Antiulcerogenic, Anti-Secretory and Cytoprotective Effects of *Piper Cubeba* (L.) on Experimental Ulcer Models in Rat

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Abstract: This paper evaluated anti-gastric ulcer and anti-secretory effects of a popular spice *Piper cubeba* L, (Family: Piperaceae) in rats. The gastric ulcer protective potential of an aqueous suspension of *Piper cubeba* (PCS) was evaluated against different acute gastric ulcer models in rats induced by pyloric ligation (Shay), hypothermic restraint stress, indomethacin and by necrotizing agents (80% ethanol, 0.2 M NaOH and 25% NaCl) induced gastric mucosal injury. *Piper cubeba* aqueous suspension (PCS) at the doses 250 and 500 mg/kg body weight administered orally (intraperitoneally in Shay rat model) showed a dose-dependent ulcer protective effects in all the above models. Besides, the PCS offered protection against ethanol-induced depletion of gastric wall mucus (GWM); replenished the reduced non-protein sulfhydryls (NP-SH) concentration and significantly replenished malondialdehyde (MDA) contents in the gastric tissue. Ethanol induced histopathological lesions of the stomach wall characterized by mucosal hemorrhages and edema was reversed by *Piper cubeba* aqueous suspension treatment. Pretreatment of rats with *Piper cubeba* provided significant protection of gastric mucosa through its antioxidant capacity and/or by attenuating the offensive and by enhancing the defensive factor.

Keywords: *Piper cubeba,* Arab Traditional Medicine, antiulcerogenic, *antisecretagogue*, cytoprotective, oxidative stress.

1. INTRODUCTION

Peptic ulcer is a common digestive disease and is considered to be a major cause of morbidity and mortality [1]. It has been postulated that gastric ulcers are caused by an imbalance between defense mechanisms such as blood flow rate, mucous/bicarbonate production, and endogenous prostaglandin enzymes, and aggressive factors such as stress, hydrochloric acid, *Helicobacter pylori*, smoking, anti-inflammatory drugs, and pepsin production. Stress appears to play a major role and leads to gastric ulcer [2]. Spices, vegetables and medicinal herbs have been recognized as a source of natural remedies for the prevention and treatment of various diseases. As they are also considered to be natural antioxidants and play a key role as chemopreventive agents [3].

Piper cubeba is the flowering vine in the family Piperaceae. Cubeb *(Piper cubeba),* or tailed pepper, is a plant in genus Piper, cultivated for its fruit and essential oil. The fruits were traditionally used as stimulant, carminative, expectorant, stomachic, and also used in the treatment of gonorrhea, especially in gleet, and in the discharge present after acute prostatitis, especially if purulent in character [4]. In addition, the antioxidant activity of 16 isolated compounds from *Piper cubeba* was identified . Also, it has been reported that fruits possesses anti-inflammatory activities [5]. Further, higher free radical scavenging activity in aqueous suspension of *Piper cubeba* fruits in comparison to *Piper nigrum* was demonstrated [3].

Piper cubeba locally known as Kababa (cubeb is common name in English) is a known medicinal plant which has been used in various countries of Europe, Middle East, Arabian Peninsula, Far Eastern countries and Indian Subcontinent. Cubeb fruits are commonly used as spice and condiment. In traditional medicine of various countries, cubeb is used for the treatment of stomach ache, diarrhea, dysentery, enteritis, gonorrhea and to relieve pain and inflammatory conditions [6]. Arabian and Unani physicians use the paste of *Piper cubeba* fruit on genitals of either sex to enhance the pleasure during coitus [7]. Recently, high antioxidant activity in *Piper cubeba* ethanol extract [3] was

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ISSN: 1927-3037/13

reported. Antimicrobial and antifungal [8], nephroprotective [9], bactericidal *Helicobacter pylori* [10] activities have been reported. An antiulcer activity of cubeba methanolic extract has also been reported [11]. *Piper cubeba* ethanol extract and (-)-cubebin and its semi-synthetic derivatives have been shown to possess bacteriostatic fungistatic effects against oral pathogens [12]. Since there is no scientific data available in the existing literature on antiulcer effect of an aqueous suspension of *Piper cubeba* (PCS) (a common dosage form against Unani and Arabian Traditional Medicine), therefore, the study was undertaken to investigate the *antisecretagogue*, antiulcer and cytoprotective activities of an aqueous suspension of *Piper cubeba* (PCS) in-vivo experimental ulcer models in rat.

