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UTILIZATION OF *MORUS NIGRA* L. LEAVES IN PRODUCTION OF FUNCTIONAL PUDDING FOR GASTRIC ULCER PROTECTION

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ABSTRACT

This study was aimed to investigate the possible gastroprotective efficacy of ethanolic extract of leaves of *Morus nigra* (EELMN) against ketoprofen-induced gastric ulcer in rats compared with omeprazole, as well as explore the possibility of incorporate EELMN in a milk pudding for the development of formulated food. Methods: Phytochemical screening and oral acute toxicity test of *Morus nigra* leaves were determined. Rats were pretreated with EELMN by two doses (250 and 500 mg/kg.day⁻¹, p.o.) or omeprazole prior to ketoprofen. The milk formulated pudding was used to study the changes in dynamic viscosity, rheological behavior, texture properties and sensory evaluation as a result of using different concentrations of EELMN. Results: EELMN exhibited a radical scavenging activity. Acute toxicity test reveals the safety profile of EELMN. Pretreatment with EELMN significantly reduced the elevation in volume and acidity of the gastric juice, ulcer score, pepsin, H⁺/K⁺-ATPase, myeloperoxidase, TNF- α and lipid peroxide, which were detected in ketoprofen group. In addition, mucus, prostaglandin E, interleukin-10,

nitric oxide and antioxidant defense system were significantly preserved in the EELMN-treated groups. These findings suggested that EELMN is a nontoxic, natural antioxidant with dose-dependent gastroprotective potential attributed to the presence of bioactive phytochemicals, which mediated the preserving of the mucosal natural defense equilibrium mechanisms. Furthermore, significant effects were observed in the texture and rheological parameters of milk pudding as formulated food, while non-significant changes were observed in the most sensory parameters. Conclusion: *M. nigra* leaves are a promising source of natural products for enhancing mucosal protection and reducing the risk developing of gastric ulcer in rats. The milk pudding was demonstrated as a formulated food to prepare a new functional food. However, further investigations especially clinical trials are required to investigate the efficacy of EELMN as functional foods for patients with long-term NSAID therapy.

Key words: anti-inflammation; antioxidant; custard; functional food; gastric ulcer; *Morus nigra*; NSAID; prostaglandin E2; pudding; rheology

INTRODUCTION

It was well documented that the first protective barrier for digested food and xenobiotics, gastric mucosal tissue. Ketoprofen, aspirin, indomethacin as well as diclofenac sodium, which are belonged to nonsteroidal anti-inflammatory drugs (NSAIDs), are the first choice for inflammatory diseases. However, their long time usages are associated with gastric ulcers (**Cheng et al., 2017**). Ketoprofen, a widely used over the counter (OTC) drug and represents one of the most common factors causing oxidative damage of the gastrointestinal mucosa in humans (**Drini, 2017**).

Gastric ulcer is a widespread gastrointestinal disease in the world, characterized by rupture of mucosal integrity. *Helicobacter pylori* infection, prolonged use of NSAIDs, alcohol consumption, and inadequate diet, play important role in the development of the gastric ulcer (**Nesello et al., 2017**). Proton pump inhibitors as omeprazole or antagonists of the type 2 histamine receptors, and other clinical drugs are widely used to management of duodenal and gastric ulcers. However, these drugs are associated with harmful side effects, including osteoporotic fracture, renal damage, rhabdomyolysis,

anemia, and deficiencies of vitamin B12. In addition, proton pump inhibitors are associated with poor ulcer healing quality and in turn ulcer recurrence (Yu *et al.*, 2017). Therefore, alternative antiulcer therapies are required.

A large amount of evidence has demonstrated that herbal extracts have shown to be an excellent choice as adjuvants, to prevent or promote an effective cytoprotective effect due to the multiple activities. They can exert simultaneously as antibiotic, anti-diabetic, antiulcerogenic, anti-inflammatory, antioxidant, and cytoprotective (El Hawary *et al.*, 2016a; Abd El-Fattah *et al.*, 2017; Escobedo-Hinojosa *et al.*, 2018).

Morus nigra, (*M. nigra*) known as black mulberry, is one of the most important and species of the genus *Morus*, Family: *Moraceae*. It has juicy fruits with extraordinary color and unique, slightly acidic flavor. Mulberry leaves are abundant at low cost and traditionally used as antioxidant, hypocholesterolemic, antidiabetic, anticancer, antimicrobial, and nephroprotective agents (Mahesh *et al.*, 2017).

Functional foods containing some health-promoting compounds beyond traditional nutrients, play an important role in enhancing human health, and reduce the risk of various diseases (Küster-Boluda and Vidal-Capilla, 2017). Milk pudding is consumed in an almost daily basis worldwide by several groups of consumer. It was observed that stability, rheological behavior, and textural properties of milk pudding are changed by the addition of polysaccharides (Pang *et al.*, 2015). Also, other investigators was confirmed that milk pudding supplemented with dietary fiber significantly improved its nutritional value as well as rheological and textural properties (Zheng *et al.*, 2017). Therefore, the present study was conducted to investigate the gastroprotective efficacy of EELMN in ketoprofen-induced gastric ulcer rat's model compared with omeprazole as a reference anti-ulcer drug. Furthermore, the efficacy of EELMN for development of functional pudding was investigated in regarding to its physicochemical, sensory, and nutritional properties.

MATERIALS AND METHODS

1. Collection and Preparation of the Leaves Extract

Fresh leaves of *M. nigra* were collected from National Organization for Drug Control and Research (NODCAR), Giza, Egypt, in April 2015. Leaves were weighted, dried in an oven at 50 °C for 48 h. the dried samples were ground and passed through a sieve (20 mesh), and stored in a refrigerator until used. The powdered leaves (1 Kg) were exhaustively extracted with 70% ethanol (6 L). The combined extract was filtered by Whatman filter paper No.42, evaporated with a rotary evaporator at 40 °C under reduced pressure, dried, labeled as EELMN (Ethanolic Extract of Leaves of *M. nigra*), and stored at 4 °C until used.

2. Chemicals

All chemicals were of analytical grade and purchased from Sigma Chem. Co., (St. Louis, MO, USA), Merck (Germany), and El Nasr Pharmaceutical Chemicals Co. (Egypt).

3. *In-vitro* Study

3.1 Preliminary phytochemical screening:

Phytochemical screening of EELMN was performed according to the methods of Association of Official Analytical Chemists (AOAC, 2016).

