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Evaluation of Hepatoprotective and Gastroprotective Activities of *Paspalidium flavidum* Leaves Extract in Experimental Animal Models

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ABSTRACT

Paspalidium flavidum (watercrown grass), a medicinal plant, is traditionally used in liver ailments and stomach problems. The hepatoprotective and gastroprotective activities of the aqueous methanol extract of *Paspalidium flavidum* (AMEPF) were studied in experimental animal models. Paracetamol and aspirin were used to induce hepatotoxicity and gastric ulcer in rats, respectively. Biochemical hepatic parameters, gastric pH, total acidity, ulcer index, percentage protection, nitric oxide and TNF-α were measured in AMEPF-treated groups. Moreover, GC-MS analysis of AMEPF was performed. Pretreatment with AMEPF improved the blood lipid profile and restored liver function tests in paracetamol-induced hepatotoxicity. While in aspirin-induced gastric ulcer, oral administration of AMEPF significantly reduced (P<0.05) the gastric lesions, total acidity and ulcer scoring index, TNF-α with upregulation of nitric oxide when compared with the Diseased group. AMEPF exhibited anti-lipid peroxidation activity. Histopathological studies were in good agreement with the biochemical findings. GC-MS analysis revealed the presence of anti-oxidant phyto-constituents, including oleic acid and 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) in AMEPF. This study suggested that aqueous methanol extract from the leaves of *P. flavidum* has beneficial hepatoprotective and gastroprotective activities related to its anti-oxidant phytochemicals.

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Introduction

The disproportion between the generation of reactive oxygen species (ROS) and the cellular antioxidant defense system is referred to as oxidative stress. The abundance of evidence linking oxidative stress to the etiology of a number of diseases has piqued the scientific community's interest in the role of antioxidants in human general health, disease control, and its management (1). Peptic ulcer disease, which is regarded as one of the modern-day epidemics, affects about 10% of the world's population (2). Scientists and medical experts are still baffled as to the exact pathophysiology of peptic ulcer, although a common ground has been offered. The imbalance between stomach aggressive factors (acid and pepsin) and mucosal defensive factors (mucus, bicarbonate, and prostaglandins) leads to the development of ulcer (3). Another crucial human health issue is liver disease. The liver is a key complex organ that detoxifies xenobiotic and impacts almost every physiological activity in the body (4). During detoxifying process, the liver is challenged to stress which can lead to liver disease, liver failure, severe complications, and even fatality. There is no medicine that can prevent liver damage or restore normal liver function once it has been exposed to toxic chemicals (5). Medicinal plants are thought to be a good source of compounds for the introduction of unique medications. Human health benefits greatly from medicinal plants (6). *Paspalidium flavidum* (watercrown grass) is a genus of tropical and subtropical plants in the grass family that grows as an annual to perennial weed. It is found in the forest undergrowth, damp places, on the edges of cultivated fields and ditches places of Punjab, Khyber Pakhtunkhwa and Kashmir in Pakistan (7). It's been used to cure skin, eyes, teeth, heart, and liver ailments, as well as headaches, dropsy, and to prevent abortion, miscarriage, and uterine discomfort after childbirth (8). This plant has reported diuretic, cardiotonic, anti-microbial, analgesic, anti-depressant and sedative biological activities (9, 10). The phytochemical analysis of *P. flavidum* presented secondary metabolites such as alkaloids, carbohydrates, flavonoids, saponins, tannins, terpenoids and phenols (11). The aim of the present study was to investigate the hepatoprotective and gastroprotective activities of the aqueous methanol extract of leaves of *P. flavidum* in experimental animal models.

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Materials and Methods

Collection of Plant

The leaves of *Paspalidium flavidum* were obtained from the botanical garden of Government College University, Lahore. Identification of the specimen was done by Professor Dr. Zaheer ud Din (GC.Herb.Bot.3629).

