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EVALUATION OF ANTIULCER AND *IN-VITRO* ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF *PSIDIUM GUAJAVA* ROOT IN ALBINO WISTAR RATS

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ABSTRACT

The study was carried out to find out the antiulcer activity of methanolic extract of *Psidium guajava*. For this study albino wistar rats(150-200g) were used .Four groups of rats were selected containing six animals in each group .The study was carried on different gastric ulcer models and ulcers were induced by pyloric ligation and aspirin .The animals were treated with Omeprazole 20mg/kg and MEPG 200 and 400mg/kg. Ulcer index was calculated in both models .In pyloric ligation model, free acid, total acid, total protein and mucin contents were also determined. In in-vitro antioxidant, Free radical scavenging (DPPH assay) activity and lipid peroxidation content were estimated. MEPG produced significant reduction in ulcer index (1.5) when compared with control (5.83) in both ulcer models .In pylorus ligated model it showed significant reduction in volume of acid secretion and increased mucin and total protein content .It also produced significant increase in Free radical scavenging and lipid peroxidation content. MEPG produces significant anti-ulcer activity which is comparable to standard drug Omeprazole. It also produces significant anti-oxidant activity which is comparable to standard ascorbic acid.

Key words: Pyloric ligation, DPPH, Lipid peroxidation, MEPG-Methanolic extract of Psidium guajava.

INTRODUCTION

Peptic ulcer disease is one of the most common gastrointestinal disorders, which causes a high rate of morbidity particularly in the population of non-industrialized countries (Falk GW, 2001). Peptic ulcer occurs due to an imbalance between the aggressive (acid, pepsin and *Helicobacter pylori*) and the defensive (gastric mucus and bicarbonate secretion, prostaglandins, innate resistance of the mucosal cells) factors (Tripathi KD, 1999). In Ayurveda, peptic ulcer mostly refers to Amlapitta or Parinamas*ula. Amlapitta* is a disease of the gastrointestinal tract, especially of the stomach. *Amlapitta* literally means, pitta leading to sour taste (Tewari PV *et al*., 1996). Number of drugs including proton pump inhibitors, prostaglandins analogs, histamine receptor antagonists and cytoprotective agents are available for the treatment of peptic ulcer.

But most of these drugs produce several adverse reaction, including toxicities and even may alter biochemical mechanisms of the body upon chronic usage (Ariyphisi I, 1986). Hence, herbal medicines are generally used in such cases when drugs are to be used for chronic periods. Several natural drugs have been reported to possess anti-ulcerogenic activity by virtue of their predominant effect on mucosal defensive factors (Sairam K *et al*., 2001; Sairam K *et al*., 2001). Psidium Guajava L. commonly known as "Atibala" in Sanskrit gives excessive tonic strength (Anonymous 1). Atibala is a stronger diuretic and heart tonic (Kirtikar KR, Basu BD, 1961). *P.guajava* reported in the Siddha system as a remedy for jaundice, piles, ulcer, leprosy, rakttapitta dosha and blood purifier (Nadkarni KM, 1976). Chemically it contains Flavonoids (quercetin), Saponins, alkaloids, tannins and phenolic compounds (Sharma PV, 1978). The following activities like analgesic, larvicidal, Hepatoprotective, and hypoglycaemic were reported.

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Recent screening of plants revealed many compounds like Flavonoids, alkaloids, Saponins, terpenoids, monoterpenoids (linalol), glycoprotein's, polysaccharides, tannins, essential fatty acids, phenolic compounds and vitamins having pronounced antioxidant, antineoplastic, anti-ulcer, anti-inflammatory and immunostimulating potential (Wagner H, 1990). Scientific literature is continuously reporting herbal drugs having anti-ulcer potential. There is need to evaluate the potential of ayurvedic remedies as adjuvant to counteract side effects of modern therapy (Wagner H, 1990). The present investigation is aimed at studying the anti-ulcer activity of the methanolic extract of leaves of Psidium guajava L. in order to justify the traditional claims endowed upon this herbal drug as a rasayana.

MATERIALS AND METHODS

Plant Material

The roots of *Psidium Guajava* L. were collected at in the local area of Shri Vishnu College of Pharmacy, Bhimavaram ,India and authenticated by Dr. Prasana kumari of the Department of Botany, DNR Degree and P.G.College, Bhimavaram, voucher specimen are for future reference.