2. MATERIALS AND METHODS

2.1. Plant Material and Preparation of Dosage Form

Piper cubeba was purchased from a local crude drugs supplier in Riyadh. The *Piper cubeb*a was identified by an expert taxonomist; the specimen was deposited in the herbarium of the Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The shade dried Fruits of *Piper cubeba* were finely powdered (particle size 70 micron) and aqueous suspension was prepared by suspending in distilled water just an hour before the experiment.

2.2. Animal and Protocol

Wistar albino male rats of either sex approximately of the same age, weighing 180-200 ± 20 g and fed standard chow diet were used. They were divided into groups of six animals each. The distribution of animals in groups, the sequence of trials, and the treatments were randomized. The solutions of the ulcerogenic drugs and necrotizing agents were freshly prepared and the animals were killed by ether euthanasia. The stomachs were removed, opened along the greater curvature, washed with saline and examined with a 6.4 x binocular magnifier and the gastric tissues were also used for biochemical estimations and histological assessment. Lesions were also assessed by two observers unaware of experimental protocol. The animal study protocol was approved by the Research and Ethics Committee of the Experimental Animal Care Society, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

2.3. Pylorus Ligated (Shay) Rats

The animals were fasted for 36 hr with access to water *ad libitum* before the pylorus was ligated under light ether anesthesia, care being taken not to cause bleeding or to occlude blood vessels [13]. PCS administered immediately after pylorus ligation by intraperitoneal injection. The animals were sacrificed 6 hr after the pylorus ligation, stomachs were removed, and contents were collected, measured, centrifuged, and subjected to analysis for titratable acidity against 0.01 N NaOH to pH 7. Each stomach was examined for lesions.

2.4. Hypothermic Restraint Stress-Induced Ulcers

The method of [14] was followed with slight modification. The animals were fasted for 36 hr with access to water *ad libitum*. One hr after receiving PCS treatment orally, they were immobilized in restraint cages and placed inside a ventilated refrigerator maintained at a temperature of 2-4°C. After 3 hr they were taken out and sacrificed. The stomachs were excised and examined for the severity of intraluminal bleeding according to the following arbitrary scale: 0, no blood detectable; 1, thin blood follows the rugae; 2, thick blood follows the rugae; 3, thick blood follows the rugae with blood clots in certain areas; and 4, thick blood [15]. After wiping the blood off, the total area of lesions in each stomach was scored.

2.5. Indomethacin-induced Gastric Ulcers

Indomethacin was suspended in 1% carboxy-methyl cellulose (CMC) in water and administered orally to the 36 h fasted rats at a dose of 30 mg/kg body weight. Control rats were treated similarly with an equivalent amount of vehicle [16]. PCS was given 30 min prior to indomethacin administration at a dose of 250 and 500 mg/kg. The animals were sacrificed 6 h after treatment. The stomachs were excised, rinsed with normal saline and examined for ulceration.

2.6. Gastric Lesions Induced By Necrotizing Agents

Each rat was administered 1 mL of a necrotizing agent (80% ethanol, 0.2 M NaOH or 25% NaCl). PCS was given 30 min before the administration of necrotizing agents. One hour after the administration of ethanol and the alkalis, the rats were sacrificed and examined for stomach lesions. The scoring of stomach lesions was as follows: Patchy lesions of the stomach induced by ethanol and hypertonic solutions were scored according to the method described by [17] using the following scale: 0 = normal mucosa; 1 = hyperemic

mucosa or up to 3 small patches; 2 = from 4 to 10 small patches; 3 = more than 10 small or up to 3 medium-sized patches; 4 = from 4 to 6 medium-sized or up to 3 large patches; 6 = from 4 to 6 large patches; 7 = from 7 to 10 large patches; 8 = more than 10 large patches or extensive necrotic zones. "Small" was defined as up to 2 mm across (max. diameter), "medium-sized" between 2 and 4 mm across and "large" more than 4 mm across.