Preparation of Plant Extract

The aqueous methanol (30:70) extract of powdered leaves of *P. flavidum* was prepared with cold maceration (12). The concentrated extract was dried in an oven at 40˚ C and semi-solid was obtained with a percentage yield of 9.48%.

Phytochemical screening of plant extract

The aqueous methanol extract of powdered leaves of *P. flavidum* was screened for secondary metabolites as described previously (13).

Animals used

Wistar rats (both male and female) of 200-250 g (6-7 weeks old) were used. The animals were kept in controlled laboratory environments (by monitoring temperature, humidity, and light/dark cycle). Prior to any investigational technique, the animals were allowed to adapt for seven days and fed standard food and water. Experimental studies were carried out in accordance with the guidelines of Institute Research Ethics Committee, Faculty of Pharmacy, The University of Lahore (IREC/2019-133).

Chemicals and reagents

All the chemicals including methanol, formalin, sodium hydroxide, phenolphthalein, carboxymethyl cellulose, paracetamol, silymarin, cimetidine and aspirin were of high quality and pure analytical grade (Sigma Aldrich).

Study design

Paracetamol induced hepatotoxicity

Total 25 animals were used. 5 groups of animals were made (n=4) as follows; Group-I: Control group that received distilled water (5 ml/kg) once a day for 7 days. Group-II: Diseased control group received paracetamol (250 mg/kg) dissolved in distilled water for 7 days on daily basis. Group-III: Standard drug treated group received silymarin (50 mg/kg) for 7 days and paracetamol was also used 3 hours post silymarin administration. Group –IV and -V: Aqueous methanol extract of *P. flavidum* (AMEPF) treated groups received (250 and 500 mg/kg respectively) followed by paracetamol. All drugs were administered orally (14).

Biochemical investigation

All animals were sacrificed and cervical decapitated 24h after the last dose, the blood was collected and centrifuged at 4000 rpm for 20 minutes. For the evaluation of blood lipid profile and liver function tests of serum, human kits with automated analyzer (Micro lab 300, Merck Germany) were used (14) .

Aspirin induced gastric ulcer

Total 25 rats were divided into five groups (four rats per group). The rats were fastened for 36 hours before studies. Group -I: Control group which received vehicle (1% CMC, 1.7 ml/kg) orally. Group –II: Diseased control group received single oral dose of aspirin (200 mg/kg). Group –III: Standard drug control group received cimetidine (50 mg/kg) followed by aspirin after 1h administration of cimetidine. Group-IV and –V: AMEPF treated groups received (250 and 500 mg /kg respectively) orally proceeded by aspirin. After four hours, the animals were sacrificed and their stomach were dissected out (15, 16).

Estimation of NO and TNF-α

The blood samples were collected by cardiac puncture from control and treated groups, and levels of NO and TNF-α were determined in serum by using ELISA Kits (Biosciences, Austria).

Determination of Lipid Peroxidation

The top most layer of stomach tissue homogenates from control rat was prepared. The stomach tissue samples were homogenized in ice cold KCl solution. The plant extract solution (5 µl) in different concentrations (0.1, 10 and 100 µg/ml) was mixed with diluted homogenate (945 µl) with addition of 1 mM ferrous sulphate (50 µl) and incubated for one hour. $HClO_4$ was added to stop the reaction and centrifuged at 10 °C for 10 min (3000 rpm). The temperature of supernatant layer was maintained at 100 °C for one hour after addition of sodium dodecyl sulfate and acetic acid buffer (pH 3.6) containing thiobarbituric acid. Butyl alcohol and pyridine (15:1) was dropped into the mixture after cooling and centrifuged for10 min (4000 rpm). The butyl alcohol–pyridine phase containing the thiobarbituric acid reactive substance was separated and its absorbance was measured at 532 nm (17).

Histopathological studies

The liver and stomach from all groups were preserved in 10 % formalin and studied for morphological changes after staining with Hematoxylin and Eosin.