Extraction

The roots were dried under shade at room temperature The shade dried, coarsely powdered roots (500 g) was successively extracted with petroleum ether (60-80^{o}C) for 7 days to remove fatty matter. The defatted marc was then subjected to soxhlet extraction with 95% methanol to obtain methanolic extract. The methanolic extract was evaporated under reduced pressure at low temperature (30^{o}C) to dryness and brownish yellow colour extracts of *P.guajava* was obtained.

Preliminary Phytochemical Screening

Methanolic extract of *Psidium Guajava* was subjected to preliminary phytochemical for the detection of various constituents (Khadelwal KR).

Experiment

Albino Wistar rat of weighing between 200-250 g was housed in groups of 5 to 6. All rats were feed with pelleted diet (Pranav Agro Industries Ltd, Sangli, India) and water ad libitum. Institutional Animals Ethics Committee(IAEC) approved the experimental protocol and care of animals was taken as per guidelines of CPCSEA, IAEC NO : **439/PO/01/a/CPCSEA.**All the drugs(Omeprazole, Aspirin) and chemicals(Ethanol, Toffer reagent and phenopthelin) used are of analytical grade .

Acute Toxicity Studies

Acute toxicity studies were performed according to organization for economic co-operation and development OECD guidelines 429 (2001). Animals were divided in groups (n=4) and fasted for 4 h with free access to water only. The MEPG extracts was administered orally in doses of 5,50,300 and 2000 mg/kg to different groups of mice and observed over 24 hr. for mortality and physical/ behavioural changes.

Assessment of Anti-Ulcer Activity

Animals were divided into 4 groups each containing six in number.

Group I : Control received only Distilled Water .
Group II : Standard received Omeprazole (20 mg/kg).
Group III : Received Methanolic extract of *Psidium Guajava* root(200mg/kg)
Group IV : Received Methanolic extract of *Psidium Guajava* root (400 mg/kg).

Pyloric ligation induced gastric ulceration

Albino rats of either sex were divided into four groups of six animals each. Animals were fasted for 24 h before the study, but had free access to water. Animals in the control group received only distilled water. Methanol extract of P. Guajava at 250 and 500 mg/kg, (p. o.) were given to the animals in the treatment group. Omeprazole (20 mg/kg) was used as a Standard . After 1h of drugs treatment, they were anaesthetized with the help of aesthetic ether; the abdomen was opened by a small midline incision below the xiphoid process. Pyloric portion of the stomach was slightly lifted out and ligated according to method of Shay *et al*., (1945), avoiding traction to the pylorus or damage to its blood supply. The stomach was replaced carefully and the abdominal wall was closed by interrupted sutures. Rats were sacrificed by an overdose of anaesthetic ether after four hours of pyloric ligation. The abdomen was opened, cardiac end of the stomach was dissected out and the contents were drained into a glass tube. The volume of the gastric juice was measured and centrifuged at 2000 rpm for 10 min. From the supernatant, aliquots (1 ml of each) were taken for the determination of pH, total and free acidity. Each stomach was examined for lesions in the fore stomach portion and indexed according to severity.

Aspirin induced gastric ulceration

Albino wistar rats weighing 150-200g are taken for this experiment. The rats are administered either the appropriate vehicle or the cytoprotective drug orally 30mins prior to administration of aspirin. This process is done for 3days and on the last day, the animals are euthanized with chloroform the stomachs are excised, cut along the greater curvature, and gently rinsed under tap water. The stomachs are stretched on a piece of foam core mat and examined under a 3-fold magnifier.

EVALUATION PARAMETERS

Collection of Gastric juice

The stomach was excised carefully keeping the esophagus closed, opened along the greater curvature and the gastric contents were removed. The gastric contents were collected in plain tubes and centrifuged at 3000 rpm for 5 min; the volume of the supernatant was expressed as ml/100 gm body weight. The mucosa was flushed with saline and observed for gastric lesions using Index dissecting microscope, ulcer score was determined.

Ulcer Scoring

After sacrificing the rat, stomach was removed and opened along the greater curvature, and washed it slowly under running tap water. Put it on the glass slide and observe under 10X magnification for ulcer. Score the ulcers as below.

0 = normal coloured stomach
0.5 = red colouration
1 = spot ulcers
1.5 = haemorrhagic streaks
2 = Ulcers ≥ 3 but ≤ 5
3 = Ulcers >5

Mean ulcer score for each animal is expressed as Ulcer Index.

