Gastroprotective Effect and Anti-Apoptotic Role of *Lawsonia inermis* Extract against Ethanol-Induced Gastric Ulcers in *Sprague Dawley*

(Kesan Gastropelindung dan Peranan Anti-Apoptosis Ekstrak *Lawsonia inermis* terhadap Ulser Gastrik Aruhan Etanol pada *Sprague Dawley*)

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ABSTRACT

Lawsonia inermis (LI) or henna has been traditionally used since antiquity for cosmetics, medicinal and healing purposes including wound healing. Even so, the proof of the gastroprotective consequences of LI is inadequate while the pharmacological mechanisms continue to be scarce. The purpose of this research was to examine the acute toxicity and gastroprotective activities of *Lawsonia inermis* L. leaves ethanol:water (80:20) extract (LILEW) in contrast to ethanol-induced gastric ulcers within rats. The acute oral toxicity experiment was performed using *Sprague Dawley* female rats (n=6) for low dose (2000 mg/kg), and high dose (5000 mg/kg) as well as vehicle (distilled water). The gastroprotective result of LILEW was performed in an ethanol-induced ulcer inside *Sprague Dawley* rats (n=6). The animals were distributed among five groups namely ulcer control, normal control, drug control-omeprazole, high dose, and low dose of LILEW (500 mg/kg and 250 mg/kg, respectively). Acute toxicity experiments with LILEW on the rats did not demonstrate any signs of mortality and toxicity up to 5 g/kg suggesting that LILEW is safe to consume. The gastroprotective effect of LILEW at doses 250 mg/kg and 500 mg/kg, as well as omeprazole (20 mg/kg), showed significant rates of inhibition as much as 70.50 \pm 1.38% (drug control), 66.67 \pm 1.42% (low dose of LILEW) and 68.45 \pm 1.53% (high dose of LILEW) of rats attenuated gastric lesions that were generated by ethanol. In conclusion, these findings confirmed that *Lawsonia inermis* L. leaves ethanol: water extract is safe to consume and able to protect against gastric ulcers.

Keywords: Acute toxicity; ethanol; gastric ulcer; gastroprotective; Lawsonia inermis L. leaves

ABSTRAK

Lawsonia inermis (LI) atau inai telah digunakan sejak zaman dahulu untuk tujuan kosmetik, perubatan dan penyembuhan luka. Walau bagaimanapun, bukti mengenai kesan perlindungan gastrik LI adalah tidak mencukupi dan mekanisme farmakologi masih kurang diketahui. Tujuan penyelidikan ini adalah untuk mengkaji ketoksikan akut dan aktiviti perlindungan gastrik ekstrak *Lawsonia inermis* L. (LILEW) etanol:air (80:20) berbanding dengan ulser gastrik yang diinduksi dengan etanol pada tikus. Ujian ketoksikan akut oral dijalankan menggunakan tikus betina *Sprague Dawley* (n=6) untuk dos rendah (2000 mg/kg), dos tinggi (5000 mg/kg) serta rujukan (air suling). Kesan perlindungan gastrik LILEW dijalankan pada ulser yang diinduksi dengan etanol dalam tikus *Sprague Dawley* (n=6). Tikus dibahagikan kepada lima kumpulan kawalan iaitu kawalan ulser, kawalan normal, kawalan ubat-omeprazole, dos tinggi dan dos rendah LILEW (500 mg/kg dan 250 mg/kg). Ujian ketoksikan akut dengan LILEW pada tikus tidak menunjukkan sebarang tanda kematian dan ketoksikan sehingga 5 g/kg, mencadangkan bahawa LILEW selamat untuk dimakan. Kesan perlindungan gastrik LILEW pada dos 250 mg/

kg dan 500 mg/kg serta omeprazole (20 mg/kg) menunjukkan kadar perencatan yang signifikan sebanyak 70.50±1.38% (kawalan ubat), 66.67±1.42% (dos rendah LILEW) dan 68.45±1.53% (dos tinggi LILEW) daripada tikus yang mengalami luka gastrik. Sebagai kesimpulan, penemuan ini mengesahkan bahawa ekstrak etanol daun *Lawsonia inermis* L. selamat untuk dimakan dan mampu melindungi daripada ulser gastrik.

Kata kunci: Daun Lawsonia inermis L., etanol; ketoksikan akut; perlindungan gaster; ulser gaster

INTRODUCTION

Peptic ulcer is a disease concerning the digestive system. It is also a medical-social issue with global economic importance resulting from its high incidence, vast geographical distribution, injury, and drug utilization. Almost 20% of the population is estimated to experience peptic ulcers during their lifetime, caused by factors including their diet, stress, smoking, alcohol intake, and specific categories of drugs (Akash et al. 2024; Bucciarelli et la. 2010; Levenstein 2000).

There are two widely known causes of peptic ulcers in patients which are *Helicobacter pylori* infection as well as prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Forte 1996; Majumdar & Looi 2024; Vijayakumar et al. 2016). Gastritis is an erosion, or soreness that takes place when the internal defensive mechanisms of the mucosal barrier are unable to effectively protect and look out for the organ (Blasco et al. 2020; Ismail et al. 2012).

There are a few synthetic drugs that are effective when treating peptic ulcers. However, the majority of the drugs when used for a long term may lead to various side effects, for instance, patients may experience gynecomastia, bradycardia, loss of libido, feeling nauseous and dizzy, an upset bowel, and dry mouth (Table 1).