Gc-Ms Analysis of P. Flavidum

GC-MS analysis of aqueous methanol extract of leaves of *P. flavidum* was performed as described previously (18, 19). Briefly, capillary column (0.25µm, 30m x 0.25mm) was used. As a carrier, helium gas was preferred. Velocity flow of column was set to 1 ml/min by using split less mode and 0.5 µL injection volume. First, the oven temperature was set at 110°C for 2 minutes then increased at a rate of 10°C per minute until the temperature reached to 200°C. After that the temperature was decreased at a rate of 5°C per minute until the temperature reached to 280°C per minute. Final temperature was maintained and set at 280°C. 70 eV ionizing voltage was used for MS and 200°C was set for quadrupole analyzer. The mass to charge range was set at 20 to 800.

Statistical Analysis

Data were expressed as mean ± standard error of mean, and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied. $P<0.05$ was considered as statistically significant.

Results

Phytochemical screening of plant extract

The preliminary phytochemical analysis revealed the

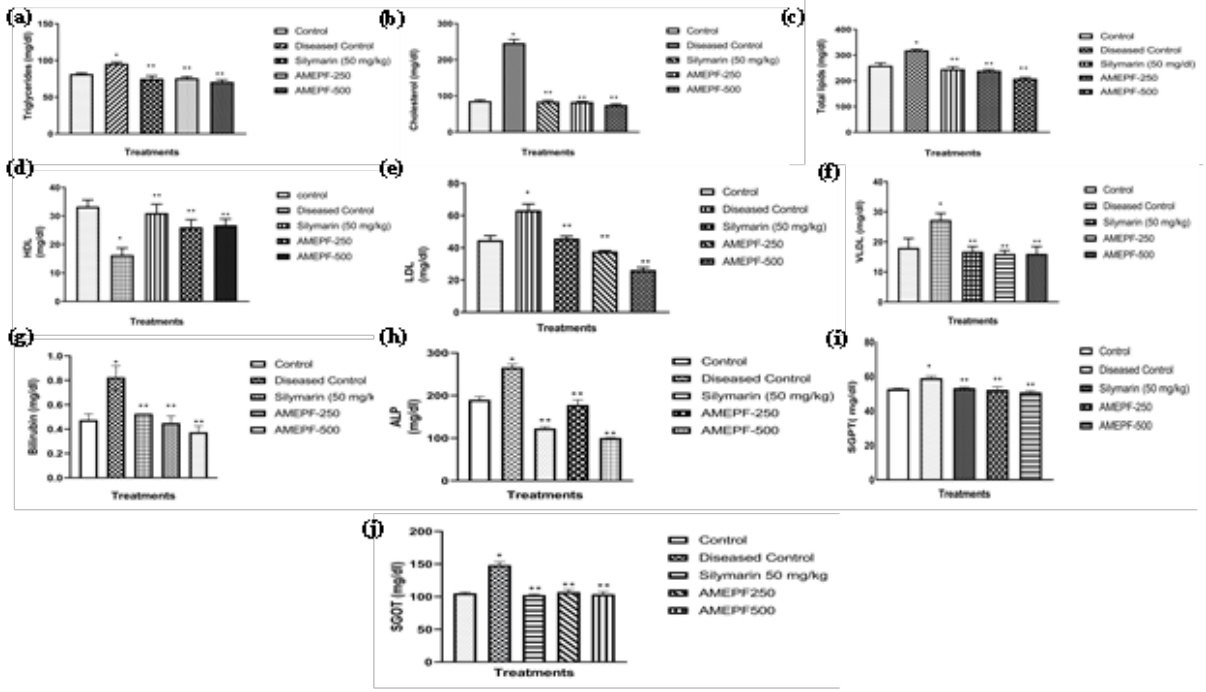

Figure 1. Effect of AMEPF (250 and 500 mg/kg) on blood lipid profile, bilirubin and liver function tests of different groups in paracetamol-induced hepatotoxicity (AMEPF; aqueous methanol extract of *Paspalidium flavidum,* HDL; high-density lipoproteins, LDL; low-density lipoproteins, VLDL; very low-density lipoproteins, ALP; alkaline phosphatase, SGPT; Serum glutamic pyruvic transaminase, SGOT; Serum glutamic oxaloacetic transaminase; n=4 where n was number of animals, $^{*}P<0.05$ compared with control, $^{**}P<0.05$ compared with Diseased control).