The percentage of ulcer protection was determined as follows: Ulcer index (UI) was measured by using following formula:

UI = UN + US + UP X 10−1

Where, UI= Ulcer Index; UN = Average number of ulcers per animal; US = Average number of severity score; UP = Percentage of animals with ulcers. Percentage inhibition of ulceration was calculated as below:

% Inhibition of Ulceration =

$$\frac{\text{(Ulcer index Control-Ulcer index Test)} \times 100}{\text{Ulcer index Control}}$$

Free acidity and Total acidity

Centrifuge the gastric contents at 1000 rpm for 10 min, note the volume. Pipette out 1 ml of supernatant liquid and dilute it to 10 ml with distilled water. Note the P^H of the solution with the help of P^H meter. Titrate the solution against 0.01N NaOH using topfers reagent as an indicator. (It is Dimethyl-amino-azo-benzene with phenolphthalein and used for detection and estimation of hydrochloric acid and total acidity in gastric fluids) Titrate to end point when the solution turns to orange colour. Note the volume of NaOH which corresponds to free acidity. Titrate further till the solution regains its pink colour. Note the total volume of NaOH which corresponds to the total acidity. Acidity (mEq/1/100 g) can be expressed as:

$$\textbf{Acidity} = \frac{Vol.of\ NaOH \times Normality \times 100}{0.1}\ \textbf{mEq/l/100 g}$$

Determination of gastric wall mucus

Gastric wall mucus was determined in Aspirin - induced ulcer models according to the method of Corne *et al.*, (1974). The glandular segments from stomachs were removed, weighed and incubated in tubes containing 1% Alcian blue solution (0.16Msucrose in 0.05M sodium acetate, pH 5.8) for 2 h. The Alcian blue binding extract was centrifuged (100 g) for 10 min and the absorbency of supernatant was measured at 498 nm. The quantity of Alcian blue extracted (_g/g of glandular tissue) was then calculated.

Determination of total protein content

Dry Weight Method

Drying a protein-containing sample in a 104' to **1M0C** oven for a few hours removes water and volatile materials. The sample is then weighed on a balance to measure the aggregate weight of protein plus whatever non-volatile material remains, such as salts and many buffers.

Materials

Protein sample
Small weighing bottle or beaker
104" to 106'C oven

1. Dry a small weighing bottle or small beaker by heating it 10 min in a **104"** to 106°C oven.
2. Cool the weighing container in a desiccator 10 min.
3. Weigh the container on a balance to the nearest 0.1 mg (tare weight).
4. Add a 0.5- to 3-ml sample of protein solution to the container.
5. Buffer salts, polysaccharides, the salt forms of amino acids, and high molecular weight pigments and sugars may not be driven off in a 4- to 6-hr drying cycle (see step 5).They should be removed from the sample by dialysis, ion-exchange chromatography, Or precipitation before it is dried (see Support Protocols 2 and 3).
6. Dry the sample and container 4 to *6* hr (or overnight) at 104" to 106°C.Usually it is not necessary to carry out more than one cycle of drying, cooling, and Weighing to approach a constant weight. Over night drying is required for samples to 5 to I0 ml.
7. Cool the container and reweigh it (dried weight).
8. Calculate the dry weight of the sample as: dry sample weight = dried weight of container and protein -tare weight.

The first dry weights mesured after 6 hr usually remain constant, but if volatile salts such **as** ammonium chloride and ammonium formate are present, the sample may have to be dried longer orfor more cycles to completely drive them off Dry weight is reliable to within -3% for net weights of 2 to 4 mg protein and to within 1% to 2% for 3mgprotein.

Histopathological studies

Stomachs were preserved in 10% formalin solution for histopathological examination .The central part of damaged or ulcerated tissue (if present) was cut off along the long diameter .If the stomach was protected from the damage then the section was taken from the basal part ,thickness of about 5µm were cut and stained with haemotoxylin and eosin .These were examined under the microscope for histopathological changes such as congestion, hemorrhage, necrosis, inflammation, infiltration, erosion and ulcers.

Determination of *in vitro* Antioxidant activity

Free radical scavenging (DPPH) assay

The free radical scavenging activity of MEPG was measured *in vitro* by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay using the method of Blois (1958). About 0.3mM solution of DPPH in 100% ethanol was prepared and 1ml of this solution was added to 3ml of the extract dissolved in ethanol at different concentrations (10–50 _g/ml). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. The IC50 value of the crude extract was compared with that of ascorbic acid, which was used as the standard.

