7	**Test for GLYCOSIDES**	
	▪ Legal's test	-
	▪ Balget's test	-
	▪ Borntrager's test	-
	▪ Modified borntrager's test	-
8	**Test for FLAVONOIDS**	

	▪ Shinoida's test	+
	▪ Ferric chloride test	+
	▪ Fluorescence test	+
	▪ Reaction with Alkali and Acids	+
	▪ Zinc, HCL reduction test	+
	▪ Lead acetate solution test	+
9	**Test for SAPONINS**	+
10	**Test for COUMARINS**	-
11	**Test for PROTEIN and PHENOLIC COMPOUNDS**	
	▪ Ferric chloride test	+
	▪ Lead acetate test	+
	▪ Gelatin test	+

➢ (+) indicates positive test result, (-) indicates negative test result

Pyloric ligation induced ulcer model was used to study the effect of extract on gastric acid and mucus secretion. The ligation causes accumulation of gastric acid in the stomach. The increase in the gastric acid causes ulcers in the stomach. The agents that decrease the gastric acid secretion and increase in the mucus secretion are effective in protecting the ulcers. The cow urine betel vine extract and Ranitidine significantly decrease the total acidity and free acidity..

Ulcer index parameters were used for the evaluation of antiulcer activity. Since the ulcer formation is directly related to factors such as reduction in gastric volume, decrease in free and total acidity. The extract at 500mg/kg showed the significant reduction in the ulcer index. The extract showed the protection of ulcer index of 53.3% and 60.6% at the dose of 250 and 500mg/kg in comparison to negative control whereas Ranitidine a reference standard showed a protection of 68.6%. The results are tabulated in Table: 2 Figure: 1. The results for gastric volume, free acidity, total acidity and pH of the gastric juice are tabulated in Table: 3 and Figure: 2, 3 and 4.

Table: 2: The effect of Cow urine Betel vine extract on ulcer index in pyloric ligation induced Peptic ulcer model

Sl no	**Treatment**	**Dose (mg/kg *b.w*)**	**Ulcer index**	**Protection (%)**
I	Negative control (Saline treated)	-	15±2.63	-
II	Positive Control (Cow urine treated)	-	8.8±1.8*	41.3%
III	Standard (Ranitidine)	50	4.7±0.4**	68.6%
IV	Test extract 1	250	7±0.6*	53.3%
V	Test extract 2	500	5.9±0.4**	60.6%

All the values are expressed in mean ± SEM. Six animals in a group (n=6) *: value significantly different at $p<0.01$ v/s. Pyloric ligation negative control, **: value significantly different at $p<0.001$ v/s. Pyloric ligation negative control.

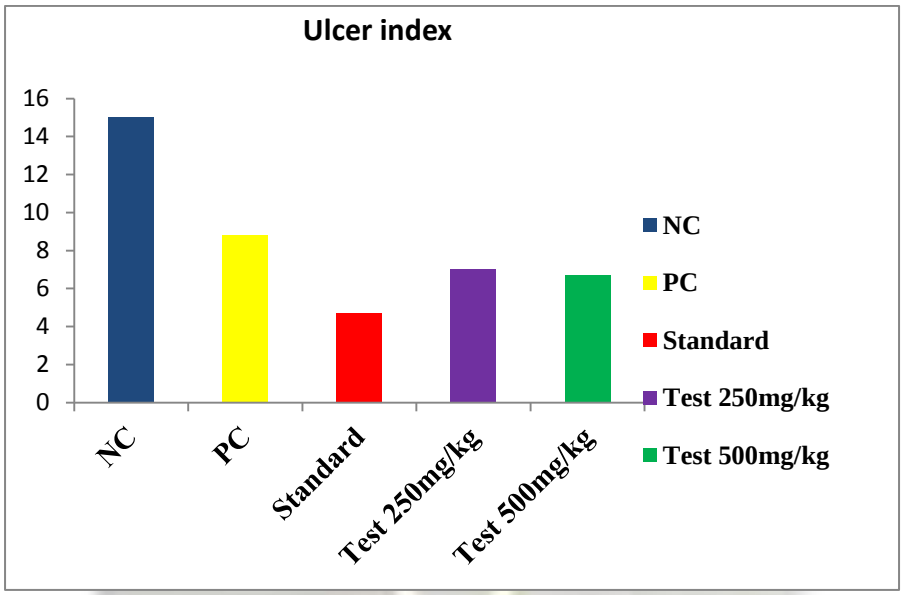

Figure 1: Effect of cow urine betel vine extract on ulcer index in Pyloric ligation induced ulcer model.