Nearly 80% of the population of the third world still use medicinal herbs as they are recognized as 'traditional healings' (Othman et al. 2016). In the time of the Prophet, henna was also used in wound treatment. Salma Umm Rafi', the freed slave woman of the Messenger of Allah (ﷺ), said: "The Prophet (ﷺ) did not suffer any injury or thorn- prick but he would apply henna to it." (Vol. 4, Book 31, Hadith 3502) (Marwat, Khan & Bhatti 2009). This is an indicator of henna's potential as a material that may be used for wound treatment. Studies have shown that henna compounds are rich in wound treatment active ingredients.

In general, *Lawsonia inermis* is regarded as a native of Asia and Africa. Particularly, it is commonly cultivated in tropical regions such as Sudan, China, Egypt, and India (Musa & Gasmelseed 2012). *Lawsonia inermis* originates from the Lythraceae family and is a small tree or tall shrub about 2.6 m in height. Henna flowers are white or red, and fruits are small with seeds in brownish capsules (Rajwar & Khatri 2011).

The antioxidative defense against the oxidative stress resulting from necrotic agents is an important aspect of supporting the gastric mucosa. A hydrogen atom can be donated by the antioxidant to decrease free radicals and prevent lipid peroxidation rummaging by the reactive oxygen species (Kattappagari et al. 2015). Several research show that *Lawsonia inermis* has excellent antioxidative capacities and the species may be a capable source of new natural antioxidants (Babili, Valentin & Chatelain 2013; Elansary et al. 2020; Goswami et al. 2011; Hsouna et al. 2011; Kumar, Kumar & Kaur 2014; Pasandi Pour & Farahbakhsh 2019).

Nowadays, herbal-based medicines are generally used as it is believed to produce low side effects. Furthermore, the growth of pharmaceutical products significantly relies on nutraceuticals (Pathan et al. 2024; Ramsay & Carr 2011; Vijayakumar et al. 2016). In literature, various medicinal plants derived from numerous taxonomic families have been examined for their anti-ulcer properties, including henna (Rouhollahi et al. 2014).

Pharmacological agents	Efficacy	Common adverse effects	References
PPIs (e.g. Omeprazole, Esomeprazole)	Highly effective in reducing acid and healing ulcers	Headaches, constipation, gastrointestinal infections	Yadav, Pandey & Mali (2024)
Histamine-2 Receptor Antagonists (e.g. Ranitidine, Cimetidine)	Effective in reducing acid secretion	Arrhythmias, blurred vision, headaches	Mubashir, Ghani & Mubashar (2022)
Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)	Effective in NSAID- induced ulcers	Gastrointestinal bleeding, mucosal damage	Zaib et al. (2023)
Antacids and Demulcents	Protect the stomach lining	Drug interactions are ineffective in preventing relapses	Pandey, Singh & Singhai (2023)

TABLE 1. Pharmacological agents used in the treatment of gastric ulcers: Efficacy and adverse effects

Henna leaves are famous for the Lawsone, 1,4-Naphthoquinone which carries a hydroxy element at C-2. It has a role as a protective agent and an antifungal agent. It is a tautomer of a naphthalene-1,2,4-trione. Henna dye is used in coloring and decorating hands, feet, skin, and hair (Chaudhary, Goyal & Poonia 2010; Kamal & Jawaid 2010). Henna is reported as one medicinal herb in Malay traditional medicine used for body ache treatments, swelling in the stomach, dizziness, inflammation, abortion, circumcision, and also for bone cancer treatments (Othman et al. 2016). Henna extracts showcased different kinds of biological activities such as fungicidal, bactericidal, antimycotic, anti-inflammatory, antipyretic, antiparasitic, analgesic, and anti-cancer activities (Abulyazid, Mahdy & Ahmed 2013; Ajitha et al. 2016; El-Hag, Al-Jabri & Habbal 2007). Natural-based products of henna, with a historical background in folk applications, deliver new therapeutic methods for treatments of numerous diseases and ailments (Goswami et al. 2011; Semwal et al. 2014). In continuation of gastroprotective potential of the medicinal plants in Malaysia (Ismail et al. 2012; Omer et al. 2015; Othman et al. 2020; Rouhollahi et al. 2014; Sim et al. 2014), an investigation was conducted, as illustrated in Figure 1, which shows the experimental design for the acute toxicity and gastroprotective of LILEW.

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MATERIALS AND METHODS

PLANT SAMPLE COLLECTION

The leaves from the *Lawsonia inermis* (LI) were compiled from Sentosa, a residential area in Bandar Baru Bangi, Selangor, Malaysia. This activity was conducted by Mr. Din Bin Md. Nor and the botanical classification was by Mr. Teoh Leong Eng, both from the Department of Chemistry, Universiti Malaya. KL5824, a voucher sample was placed inside the herbarium of the Department of Chemistry, Faculty of Science, Universiti Malaya (Othman et al. 2020).

EXTRACTION OF *Lawsonia inermis* L. LEAVES ETHANOL:WATER (80:20) EXTRACT (LILEW)

The extraction was performed based on the technique from Gondokesumo et al. (2019) with a minor modification. The fresh leaves of LI were washed, dried, and ground into powder. The powdered leaves were then derived using aqueous extraction of LI (300 g) with the ratio of ethanol:water (80:20) (Table 2) (Othman et al. 2020). Afterward, the compound was filtered and then freezedried. The crude extract was stored at -20 °C till further analyses could be done.