3.2 Determination of total phenolic and flavonoids contents:

Total phenolic content of EELMN was assayed using Folin-Ciocalteu's reagent (**Singleton et al., 1999**), and expressed as mg of gallic acid equivalents (GAE) per gram of dried extract according to the equations obtained from the calibration curve of gallic acid ($y=0.008x + 0.0007$, $R^2=0.9975$) Meanwhile, total flavonoid content of EELMN was conducted according to the aluminum chloride calorimetric method (**Sharma et al., 2014**), and expressed as mg of quercetin equivalents (QE) per gram of dried extract using the equations obtained from the calibration curve of quercetin ($y=0.0244x + 0.0094$, $R^2=0.9976$).

3.3 Radical scavenging potential

The antioxidant activity is expressed as radical scavenging potential which was evaluated by determining the ability of EELMN to inhibit 2,2'-diphenyl-1-picrylhydrazyl (DPPH[•]) radical (**Blois,**

1958). The % inhibition of DPPH[•] by different concentrations (0-100 µg/mL) of the EELMN was comparing with respect to ascorbic acid as antioxidant reference drug, respectively. All measurements were done in triplicate and the % inhibition expressed as IC₅₀. The IC₅₀ value was defined as the concentration of the EELMN or reference agent that exhibit 50% inhibition to DPPH. The lower IC₅₀ value indicates the higher activity.

4. In-vivo Study

4.1 Animals

Female Sprague Dawley rats weighing 180±20 g were provided from animal house of National Organization for Drug Control and Research (NODCAR), Giza, Egypt. Animals were housed in cages under standard conditions of a 12/12 hours dark/light cycle, temperature 25±2°C and relative humidity. They were allowed free access to water and food, and left for a week for acclimatization. The experimental design was conducted in accordance with the guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 2011). The experimental protocol was approved by the Institutional Ethics Committee of NODCAR, Giza, Egypt.

4.2 Acute oral toxicity test

Acute oral toxicity study of EELMN was conducted according to the Organization for Economic Cooperation and Development guidelines no 425 (**OECD, 2001**). Overnight fasted animals were received a single oral dose of the tested extract (1000, 2000, 3000, 4000, and 5000 mg/Kg b.wt.). Then the animals were observed for morphological behavior, signs of toxicity and mortality for 24 hours.

4.3 Experimental design

Animals were randomly divided into five groups, each of eight rats as follows:

Group 1: Control group; received the vehicle (carboxymethyl cellulose 1%, in water) for 3 weeks.

Group 2: ketoprofen- induced gastric ulcer group; received a vehicle for 21 consecutive days, and on the last day ketoprofen (50 mg/Kg body weight, p.o) was administrated on 24-hours empty stomach (**Cheng et al., 2013**).

Group 3-4: EELMN-treated groups; pretreated daily with a sole concentration of EELMN (250 and 500 mg/kg/day, p.o respectively), for 21 consecutive days, and simultaneously received ketoprofen on the last day as group 2.

Group 5: Omeprazole-treated group received a freshly prepared omeprazole (Omz) at a dose equivalent to human recommended dose of 20 mg/day, p.o for 3 weeks, and simultaneously with ketoprofen on the last day as group 2 (**Paget and Barnes, 1964**).

4.4 Macroscopic analysis of the gastric mucosa

All animals were sacrificed after 24 hours of ketoprofen. Stomachs were rapidly dissected out. Stomachs opened along the greater curvature, and rinsed with normal physiological saline solution (0.9% of NaCl). Stomachs fixed on the dissection plate, photographed with a digital camera. The glandular mucosa examined with magnifying lens (x10) to quantify the ulcer lesions. The mucosal lesions were scored as follows: 0: Normal colored stomach, 0.5: Red coloration, 1: Spot ulcer, 1.5: Hemorrhagic streaks, 2: Deep ulcers and 3: Perforation. The protective efficacy of EELMN was expressed as percentage of ulcer inhibition (**Jarosz et al., 2017**).

4.5 Biochemical analysis

4.5.1 Determination of acid secretory parameters

Volume and pH

The gastric fluid contents of each stomach were collected in a graduated centrifuge tube and centrifuged at 3000 rpm for 15 minutes. Its volume was measured and expressed as ml/100g b.wt (**Sen et al., 2013**). The pH of the supernatant was measured.

4.5.1.2 Free and total acidity

Free and total acid output was determined by titrating with 0.01N NaOH, using Topfer's reagent and phenolphthalein as indicators (**Zakaria et al., 2016**) and expressed as molar equivalent (mEq)/L.

4.5.2 Determination of adhered mucus to gastric wall

The gastric barrier mucus (mucin) adhered to gastric wall was estimated spectrophotometric method (Unicam- UV-Vis-Spectrometry, Japan), (**Corne et al., 1974**), and expressed as µg of alcian blue/g glandular tissue.

4.5.3Determination of gastric pepsin activity

Pepsin activity in gastric juice was assayed according to the method of (Anson, 1938), as modified by (Chen *et al.*, 2009) and expressed as μM of tyrosine liberated/ml.

4.5.4Determination of gastric mucosal H^+/K^+ -ATPase and myeloperoxidase activities

The gastric mucosa tissues scraped from the gastric wall by a glass slide, weighed, homogenized in ice cold phosphate buffered saline and centrifuged at 3000 rpm for 15 min. The gastric mucosa H^+/K^+ -ATPase activity was measured according to the method of (Gallagher and Leonard, 1982), as modified by (Sze, 1985), and expressed as nM of inorganic phosphate liberated/min/mg protein. Meanwhile, myeloperoxidase (MPO) activity was measured by following the oxidation of O-dianisidine dihydrochloride by H_2O_2 (Bradley *et al.*, 1982), and expressed as unit per milligram protein.

4.5.5Determination of gastric mucosal Prostaglandin E_2 , inflammatory markers, and nitric oxide

The gastric mucosal level of Prostaglandin E_2 (PGE_2) was investigated according to the Biotrak enzyme-linked immunosorbent assay (ELISA) assay kit. Results were expressed as pg/mg protein. While, the mucosal concentration of Tumor necrosis factor- α ($\text{TNF-}\alpha$) and interleukin-10 (IL-10) were assayed by rat ELISA kits (R&D Systems, UK), according to the manufacturer's instructions. Mucosal nitric oxide (NO) was estimated by colorimetric method (Montgomery and Dymock, 1961).

4.5.6Determination of gastric mucosal oxidative stress

Gastric mucosal superoxide dismutase (SOD) and catalase (CAT) were determined according to the methods of Nishikimi *et al.*, (1972), and Aebi, (1984). Malondialdehyde (MDA) in terms of thiobarbituric acid reacting substances and reduced glutathione (GSH) were assayed according to Uchiyama and Mihara, (1978), and Ellman, (1959), respectively. The protein content in gastric homogenate was estimated by the method of (Lowry *et al.*, 1951).