2.7. Determination of Gastric Wall Mucus (GWM)

Gastric wall mucus was determined according to the modified procedure of [16]. The glandular segment of the stomach was separated from the rumen of the stomach, weighed, and transferred immediately to 10 mL of 0.1% w/v Alcian blue solution (in 0.16 mmol/l sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for 2 h in Alcian blue, and excess dye was removed by two successive rinses with 10 mL of 0.25 mmol/L sucrose, firstly after 15 min and then after 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mmol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract were then vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 rpm /min for 10 min and the absorbance of the aqueous layer was recorded at 580 nm. The quantity of Alcian blue extracted per gram of wet glandular tissue was then calculated.

2.8. Estimation of Non-Protein Sulfhydryl (NP-SH) in Gastric Tissue

Gastric mucosal non-protein sulfhydryls were measured according to the method of [19]. The glandular part of the stomach was homogenized in ice-cold 0.02 mmol/L ethylenediaminetetraacetic acid (EDTA). Aliquots of 5 mL of the homogenates were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10 min and centrifuged at 3000 rpm/min. Two milliliters of supernatant were mixed with 4 mL of 0.4 mol/L Tris buffer at pH 8.9. 0.1 mL of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was measured within 5 min of DTNB addition at 412 nm against a reagent blank.

2.9. Estimation of Malondialdehyde (MDA) in Gastric Tissue

The method reported by Utely *et al.* [20] was followed. The animals were killed 1 h after ethanol administration. The stomachs were removed and each was homogenized in 0.15 mol/L KCl (at 4°C) in a Potter-Elvehjem type C homogenizer to give a 10% w/v homogenate. Aliquots of homogenate 1 mL in volume were incubated at 37°C for 3 h in a metabolic shaker. Then 1 mL of 10% aqueous TCA was added and mixed. The mixture was then centrifuged at 800 g for 10 min. One milliliter of the supernatant was removed and mixed with 1 mL of 0.67% 2-thiobarbituric acid in water and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 mL distilled water. The absorbance of the solution was then read at 535 nm. The content of malondialdehyde (nmol/g wet tissue) (index of the magnitude of lipid peroxidation) was then calculated, by reference to a standard curve of malondialdehyde solution.

2.10. Histopathological Evaluation

Gastric tissue samples were fixed in neutral buffered formalin for 24 h. Sections of gastric tissue were histopathologically examined to study the ulcerogenic and/or anti-ulcerogenic activity of PCS. The tissues were fixed in 10% buffered formalin and processed using a tissue processor. The processed tissues were embedded in paraffin blocks and sections about 5 μm thick were cut using an American optical rotary microtome. These sections were stained with haematoxylin and eosin using routine procedures [21]. The slides were examined microscopically for pathomorphological changes such as congestion hemorrhage, edema, and erosions using an arbitrary scale for severity assessment of these changes.

Statistical Analysis

Values in tables and figures are given as mean ± SE. Data were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests.

3. RESULTS

An increased accumulation of gastric secretory volume, titratable acidity and ulceration in 6 hr., pylorus ligated Shay rats have shown significant inhibition of gastric secretory volume, acidity and ulceration in the animals treated with *Piper cubeba* (PCS) evident by marked decrease in volume of gastric content (mL), titratable acid (mEq/L), and ulcerative index as shown in Table **1**. The results obtained were statistically significant.

Animals subjected to restraint plus cold for 3 hr showed the presence of considerable ulcerogenicity as

Table 1: Effect of *Piper cubeba* Suspension on the Volume of Gastric Secretion, Titratable Acidity and the Degree of Ulceration in 6-hr Pylorus Ligated (Shay) Rats

Treatment	Dose (mg/kg, i.p.)	Mean ± S.E.		
		Volume of gastric content (mL)	Titratable acid (mEq/L)	Ulcer index
Control	–	4.91 ± 1.16	137.22 ± 2.00	0.66 ± 0.33
PCS	250	1.41 ± 0.52*	127.49 ± 2.84	0.00 *
PCS	500	1.58 ± 0.95*	1.66 ± 1.00***	00***

Six animals were used in each group. **P < 0.05; **P < 0.001. ANOVA, followed by Dunnett's multiple comparison tests.

indicated by ulcerative index (18.16±1.04) in the form of hemorrhagic mucosal lesions in the stomach, which were confined to the glandular segment only the intraluminal bleeding score was about (1.50±0.2) in case of control. Treatment with *Piper cubeba* 250 mg/kg and 500 mg/kg produced a significant and dose-dependent inhibition of ulceration index and intraluminal bleeding score. PCS 250 mg/kg treated rat showed slight reduction in intraluminal bleeding score and ulceration index which is respectively 1.33±0.33 and 13.66±0.42** as compared to control while PCS 500 mg/kg which showed marked reduction in intraluminal bleeding score and ulceration index which is respectively 10.00±1.18***, and 0.50±0.22* results were statically significant (Table **2**).