FIGURE 1. Experimental design of acute toxicity and gastroprotective study of LILEW

EXPERIMENTAL ANIMALS

Healthy female *Sprague Dawley* rats (220-250 g) were acquired from the Animal Experimental Unit, Faculty of Medicine, Universiti Malaya. The animals have been housed in a structured environment (12 h light: 12 h dark, temperature 24 ± 2 °C; 5 to 6 animals per cage). The rats were given a rat chow diet as well as tap water *ad libitum*. The experimental operations were approved by the Faculty of Medicine Animal Care and Use Committee (FOM IACUC), and Universiti Malaya ethics no. 2017-201205/IBS/R/SNSAR.

ACUTE TOXICITY STUDY

Eighteen Sprague Dawley females were separated into groups of three, group 1 as the vehicle was fed orally with 10 % Tween 20, the second low-dose group was provided with a low-dose of LILEW (2000 mg/kg), and the third group received a high-dose of LILEW (5000 mg/kg). The mixture was fed to the rats by gavage orally and the LILEW toxicity procedure was carried out at 30 min, 2 h, 4 h, 24 h, and 48 h. All rats were fasted overnight before the test (only water was allowed except for the last 2 h). Food was delayed after dosing for another 1 to 3 h. During the twoweek duration (14 days), any symptoms of mortality and toxicity were further reported. On the 15th day, the animals were euthanized with a dosage of 10 mg/kg of ketamine and xylazine for hematological along with histological analysis. Each group's blood serums were sent to the 4th floor, East Tower, Universiti Malaya Medical Center Laboratory for biochemical profiling. Lastly, a histopathology analysis was conducted on the kidneys and liver of the rats (Saremi et al. 2020).

ETHANOL-INDUCED GASTRIC ULCER STUDY

The female *Sprague Dawley* rats were distributed among 5 groups that consisted of 6 rats randomly and fasted for

24 h (water was available for the last 2 h). As in Table 3, the groups were numbered 1-5, and all the rats were included according to the experimental design. After 1 h, the rats were given a xylazine and ketamine injection and the stomachs of the rats were handled accordingly for analysis.

LOSS DETERMINATION OF MUCOSAL CONTENT AND GASTRIC JUICE ACIDITY

In order to extract the juice, each of the rats' stomachs was attentively cut off from their larger curvature. The supernatant was acquired by centrifugation at 1008 g for 10 min and was screened for the pH (Saremi et al. 2020).

MACROSCOPIC ANALYSIS OF ULCER

Numerous research have shown that ethanol-induced gastric mucosal ulcers have been characterized as elongated hemorrhagic ulcer bands aligned with the long stomach axis. The luminal surface assessment was used to determine hemorrhagic damage to the stomach. Each pre-treatment protective potential (p %) was measured by a planimeter (10×10 mm²) along with a dissecting or stereoscopic microscope (1.8×) in which the control area of the ulcer (UC) and the control area of the ulcer (UT) was measured. As previously defined in depth by Rouhollahi et al. (2014), the ulcer calculation was carried out.

$$p \% = \frac{UC - UT}{UC} x 100$$

HISTOLOGICAL EVALUATION OF GASTRIC ULCER

Hematoxylin and eosin staining

Formalin moderated with phosphate (10%) was implemented to fix stomach wall samples at ambient temperatures. Afterward, the samples were tissueprocessed (dehydration, clearance, and paraffin infiltration)

TABLE 2. The yield of Lawsonia inermis L. (Malaysia) leaves ethanol:water (80:20) extract (LILIEW)

Code	Leaves	Extract	Weight (crude)
Leaves KL5824	300 gram	LILEW	36 gram

TABLE 3. The	e experimental	design and	specification	of t	he animal	investigatio	n
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Groups	Description	Pre-Treatment (5 mL/kg)	Treatment (5 mL/kg)
1	Normal control	10% Tween 20	RO water
2	Ulcer control	10% Tween 20	Absolute ethanol
3	Drug control	Omeprazole (20 mg/kg)	Absolute ethanol
4	Low dose LILEW	Low dose (250 mg/kg)	Absolute ethanol
5	High dose LILEW	High dose (500 mg/kg)	Absolute ethanol

by a tissue-processing system (Leica, Solms, Germany), accompanied by paraffin-embedding. The stomach tissues were then divided at a 5 μ m thickness and marked with hematoxylin and eosin (H&E) for further histological evaluation (Rouhollahi et al. 2014; Saremi et al. 2020).

Glycoprotein-PAS staining

For the purpose of obtaining a scientific understanding of the gastric epithelial mucus secretion and improved evaluation for any changes and developments in either basic or acidic glycoproteins, the segments per stomach wall were stained with a periodic acid-Schiff (PAS) (Saremi et al. 2020).

Immunohistochemistry

BAX (BCL-2), and Caspase 3 immunostaining were performed according to the instructions of the Dako kits (Dako Cyomation, Carpinteria, USA) manufacturer (El-Sisi et al. 2020; Yuan et al. 2016).

Statistic analysis

All the values have been stated as mean \pm SD. Statistically notable variations among each group were decided using a one-way ANOVA which was followed with multiple reference tests of Tukey's post hos. The value of p<0.05 was assumed to be significant.