presence of cardiac glycosides, carbohydrates, saponins, alkaloids, phenols, flavonoids and tannins, while terpenoids and steroids were not found in the aqueous methanol extract of *P. flavidum.*

Paracetamol induced hepatotoxicity

Effect of P. Flavidum on blood lipid profile

The blood lipid profile was measured for the Control group, Diseased Control, Standard drug (silymarin) treated, and AMEPF-treated groups. The level of triglycerides, cholesterol, total lipids, low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) was increased ($P<0.05$) in the diseased control as compared with the control group. However, there was a significant decrease ($P<0.05$) in HDL in the diseased control group. Pre-treatment with silymarin and AMEPF (250 and 500 mg/kg) restored the blood lipid profile (Figure 1).

Effect of P. Flavidum on liver function tests

The liver function tests, including bilirubin, alkaline phosphatase (ALP), Serum glutamic pyruvic transaminase (SGPT) and Serum glutamic oxaloacetic transaminase (SGOT), were determined in serum. Hepatic damage caused by paracetamol elevated ($P<0.05$) levels of bilirubin, ALP, SGPT and SGOT as shown in Figure 1. However, there was a remarkable decline ($P<0.05$) in the levels of bilirubin, ALP, SGPT, and SGOT in AMEPF (250 and 500 mg/kg) and silymarin-treated groups.

Histopathological studies

The histopathological abnormalities of the liver from all five groups were studied. The liver cells appeared intact, with normal architectural integrity in the Control group, while paracetamol treatment caused necrosis, fibrosis, inflammation and morphological changes. AMEPF (250 and 500 mg/kg) treated groups represented reversion of inflammation and abnormal changes (Figure 2).

Aspirin-induced gastric ulcer

Effect of P. Flavidum on stomach pH and total acidity

The pH and total acidity of gastric contents were determined. It was observed that the pH of gastric contents had been decreased and corresponding increased total acidity

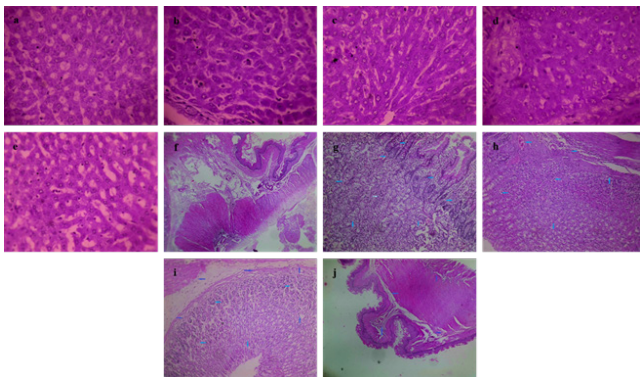

Figure 2. Histopathological studies of liver from Control, Diseased control, Standard drug and AMEPF (250 and 500 mg/kg) treated groups of rats in paracetamol-induced hepatotoxicity (a) hepatic cells appeared intact and normal in architecture in Control (b) hydropic degeneration, necrosis of hepatocytes with neutrophilic infiltration in Diseased group (c) normal parenchymal architecture with no malignancy in standard drug treated (d) mild degeneration with dilated sinusoids in AMEPF (250 mg/kg) and (e) near to normal hepatocytes, no necrosis, apoptosis or inflammation in AMEPF (500 mg/kg) treated groups of rats. Histopathological studies of stomach mucosa from different groups of rats in aspirin-induced gastric ulcer (f) intact normal mucosal linings, no inflammation or malignancy in Control (g) reddish mucosa, inflammatory cells infiltration, hyperplastic parietal cells in Diseased (h) partially healed gastric mucosa, no inflammation in Standard drug (i) mild inflammation with mild hypertrophied parietal cells in AMEPF (250 mg/kg) and (j) almost normal intact gastric mucosa with normal architecture in AMEPF (500 mg/kg) treated groups of rats (AMEPF; aqueous methanol extract of *Paspalidium flavidum*).