Administration of indomethacin resulted in the production of gastric lesions mainly in the glandular segment of the stomach of rats its ulcerative index was 26.83 ± 6.99. Pretreatment of animals with PCS 250 mg/kg and 500 mg/kg decrease the intensity of gastric mucosal damage induced by indomethacin as indicated by ulcerative index (6.33 ± 2.84**) in higher dose group (Table **3**). However, in the lower dose (250 mg/kg) group the protection was not statistically significant.

Necrotic patches of the stomach, induced by noxious chemicals were found to be significantly reduced in the groups of animals pretreated with PCS at both doses, as indicated by ulcerative index (Table **4**).

Lowered gastric wall mucus was observed in the animals treated with 80% ethanol and this depletion of wall mucus was significantly reversed by pretreatment with PCS (Figure **1**).

As depicted in Figure **2**, MDA levels in the gastric mucosa used as an index of lipid peroxidation were significantly lower in the group treated only with ethanol than in the untreated control group. PCS at the dose of (500 mg/kg) significantly replenishes the MDA content of the gastric tissue.

As depicted in Figure **3**, the gastric mucosal NP-SH contents were significantly lower in the group treated

Table 2: Effect of *Piper cubeba* Suspension on Hypothermic Restraint Stress- Induced Intraluminal Bleeding and Gastric Lesion in Rats

Treatments	Dose (mg /kg)	Gastric lesion ulcer index	Intraluminal bleeding score
Control(Distilled water)	—	18.16±1.04	1.50±0.2
PCS	250	13.66±0.42**	1.33±0.33
PCS	500	10.00±1.18***	0.50±0.22*

Six rats were used in each group. *P < 0.05,** P < 0.01,***P < 0.001. ANOVA, followed by Dunnett's multiple comparison tests.

Table 3: Effect of *Piper cubeba* Suspension on the Gastric Mucosal Damage Induced by Indomethacin in Rats

Treatment	No. of Animals	Dose (mg/kg, p.o.)	Ulcer Index (Mean ± S.E.)
Control	6	–	26.83 ± 6.99
PCS	6	250	10.33 ± 5.01
PCS	6	500	6.33 ± 2.84**

Six rats were used in each group. *P < 0.05,** P < 0.01,***P < 0.001. ANOVA, followed by Dunnett's multiple comparison tests.

Table 4: Effect of *Piper cubeba* Suspension on the Gastric Lesions Induced by Various Necrotizing Agents in Rats

Treatment (n = 6)	Dose (mg/kg, p.o.)	Ulcer index (Mean ± S.E.)		
		80% EtOH	0.2M NaOH	25% NaCl
Control	–	7.83 ± 0.33	7.83 ± 0.16	7.80± 0.20
PCS	250	7.00 ± 0.63***	4.60 ± 0.55***	4.50 ± 0.61***
PCS	500	2.60 ± 0.67***	3.66± 0.47***	2.16 ± 0.30***

Six rats were used in each group. *P < 0.05,** P < 0.01,***P < 0.001. ANOVA, followed by Dunnett's multiple comparison tests.