RESULTS

ACUTE TOXICITY EVALUATION

In the acute toxicity examination, both of the groups of rats were alive and were not exhibiting any noticeable indication of toxicity and irregularity with a low dose of 2000 mg/kg alongside a high dose of 5000 mg/kg. In contrast to the control group, there had been no demonstrable signs of renal toxicity or hepatic among the groups that were treated. This test showed that when administered orally up to 5000 mg/kg, the plant is harmless and has no toxicity. Certain anti-ulcer drugs, have been shown to increase the quantity of gastric mucosa, such as sodium salicylate (Al Batran et al. 2013; Jia et al. 2023; Robert et al. 1984). There was no indication of weight loss for a period of 14 days and no irregular behaviour was found. As seen in Table 4, it is reported that serum biochemical parameters are normal. There were no major differences induced during the hematological examination of the kidney and liver in the group treated relative to the vehicle (Figure 2).

THE GASTRIC CONTENT PH AND MUCUS PRODUCTION DETERMINATION

In the ethanol-induced gastric lesion model, the antiulcer exercise of the *Lawsonia inermis* leaf extract is shown in Table 5. As seen in Table 5, the gastric substance's acidity of rats treated orally using ethanol substantially grew

in accordance with the normal control group. The rats treated with LILEW are capable of reducing Omeprazole (20 mg/kg) according to substance control.

MACROSCOPIC EVALUATION OF GASTRIC LESIONS

Absolute ethanol has resulted in large noticeable hemorrhagic lesions inside the gastric mucosa. Experimental results showed that the rat stomach treated with LILEW extract and omeprazole showed particularly compact areas of stomach ulceration rather than control ulcers prior to absolute ethanol treatment.

As shown in Figure 3 gross evaluation, low-dose, and high-dose stomach pre-treatment had distinctly minimized areas of the formation of gastric ulcers. The frequency of ulcer area was decreased with LILEW at doses of both 250 mg/kg and 500 mg/kg. It is shown that LILEW in both concentrations assisted in flattening the gastric mucosal folds inside the rats.

LILIEW significantly prevented the development of ulcers caused by absolute ethanol and specifically dosedependently minimized the damage to the gastric mucosa, i.e., LILEW notably extinguished the development of the ulcers. The gastric ulcer inhibition development at low and high doses of LILIEW was significant as well as equivalent to that seen among the omeprazole treatment group (Figure 3 & Table 5).

HISTOLOGICAL GASTRIC ULCER EVALUATION

H and E stain

Histological observation of Group 1 (orally fed 10% Tween 20) indicated that no signs of disruption on the surface of epithelium occurred, although the histological analysis indicated considerable damage effects to the gastric mucosa in Group 2 (Ulcer Control fed with Absolute Ethanol) accompanied by widespread necrosis and submucosal layer leucocyte (white blood cell) infiltration, including necrotic lesions that penetrate deep inside the mucosa (Figure 4).

Group 3 (omeprazole) demonstrated a slight disturbance of penetration of leucocytes and edema of the surface epithelium into the submucosal layer. Group 4 (low dose of LILEW) demonstrated major disruption disturbance regarding the surface epithelium, including submucosal edema along with infiltration of the leucocyte. Group 5 (high dose of LILIW) demonstrated mild disruption of edema and submucosal layer leucocyte infiltration, but no surface epithelium disturbance. These findings showed that cytoprotective effects were exerted by plant extracts with a dependent manner of doses.

Glycoprotein-PAS staining

Gastric mucosa showed increased PAS staining strength in animals pretreated with high doses of LILEW or omeprazole (groups 3 and 5) compared to rats pretreated with group 2 and low doses of LILEW, suggesting a glycoprotein content growth of gastric mucosa in the pre-treated rats (Figure 5).

a) Effects of LILEW on kidney biochemical parameters								
Group	Sodi	um Pota	ssium	Chloride	CO ₂	Anion	Urea	Creatinine
	(mmc	ol/L) (mm	ol/L)	(mmol/L)	(mmol/L)	(mmol/L)	(mmol/L)	(µmol/L)
2000 mg/kg	139±0.0	00 5±0.1	0 10	0 ± 0.00	28±1.00	16±1.00	6.2 ± 0.20	31±1.00
5000 mg/kg	140±0.0	00 4.25±	0.15 10	0.5 ± 0.00	28±0.00	15.5±0.50	5.35±0.65	30.5±2.50
Vehicle	139±1.(00 4.35±	0.25 99	.5±0.50	27±0.00	16.5±0.50	6.85±0.25	29±1.00
		b) E	ffects of LI	LEW on liver	biochemica	l parameters		
Group	Albumin	Total Bilirul	oin A	Ikaline	Ala	anine	G-Glutam	yl Transferase
	(g/L)	(umol/L)	Phosp	hatase (U/L)	Aminotran	sferase (U/L)	(U/L)
2000 mg/kg	15.5±0.50	<2	9	1±1.00	43=	±1.00		<6
5000 mg/kg	16.5±0.50	<2	10	4.5±1.00	43.0)5±1.5		<6
Vehicle	15.5±0.50	<2	8	.5±1.50	41=	±1.00		<6
c) Effects of LILEW on hematology profile examination								
Group	HGB (g/L)	HCT (L/L)	RBC	MCV (fl)	MCH (pg) MCHC (g/	′L) RDW (%	6) WBC
			(10^12/L	.)				(10^9/L)
2000 mg/kg	156.5±5.5	$0.495 {\pm} 0.015$	8.34±0.24	59.0 ± 0.00	18.75±0.15	317.0±2.0	12.55±0.3	5 7.75±1.45
5000 mg/kg	162.5±1.5	0.150 ± 0.00	8.66 ± 0.01	59.0 ± 0.00	18.75±0.15	318.0±2.0	12.60±0.0	$0 8.00 \pm 0.60$
Vehicle	159.5±4.5	0.495±0.015	8.60±0.21	57.5±0.50	18.55±0.05	320.5±1.50) 12.8±0.20	8.20±1.00
Hemoglobin (1)(D) Hamataarit	(IICT), Dad Plaad	Calle (DDC): M	Icon Compusciale	" Volumo (MCV	Maan Comu	ular Hamaalahi	n (MCID: Moon