5.Utilization of EELMN for Development of Functional Pudding

5.1Preparation of formulated pudding

The pudding was prepared by using of egg solution and whole milk at the w/w ratio of 1:2, and sucrose (15%) as the described by previous study (**Sun *et al.*, 2007**). The fresh egg solution and vanilla (1%) was hand blender for 10 min and filtered. Mix the egg solution with sugar–milk, and degassed them for 30 min. This solution was heated in boiling water bath. After cooking for 30 min, the pudding was immediately cooled in ice bath and kept in a refrigerator at $6\pm1^{\circ}\text{C}$ for analysis. Three pudding formula (A-C) were prepared including, Control (A): milk: egg (2:1 w/w) and vanilla, Products (B & C): milk: egg (2:1 w/w), vanilla, and 2.7g or 5.4 g of EELMN / 100 ml pudding that equivalent to tested dose of 250 and 500 mg/Kg body weight of rats (**Paget and Barnes, 1964**).

5.2Physical properties of formulated pudding

5.2.1Determination of Texture:

Texture profile analysis (TPA) of the tested samples were conducted with universal testing analyzer machine (Cometech, B type, Taiwan) provided with software. Back extrusion cell with 35 mm diameter compression disc was used. Two cycles were applied, at a constant cross head velocity of 1mm/s, to 30% of sample depth, and then returned. From the resulting force-time curve, the values for texture attributes, i.e. Firmness, Cohesiveness, Gumminess, Chewiness, Springiness, Resilience, and Adhesiveness were calculated.

The principle of the texture profile analysis test is compressed and decompressed a sample two times by a platen attached to the drive system. The height of the force peak on the first compression cycle was defined as hardness. The ratio of the positive force areas under the first and second compressions (A_2/A_1) was defined as cohesiveness. The distance that the foods recover diets height during the time that elapsed between the end of the first bite and the start of the second bite was defined as springiness (originally called elasticity). Two other parameters were derived by calculation from the measured parameters: gumminess was defined as the product of hardness $\times$ cohesiveness; chewiness was defined as the product of gumminess $\times$ springiness (**Bourne, 2002**).

5.2.2 Determination of viscosity:

Viscosity was determined by Brookfield rheometer viscometer spindle-TB according to standard / official methods. The results relate only to the items tested sample were taken by client. This test certificate will not be reproduced except in full without the written approval of the laboratory. Estimated uncertainty of measurement to be added was according to customer's request.

5.2.3 Sensory evaluation :

The puddings were prepared by incorporating of different concentrations of EELMN were evaluated for their sensory characteristics. Ten panelists from the staff members of NODCAR were asked to score coded samples for color (10), taste (10), clarity (10), texture (10), graiaess (10), bleeding (10) and total score (100) as previously described (**Ebeid *et al.*, 2003**).

6.Statistical Analysis

The obtained data were expressed as mean $\pm$ standard error (S.E.) of 8 animals. The results were statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test using software of Statistical Package for the Social Sciences (SPSS Statistics version 25; USA). The presence of different superscripts over the column indicates significant difference at $P < 0.05$.

RESULTS AND DISCUSSION

Usage of ketoprofen is associated with oxidative injury to the gastrointestinal mucosa (**Cheng *et al.*, 2014; Singh *et al.*, 2018**). Consequently, there is a growing interest and need to find natural source with antioxidant and antiulcer potentials from medicinal plants to provide a proper protection from gastric ulcer induced by NSAIDs (**Drini, 2017**). The present study reveals for the first time that EELMN could be consider as a promising source of natural product with potential gastroprotective effect against ketoprofen-induced gastric ulcer. Furthermore, the present study confirmed that EELMN enhances the organoleptic properties and increases the nutritional value of resultant functional pudding.

1.Phytochemical screening of EELMN

The preliminary qualitative phytochemical screening (**Table 1**) reveals the presence of a variety of naturally occurring bioactive phytochemicals in the EELMN as phenolics, flavonoids, tannins, alkaloids, and terpenoids. The presence of different kinds of bioactive compounds can act in synergism manner to perform different biological and pharmacological activities through several pathways.

Table 1. Preliminary phytochemical screening of EELMN

Constituents	EELMN
Phenolics	+
Flavonoids	+
Tannins	+
Alkaloids	+
Terpenoids	+
Saponins	-
Steroids	-
Cardiac glycosides	-
Anthraquinones	-
Volatile oils	-
(+): presence,	(-): absence

2.Total phenolic and flavonoid contents and antioxidant potential

The data depicted in **Table 2** illustrated the total phenolic and flavonoids contents of the EELMN are 84.7 mg GAE/g and 22.9 mg QE/g, respectively calculated by the equations obtained from calibration curve of gallic acid and quercetin, respectively. Furthermore, EELMN exhibited a high radical scavenging activity towards DPPH radicals that reflatess the antioxidant potential of the tested extract as compared with ascorbic acid (as standard antioxidant agent). From the results of IC₅₀ indicated that the antioxidant potential of 19.8 µg/ml of EELMN was equivalent to 12.3 µg/ml of ascorbic acid. Therefore this study suggested that EELMN could be considered as a promising natural antioxidant agent can be used as health promoting agent as well as good alternative for synthetic antioxidants in food products.

In this concept, previous studies have reported that the health promoting ability of medicinal plants is attributed to their bioactive

phytochemicals contents mainly phenolics and flavonoids (**Sokkar et al., 2013; El Hawary et al., 2016b**). Also, it has been demonstrated that lipid peroxidation causes destruction of the essential components, result in decreasing the nutritional value and formation of potentially toxic compounds with harmful effects to human health (**Pérez-Andrés et al., 2018**).

Table 2. Total phenolic and flavonoid contents and radical scavenger potential of EELMN

Total phenolics (mg GAE/g dry extract)	Total flavonoids (mg QE/g dry extract)	DPPH scavenger potential IC ₅₀ (µg/ml)	
		EELMN	Ascorbic acid
84.7 ± 1.61	22.9 ± 1.35	19.8 ± 0.72	12.3 ± 0.48

The data represent the mean ± S.E.(n=3)

3.Acute oral toxicity test

In the present study, acute oral toxicity test showed no mortalities or signs of toxicity up to a dose level of 5 g of EELMN/kg body weight confirmed the safety of the tested extract. Consequently, two doses of one twentieth and one tenth of the maximum tested dose of the EELMN that equivalent to 250 and 500 g/kg body weight, were selected to evaluation of gastroprotective efficacy of EELMN against ketoprofen-induced gastric ulcer in rats.