Table 3: Effect of Cow urine Betel vine extract on Gastric volume, pH and Total acidity.

Treatment	Volume of gastric content (ml/100g)	pH of gastric content	Free acidity (µeq/100g)	Total acidity (µq/100g)
Negative control(saline treated)	3.5±0.09	1.6±0.09	48.2±0.12	71.8±0.14
Positive control (Cow urine treated)	2.88±0.17**	2.18±0.10*	41.2±0.10**	60.6±0.18**
Standard (Ranitidine)	2.12±0.13***	5.04±0.14***	29.6±0.14***	44.2±0.77***
Test extract 1 (250mg/kg)	2.14±0.08***	4.30±0.19***	34.4±0.12***	50.0±0.31***
Test extract 2 (500mg/kg)	1.98±0.11***	4.80±0.11***	30.8±0.16***	45.6±0.45***

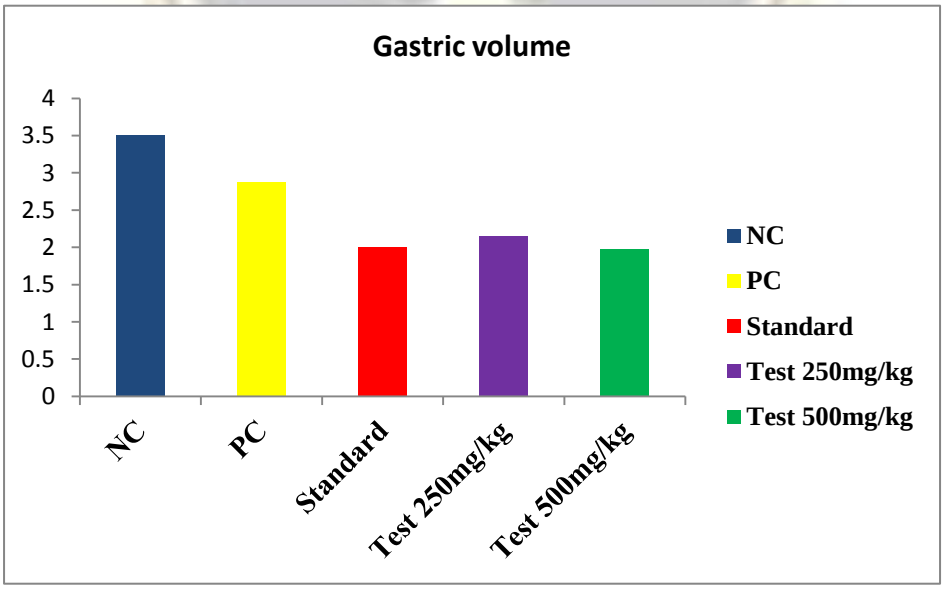

Figure 2: Effect of Cow urine Betel vine extract on Gastric volume.