TABLE 4. Effect of LILEW on (a) Kidney biochemical parameters, (b) liver biochemical parameters, and (c) lipid profile examination

Hemoglobin (HGB); Hematocrit (HCT); Red Blood Cells (RBC); Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC); Red Cell Distribution Width (RDW); White Blood Cells (WBC)

The value represents means \pm SD (n = 6) per group. The value of p<0.05 was considered as significant

Unit: picogram (pg); millimoles per liter (mmol/L); gram per liter (g/L); micromole per liter (µmol/L), units per liter (U/L); femtolitre (f/L)



FIGURE 2. Histopathology of the kidney (first row) and liver (second row) in the acute toxicity experiments in rats that are treated with vehicle, LILEW (low dose of 2000 mg/kg) and LILEW (high dose of 5000 mg/kg). The outcome showed no major variations within the anatomy of the kidney and the liver among the treated and the controlled groups (100 μ m)

Animal group	pН	Ulcer area (mm) ²	Inhibition (%)
Normal control	4.23±0.17	0	0
Ulcer control	$1.99{\pm}0.36$	766.3±35	0
Drug control	4.85±0.17*	226±7.35	70.50±1.38
Low dose (250 mg/kg)	5.4 ± 0.40	256±14.21*	66.67±1.42
High dose (500 mg/kg)	4.8±0.20*	241±6.74*	68.45±1.53

TABLE 5. LILEW's gastroprotective effect against gastric injury that is induced with ethanol

Values are shown as mean \pm SEM. (*) shows significance in the P < 0.05 versus the ulcerated pretreatment group versus the indomethacin (ulcerated) group



FIGURE 3. Gross assessment findings showed that low-dose (250 mg/kg) and high-dose high-dose (500 mg/kg) of LILEW pre-treated omeprazole (drug control) in rats had a markedly reduced region of gastric ulcer development relative to ulcer control

BCL-2 staining

Downregulation of the BCL protein was demonstrated by the immunohistochemical staining of the gastric mucosa in pretreated rats with a low dose and a high dose of LILEW substance or omeprazole (BCL-2) (Figure 6).

BAX staining

Downregulation of the BAX protein was demonstrated by immunohistochemical staining of the gastric mucosa in pretreated rats with a low dose and a high dose of LILEW substance or omeprazole (BAX) (Figure 7).

Caspase-3

It was shown that the normal control group showed intact mucosa along with no signs of gastric ulcer formation, based on the histopathological test of gastric sections. For the Caspase-3 antibody, the normal group showed a negative immunostaining. However, the ulcer group showed positive immunostaining for the Caspase-3 antibody. Drug control showing positive immunostaining for Caspase-3 antibody. Low dose showing positive immunostaining for Caspase-3 antibody (Moderate). Additionally, the high dose showed a positive immunostaining outcome for



FIGURE 4. Histological examination in pre-treatment rats: drug control, with low dose (250 mg/kg) and high dose (500 mg/kg) along with monitoring of ulcers. The normal histological structure was shown by the normal control group. (H and E stain) (Scale bar = 200 μm)

PAS



FIGURE 5. Glycoprotein-PAS staining complex activity on gastric tissue: control of the drug, low dose, and high dose, compared with the control of the ulcer. The normal histological structure has been demonstrated by the normal control group (Scale bar = $200 \ \mu m$)

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FIGURE 6. BCL-2 staining complex activity on gastric tissue: control of the drug, low dose, and high dose, compared with control of the ulcer. The normal histological structure has been demonstrated by the normal control group (Scale bar = $100 \ \mu m$)

BAX



FIGURE 7. BAX staining complex activity on gastric tissue: control of the drug, low dose, and high dose, compared with control of the ulcer. The normal histological structure has been demonstrated by the normal control group (Scale bar = $100 \ \mu m$)

the Caspase-3 antibody showing maximum expression of activated caspase-3. The results found indicated, that pretreatment with a high dose pretreatment essentially inhibits Caspase-3 immune signals which is demonstrated in Figure 8.

DISCUSSIONS

Topical pure henna is generally safe and well-tolerated in humans, but oral and topical henna with additives like paraphenylenediamine (PPD) have many side effects, some of which are life-threatening (Güdeloğlu & Erdur 2020). Ingesting henna, especially with additives like PPD, can lead to severe systemic toxicity, including rhabdomyolysis and renal failure, with a high mortality rate. The presence of PPD in henna can cause serious allergic reactions and discoloration of body fluids. Therefore, it is important to be cautious when using henna products, especially in children and individuals with glucose-6-phosphate dehydrogenase deficiency (Aktas Sukuroglu, Battal & Burgaz 2017). A study on potential henna mouthwash shows promise as an alternative to chlorhexidine mouthwash for Oral Lichen Planus (OLP) treatment, with comparable efficacy in the short term. However, more extensive studies are required to confirm these findings and ensure safety (Kakoei et al. 2022).

