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Evaluation of gastro-protective effect of the hydro-alcoholic extract of *Juglans regia*. L leaves in experimental animals

Kumaraswamy Dabburu[1], Suresh Babu Kondaveeti[2*], Sarath Babu.K[3.]
[1] Lecturer,Department of Pharmacology, SRM Medical College, Kattankalathur-602239, Tamilnadu.
[2] Lecturer, Department of Biochemistry, MAPIMS&R-603319, Tamil Nadu.
[3] Lecturer,Department of Pharmacology, SMIMS&R, Marthandam-603453, Tamilnadu

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ABSTRACT

The aim of our study was to evaluate the gastro protective effect of aqueous extract of *Juglans regia*.L leaves in albino rats. Albino rats of wistar variety weighing 140-165gms were used in the experiment. The sexes were evenly divided into different treatment groups. The aqueous leaf extract of *Juglans regia*.L was investigated for its anti- ulcer activity against pylorus ligation, aspirin induced and ethanol induced gastric ulcer in rats at 500mg/kg body weight p.o. Histopathological assessment of rat stomach was carried out. A significant reduction ($p<0.01$) in ulcer index was seen in leaf extracts of *Juglans regia*.L treated rats of pylorus ligation, aspirin induced and ethanol induced gastric ulcer models. The gastro protective effect was further confirmed by histopathological examination of rat stomach. Thus the present study concludes the *Juglans regia*.L leaf extract having potential gastro protective effect in the three models tested.

INTRODUCTION

Peptic ulcer disease is one of the most common set of disorders characterized by well circumscribed mucosal defects, found only in portions of the gastrointestinal tract that are exposed to the acid and pepsin component of gastric juice in the stomach and the duodenum. Humans have suffered from abdominal symptoms of discomfort, gnawing, burning or even blood in stool since times immemorial. Ayurveda, the ancient Indian medical science describes it as 'Amlapitha' or 'Parinamasula'. However, peptic ulcer was established in autopsy for the first time in the 16th century. Since then, there have been continuous efforts to unearth the pathophysiology of ulcer production and discover newer remedies for the human suffering caused. Peptic ulcer disease results from an imbalance between defensive factors that protect the mucosa and offensive factors that disrupt the important barriers. The mucosal barrier, under normal conditions is maintained by local defense mechanisms like alkaline secretions, mucosal hydrophilicity, rich mucosal blood flow, rapid epithelial cell renewal, mucosal sulfhydryl and increased resistance of gland cells in deep mucosa to acid and peptic activity by Konturej et al., 2003. Both hydrochloric acid and pepsin tend to damage the gastric mucosa. In 1980s, the advent of Helicobacter pylori (H. pylori) introduced a dramatic change in the concept of causation of peptic ulcer. H. pylori infection is present in virtually all patients with duodenal ulcers and about 70% of those with gastric ulcers from Robbins and Cotran; Pathologic basis of diseases, 7th edition. Thirty years back, peptic ulcer disease was mostly prepared by antacid preparations and surgical procedures which were highly risky. In 1979, a major breakthrough was made by the introduction of acid suppressive drugs like histamine (H2) receptor blocker like cimetidine and ranitidine. Apart from this other drugs present for PUD are proton pump inhibitors like omeprazole, anticholinergics, ulcer protective agents, ulcer healing agents and antibiotic therapy for H.Pylori.

However, post marketing surveillance of these drugs has shown development of tolerance, relapses and side effects. This has led to many initiatives at developing newer anti ulcer agents.

* *Corresponding Author*
Suresh Babu Kondaveeti
Lecturer,Department of Biochemistry
MAPIMS, Melmaruvathur-603319
Ph-09786668848

This exhaustive search has also been extended to many herbs which are already in use, though in crude forms either for the same condition or for other diseases through efforts made to scientifically judge their utility.