Lipid peroxidation inhibition assay

Procedure

Induction of lipid peroxidation by ascorbate system:

Inhibition of lipid peroxidation was determined by method developed by Ohkawa H *et al*., 1979.

Rat liver tissue weighing 10 gm was homogenized with a polytron homogenizer in ice –cold Tris –Hcl buffer to produce a 25% w/v homogenate. The homogenate was centrifuged at 4000 rpm for 10 min. An aliquot of supernatant 0.1 ml was mixed with 0.1 ml of plant extract of different concentrations followed by addition of 0.1ml of potassium chloride (30 mM) , 0.l ml of ascorbic acid (0.06mM) and 0.1 ml of ammonium ferrous sulphate (0.16mM) and were incubated for 1 hour at 37 ^{0} C. The reaction mixture was treated with 0.2 ml of sodium dodecyl sulphate (8.1%), 1.5 ml of thiobarbituric acid (0.8%) and 1.5ml of 20% acetic acid (PH 3.5) .The total volume was then made up to 4 ml by adding distilled water and kept in an oil bath at 100^0 C for 1 hour. After the mixture had been cooled, 1 ml of distilled water and 5ml of 15:1 v/v butanol -pyridine mixture were added. Following vigorous shaking, the tubes were centrifuged at 4000 rpm for 10 min and the absorbance of the organic layer containing the thiobarbituric acid reactive substances (TBARS) was measured at 532 nm.

A control was prepared using 0.1 ml of respective vehicle in the place of plant extract /ascorbic acid .The percentage inhibition of lipid peroxidation by the extract was determined by comparing the absorbance values of the control and the experimental tubes as calculated for Nitrous oxide scavenging assay.

STATISTICAL ANALYSIS

The results were expressed as mean ± SEM (n=6) .Statistical analysis was performed using one way ANNOVA followed by Dunnett's comparison test T.
P-values (expressed) calculated against Ulcer group and $p<0.001$ were considered.

RESULTS

PRELIMINARY PHYTOCHEMICAL SCREENING

The MEPG were found to contain carbohydrates,, amino acids, Saponins, Flavonoids, tannins and phenolic compounds.

Acute Oral Toxicity Study

Acute oral toxicity was carried out by up-down regulation method. It is found that MEPG were safe at limit dose 2000 mg/kg with no mortality in studied subjects.1/10th of these doses i.e. 200 mg/kg and 400 mg/kg were used in the subsequent study respectively.

Table 1. Pyloric Ligation Induced Gastric Ulceration

Groups	Gastric Volume (ml)	pH	Free Acidity	Total Acidity	Mean Ulcer Index	%Protection
Group I (Control)	9.5±0.02	2.5±0.2	102±0.2	115±0.55	5.83±0.33	_
Group II (Standard)	5.2±0.5	4.2±014	31±0.22	39±0.44	1.0±0.36***	88.8±0.15
Group III (Test I)	6.8±0.04	3.2±008±	60±0.23	78±0.25	2.5±0.08	55.5±0.21
Group IV (Test II)	5.5±05	3.7±0.2	65±06	65±0.55	1.5 ± 0.50**	77.5±0.21

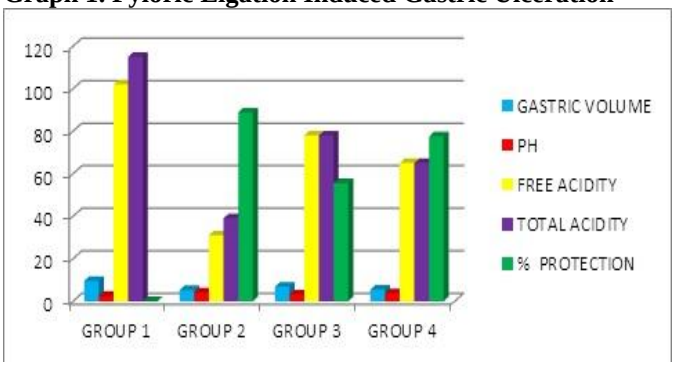
Table 2. Aspirin induced gastric ulceration