4.Evaluation of the gastroprotective efficacy of EELMN against ketoprofen-induced gastric ulcer in rats :

It is well established that gastric ulcer results from disturbance in the balance between the natural gastric mucosal defense system involved mucus barrier, sufficient blood flow and secretion of bicarbonate of the gastric mucosa, and aggressive factors involved gastric acid secretion, pepsin activity, and reactive oxygen species (**Cheng et al., 2017**). It was reported that the induction of gastric ulcer by non-selective NSAIDs as ketoprofen is a multifaceted process includes: 1. inhibition of the cyclooxygenase, 5-lipoxygenase and synthesis of prostaglandins, 2. overproduction of pro-inflammatory cytokines, 3. enhancement of oxidative stress (**Drini, 2017**). On the other hand, recent evidence reveals that natural products can exhibit antiulcerogenic activities through prophylactic or therapeutic or by

both ways (**Singh *et al.*, 2018**). On these concepts, the present study provide evidence that the potential role of EELMN for the prevention of ketoprofen induced- gastric ulcer in rats may be mediated by strength the gastric mucosal defense system and inhibits the aggressive factors.

4.1 Effect of EELMN on gastric mucosal defense factors :

Gastroprotective effect of EELMN was manifested from the degree of mucosal damage (ulcer score) evoked by ketoprofen intake. Macroscopic appearances of the gastric mucosa illustrated in **Figure 1(a-e)**. In agree with the previous study, untreated ketoprofen- group showed superficial and deep ulcers (**Shientag *et al.*, 2012; Cheng *et al.*, 2014**). In contrast, pretreated with EELMN significantly decreased ($P < 0.05$) the ulcer scores in a dose-depended manner as compared with untreated ketoprofen- group. Furthermore, the percentage of ulcer inhibition of rats pretreated with EELMN was not significantly different ($P > 0.05$) from that of the omeprazole group at dose of 500 mg/Kg (**Table 3**).

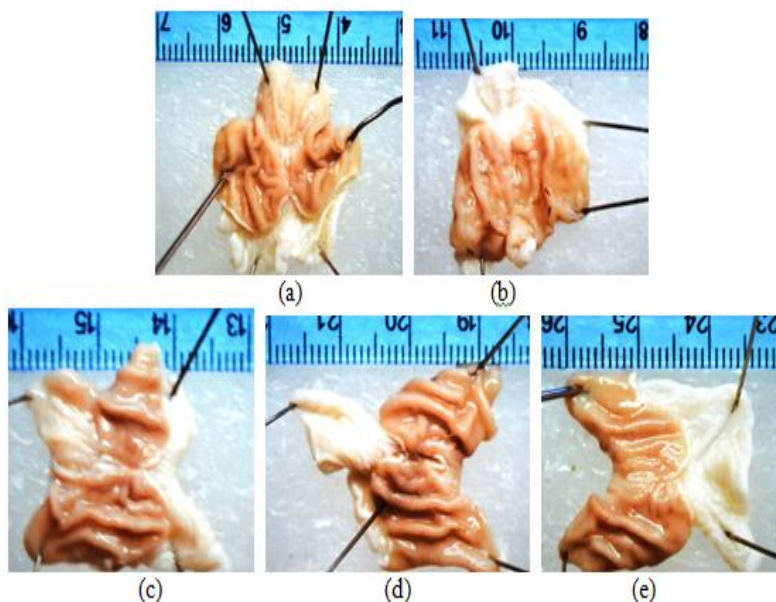


Figure 1. Photography appearance of the stomach in control and treated groups

a. Control group showing normal intact covering gastric mucosa.

b. ketoprofen- induced gastric ulcer group showing focal hemorrhagic ulcerative strcakes in mucosal surface.

c-d. EELMN treated groups showing mild to normal intact mucosal surface

e. Omeprazole-treated groups showing nearly normal intact mucosal surface.

In addition, **Table 3** shows the effect of different treatments on gastric acid secretory parameters. Ketoprofen- induced gastric ulcer group showed significant ($P < 0.05$) increase in volume, and free and total acidity of gastric juice by 124.1, 76.1, and 119.2%, respectively as compared to the control group. However, pretreatment with EELMN at doses of 250 mg/kg and 500 mg/kg resulted in a significant ($P < 0.05$) and dose-dependent decrease in the gastric volume by 21.3 and 36.7 %, free gastric acidity by 23.3 and 38.3%, and total acidity by 34 and 47%, respectively as compared to the ketoprofen- induced gastric ulcer group. These findings reveal that the high dose of EELMN (500 mg/kg b.w.) is more effective in inhibiting the gastric ulcers, which was nearly similar to that of omeprazole treated- group.

Table 3. Effect of different treatments on ulcer score, ulcer inhibition percent, and gastric acid secretory parameters

Groups	Ulcer Score (number)	Ulcer inhibition (%)	Gastric acid secretory parameters			
			Volume (ml)	pH	Free acidity (mEq/L)	Total acidity (mEq/L)
Control	---	---	1.31 ± 0.06 ^d	2.02 ± 0.13 ^a	47.4 ± 1.71 ^c	66.8 ± 1.19 ^d
Ketoprofen	13.44 ± 0.75 ^a	---	2.94 ± 0.20 ^a	1.11 ± 0.07 ^c	83.5 ± 3.13 ^a	146.4 ± 4.20 ^a
EELMN (L.D) + Ketoprofen	5.87 ± 0.52 ^b	56.3 ± 3.89 ^c	2.31 ± 0.09 ^b	1.65 ± 0.08 ^b	64.1 ± 1.82 ^b	96.8 ± 2.77 ^b
EELMN (H.D) + Ketoprofen	2.88 ± 0.24 ^c	79.1 ± 1.85 ^b	1.86 ± 0.06 ^c	1.88 ± 0.05 ^{ab}	51.5 ± 1.31 ^c	78.1 ± 2.12 ^c
Omeprazole + Ketoprofen	1.63 ± 0.26 ^c	87.9 ± 1.96 ^a	1.53 ± 0.07 ^d	1.95 ± 0.07 ^a	48.6 ± 1.47 ^c	71.5 ± 1.87 ^c

The data represent the mean of 8 rats ± S.E. The presence of different superscripts over the column indicates significant difference between means tested by a one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons test (DMCT) at $P < 0.05$. Abbreviations: EELMN: ethanolic extract of leaves of *Morus nigra*; L.D: low dose; H.D: high dose.