The vast biodiversity of Indian forests provides several plants, which are mentioned in Ayurveda for medicinal care. *Juglans regia* L. the royal species from family Juglandaceae has been used in traditional medicines from ancient times. It is a frost tender deciduous tree growing up to 40-60m. It is found in the Himalayan regions in India.

The walnuts consume extensively as a food, which are rich unsaturated fatty acids and its leaves has been widely used in traditional medicine for the treatment of skin inflammations, hyperhidrosis and ulcers and for its antidiarriec, antihelmintic, antiseptic and astringent properties by Almeida et al., 2008. All parts of the plant; stem, bark, leaves, fruits, seeds, seed oil are used in folk medicines to treat variety of health disorders including cancer by Bown et al. Walnut has been widely used as herbal medicine in the treatment of diabetes and in folk medicine to treat prostate and vascular disturbance.

Antiradicalar and antibacterial activities have also described for different J. regia cultivars by Asgary, Spaccarotella, Pereira et al., 2008,2007. In addition, Plant-derived products can also be used as antimicrobial agents, with phenolics and polyphenolic having major interest. In the present study ethanolic extract of *Juglans regia* L. leaves, evaluated for the in vitro gastroprotective properties using various experimental models.

MATERIALS & METHODS

Sample preparation and extraction procedure

The *J. regia* leaves were collected during November 2011 from gardens located in eastern part of Tamilnadu. authenticated by, Prof & H.O.D, Dept. of Botany, Sri Andavan College of Sciences, Thiruvanaikkaval, Trichy. and voucher specimen was 2-10 HMRC. The experiments were carried out after the approval from the MAPIMS&R animal ethical committee (Regd. No. MAPIMS /1058/PO/ac/10/CPCSEA) and the approved experimental design no. (proposal No 332, dated 22/08/2010).

The leaves of healthy plants were plucked, washed thoroughly under running tap water, dried outside in the shade for 5 days and then ground into the fine powder using an electric mixer. The powdered plant material (700 g) was soaked in 90% ethanol at room temperature for 24 hours, a procedure repeated twice. The mixture was filtered using Whatman No. 1 filter paper. The filtrate was evaporated in the soxhlet apparatus to obtain 80.4 g extract powder. The extract was stored in a refrigerator at 2–8oC to be used in subsequent experiments.

Animals

Albino rats of wistar variety weighing 140-165gms were used in the experiment. The sexes were evenly divided into different treatment groups.

Acute toxicity test LD50

Animals were housed in well ventilated room (temperature 23± 20C, humidity 65-70% and 12h light/dark cycle) in animal house, MAPIMS. The animals were given standard rat pellets and tap water and libitum. The acute toxic study was used to determine a safe dose for the *Juglans regia*.L extract. Thirty rats (6 males) in each group were assigned equally each into 5 groups (0.25% w/v, 5 ml/kg); 100,200 and 300 mg/kg of *Juglans regia*.L leaf extract preparation, respectively. The animals were fasted overnight (food but not water) prior to dosing. Food was withheld for a further 3 to 4 h after dosing. The animals were observed for 30 min and 2, 4, 8, 24 and 48 h after the administration for the onset of clinical or toxicological symptoms. Mortality, if any was observed over a period of 2 weeks. The acute toxicity LD50 was calculated as the geometric mean of the dose that resulted in 100% lethality and that which caused no lethality at all. The animals were sacrificed on the 15th day. Hematological and serum biochemical parameters were determined by following standard methods.