Groups	Gastric Volume (ml)	pH	Free Acidity	Total Acidity	Mean Ulcer Index	% Protection	Gastric Wall Mucus µG/G	Total Protein Content (Mg/100mg)
Group I (Control)	1.5±0.05	2.5±0.45	116±0.16	120±0.55	6.33±0.13	_	280.39±11.2	8.34±0.24
Group II (Standard)	0.7±0.13	4±0.32	32±0.8	42±0.8	1.33±0.61***	88.8±0.13	361.11±9.61	14.50±0.17
Group III (Test I)	1.2±0.12	3±0.35	65±0.44	70±0.25	2.5±0.1	63.6±0.21	310.0±5.60	10.50±0.50
GROUP IV (Test II)	0.9±0.13	3.8±0.16	55±0.12	59±0.06	2.0 ± 0.00**	72.88±0.06	342.32±9.61	12.48±0.17

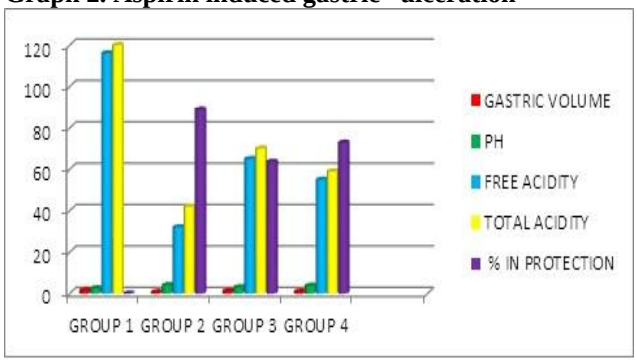
Table 3. *In vitro* anti oxidant activity

Sample	Concentration (µg/ml)	DPPH	Inhibition of lipid peroxidation (%)
Ascorbic acid	5	48.1±0.26	30.1±0.31
	10	55.2±0.75	42.9±0.15
	25	56.6±0.66	51.6±0.1
	50	64.8±2.05	63.6±0.21
	100	84.9±0.53	80.9±0.2
Methanolic extract(400mg/kg) of Psidium Guajava	5	35.5±0.55	20.9±0.13
	10	70.05±0.8	31.1±0.06
	25	84.4±0.1	48.2±0.25
	50	88.6±0.8	62.4±0.4
	100	95.6±0.5	74.6±0.1

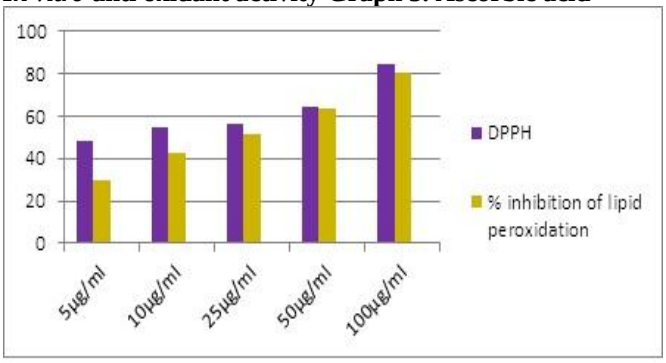
Graph 1. Pyloric Ligation Induced Gastric Ulceration



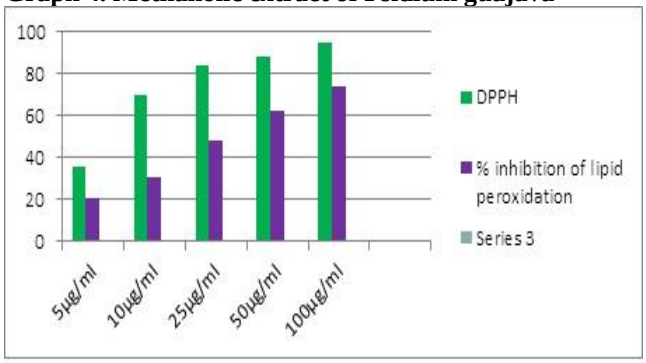
Graph 2. Aspirin induced gastric ulceration