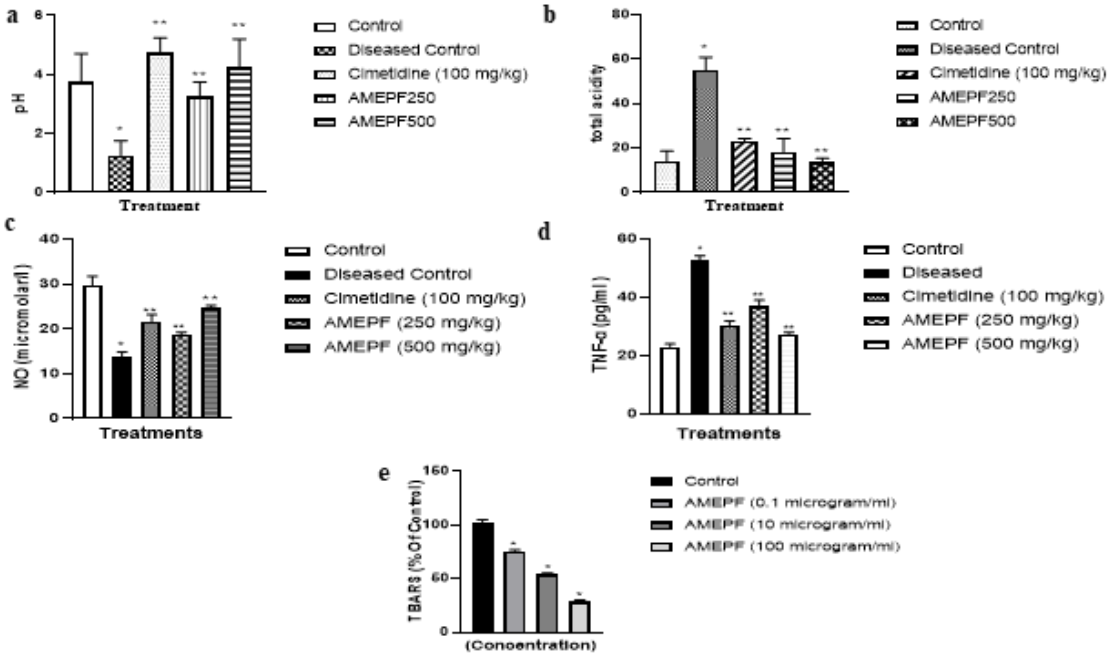

Figure 3. Determination of pH (a) and total acidity of gastric contents (b) from Control, Diseased, Standard drug and AMEPF (250 and 500 mg/kg) treated rats in aspirin-induced gastric ulcer and effect of AMEPF (250 and 500 mg/kg) on nitric oxide (c) and TNF-α (d) serum levels, and on TBARS production (e) (AMEPF; aqueous methanol extract of *Paspalidium flavidum,* n=4 where n was number of animals, $^{*}P<0.05$ compared with control, $^{\#}P<0.05$ compared with Diseased control).

($P<0.05$) in the Diseased group when compared to the Control group and pH was raised with low ($P<0.05$) total acidity in AMEPF (250 and 500 mg/kg) and cimetidine (50 mg/kg) treated groups as shown in Figure 3.

Estimation of NO and TNF- α

Treatment with aspirin decreased nitric oxide and increased TNF-α levels in blood serum which have been restored to normal by AMEPF (250 and 500 mg/kg) and cimetidine administration (Figure 3).

Determination of lipid peroxidation

The effect of AMEPF on lipid peroxidation was investigated in stomach tissue homogenates. It has been observed that AMEPF (0.1, 10 and 100 µg/ml) reduced the production of TBARS in a concentration-dependent fashion *in vitro* analysis (Figure 3).