Pylolus ligation method

Animals were divided into 3 groups with 6 animals in each group. All the animals in group A received normal saline & group B receives Omeprazole(20mg/kg) as standard & group C receives *Juglans regia*.L (500mg/kg) (Al-Yahya et al. 1989) for 7 days along with standard diet before pylorus ligation. On the seventh day, half an hour after saline or drug treatment in 36 hours fasted rats, pylorus was ligated under light ether anaesthesia as per the method Shay et al., 1945. Post operative period was deprived of food and water and after 6 hours animals were sacrificed by ether overdosing and stomach was dissected out after ligating its cardiac end and cut open along the greater curvature, stomach contents are collected and measured for volume, centrifuged and subjected to analysis for total acidity and inner surface is examined for any ulceration both macroscopically and microscopically. The ulcer index was calculated as by the method of Ganguly and Bhatnagar et al., 1973.The gastric juice was collected after 6 hours of pyloric ligation as described by Sanyal et al., 1971.The total acidity of the gastric juice was determined as per Kulkarni et al., 1999.

$$\text{Acidity} = \frac{\text{volume of NaOH} \times \text{Normality} \times 100 \text{ m Eq/l.100gm}}{0.1}$$

(Hawk et,.al 1947)

Aspirin induced method

Aspirin is suspended in 1% carboxy methyl cellulose in water (20 mg/ml) and administered orally (gavage) in a dose of 500 mg/kg in 36 hours fasted rats in all the 3 groups. Along with aspirin Group-I receives normal saline as control, Group-II receives Omeprazole (20mg/kg) as standard & Group-III (A,B,C) receives *Juglans regia*.L extract (100,200,300mg/kg). Four hours later the animals are sacrificed. The stomachs are removed and opened along the greater curvature to determine the ulcer index

(Parmer et al., 1993). The ulcer index was determined using the formula.

Ulcer index =10/X (Ganguly et,.al 1969)

where X =total mucosal area/total ulcerated area.
The ulcers were given scores based on their intensity as follows: 0/Xno ulcer, 1/X superfacial mucosal erosion, 2/X deep ulcer or transmural necrosis, 3/X perforated or penetrated ulcer.

Ethanol induced method

Group-I received 1ml ethanol (99.9%) and was taken as control; Group-II received Omeprazole 20mg/kg once daily orally for six days and 30min prior to ulcerogen on the seventh day. Group-III (A,B,C) received *Juglans regia*.L extract (100,200,300mg/kg). once daily for seven days and 30min prior to ulcerogen on seventh day.

The animals in all the groups were fasted for 24hrs prior to the administration of ulcerogen, with water ad libitum. Animals were sacrificed one hour after the administration of ethanol and stomach was dissected out and examined for ulceration. Ulcer indices were calculated as described by Parmer et al., 1993.

Statistical analysis

Results were analysed by one way analysis of variance (ANOVA), followed by Dunnet's multiple comparison test.

All the results are expressed as Mean ± SD. Significance was established when the probability value was less than 0.05. (Ghosh M.N.)

Histopathology

After macroscopic examination, the stomachs were immersed in 10% formalin solution for 24 hours. A strip of gastric wall was cut from the forestomach to the pylorus through the entire glandular mucosa, necessarily including red streaks or sites of ulceration. This sample was subsequently processed for the preparation of sections (4-5mm thick) after embedding in paraffin wax and staining with haematoxylin and eosin using routine techniques.

RESULTS

In pylorus ligation induced gastric ulcer *Juglans regia*.L has shown significant reduction in ulcer index when compared to that of the control. Whereas the decrease in gastric secretion volume & total acidity are not significant. It is also observed that ulcer index is significantly high in aspirin induced & ethanol induced peptic ulcer group as compared to the *Juglans regia*.L extract treated group which shows marked reduction($p<0.01$) in gastric lesions. In histopathological examination of stomach specimens of control group from all the models it was seen that there was extensive gastric damage, even involving all the layers of the stomach wall in some regions. The mucosal epithelial cells were completely eroded and there was severe infiltration by inflammatory cells. The submucosal layer was edematous and engorged blood vessels could be seen. The muscular layer was also edematous. However the groups treated with *Juglans regia*.L extract and ranitidine did not show any such findings of that extensive gastric damage.