***In vitro* anti oxidant activity Graph 3. Ascorbic acid**



Graph 4. Methanolic extract of Psidium guajava

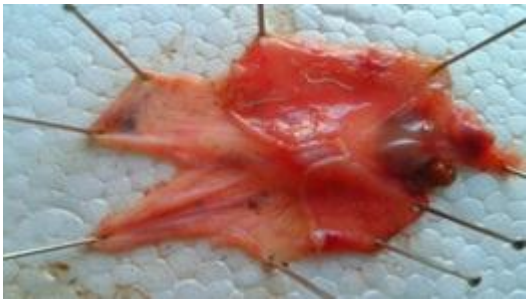


MICROSCOPICAL VIEW OF RAT STOMACH

Pyloric ligation method

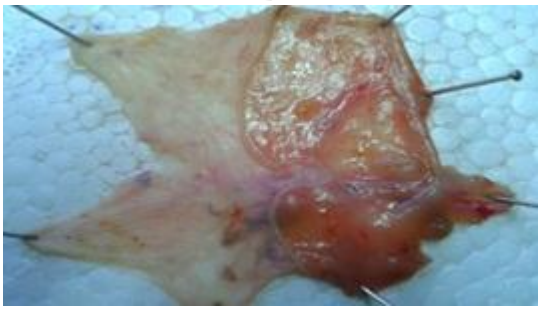
Fig 1. Microscopical view of rat stomach

A



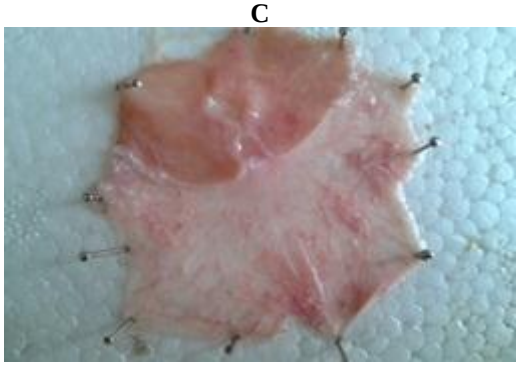
Group I control

B



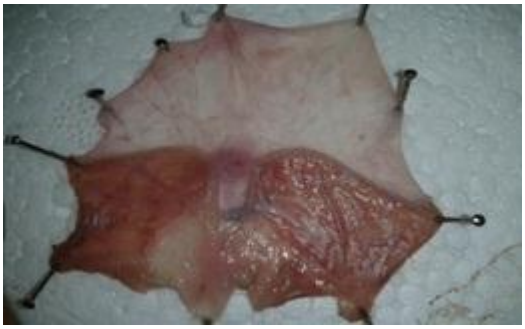
Group II(Omeprazole 20mg/kg)

C



Group III (MEPG 200mg/kg)

D



Group IV (MEPG 400mg/kg)

Aspirin induced gastric ulcerationFig 2. Microscopical view of rat stomach

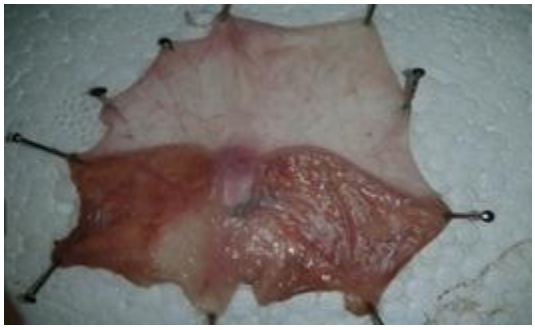
A

Group I control

B

Group II(Omeprazole 20mg/kg)

C



Group III (MEPG 200mg/kg)

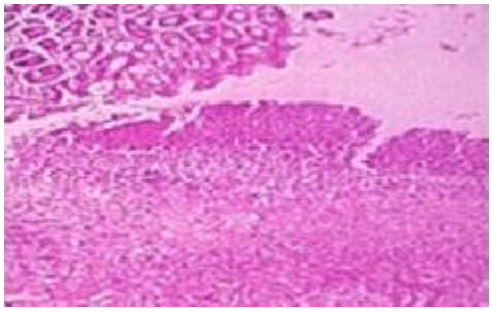
D

Group IV (MEPG 400mg/kg)

Histopathalogical studies Pyloric ligation

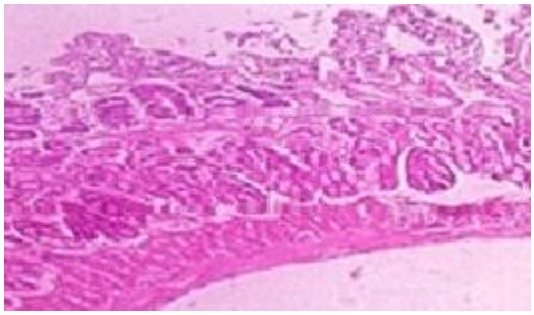
Fig 3. Histopathology of rat stomach A-Control , B- Standard (Omeprazole) C- MEPG200mg/mg , D- MEPG 400mg/kg