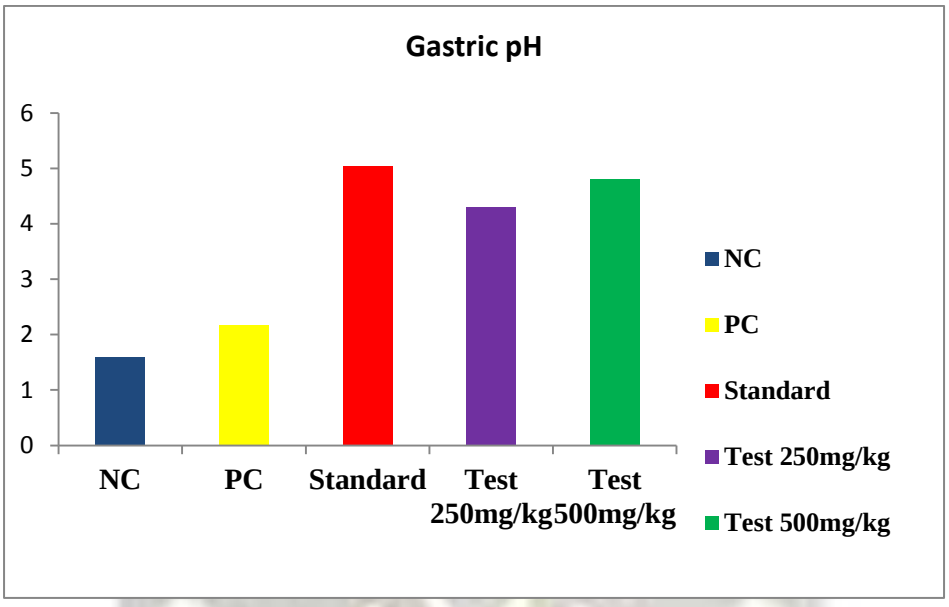

Figure 3: Effect of Cow urine Betel vine extract on Gastric pH.

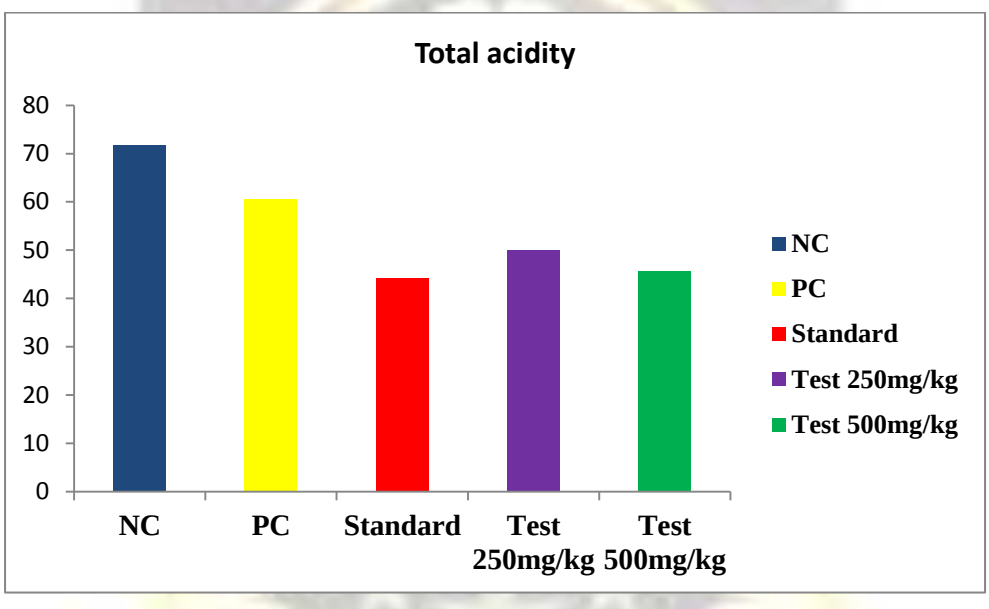

Figure 4: Effect of Cow urine Betel vine extract on Total acidity.

Although a number of antiulcer drugs such as H_2 receptor antagonists, proton pump inhibitors and cyto protectives are available for the treatment of gastric ulceration but all these drugs have side effects and limitations. Complementary and alternative medicines were in use to treat peptic ulcer from hundreds of years. These medicines are considered safer because of the natural ingredients with no side effects [13].

The flavonoids present in the leaf extract are well known for its antiulcer activity and cow urine acts as an enhancer of the ulcer healing activity. The combination of cow urine with extract of the betel vine synerges the ulcer healing activity and is almost equal to the Standard ranitidine. The

ulcer healing effect may be due to the anti-secretary and cyto-protective activity of the constituents present in extract, as evident by a decrease in the gastric acidity and decrease in the ulcer index [14].