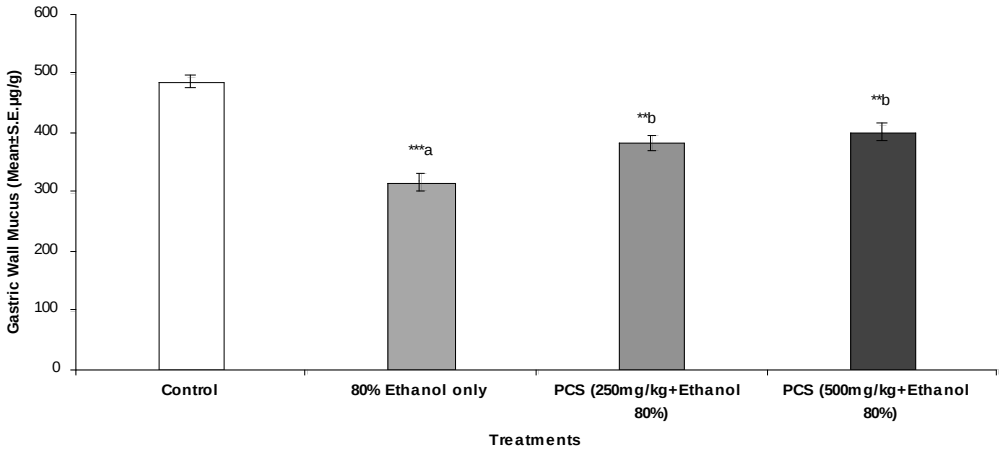

Figure 1: Effect of *Piper cubeba* suspension on gastric wall mucus concentration in gastric ulcer induced by 80% Ethanol. All values represent mean ± SEM. **p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test.
[a]As compared with control group.
[b]As compared with 80% ethanol group.

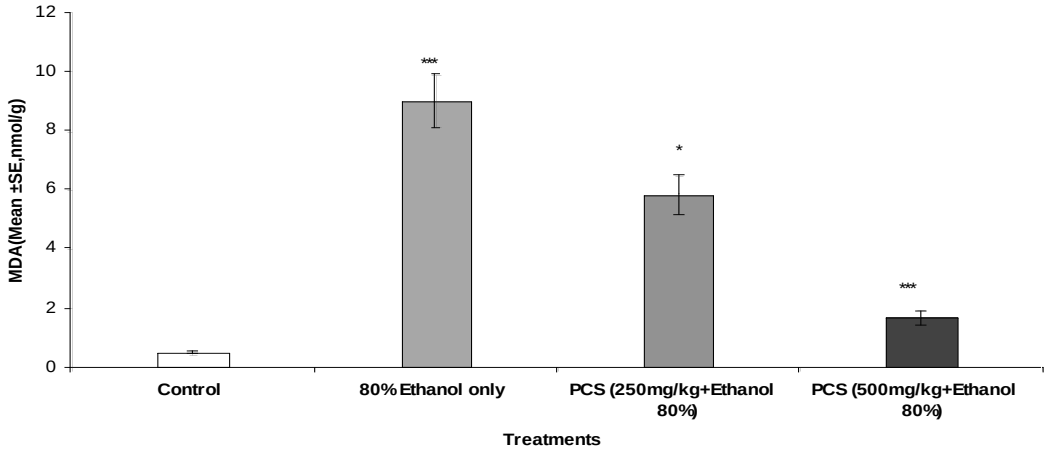

Figure 2: Effect of *Piper cubeba* suspension on MDA concentration in gastric ulcer induced by 80% ethanol
All values represent mean ± SEM. *p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test.
[a]As compared with control group.
[b]As compared with 80% ethanol only group.

only with ethanol than in the untreated control group. PCS at the dose of (500 mg/kg) significantly increased the gastric mucosal NP-SH contents therefore the pretreatment of PCS significantly reversed the depletion of NP-SH content in gastric tissue.

The histopathological results on gastric tissue showed that ethanol treatment caused congestion, hemorrhage, edema, necrosis, inflammatory changes, mucosal erosion and ulceration. Pretreatment with PCS showed marked reduction in all indices in dose