Also, **Figure 2** illustrated that ketoprofen- induced gastric ulcer group exhibited a significant ($P < 0.05$) decrease in the gastric mucin content by 41.6 % as compare with control group that attributed to the presence of more lesions. On contrast, oral administration of EELMN at two doses (250 or 500 mg/kg/day) or omeprazole enhanced the production of gastric mucin in dose dependent manner by 26.0, 39.2 and 52.8%, respectively as compared with ketoprofen- induced gastric ulcer group. Consistent with these results, it was demonstrated that the gastric mucosal barrier consists of protective factors mainly mucin against auto digestion of the mucosal wall by gastric acid and pepsin. In addition, the accumulated gastric juice enhances the breakdown of the mucin (a mucosal defense barrier) and formation of gastric ulcer (**Balogun et al., 2018**). By this concept, our results supported that the pathogenic effect of ketoprofen on gastric mucosal lesions is attributed to increase the secretion of gastric juice (hydrochloric acid), resulted in increased gastric volume, reduced pH, and increased free and total acidity, and treatment with EELMN strongly inhibited gastromucosal injury induced by ketoprofen. In addition, the obtained results suggested that the mechanism by which EELMN produces gastroprotective effect on gastric ulcer induced by ketoprofen might

be due to its ability to inhibit gastric acid secretion (**Cheng *et al.*, 2014; Li *et al.*, 2017**) and strengthen of the mucosal defense system (**Nesello *et al.*, 2017**). Furthermore, the productive effect of EELMN at doses of 250 and 500 mg/kg was evident by the decreased the aggressive pepsin secretion in the gastric juice by 25% and 34%, respectively. Meanwhile, administration of omeprazole was decreased the pepsin activity by 50% as compared to ketoprofen- induced gastric ulcer group.

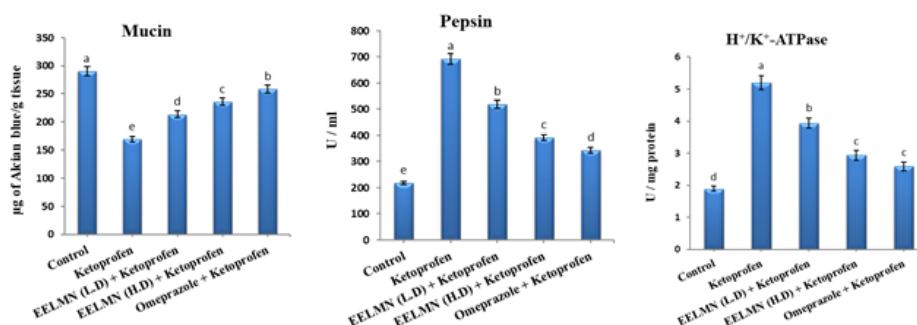


Figure 2. Effect of different treatments on gastric mucin, pepsin and H⁺/K⁺-ATPase

The data represent the mean of 8 rats $\pm$ S.E. The presence of different superscripts over the column indicates significant difference between means tested by a one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons test (DMCT) at $P < 0.05$. Abbreviations: L.D: low dose; D.D: double dose; U: One unit of H⁺/K⁺-ATPase activity is equivalent to 1 μ M inorganic phosphorus produced from ATP breaking that by 1 mg of ATP enzymes per hour.

It's well known that omeprazole is a member of proton pump inhibitors agents. This is a class of anti-secretory agents that target gastric H⁺/K⁺-ATPase in the parietal cells and block it (**Asnaashari *et al.*, 2018**). These proton pump inhibitors have been used as strong acid suppression agents in clinics for over 20 years and are mainly used in the treatment of peptic ulcer disease (**Yu *et al.*, 2017; Singh *et al.*, 2018**). In the present study, the obtained results in **Figure 2** illustrated that omeprazole inhibited the H⁺/K⁺-ATPase enzyme activity by 50%, while H⁺/K⁺-ATPase activity decreased significantly in EELMN (250 and 500 mg/kg b.w)- treated groups by 24% and 34%, respectively as compared to ketoprofen- induced gastric ulcer

group. This result supporting the hypothesis that EELMN exert gastroprotection also *via* inhibiting the proton pump function.

4.2 Effect on mucosal prostaglandin E2 and inflammatory mediators

The obtained results provide more evidences demonstrates that there are different possible mechanisms can be suggested for the gastroprotective potential of EELMN. The data depicted in **Table 4** illustrates the effect of different treatments on prostaglandin E2, TNF- α , IL-10 and MPO. It's well documented that prostaglandins are involved in the regulation of a variety of gastrointestinal functions, including blood flow, and secretion of acid, mucus, and hydrochloric acid (**Motawi et al., 2012; Singh et al., 2018**). In the current study, EELMN at two doses (250 and 500 mg/kg b.w.) manifested significant ($P < 0.05$) reduction in the aggressive gastric mucosal level of PGE2 by 29% and 51%, respectively, while significant ($P < 0.05$) decreased by 60% after administration of omeprazole as compared to ketoprofen- induced gastric ulcer group.

Also, treatment of gastric ulcer by EELMN (250 and 500 mg/kg b.w.) resulted in a significant decrease in the gastric mucosal TNF- α level by 35, and 57%, respectively, while IL-10 level increased by 44% and 61%, respectively versus to ketoprofen- induced gastric ulcer group (**Table 4**). Omeprazole caused also a significant decrease in the gastric mucosal TNF- α level by 71%, and a significant increase in the IL-10 level by 78%. These results suggested that the gastroprotective effect could well be promoted by the suppression the release of proinflammatory cytokines (TNF- α) and enhance the anti-inflammatory cytokine (IL-10). Our results demonstrating the possible control of the complex inflammatory cascade reaction to management of gastric ulcer (**El-Naga, 2015; Saiah et al., 2018**).

The data represent the mean of 8 rats $\pm$ S.E. The presence of different superscripts over the column indicates significant difference between means tested by a one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons test (DMCT) at $P < 0.05$. Abbreviations: EELMN: ethanolic extract of leaves of *Morus nigra*; L.D: low dose; D.D: double dose; PGE2: Prostaglandin E2; TNF- α : Tumor necrosis factor- α ; IL-10 interleukin-10; MPO: myeloperoxidase; One unit of MPO activity is defined as the amount of enzyme that degrades 1 μ M of peroxide per minute at 25°C.

Furthermore, MPO activity is used as a marker for neutrophil infiltration into tissues (Scoparo et al., 2016). The current study revealed that administration of omeprazole reversed the effects of ketoprofen on the activity of myeloperoxidase in the gastric mucosa and consequently decreased the level of reactive species of oxygen. Similarly, EELMN in dose (500 mg/kg b.w) but not (250 mg/kg b.w) also did that by 40%, displaying that a protective effect EELMN on myeloperoxidase activity is in a concentration-dependent manner (**Table 4**).

Various studies have focused on the role of NO in maintaining mucosal integrity (Borhade et al., 2012). Like prostaglandins, NO is a vasodilator that has been reported to increase mucosal blood flow, stimulate mucus secretion and inhibit neutrophil adherence. In animals NO-releasing NSAIDs produce less gastric damage than their parent drugs and they even promote ulcer-healing (**Fornai et al., 2011; El-Naga, 2015**). In the present study, the mucosal level of NO was decreased significantly ($P<0.05$) in ketoprofen-induced ulcer group (**Table 4**). However, pretreated with EELMN at two doses showed increase mucosal NO that exhibited a healing-promoting effect on gastric ulcers.