Histopathology:

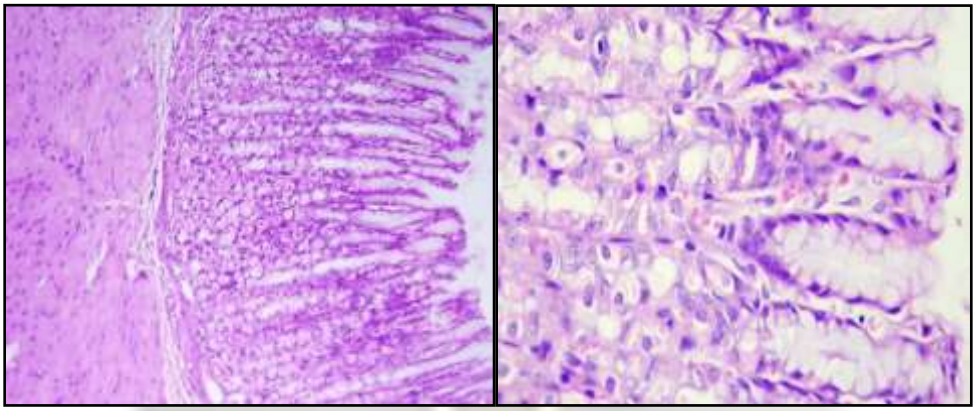

Figure 5: Histo-pathological study of Negative control (Saline treated) group in Pylorus ligation induced ulcer model.

After 4 hours of pyloric ligation the reports of histo-pathological studies of the stomach shows diffuse mucosal ulceration. The epithelial cell regeneration with secretion and few congested vascular spaces, severe sub-mucosal edema. The muscularispropria appears intact and it is shown in the Figure 5.

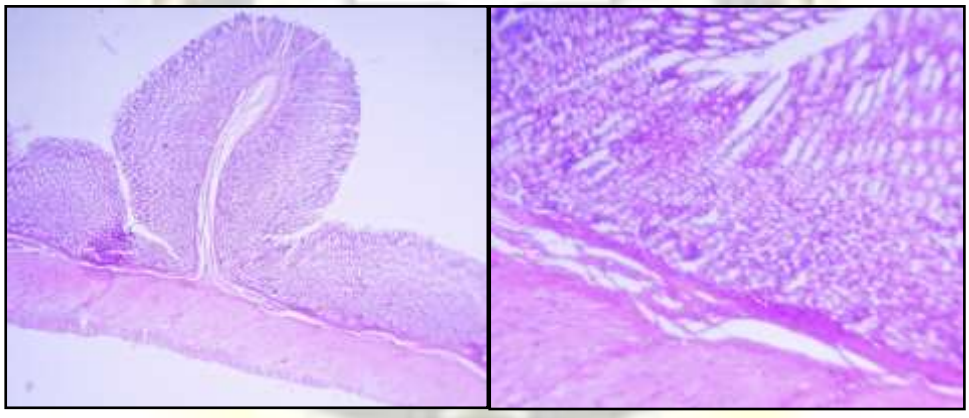

Figure 6: Histo-pathological study of positive control(Cow urine treated) group in Pylorus ligation induced ulcer model.

After 4 hours of pyloric ligation the reports of the histopathological studies of the stomach shows intact gastric mucosa. The mucosal layer, sub mucosal layer, mucosal and serosal layers appear within normal limits and are showed in the Figure 6.

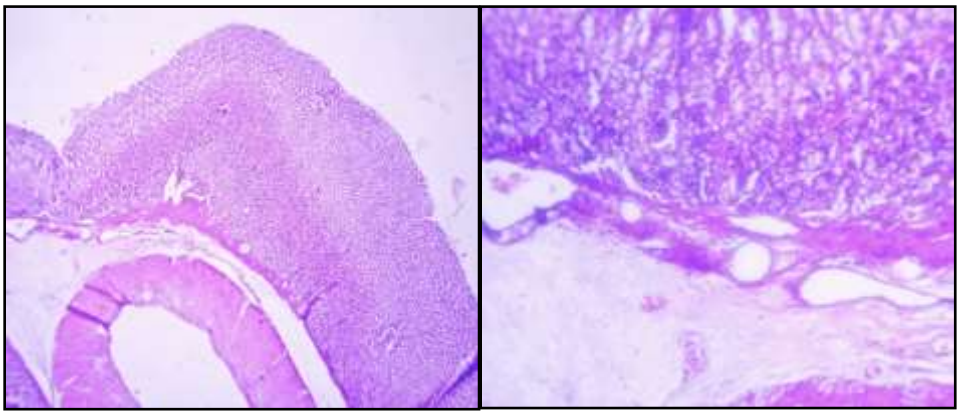

Figure: 7 Histo-pathological study of Standard (Ranitidine treated) group in Pylorus ligation induced ulcer model.