Macroscopic evaluation of stomach

The gastric tissue samples were macroscopically examined for any change in the color of gastric mucosa, damage and hemorrhagic lesions. It was noticed that AMEPF (250 and 500 mg/kg) treatment has suppressed ($P<0.05$) gastric mucosal damage, hemorrhage and ulcer induced by aspirin (Table I).

Effect of P. Flavidum on Ulcer Index and percentage protection

Aspirin administration induced gastric damage with a high ulcer index that, with the pretreatment of AMEPF (250 and 500 mg/kg) has been reduced ($P<0.05$) as shown in Table 2. The percentage protection produced by AMEPF (50%) was comparable with cimetidine.

Histopathological studies of stomach

The histopathological studies supported our findings that groups treated with AMEPF improved aspirin induced edema, necrosis and inflammatory changes in gastric mucosa (Figure 2).

Gc-Ms Analysis of Aqueous Methanol Extract of P. Flavidum

The GC-MS analysis of aqueous methanol extract of *P. flavidum* revealed a number of phyto-components shown in Table 3 and Figure 4.

Discussion

Paracetamol induced hepatotoxicity

The goal of this study was to investigate the ability of *P. flavidum* to protect the liver against paracetamol-induced damage. Paracetamol is a well-known hepato-toxin that is commonly used in experimental cells or tissue models of hepatic injury. The intensity of hepatotoxicity has been associated with increase in enzyme levels such as AST, ALT, ALP, bilirubin, albumin, and triglycerides (20). Since managing liver diseases is difficult, and the accessible drugs to treat liver diseases have efficacy and safety limitations, there is increased interest in finding new, more potent, safer, and less expensive hepatoprotective drugs.

Table 1. Macroscopic Evaluation of Stomach. Macroscopic evaluation of stomach of rats in aspirin-induced gastric ulcer for the colour change, spot, haemorrhage, ulcer formation, and perforation (AMEPF; aqueous methanol extract of Pasalidium flavidum, n=4 where n was the number of animals, $^{*}P<0.05$ compared with Control, $^{\#}P<0.05$ compared with Diseased Control).

Parameters	Control	Diseased control	Standard drug treated	AMEPF (250 mg/kg)	AMEPF (500 mg/kg)
Ulcer score (Mean±SEM)	0.0±0.0	1.4±0.2*	0.6±0.5**	0.9±0.8**	0.4±0.8**

Table 2. Effect of *P. flavidum* on Ulcer Index.

Groups	Un	Us	Up	UI	Protection (%)
Control	0	0	0	0±0.00	0
Diseased Control	15	3	100	28±2.54*	0
Standard treated	10	2	50	17±0.49#	39.3
AMEPF (250mg/kg)	8.2	1	50	14.2±2.11#	49.3
AMEPF (500mg/kg)	8	1	50	14±1.09#	50

Calculation of ulcer index (UI) from stomach of Control, Diseased Control, Standard drug, AMEPF (250 and 500 mg/kg) treated group of rats in aspirin induced gastric ulcer (*P<0.05; compared to the normal control group, #P<0.05 compared to disease control group; n=4 where n was number of animals), (Un = average no of ulcers per animal, Us = average no of severity of scores, Up = percentage of animals with ulcer, AMEPF; aqueous methanol extract of Paspalidium flavidum).

Table 3. Different phytochemicals found in AMEPF.

Peak No.	Retention Time (min.)	Molecular weight	Molecular formula	Phyto-components
1	8.22	206	$C_9H_9F_3O_2$	2,3-dimethyl-5-(trifluoromethyl)-1,4-benzenediol
2	9.35	282	$C_{18}H_{34}O_2$	Oleic acid
3	12.58	278	$C_{16}H_{22}O_4$	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl)

Phytocomponents with their retention time, molecular weight and formulae.