Table. 1: Effect of *Juglans regia*.L leaf extract on gastric secretion, total acidity & ulcer index in Pylorus ligated rats, aspirin induced method & ethanol induced methods:

Grp	Treatment	Gastric secretion Mean ± SD	Total acidity (mEq/l/100gm)	Ulcer Index Mean ±SD	Ulcer Index Mean ± SD (By aspirin induced method)	Ulcer Index Mean ±SD (By ethanol induced method)
I	Normal saline	3.633 ± 0.62	89.53 ± 4.35	0.1809 ± 0.048	0.4305 ± 0.06	0.5476 ± 0.07
II	Omeprazole (20mg/kg)	1.816 ± 0.360 *	54.26 ± 4.68 *	0.0274 ± 0.0045*	0.0736±0.01*	0.2879 ± 0.05*
III.A	*Juglans regia*.L (100mg/kg)	2.343 ± 0.210 *	78.6 ± 3.325 *	0.0316 ± 0.0019*	0.0625± 0.01*	0.2112 ± 0.02* LD_{50}=90.14%
B.	*Juglans regia*.L (200mg/kg)	2.683 ± 0.286 *	79.6 ± 3.55 *	0.0416 ± 0.0049*	0.0654± 0.01*	0.2208 ± 0.02* LD_{50}=95.45%
C.	*Juglans regia*.L (300mg/kg)	2.983 ± 0.491 *	83.6 ± 4.35 *	0.0516 ± 0.0089*	0.0797± 0.01*	0.2318 ± 0.04* LD_{50}=99.25%

Values are mean ± SD; (n = 6), * $p < 0.01$ when compared with control, + $p>0.05$ when compared with control.

Fig. 1: *Juglans Regia*.L

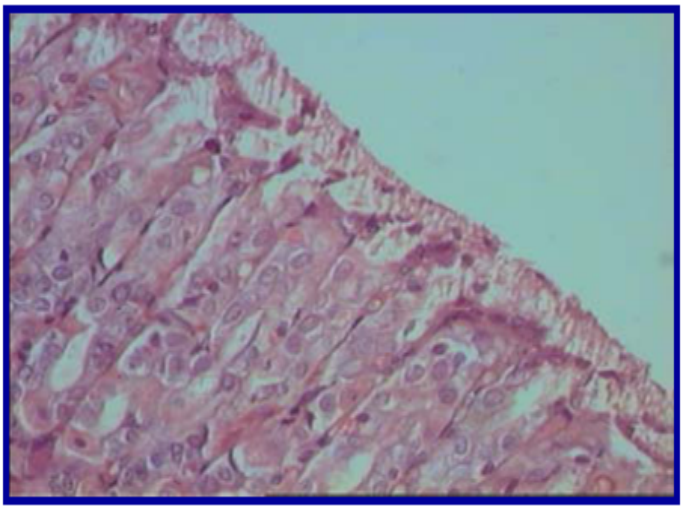

Fig. II a: Section of stomach from Group-I Normal Saline treated rat showing normal epithelium, lamina porperia and muscularis mucosa.

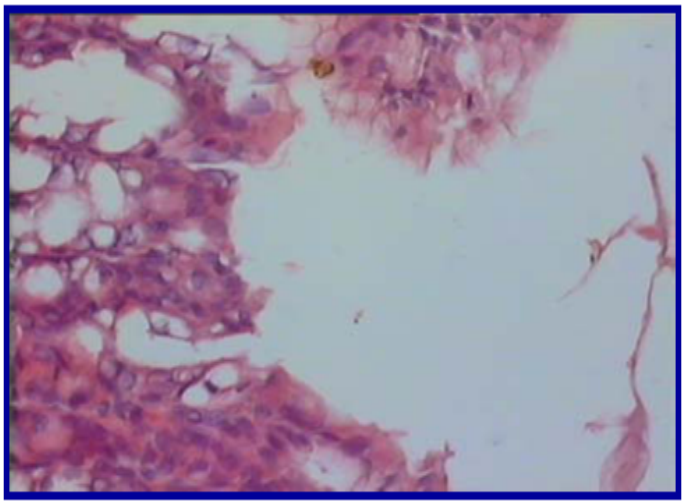

Fig. II b: Section of stomach from Aspirin – Ethanol treated rat showing extensive injury in the epithelial layer of the mucosa. The lamina propria were greatly damaged with hemorrhagic areas.