After 4 hours of pyloric ligation the reports of the histopathological studies of the stomach shows intact gastric mucosa. The mucosal layer shows both degenerating and regenerating epithelium. The submucosal layer shows mild edema with mild mononuclear inflammatory infiltration in mucosa and few congested vascular spaces. The mucosal and serosal layers appear within normal limits and are showed in the Figure 7.

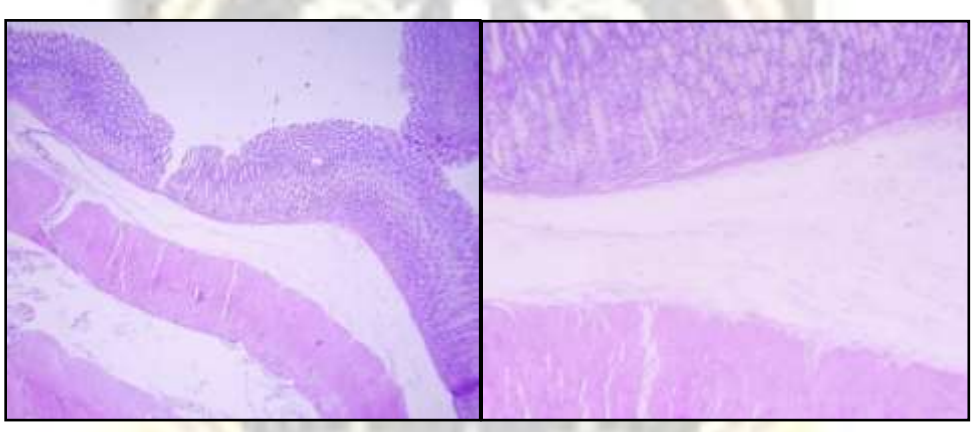

Figure 8: Histo-pathological study of Cow urine Betel vine extract (250mg/kg) in Pylorus ligation induced ulcer model.

After 4 hours of pyloric ligation the reports of the histopathological studies of the stomach shows intact gastric mucosa. Most of the mucosal layer shows regenerating epithelium. The submucosal layer shows mild edema with scattered mononuclear inflammatory infiltration. The mucosal and serosal layers appear within normal limits and are shown in the Figure 8.

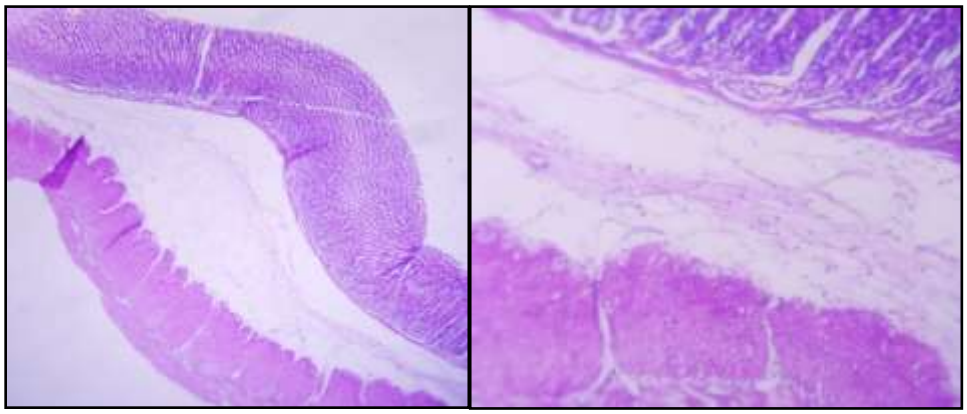

Figure 9: Histo-pathological study of Cow urine Betel vine extract (500mg/kg) in Pylorus ligation induced ulcer model.

After 4 hours of pyloric ligation the reports of the histopathological studies of the stomach shows intact gastric mucosa. Most of the mucosal layer shows both degenerating and regenerating epithelium. The sub mucosal layer shows moderate edema with mixed inflammatory infiltration. The muscular and serosal layer appears within normal limits and is shown in the Figure 9.

CONCLUSION

From the review of literature and results of the treatment suggest that the Cow urine processes Anti-ulcer activity and the combination of Cow urine with betel vine showed a synergistic effect in the model selected for ulcer healing.

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