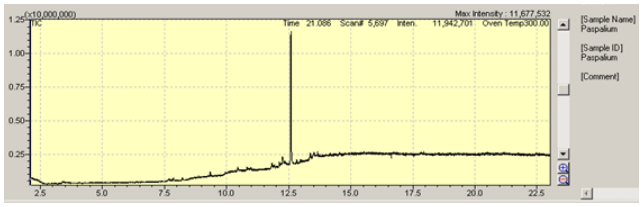

Figure 4. Gas- Chromatograph of aqueous methanol extract of *Paspalidium flavidum.*

Medicinal plants used in traditional medicine are the most promising sources of hepatoprotective agents in this regard (21). *P. flavidum* is a genus of grasses that grows as an annual to perennial weed. To determine its hepatoprotective effect, liver function tests (LFTs), plasma proteins and blood lipid profile was investigated.

When compared to control group, the diseased group had a substantial increase in serum TC, TG, VLDL and LDL, but a significant decrease in serum HDL. However, concurrent administration of paracetamol and AMEPF (250 and 500 mg/kg) lowered blood TC, TG, VLDL and LDL while significantly increasing serum HDL in diseased group. Paracetamol appears to affect lipoprotein metabolism, resulting in changes in cholesterol metabolism. The availability of free acid, slower hepatic release of lipoprotein, and enhanced esterification of free acids may all contribute to the higher blood level of TG in diseased group (22). By suppressing free radicals on hepatic cells, AMEPF revealed an esterification action, resulting in a hepatoprotective benefit. These findings are in parallel with previous studies (23)

Hepatic aminotransferases such as alanine transaminase (ALT) and aspartate aminotransferase (AST) are known as critical markers for hepatocellular damage. SGPT (also known as ALT) is an enzyme that catalyzes the conversion of alanine to pyruvate and glutamate. So, ALT is more specific to the liver and a stronger indicator of liver impairment. Serum ALP and bilirubin levels are linked to hepatic cell activity. Higher production of ALP in the presence of elevated biliary pressure causes an increase ALP in blood. Damage of hepatic cells elevates serum glutamic-oxaloacetic transaminase (SGOT) (also known as AST) level in serum (24). The level of bilirubin, ALP, SGPT and SGOT was found increased in paracetamol induced hepatic injury which was restored to normal in AMEPF (250 and 50 mg/kg) treated groups showing the hepatic cell membrane stabilizing potential of *P. flavidum*. The findings of present study agreed with previuos study (25, 26).

Histopathological findings indicated the normal, intact hepatic cells by control group. However, paracetamol caused diffuse granular degeneration, mild multifocal and periportal lymphocytic infiltration in centrizonal area. Silymarin and AMEPF (250 and 500 mg/kg) improved the morphological damage, supporting the biochemical findings of the study.

Aspirin induced gastric ulcer

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to trigger gastric mucosal apoptosis and the production of reactive oxygen species (ROS), which are considered to be the primary causes of gastric ulcer (27). Aspirin, an NSAID, binds to a site near mitochondrial electron transport chain component, the complex I and ubiquinone to produce reactive oxygen species (ROS) (28). Due to a variety of side effects, toxicity, and the high cost of currently available anti-ulcer drugs, it is necessary to find more affordable, effective, and alternative sources of treatment. The present study was also aimed to determine the gastro-protective potential of *P. flavidum* in aspirin induced gastric ulcer. The pH, total acidity of gastric contents, ulcer index and percentage protection were estimated.

Normally, pH of gastric contents of rats varies between 3.2 to 3.9 (29). In aspirin induced gastric ulcer model, pH of gastric contents of stomach from *AMEPF* (250 and 500 mg/kg) treated groups had been escalated towards more basic when compared with diseased rats. The total acidity of gastric contents of stomach from rats treated with AMEPF (250 and 500 mg/kg) and standard drug (cimetidine) was declined significantly as compared to Diseased control group. These results showed the same tendency as

in previous study (16, 30).