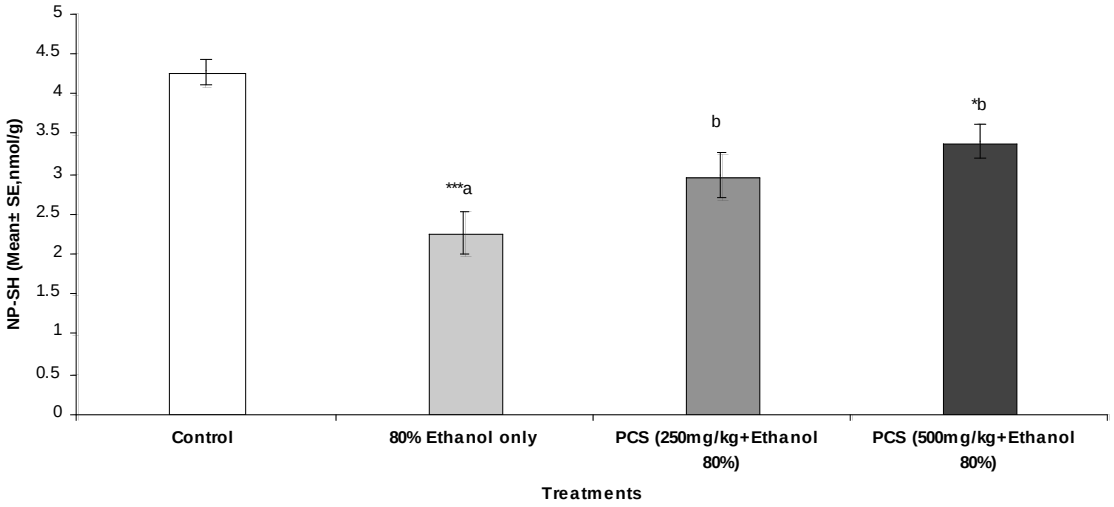

Figure 3: Effect of *Piper cubeba* suspension on NP-SH concentration in gastric ulcer induced by 80% Ethanol. All values represent mean ± SEM. *p<0.01; ***p<0.001; ANOVA, followed by Dunnett's multiple comparison test.
[a]As compared with control group.
[b]As compared with 80% ethanol only group.

Table 5: Effect of PCS on Ethanol-Induced Histopathological Legions Mucosa of Rats

Treatment and dose (mg/kg bw/day)	Histopathological Lesions induced by 80% ethanol							
	Congestion	Haemorrhage	Edema	Necrosis	Inflammatory changes	Dysplastic changes	Mucosal erosion	Ulcerations
Control (distilled water) – (1mL/rat)	-	-	-	-	-	-	-	-
Ethanol 80% (1mL/rat)	++	++	++	++	+++	++	+++	++
PCS 250+ Ethanol 80% (1mL/rat)	-	-	+	+	+	-	+	-
PCS 500 Ethanol 80% (1mL/rat)	-	-	-	-	-	-	-	-

- = Normal, + = Moderate effect, ++ = Severe effect, +++ = Intensely Severe effect.

dependent manner (Table **5**) which further confirmed that pretreatment with PCS possess reduces the intensity of ethanol-induced various indices of the gastric mucosa.

4. DISCUSSION

The results of the current study clearly indicate that the *Piper cubeba* suspension (PCS) pretreatment produced a significant anti secretory, antiulcer and cytoprotective effect in rats. The significant reduction in basal gastric acid secretion and ulceration by PCS after pyloric ligation indicates towards an ulcer preventive property of the suspension [22]. Furthermore, gastric acid is known to be an important factor in the formation of gastric lesions by pylorus ligation [13]. Various factors contribute to regulate gastric acid secretions including vagus activity, histaminergic, cholinergic, proton pump and post synaptic receptors [23]. The obtained results clearly demonstrate that the PCS suppressed the aggressive factor, the gastric acid secretion. The suspension exerted antiulcerogenic effect that may be related to the antisecretory action of the acid is the major cause in developing gastric ulcers [24]. Our findings are in agreement with earlier reports in which the methanolic extract of *Piper cubeba* was found to decrease gastric acid secretions and ulceration through its potent antisecretory activity, but this antisecretory effect may not be the sole factor responsible for its antiulcerogenic activity [25].

Cold plus restraint stress is commonly used as an experimental model to inflict acute stomach injury in rats [14, 26] because of its reliability and reproducibility [27]. Enhanced gastric acid secretions [28] disturbance in microcirculation of gastric mucosa [29] impairing gastric acid secretions and motility [30], are believed to

be the pathogenic factor in the formation of stress induced gastric lesions, which develop as a result of vagus nerve stimulation which causes the promotion of gastric acid secretion [28]. These phenomena are often termed the aggressive factor [31]. Treatment of animal with the PCS inhibited the formation of stress induced gastric ulceration further supporting that the PCS may strengthen the gastric mucosal defensive factors. The observed antiulcer activity in this model might be attributed to the antiscretagogue effect of the cubeba suspension. The PCS treated rats were found to prevent ethanol induced gastric wall depletion. The cubeba suspension restored the depleted wall mucus. Alcian blue dye is capable to bind negatively charged materials in the stomach. The increment in the concentration of alcian blue suggested the protective effect of orally administered PCS. This protective effect may be *via* the generation of protecting complexes between the PCS and mucus coat, which provides a shield against noxious agents introduced to the gastric mucosa [32, 33]. The observed gastric mucosal protection by PCS treatment may be partly due to the improvement in mucus content which might play a role in ulcer prevention.