The evaluation of LILIEW in this study for acute toxicity was negative for indications of mortality or toxicity with the doses used, from the low dose (2000 mg/kg) to the high dose (5000 mg/kg). Based on Figure 2, the intact glomeruli and well-preserved tubular structures in the kidney tissues of rats treated with Vehicle, LILEW at 2000 mg/kg, and LILEW at 5000 mg/kg during acute toxicity experiments have potential toxicological implications. The intact glomeruli and well-preserved tubular structures indicate the absence of necrosis, inflammation, or degeneration in the kidney tissues. These findings support the potential of henna in gastroprotective studies. A previous study conducted by Khantamat et al. (2021) on an aqueous extract of Thai henna leaf showed non-cytotoxicity to human immortalized keratinocyte cells, peripheral blood mononuclear cells, and murine macrophage RAW 264.7 cell line. However, extracts of henna leaves in chloroform/ methanol (7:3) and methanol alone have shown significant cytotoxicity at higher concentrations. These extracts were particularly toxic to Vero cells, indicating the presence of cytotoxic compounds (Manuja et al. 2021). Adulterated henna products, especially those containing synthetic chemicals like PPD, are more likely to cause severe allergic reactions and contact dermatitis (Güdeloğlu & Erdur 2020). While natural henna is generally safe when used in its pure form, the presence of synthetic additives like PPD significantly increases the risk of toxic effects. Awareness and careful selection of henna products are essential to avoid adverse health outcomes. There have been reports of adverse reactions, particularly in individuals with glucose-6-phosphate dehydrogenase deficiency or when henna is adulterated with harmful substances (Semwal et al. 2014). However, caution is advised due to potential adverse reactions and the risk of adulteration. More *in vivo* studies are needed to fully validate its pharmacological benefits.

Henna contains various bioactive compounds such as flavonoids and phenolics, including chlorogenic acid, ellagic acid, and rutin, which contribute to its wound healing properties. These compounds exhibit antioxidant and antimicrobial activities, further aiding the healing process (Alsalamah et al. 2023). Studies have shown that henna-based formulations have the potential for dermatological, cosmetic, and therapeutic applications due to the presence of phenolic compounds that aid in wound healing and re-epithelialization (El Massoudi et al. 2023). Henna leaves contain significant amounts of phenolic and flavonoid compounds, which contribute to their antioxidant and anti-inflammatory activities. These properties were evidenced by the scavenging of ABTS and DPPH radicals and the reduction of nitric oxide (NO) levels in lipopolysaccharide LPS-induced cells (Khantamat et al. 2021). Compounds from the phenolic group have shown a role in antioxidant activity, among such studies are such as Aqil, Ahmad and Mehmood (2006), Hosein and Zinab (2007), Hsouna et al. (2011), Kumar et al. (2016), and Elansary et al. (2020). Several active compounds contribute to antioxidant activity, making them valuable in wound treatment. Notable examples include Apiin (8), Lalioside (11), Luteolin-7-O-Glucoside (14), Lawsone (15), Cosmosiin (18), and Apigenin (24) (Othman et al. 2020).

The lawsone compound has demonstrated greater efficacy as a wound-healing agent, particularly when utilized topically in an ointment form (Lozza et al. 2019; Nayak et al. 2007). Additionally, the antimicrobial properties of luteolin-7-O-Glucoside, isolated from henna oil, exhibit antibacterial activity against *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, and group B streptococcus (Khazaeli et al. 2019).

Furthermore, henna contains compounds like lawsone, which have anti-inflammatory and antioxidant properties. These properties help reduce inflammation and oxidative stress at the wound site, promoting faster healing (Alsalamah et al. 2023). Henna compounds aid in the re-epithelialization process, which is the formation of new epithelial tissue over a wound. This is facilitated by the presence of bioactive compounds that promote cell proliferation and migration (Daneshmand et al. 2024). Wound healing involves a complex process of restoring normal skin structure and function, coordinated through four steps: homeostasis, inflammation, proliferation, and regeneration (Ghomi et al. 2022). Gastric ulcer healing is a regeneration process involving cell dedifferentiation, proliferation, migration, re-epithelialization, granulation tissue formation, angiogenesis, and tissue remodeling, resulting in scar formation (Tarnawski & Ahluwalia 2021). Both wound healing and gastric ulcer healing encompass inflammation as a crucial phase. The inflammatory phase

Caspase-3

Group	20x	60x
Normal control		
Ulcer control		
Drug control		
Low dose (250mg /kg)		
High dose (500mg /kg)		

FIGURE 8. Caspase-3 staining complex activity on gastric tissue: control of the drug, low dose, and high dose, compared with the control of the ulcer. The normal histological structure has been demonstrated by the normal control group (Scale bar = $200 \ \mu m$)

involves hemostasis and the release of growth factors and cytokines to initiate the healing cascade (Criollo-Mendoza et al. 2023). Gastric ulcer healing is regulated by various gastrointestinal (GI) hormones such as gastrin, cholecystokinin(CCK), and orexigenic peptides like ghrelin, orexin-A, and obestatin. These hormones play crucial roles in cell proliferation, migration, and survival through signaling pathways like Ras, MAPK, PI-3K/AKT, and PLC- γ (Tarnawski & Ahluwalia 2012). Additionally, henna extracts have been found to exert physiological effects on gastric pH by decreasing gastric acid secretions and total acidity, ultimately creating a less acidic environment that supports ulcer healing. These findings are consistent with the report by Goswami et al. (2011) regarding the impact of henna extract on gastric pH.