Table 4. Effect of different treatments on PG E2, TNF- α , IL-10, MPO, and NO

Groups	PGE2 (pg/g tissue)	TNF- α (pg/g tissue)	IL-10 (pg/mg tissue)	MPO (U/mg protein)	NO (μ mol/mg tissue)
Control	206.0 $\pm$ 12.8 ^d	56.8 $\pm$ 1.77 ^d	2.88 $\pm$ 0.11 ^a	1.24 $\pm$ 0.09 ^c	12.3 $\pm$ 0.46 ^a
Ketoprofen	559.9 $\pm$ 16.6 ^a	239.3 $\pm$ 7.48 ^a	1.55 $\pm$ 0.06 ^d	2.62 $\pm$ 0.18 ^a	6.81 $\pm$ 0.21 ^d
EELMN (L.D) + Ketoprofen	395.2 $\pm$ 14.9 ^b	156.3 $\pm$ 4.89 ^b	2.23 $\pm$ 0.09 ^c	2.22 $\pm$ 0.15 ^b	8.42 $\pm$ 0.27 ^c
EELMN (H.D) + Ketoprofen	272.6 $\pm$ 7.30 ^c	101.8 $\pm$ 3.18 ^c	2.49 $\pm$ 0.10 ^{b,c}	1.56 $\pm$ 0.11 ^c	9.92 $\pm$ 0.30 ^b
Omeprazole + Ketoprofen	223.1 $\pm$ 5.05 ^d	69.2 $\pm$ 2.16 ^d	2.76 $\pm$ 0.11 ^{a,b}	1.46 $\pm$ 0.10 ^c	10.7 $\pm$ 0.41 ^b

The data represent the mean of 8 rats $\pm$ S.E. The presence of different superscripts over the column indicates significant difference between means tested by a one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons test (DMCT) at $P < 0.05$. **Abbreviations:** EELMN: ethanolic extract of leaves of *Morus nigra*; L.D: low dose; D.D: double dose; PGE2: Prostaglandin E2; TNF- α : Tumor necrosis factor- α ; IL-10 interleucin-10; MPO: myeloperoxidase; One unit of MPO activity is defined as the amount of enzyme that degrades 1 μ M of peroxide per minute at 25°C.

4.3 Effect on gastric mucosal oxidative stress :

Multiple mechanisms appear to be involved in the induction of gastric ulcer by ketoprofen including oxidative stress. The enhanced formation of reactive oxygen species (ROS) such as hydrogen peroxide and molecular oxygen has been implicated in the development gastric ulcer (Cheng *et al.*, 2014; Abd El-Ghffar *et al.*, 2018). Previous study demonstrated that ketoprofen induced cellular damage mediated by oxidative stress as evidenced from increased the levels of lipid peroxidation and ROS associated with decreased the levels of intracellular antioxidants (Cheng *et al.*, 2013; El-Naga, 2015). In the present study, our result reveals that treatment with EELMN (250 and 500 mg/kg b.w.) not only decreased the levels of MDA by 14 and 36%, respectively, but also increased the antioxidant defense enzymes as SOD by 11 and 67%, and CAT by 12.4 and 28.7%, respectively (Figure 3). In addition, EELMN also significantly ($p < 0.05$) increased GSH by 11.7 and 32.6%, suggesting that polyphenols play an important role in preventing gastrointestinal oxidative injury (Cheng *et al.*, 2013). Similarly, omeprazole reversed the effects of ketoprofen on SOD, CAT, MDA and GSH in the gastric

mucosa. As consequence, these results suggest that EELMN provide effective protection against NSAIDs-induced oxidative injury to gastrointestinal tissue (Cheng *et al.*, 2017).

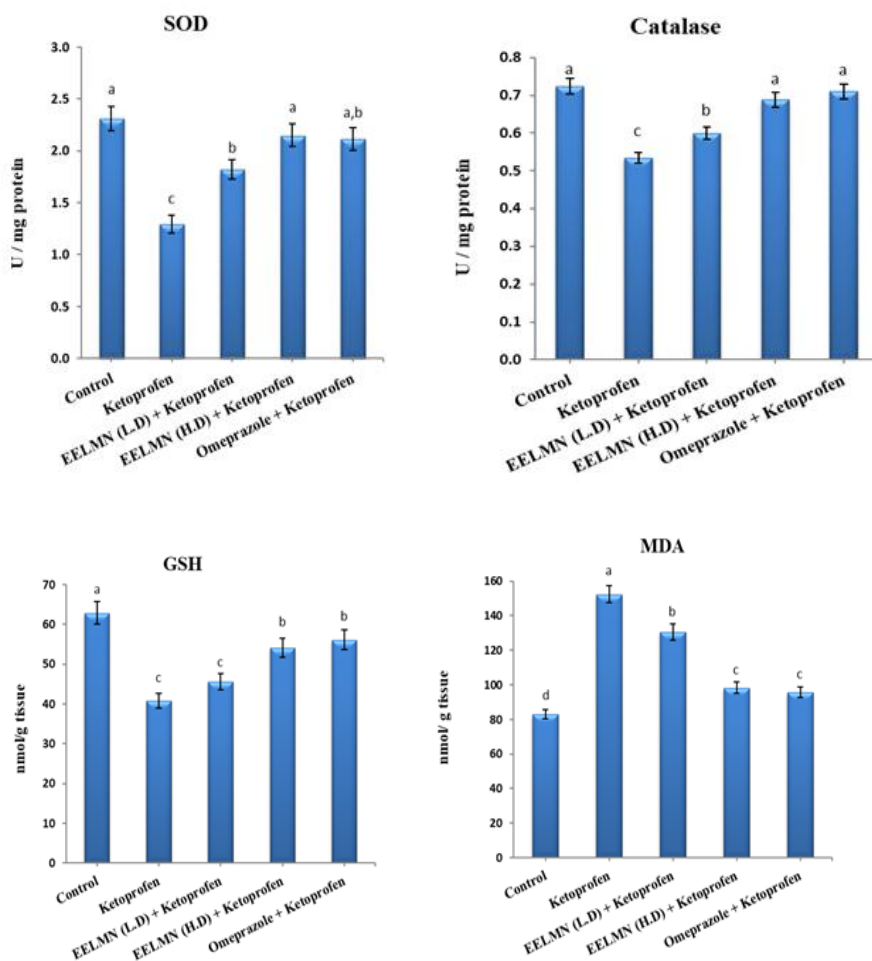


Figure 3. Effect of different treatments on gastric mucosal oxidative stress

The data represent the mean of 8 rats $\pm$ S.E. The presence of different superscripts over the column indicates significant difference between means tested by a one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons test (DMCT) at $P < 0.05$. Abbreviations: EELMN: ethanolic extract of leaves of *Morus nigra*; L.D: low dose; D.D: double dose; a: significantly different from control group; SOD: superoxide dismutase; CAT: catalase; GSH; reduced glutathione; MDA: malondialdehyde.