A



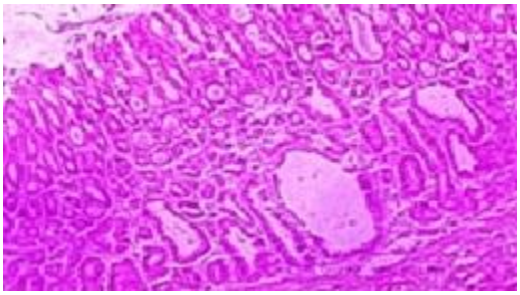
control

B



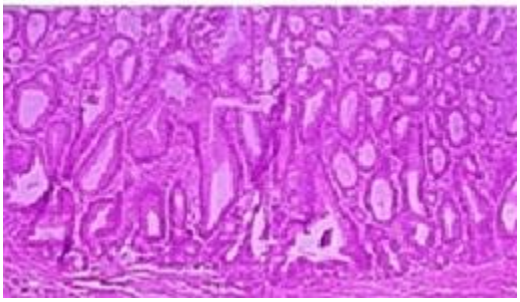
Standard

C



MEPG 200mg/kg

D

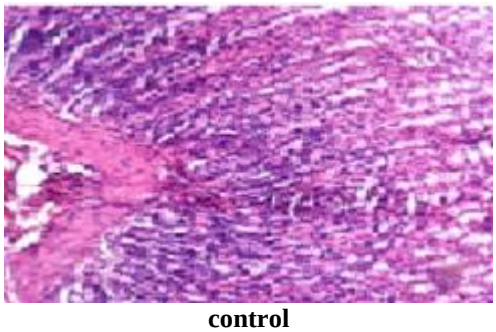


MEPG 400mg/kg

Aspirin induced ulcer method

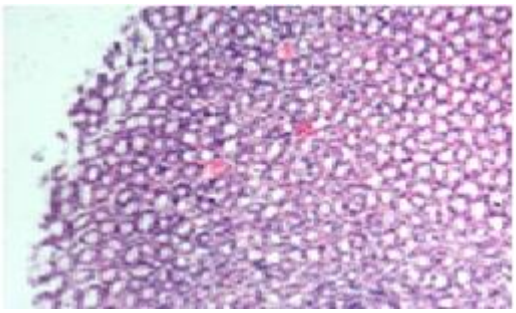
Fig 4. Histopathology of rat stomach Control ,B- Standard(Omeprazole) , C-MEPG200mg/kg, D-MEPG 400mg/kg

A



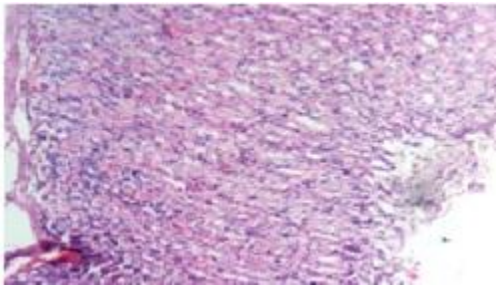
control

B



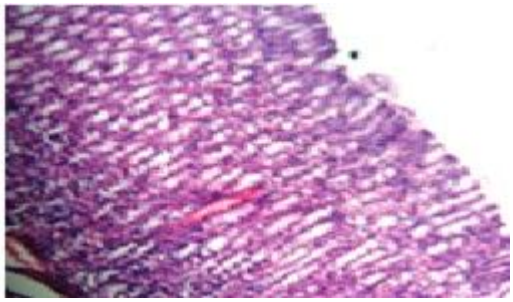
Standard

C



MEPG 200mg/kg

D



MEPG 400mg/kg

DISCUSSION

The etiology of peptic ulcer is unknown in most of the cases, yet it is generally accepted that it results from an imbalance between aggressive factors and the maintenance of mucosal integrity through the endogenous defence mechanisms. To regain the balance, different therapeutic agents including plant extracts may be used. *P. guajava* root extract is one such herbal drug used in the present study primarily to evaluate the anti-ulcerogenic in pylorus ligation and aspirin induced ulcers in rats. The causes of gastric ulcer pyloric ligation are believed to be due to stress induced increase in gastric hydrochloric acid secretion and/or stasis of acid and the volume of secretion is also an important factor in the formation of ulcer due to exposure of the unprotected lumen of the stomach to the accumulating acid. Pylorus ligation induced ulcers are due to auto digestion of the gastric mucosa and breakdown of the gastric mucosal barrier. These factors are associated with the development of upper gastrointestinal damage including lesions, ulcers and life threatening perforation and hemorrhage.