According to Goswami et al. (2011), henna leaf extracts have shown significant anti-ulcer activity in various gastric ulcer models in rats. The ethanolic extract, in particular, demonstrated a dose-dependent ulcer protective effect, reducing ulcer formation by up to 94% in ethanol-induced ulcers and 56% in cold-restraint stress-induced ulcers. The ethanolic extract again showed notable protection, although the efficacy was lower compared to ethanol-induced ulcers. Another research, henna also showed hepatoprotective effects, where it protected against acetaminophen-induced hepatotoxicity in rats. This study used two doses (100 mL/kg and 400 mL/kg) and found that higher doses provided better protection, as evidenced by normalized ALT and AST levels (Ishtiaq et al. 2022).

The gastroprotective effects are likely due to the presence of phenolic compounds and tannins, which can enhance mucosal defense mechanisms and reduce oxidative stress. More studies are needed to assess the safety and efficacy of henna's bioactive compounds in gastroprotection (Alsamahi et al. 2023). Detailed investigations into the specific mechanisms by which henna exerts its gastroprotective effects are essential. Therefore, this study was conducted using a henna extract formulation consisting of 80% ethanol and 20% water, focusing on the observation of mechanisms involving the BAX protein, BCL-2 pathway, Glycoprotein-PAS, and Caspase-3 antibody.

Apoptosis is an organized process in which immune cells in tiny membrane packets prepare the contents of the cells for 'garbage collection'. During the development, apoptosis eliminates the cells, kills cells that may be cancerous and infected with viruses, and restores the body's balance (Roche Applied Science). Cells go through 'cellular suicide' when they get possession of certain cues in programmed cell death. Apoptosis proves beneficial to the organism as a whole, but it involves the death of cells. Cells that experience apoptosis indicate characteristic features that are morphological and biochemical. The features consist of chromatin accumulation, cytoplasmic and nuclear consideration, the mitochondria start being leaky because of the formation of pores that include proteins within the BCL-2 family, and activation of the Caspase cascade. These apoptotic bodies are promptly acknowledged as well as phagocytized by either adjacent epithelial or macrophage cells *in vivo* (Uehara, Elmore & Szabo 2018). In this study, an increase in gastric pH was observed (Table 5), with minimum apoptosis presenting a marked declaration for the activated Caspase-3 within all the cells, and maximum expression of activated Caspase-3. Our findings showed that Omeprazole pretreatment substantially inhibits Caspase-3 immune signals of activated Caspase-3, as demonstrated in Figure 8.

Periodic Acid-Schiff (PAS) staining is used to detect glycoproteins, which are essential in various biological functions, including the gastrointestinal (GI) tract. PAS staining highlights glycoproteins by staining them magenta, making it easier to identify and study their distribution and abundance in tissues (Valdivia et al. 2019). Glycoprotein-PAS staining plays a crucial role in identifying glycoproteins and specific carbohydrate moieties in the context of gastrointestinal function, providing insights into the immune response and potential serodiagnosis in gastrointestinal infections. The staining results offer valuable information about the presence of O-glycans and N-glycans, which are key considerations in understanding the immune response and potential serodiagnosis in gastrointestinal infections. In Figure 5, the magenta color is observed in PAS staining, a positive indicator of the presence of neutral mucins (Arman & Üçüncü 2017). Omeprazole, a gastric acid secretion blocker, has been shown to improve the gastric mucosal surface and reduce the severity of mucosal inflammation and hemorrhaging in an ethanol-induced gastritis rat model (Kengkoom et al. 2017). From Glycoprotein-PAS staining Figure 5, indicated by increased PAS staining strength in the gastric mucosa of rats pretreated with high doses of LILEW (500 mg/kg) with omeprazole (20 mg/kg) indicates enhanced mucus production and glycoprotein content, leading to better protection against gastric mucosal damage compared to low doses of LILEW (250 mg/kg). The comparison between high and low doses of LILEW suggests that the gastroprotective effects and mucus production are dose-dependent, with higher doses providing more significant protection and increased PAS staining. The intense PAS staining indicates a higher glycoprotein content in the gastric mucosa, which is crucial for maintaining the integrity and function of the gastric barrier. This can help in preventing and healing gastric ulcers. High doses of LILEW and omeprazole have been shown to reduce gastric lesions and enhance mucosal defense mechanisms, such as upregulating protective proteins like Hsp70 and downregulating pro-apoptotic proteins like Bax (Omar, Nordin & Hassandarvish 2017).

Cells experiencing apoptosis show characteristics of the morphological and biochemical features. Caspase-3 (detects the programmed death cell) is the ultimate killer of apoptosis, IHC to the effective formation of Caspase-3 (active casp-3) was operated to examine apoptosis in sections of paraffin from numerous tissues (Figure 8). Various protein targets of operational caspases are biologically vital apoptotic displays of biochemical as well as morphological conversions correlated with apoptosis (El-sisi et al. 2020).