5. Development of Functional Pudding Contained EELMN :

In the present study, the effect of EELMN at two different ratios on physical properties of pudding (texture, viscosity and sensory evaluation) was evaluated.

5.1 Evaluation of the effect of EELMN on the Texture properties :

In the present study, the effect of different concentrations of *Morus nigra* L extract (2.7 and 5.4 g of EELMN/100 ml) on the texture profile of the prepared pudding was presents in **Table 5**. The firmness dramatically fallen by 51.24 and 61.19%, while cohesiveness increased up to 17.34 and 39.53%, the gumminess was significantly decreased by 42.61 and 46.03 % in formula B and C, respectively as compared to control (A). Also, there significant increase in springiness by 17.86 and 22.80%, while a significant decrease were observed in chewiness by 34.44 and 35.5%, resilience decreased to 70.37 and 29.01%, and adhesiveness reached to 85.52 and 80.15% as a result of the addition of EELMN at concentration of 2.7 and 5.4% respectively.

Recent study confirmed that the addition of 2 g of bamboo shoot dietary fiber /100 g effectively improved the textural and rheological properties of milk pudding (**Zheng et al., 2017**). From the obtained data, it could be said that, the addition of EELMN to the pudding caused significant ($P < 0.05$) effect on the texture profile of pudding.

Table 5. Texture properties of pudding prepared using various concentrations of EELMN

Treatments	Firmness (N/m)	Cohesiveness	Gumminess (g)	Springiness	Chewiness (ml)	Resilience	Adhesiveness (g/Sec)
A	2.010 ± 0.006 ^a	0.640 ± 0.006 ^b	1.286 ± 0.003 ^a	0.750 ± 0.031 ^b	0.990 ± 0.0009 ^a	0.162 ± 0.003 ^a	8.075 ± 0.0003 ^a
B	0.980 ± 0.006 ^b ↓ (51.24%)	0.751 ± 0.002 ^{ab} ↑ (17.34%)	0.738 ± 0.002 ^b ↓ (42.61%)	0.884 ± 0.003 ^a ↑ (17.86%)	0.649 ± 0.002 ^b ↓ (34.44%)	0.048 ± 0.002 ^a ↓ (70.37%)	1.169 ± 0.0002 ^b ↓ (85.52%)
C	0.780 ± 0.006 ^c ↓ (61.19%)	0.893 ± 0.002 ^a ↑ (39.53%)	0.694 ± 0.003 ^b ↓ (46.03%)	0.921 ± 0.003 ^a ↑ (22.80%)	0.639 ± 0.003 ^b ↓ (35.35%)	0.115 ± 0.003 ^a ↓ (29.01%)	1.603 ± 0.0004 ^b ↓ (80.15%)

The data represent the mean ± S.E. The presence of different superscripts over the column indicates significant difference between means tested by a one-way analysis of variance (ANOVA) followed by Duncan multiple comparisons test (DMCT) at $P < 0.05$. Abbreviations: A: (control); milk: egg (2:1 w/w) and vanilla, B: milk, egg (2:1 w/w) and vanilla + 2.7g EELMN/ 100ml puddings, EELMN: ethanolic extract of leaves of *Morus nigra*; C: milk, egg (2:1 w/w) and vanilla 5.4 g EELMN/ 100ml puddings.

5.2 Evaluation of the effect of EELMN on the Rheological parameters

Effect of increasing of shear rates (sec^{-1}) on the dynamic viscosity (C_p) was determined and the data are presents in (**Figure 4**). The control pudding sample had a higher dynamic viscosity value than, the other prepared samples at low shear rate. With increasing the shear rate value the behavior of control sample was changed. However, the treated pudding sample with 5.4% EELMN had the highest dynamic viscosity values at all shear rates compared to other samples. In contrary, the prepared sample with 2.7% EELMN had the lowest dynamic viscosity values at all shear rate values. But the difference between its values and those values of control sample was closed with increasing the shear rate values.

In the present study, the flow behavior index (N) determined from the slope of the double logarithmic plots of γ and σ . Consistency index (K) calculated from the same plots by reading σ value at shear rate ranging from 1.05 to 6.33 sec^{-1} . The results for the control and different studied pudding samples were given in **Table 6**. The coefficient (R^2) for the regression analyses of the log shear stress-log shear rate data was ranged between 0.7711 and 0.9818. It could be indicated, the good fit exist between the experimental data and model. The flow behavior indices (N) of the different tested samples were less than 1.0 (ranging between 0.13 and 0.29) indicating their pseudoplastic nature.

Also, the K value of the control and other prepared samples using 2.7% and 5.4% EELMN showed the highest value being 6.76. K values for sample prepared using 5.4% the control pudding sample and the prepared sample using 2.7% became in the second and third order being 5.88 and 5.01, respectively. According to the apparent viscosity data the control pudding sample and the sample prepared using 5.4% had a value 1.9 which higher than the sample prepared using 2.7% which had a value of 1.8.

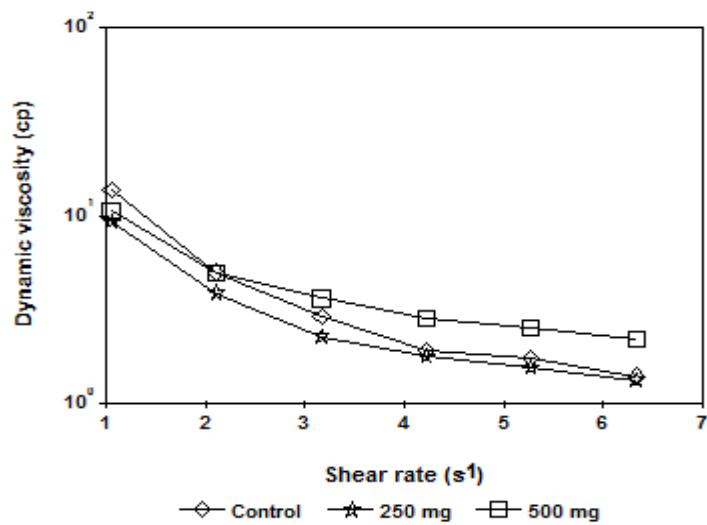


Figure 4. Flow curves of control and samples A and B
at shear rate from 1.056 to 6.330 sec⁻¹

Table 6. Consistency index (*K*), flow behavior index (*n*) and apparent viscosity (*μ*)

Treatments	<i>K</i>	<i>n</i>	<i>μ</i>	<i>R</i> ²
Control	5.88	0.23	1.9	0.7711
A	5.01	0.29	1.8	0.9818
B	6.76	0.13	1.9	0.9709

Abbreviations: A: control, milk: egg (2:1 w/w) and vanilla, B: milk, egg (2:1) and vanilla + 2.7g EELMN/ 100ml puddings
C: milk, egg (2:1) and vanilla + 5.4 g EELMN/ 100ml puddings.