Methanolic extract of psidium guajava of anti ulcerogenic activity of MPEG was studied in aspirin induced gastric mucosal damage model in swiss albino rats. This model was chosen because NSAID abuse is the main exogenous cause of refractory peptic ulcer constituting 39% of the cases of peptic ulcer. NSAIDs produce a spectrum of injury to the gastrointestinal mucosa, from haemorrhages and petechiae to erosions and ulcers. Aspirin is known to inhibit PG cycloxygenase, leading to reduced production of PGE and endothelial PGI. This causes vasoconstriction, inhibition of platelet aggregation (enhanced bleeding) and contributes to the enhanced acid secretion. It can also cause mast cell degranulation resulting in the release of histamine. Tissue damaging free radicals which are produced from the conversion of hydroperoxy to hydroxy fatty acids further contribute to cell destruction. In our study *Psidium guajava* significantly reduced the ulcers induced by aspirin and results were comparable with omeprazole.

The antiulcer property of *P.guajava* in pylorus ligation mode and aspirin induced model is evident from its significant reduction in free acidity, total acidity, number of ulcers and ulcer index. *P.guajava* treated animals significantly inhibited the formation of ulcers in the pylorus ligated rats and also decreased both the concentration and increased the pH,and increased the gastric wall mucus and protein content of the gastric mucosa so it is suggested that *P.guajava* can suppress gastric damage induced by aggressive factors. Aspirin-induced ulcer is mediated through tissue damaging free radicals (Scheiman, 1996), which are produced from the conversion of hydroperoxyl to hydroxy fatty acids, which leads to cell destruction. The hydroperoxyl fatty acids are generated from the degeneration of mast cells and generalized lipid peroxidation accompanying cell damage (Van Kolfschten *et al*., 1983). So MEPG significantly increase the free radical scavenging and lipid peroxidation inhibition .Hence it produces antioxidant activity.

Omeprazole the proton pump inhibitor play an important role in the reduction of gastric volume and total acidity and thus perform a cytoproective effect. From references it is observed that by comparing the effect of various clinical agents on healing of ulcers induced by aspirin, We observed that among different anti-secretory and cytoprotective agents, omeprazole was found to be most effective drug. Omeprazole produced highest protection of 89.74% followed by misoprostol, ranitidine and sucralfate. These inducing methods of gastric lesions are rapid and convenient way of screening plant extracts for antiulcer potency and cytoprotection in macroscopically and microscopically visible lesions.

The preliminary phytochemical analysis of *P.guajava* extract showed the presence of flavonoids, triterpenoids, carbohydrates phenols,sapponins and tannins. The significant increase in the antiulcer activity of *P.guajava* could be attributed to the presence of flavonoids (quercetin), , tannins, saponin glycosides and phenolic compounds. Flavonoids are among the cytoprotective materials for which antiulcerogenic efficacy has been extensively confirmed. It is suggested that, these active compounds would be able to stimulate mucus, bicarbonate and the prostaglandin secretion and counteract with the deteriorating effects of reactive oxidants in gastrointestinal lumen. [25] So the antiulcer activity of *P.guajava* may be attributed to its flavonoids content. The results of the present study suggest that the methanol extract of *P.guajava* root may be beneficial in the treatment of gastric lesions. Further studies to identify the active moieties and elucidation of the mechanism of action are recommended.

CONCLUSION

Among the two doses (200 and 400 mg/kg) of 400mg/kg MEPG produces significant antiulcer and antioxidant activity. MEPG produces significant anti ulcer activity which comparable to standard drug Omeprazole. It also produces significant anti oxidant activity which comparable to standard ascorbic acid. The methanolic extract of MEPG produced more significant anti ulcer activity and antioxidant activity in pyloric ligation induced model than aspirin induced model.

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