Apoptosis is promoted by BAX, whereas the BCL-2 restrains this procedure. The imbalance of anti-apoptotic protein expressions of the BCL-2 family as well as the apoptotic BAX proteins within stress ulcers can lead to apoptosis. The proteins are accountable for the defense of homeostatic cells. Environmental and physiological injury processes and the research on this protein may supply interesting evidence for elucidating potential mechanisms of action through the conservation of the normal protein's structure and the mending or eradication of proteins that are damaged (Shamas-Din et al. 2013). Moreover, the immunohistochemical staining suggested that the BAX protein expression had been downregulated in the pretreated rats that consisted of the LILEW mixture.

Ethanol administration upregulates the levels of proapoptotic markers, such as Bax, and downregulates the anti-apoptotic marker BCL-2 in gastric tissue. Ethanolinduced increases in the BAX/BCL-2 ratio in gastric tissue can be blunted by pretreatment with certain compounds, such as carvacrol and Aloe vera gel, which possess antiapoptotic activities (Badr et al. 2023).

The treatments demonstrate a clear dose-dependent increase in BCL-2 expression, as evidenced by the immunohistochemical staining intensity shown in Figure 6. The high dose (500 mg/kg) exhibits robust and consistent staining, closely resembling the levels observed in the drug control group, suggesting strong anti-apoptotic activity and effective protection against cellular stress. In comparison, the low dose (250 mg/kg) shows moderate staining, indicating partial restoration of BCL-2 expression, albeit at a lower intensity than both the high dose and drug control. These results highlight the potential efficacy of higher doses in mimicking the therapeutic effects of the drug control (omeprazole - 20 mg/kg), while the lower dose still provides measurable benefits in enhancing cellular survival.

Figure 7 shows the reduction in the BAX ratio associated with decreased apoptosis and enhanced cell survival. Histopathological examinations confirm that treatments reducing the BAX ratio led to less gastric mucosal damage and fewer lesions, indicating effective protection against ethanol-induced gastric ulcers. The BAX/BCL-2 ratio is a critical factor in ethanol-induced gastric ulcers, with a higher ratio promoting apoptosis and ulcer formation. Treatments that lower this ratio can significantly protect the gastric mucosa by reducing cell death and enhancing tissue repair mechanisms (Badr et al. 2023).

Conventional treatments like proton pump inhibitors (PPIs), H2 receptor antagonists, and other medications are

effective but often come with higher costs and potential side effects. (Liu & Tang 2020). Studies on other herbal treatments and alternative therapies suggest that they can be cost-effective, especially when considering the lower incidence of side effects and the reduced need for additional medications (Prayoga et al. 2024). The general trend indicates that herbal and alternative treatments can be more cost-effective compared to conventional drugs due to lower side effects and overall treatment costs.

CONCLUSION

To conclude, the present analysis showed that the two experiments observed acute toxicity and gastroprotective properties of the Lawsonia inermis L. leaves ethanol:water (80:20) extract was effective in reducing injury to the ethanol-induced gastric mucosal in rats. The extract significantly reduced gastric lesions, decreased gastric secretion and acidity, and restored mucin content, indicating its potential as a therapeutic agent for gastric ulcers. Henna as a treatment for wounds has been shown as efficient in several studies that have been performed, whether external wounds or internal wounds, as in the treatment of ulcers. To some extent, this study has shown that henna extract ethanol: water (80:20) can treat ulcer wounds safely. From the pharmacology view, henna extract composed based on water (20%) and ethanol (80%) was indicated as safe against Sprague Dawley rats without showing any signs of acute toxicity such as diarrhea, lethargy, or demise. The findings of biochemical parameters support this whereby the study indicated a normal function of the liver and kidney after feeding.

These findings also confirm that the presence of active mixtures plays a part in antioxidant activities, hence the presence of a large number of phenolic compounds assists in reducing free radicals and preventing the occurrence of ulcers. Gastroprotective experiments show that henna extract acts as a barrier to the occurrence of ulcers when exposed to absolute ethanol. Findings from the experiments also contributed to the growth of safer and natural-based health products, for instance, ulcer wound treatments (mouth/stomach) and treatments of skin diseases. The findings support the use of Lawsonia inermis L. traditionally for ulcer treatments. Previous studies have also shown henna extracts (aqueous, chloroform, and ethanol) demonstrated significant antiulcer activity in rat models when administered orally. These extracts reduced gastric acid secretion and ulcer index, indicating potential benefits for gastric ulcers. However, this involves systemic absorption rather than topical application. Thus, henna cannot be recommended as a topical treatment for gastric ulcers without absorption based on current evidence.

In conclusion, the acute toxicity of *Lawsonia inermis* L. leaves ethanol:water (80:20) extract in rats was found to be moderate, and the extract demonstrated potential therapeutic effects on ethanol-induced gastric ulcers in rats through its anti-inflammatory, antioxidative, and anti-apoptotic mechanisms. Henna exhibits promising anti-ulcer properties through its ability to reduce gastric acid secretion and enhance mucosal defenses, making it a potential natural treatment for gastric ulcers. However, it is important to note that while the extract showed promise in animal studies, further research is needed to explore its potential for clinical applications. Moreover, it provided valuable information regarding the safe usage of this traditional medicinal-based plant which is referred to inside the ethnobotany of medicinal stomachache as well as bloating in Malay Medicine (Werner 2002) book Medicine in Malay Villages vol. 2.

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