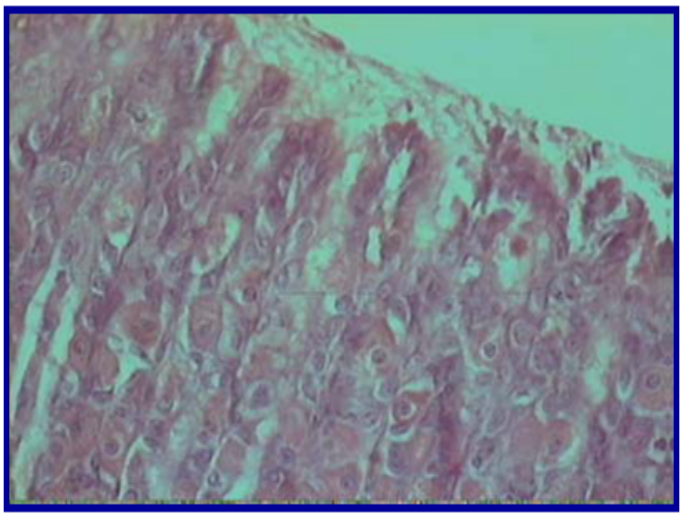

Fig. II c: Section of stomach from Group-II Omeprazole treated rat showing continuous mucosal layer and formation of the epithelial layer showing regeneration of cells.

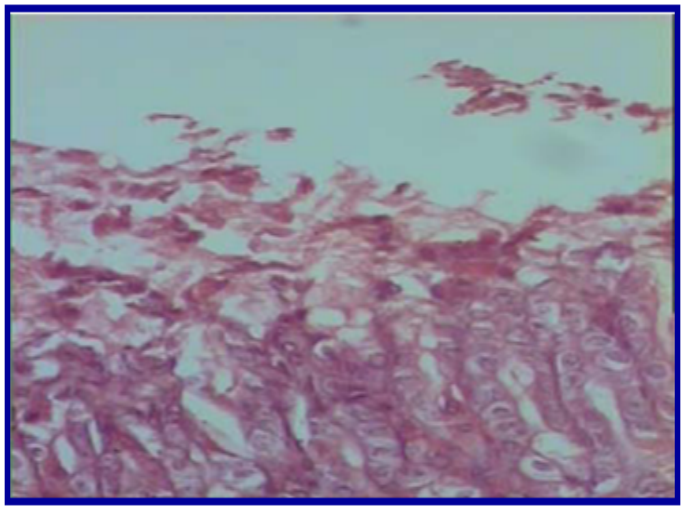

Fig. II d: Section of Stomach from Group- IIIA *Juglans regia*.L (100mg/kg) treated rat showing damage in the epithelial layer of mucosa with moderate infiltration.

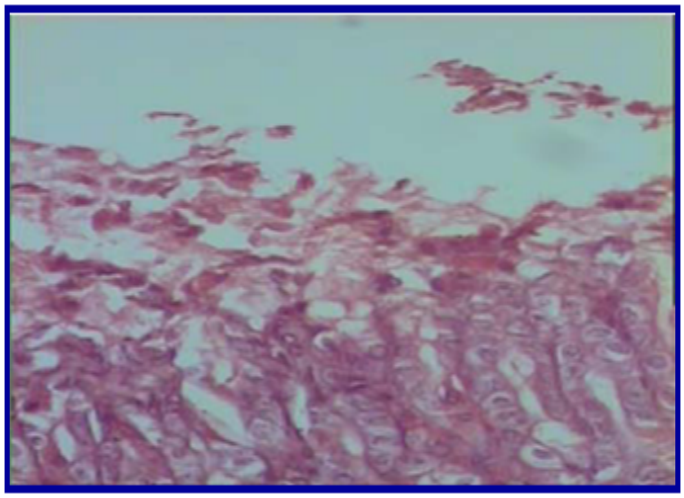

Fig. II d: Section of Stomach from Group- IIIB *Juglans regia*.L (200mg/kg) treated rat showing damage in the epithelial layer of mucosa with moderate infiltration.