5.3 Evaluation of the effect of EELMN on the sensory evaluation :

Sensory evaluation of pudding incorporate with EELMN at two levels 2.7 and 5.4% was evaluated and the results are presented in **Table 7**. It could be observed that no significant differences are found concerning taste, texture, bleeding and Graiaess among different pudding samples including control one. These findings mean that adding extract either 2.7 or 5.4% EELMN didn't affect most sensory criteria. On the other hand, color was significantly affected by addition of EELMN at the two levels ($P < 0.05$). On the other side; adding of 2.7% didn't affect the clarity of the prepared pudding. This finding proved that using 2.7 and 5.4% of EELMN did not negatively affect most of sensory characteristics of pudding, which means that it can be considered a promising ulcer protection functional food.

Table 7. Sensory evaluation of pudding prepared using two concentrations of EELMN

Pudding Formula	Sensory attributes						Total score (100)
	Color (10)	Taste (10)	Clarity (10)	Texture (10)	Graiaess (10)	Bleeding (10)	
A	9.3 ^a	8.8 ^a	9.2 ^a	9.1 ^a	8.7 ^a	9.0 ^a	72.6 ^a
B	7.7 ^b	9.0 ^a	8.5 ^{ab}	9.2 ^a	8.5 ^a	8.9 ^a	69.6 ^{ab}
C	7.7 ^b	8.9 ^a	8.0 ^b	9.0 ^a	8.4 ^a	8.4 ^a	67.9 ^b

A (control); milk, egg (2:1 w/w) and vanilla; B : milk, egg (2:1) and vanilia + 2.7g EELMN/ 100ml puddings; C : milk, egg (2:1) and vanilia 5.4 g EELMN/ 100ml puddings. Means followed by different superscripts are significant different at $P < 0.05$ between treatments.

Conclusion

Taking together, the gastroprotective effect of high dose of EELMN in ketoprofen-induced ulcers was comparable to that of omeprazole. Therefore, the promising results from this investigation are strongly confirmed that EELMN exhibited anti-inflammatory, antioxidant, and in turn gastroprotective potential. Furthermore, incorporation of EELMN in foods can be considered cost effective, as it is abundantly available throughout the year. *Morus nigra* leaves are a safe, natural gastroprotective agent that could be used in processed foods. Further studies still needed to establish the role of EELMN for patients who take NSAIDs for a long time or those unable to tolerate the undesirable effect of NSAIDs.

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Authors' Contributions

All authors had equally contributions.

Conflict of Interest

The authors declare no conflict of interest.

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استخدام اوراق نبات التوت الاسود فى تصنيع بودنج وظيفى للحماية من قرحة المعدة

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يهدف البحث الى دراسة مدى فاعلية مستخلص أوراق نبات التوت الاسود فى الحماية من حدوث قرحة المعدة الناتجة عن تناول بعض العقاقير وذلك مقارنة بعقار الاومرازول المستخدم لذات الغرض مع استخدام المستخلص النباتى كمصدر طبيعى بنسب مختلفة فى تصنيع البودنج كغذاء وظيفى يستخدم للحماية من حدوث تلك القرحة.

تم عمل تحاليل كيميائية للمجموعات الفعالة فى المستخلص النباتى (EELMN) والذى يشمل المواد الفينولية والفلافونيد الكلية والنشاط الكاسح للشقوق الحرة مع تقييم السمية الحادة عن طريق الفم وقد شملت الدراسة البيولوجية اعطاء الجرذان جرعتين مختلفتين من المستخلص النباتى (250 ، 500 ملجم / كجم من وزن جرذ) ومجموعة بعقار الأومرازول عن طريق الفم يوميا ولمدة ثلاثة اسابيع مع معاملتهم بعقار الكيتوبروفين (50 ملجم / كجم) والمسبب للقرحة . تم تقدير التغيرات البيوكيميائية المختلفة التى أظهر فيها المستخلص النباتى نشاطا جيدا فى كسح الشقوق الحرة وأيضا فى إختبار السمية الحادة أظهر امانة وسلامه استخدامه حتى جرة تصل الى 5جم/ كجم .

كما أظهرت النتائج ان معاملة الجرذان بالمستخلص النباتى أدت إلى خفض كبير فى حجم ودرجة حموضة العصارة المعدية ودرجة القرحة المعدية وكذلك نشاط إنزيم الببسين ، $H^+/K^+-ATPase$ والميلوبيروكسيديز ودليل الالتهاب ($TNF-\alpha$) ومقدار التاكسد الفوقى للدهون والتى أتضح إرتفاعهم فى المجموعة المعاملة بالكيتوبروفين . بالإضافة الى ذلك أظهرت القياسات المحافظة على كمية المادة المخاطية المبطنة لجدار المعدة ونسبة البروستاجلاندين E ومضاد الالتهاب (الانترلوكين- 10) وكذلك الحفاظ على النظام الدفاعى

لمضادات الاكسدة وذلك فى المجموعات المعاملة بالمستخلص النباتى.

كما تم استخدام المستخلص النباتى فى تصنيع البودنج كغذاء وظيفى مع دراسة التغيرات فى كلا من اللزوجة الديناميكية والسلوك الريولوجى والتقييم الحسى فى المنتج.

مما سبق ثبت أن مستخلص نبات أوراق التوت الاسود (EELMN) يعتبر أحد مضادات الأكسدة الطبيعية الامنة ، لديه القدرة على الحماية من القرع المعدية التى يسببها تناول عقار الكيتوبروفين . ويرجع هذا النشاط الى محتوى المستخلص الكحولى من الفينولات والفلافونويدات والقلويدات والتانينات التى تودى الى الحفاظ على أليات توازن الدفاع الطبيعى للمادة المخاطية المبطنة لجدار المعدة. وأوضحت هذه الدراسة امكانية استخدام هذا المستخلص كمصدر طبيعى واعد لتعزيز حماية الغشاء المخاطى والحد من مخاطر قرحة المعدة الناجم عن بعض العقاقير المسببة . كما اوضحت هذه الدراسة امكانية استخدام هذا المستخلص فى انتاج غذاء وظيفى يستخدمها فى ذات الغرض.

وأوصت هذه الدراسة بضرورة اجراء تجارب سريرية لهذه النوعية من المنتجات الغذائية لتحديد امكانية استخدامها فى الوقاية من حدوث القرع المعدية الناتجة عن العلاج بالادوية المضادة للالتهابات.