PCS showed a significant reduction in gastric mucosal damage induced by indomethacin. Indomethacin is a well-known gastric mucosal barrier breaker [34]. It acts by inhibiting the prostaglandin biosynthesis [35–37], decreasing gastric cyclooxygenase activity [38] and increasing acid secretion [39] and gastric mucosal erosions and ulceration [40]. On the other hand an increase in certain endogenous prostaglandins can provide strong gastric mucosal resistance against ulcerogenic agent such as non-steroidal anti-inflammatory drugs (NSAIDs) including indomethacin [41]. In the present study cubeba suspension was found to produce significant diminution of gastric mucosal injury induced by indomethacin, indicating probable local increase in prostaglandins biosynthesis. The improvement in mucus content might play a role in ulcer prevention along with prostaglandin mediation, which cannot be ruled out [42]. Furthermore, PCS has also shown the ability to prevent gastric lesions induced by noxious chemicals including 80% ethanol and strong alkalis. Ethanol can affect gastric intramucosal mucus either by mobilizing it through its biochemical property or by inducing structural damage to the glandular mucosa and oxidative stress which results in gastric mucosal damage [43, 44]. It has been found that oxygen-derived free radicals are implicated in the mechanism of acute and chronic ulceration in the gastric mucosa [45] and scavenging of these free radicals can play an appreciable role in healing these ulcers. In this study the treatment of rats with *Piper cubeba* significantly decreased the ethanol induced elevated concentration of MDA; an end product of lipid peroxidation caused by a free radical mediated injury in gastric tissue. This finding further confirms that PCS possesses an antioxidant potential [46, 47]. Non protein Sulfhydryl (NP-SH) is thought to be involved in protecting gastric mucosa against various chemical agents [48]. Our observations showed a significant reduction in the NP-SH content of gastric mucosa after 80% ethanol administration. However, pretreatment with PCS prevented this depletion. An elevated NP-SH level is reported to protect gastric damage against various noxious chemicals [49]. These findings clearly showed the possible involvement of NP-SH in the ulcer protective potential is through the antioxidant properties of PCS, thus, PCS treatment appears to strengthen the mucosal barrier, which is the first line of defense against endogenous and exogenous ulcerogenic agents. Gayatri *et al.* [3] showed that the *Piper cubeba* to contain an appreciable amount of monoterpenes, sesquiterpenes, glycosides, alkaloids, tannins, phenolics and other principal secondary metabolites in ethanolic extract of *Piper cubeba.* Monoterpenes such as α-Pinene, β-Pinene, (-)-Limonene and 1,8–Cineole are known antioxidants. Piperine other constituent of *Piper cubeba* also possess anti-inflamatory, antiulcerogenic; anti-secretory as well as antioxidant properties.

The present study establishes the antiulcerogenic, anti-secretory and cytoprotective properties of PCS, substantiates its use against gastric disorders in Unani and Arab traditional medicine. The effects of PCS are possibly PG-mediated and/or through its free radical scavenging and anti-secretory properties. PCS also exhibited its protective effect on various histopathological indices, which further supports its antiulcer properties [22, 25].

5. CONCLUSION

The present observations demonstrate that the gastro protective efficacy of the *Piper cubeba* are probably due to its antisecretory and antioxidant nature by which it strengthens mucosal defensive factor and that the role of prostaglandin mediation cannot be avoided.

ACKNOWLEDGEMENT

The authors are thankful to Research Center of College of Pharmacy, King Saud University and the

Deanship of the Scientific Research, King Saud University Riyadh, Saudi Arabia for their support.

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Received on 24-11-2013 Accepted on 04-12-2013 Published on 31-12-2013

DOI: http://dx.doi.org/10.6000/1927-3037.2013.02.04.4