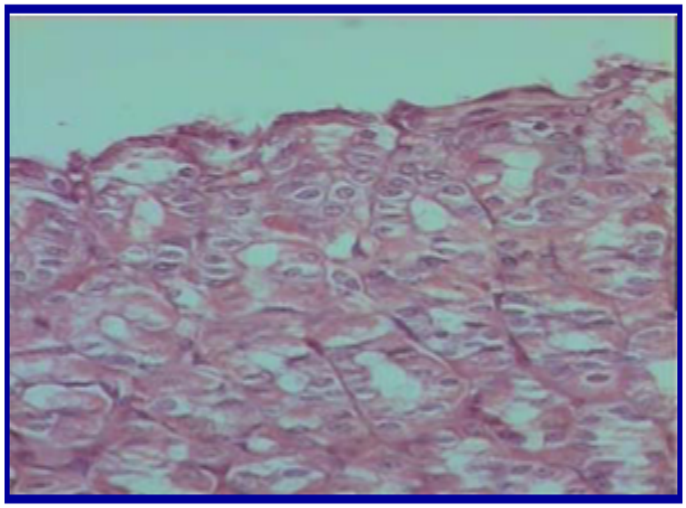

Fig. II d: Section of Stomach from Group- IIIC *Juglans regia*.L (300mg/kg) treated rat showing damage in the epithelial layer of mucosa with less infiltration.

DISCUSSION

In the present study there is significant reduction ($p < 0.01$) in ulcer index in all the three models of gastric ulcer induction namely pylorus ligation, aspirin induced and ethanol induced models. Increased vascular permeability as a result of endothelial lesions occur very early (within 1min) in ethanol induced injury by Szabo et al., 1985. Further, gastric lesions caused by ethanol have been attributed to free radical damage which results in lipid peroxidation products a study conducted by Desai et al., 1997.

Juglans regia.L has shown significant protection against ethanol induced gastric lesions. *Juglans regia*.L seeds has been shown to inhibit lipid peroxidation by scavenging superoxide and hydroxyl radicals as seen in a study by Cao et al., 1993. *Juglans regia*.L leaf extract might have protected the gastric mucosa probably by the above mechanism.The alcoholic extract inhibited ulceration by inhibiting output volume and total acidity. The ulcer healing activity of the plant extract may be due to antisecretory property associated with an enhancement of the local healing process, which was comparable with the standard drug ranitidine (H2-antagonist). Flavonoids are reported to have antiulcer activity. Aerial parts of *Juglans regia*.L are reported to contain flavonoids by Amaral et al., 2005. In the present study the preliminary phytochemical investigation of the alcoholic extract of Bermuda grass showed the presence of flavonoids, which may be responsible for the antiulcer property. Thus from the above discussion it can be seen that *Juglans regia*.L has antiulcer activity and gastroprotective effect by more than one mechanism. The Indian medicinal plants deserve special attention as they have long history of use in traditional and Ayurvedic medicinal systems and are largely devoid of adverse effects and toxicity. *Juglans regia*.L is one such plant with multitude of medicinal properties giving us reason to cheer and engage in more such studies.

CONCLUSION

Thus the present study concludes the *Juglans regia*.L leaf extract have potential gastro protective effect in ulcerative colitis & gastro protective effect. Most of the anti-secretory drugs reduce acid secretion, thus giving immediate symptomatic relief, but there are reports of adverse effects and relapses in the long run. On the contrary natural drugs mostly augment the defensive factors and may be slow in activity but are reliable and safe. Hence use of natural drugs alone or with combination with other drugs should be seriously considered.

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