Aspirin, by inhibiting prostaglandins synthesis, is regarded as mucosal breaker that induces back diffusion of gastric H^+ and produces mucosal lesions. The loss of gastroprotective effect of prostaglandins against acid and pepsin leads to development of gastric lesions within a few hours after aspirin administration (31). Pre-treatment with AMEPF reduced the number of gastric lesions induced by aspirin. Ulcer index was also reduced remarkably in *P. flavidum* (250 mg/kg and 500 mg/kg) methanol extract treated groups as compared to diseased group. These findings supported that *P. flavidum* might be involved in mucosal defensive mechanisms (16, 31).

The percentage of total surface area damage of stomach linings is termed as ulcer index. It is an indicator of mean ulcer score for each animal (32). It was found that significant reduction in ulcer index and improvement in percentage protection by AMEPF in aspirin treated rats. This result might be due to the protective effect of *P. flavidum* against aspirin induced gastric ulcer.

The pathogenesis of aspirin induced gastric ulcer involves reduced prostaglandins synthesis and angiogenesis, induction of oxidative stress and inflammatory mediators such as TNF-α, and production of reactive oxygen species (ROS). ROS cause lipid peroxidation of stomach wall as well (33). In this study, rats treated with aspirin presented a rise in TNF-α with low NO levels which were normalized after treatment with AMEPF and cimetidine. Upregulated TNF-α by aspirin stimulates caspase-3 in stomach that induces mucosal injuries (34). Furthermore, it has been reported that NO is helpful is maintaining gastric mucosal integrity and has ulcer healing property via different mechanisms (35). Anti-lipid peroxidation potential of AMEPF might also be responsible to minimize the deleterious effects of aspirin on gastric mucosa.

Histological examination of the rat stomach mucosa of Control group revealed intact normal mucosal lining and folds. The submucosal glands were arranged in arrays. No inflammatory reaction or malignancy was seen. While ulcerated reddish mucosal surface with presence of punctate ulcers in submucosa was found in diseased group. There was inflammatory infiltrate in mucosa and submucosa. However, histological examination of the *P. fflavidum* extract treated group showed healed reddish mucosal lining and minimal inflammatory infiltrate in submucosa. No punctate ulcers were present. The muscle coats appeared in sequence. No inflammatory reaction or malignancy was seen.

The cardiac glycosides, carbohydrates, saponins, alkaloids, flavonoids, phenols and tannins were present in the preliminary phytochemical analysis of *P. flavidum.* These secondary metabolites might be responsible for pharmacological activities of *P. flavidum*. However, three different phytochemicals including 2,3-dimethyl-5-(trifluoromethyl)-1,4-benzenediol, Oleic acid, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) were found in AMEPF by GC-MS analysis (Table). 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) exhibited antioxidant and anti-inflammatory activities (36) while oleic acid presented stability against oxidation and enhanced anti-oxidative effect can be achieved when it is used in combination with other anti-oxidants (37). Thus, hepatoprotective and gastroprotective effects of *P. flavidum* might be due to its phytochemicals with anti-oxidant properties. There is leaf extract of many medicinal plants (38-43) that have medicinal and therapeutic effects and need to be tested.

Conclusions

It is concluded that *P. flavidum* showed hepatoprotective activity by restoring liver function tests and blood lipid profile. The gastroprotective potential of plant involved improvement in stomach histology, reducing acidity and ulcer index. This potential might be due to the anti-oxidant phytochemicals present in plant extract. Therefore, further studies are required to isolate these biologically active principles and determine their exact mechanism of action along with their pharmacokinetic parameters.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authors contribution

Sana Ismail and Ushna Shabbir; collection of data, Irfan Anjum; Conception, study design, data interpretation, Muhammad Naveed Mushtaq; Study design, data validation; Saima and Asma Razzaq; facilitated data collection, Haseeb Ahsan; data interpretation, Hafiz Muhammad Ramzan and Alina Shoukat; initial manuscript writing. All the authors have read final version of manuscript and approved its contents.

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