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Evaluation of the safety, gastroprotective activity and possible mechanisms of action of *Ziziphora hispanica* L. methanol extract

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ABSTRACT

This study aims to evaluate the gastroprotective effects of *Z. hispanica* methanol extract (ZME) in an ethanol-induced gastric ulcer model in rats, assess its safety profile, and investigate its chemical composition. Methods: A methanol extract was prepared by macerating 100 g of powdered aerial parts of *Z. hispanica* in 1000 mL of 85% methanol for 72 h. Total polyphenol content was quantified. Gastric ulcers were induced in Wistar rats using a single oral dose of 70% ethanol. Rats were pre-treated with ZME (50, 250, or 500 mg/kg) or ranitidine (50 mg/kg) as a reference control. Gastric tissues were examined for ulcerative lesions, and the percentage of protection was calculated. Gastric mucus production was also evaluated to explore the extract's mechanism of action. ZME significantly reduced ethanol-induced gastric mucosal damage in a dose-dependent manner. The highest dose (500 mg/kg) provided 97.18% protection. Additionally, ZME significantly enhanced gastric mucus secretion, exceeding that of the positive control. The extract's bioactivity is attributed to its phenol and flavonoid constituents. ZME exhibits strong gastroprotective properties by reinforcing the gastric mucus barrier. These findings validate the traditional use of *Z. hispanica* for gastrointestinal relief and suggest its potential as a natural therapeutic agent for the management of gastric ulcers.

1. Introduction

A peptic ulcer is a chronic, recurring gastrointestinal disorder that can affect the oesophagus, stomach, and duodenum. It is one of the most frequent illnesses of the gastrointestinal system, affecting 5 to 20% of the general population [1]. The development of the disease is widely recognised to stem from an imbalance between the harmful and protective factors of the mucosa. Potential aggressive factors include *Helicobacter pylori*, gastric acid, pepsin, non-steroidal anti-inflammatory drugs (NSAIDs), smoking, stress, and alcohol consumption [2].

Abbreviations: CAT, catalase activity; CMC, Directory of open access journals; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; GSH, Glutathione; HPLC-TOF/MS, High-Performance Liquid Chromatography – Time-of-Flight Mass Spectrometry; IC_{50}, Half Maximal Inhibitory Concentration; ME, Methanol Extract; NSAIDs, Non-Steroidal Anti-Inflammatory Drugs; OECD, Organisation for Economic Co-operation and Development; SOD, Superoxide Dismutase; TPC, Total Phenolic Content; ZME, *Ziziphora hispanica L* methanol extract.

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Conversely, defensive agents may consist of mucus, bicarbonate, prostaglandin, blood flow, nitric oxide, the antioxidant system, and epithelial cell regeneration [3,4]. The contemporary pharmacological management of peptic ulcers predominantly depends on proton pump inhibitors and H2-receptor antagonists. Regrettably, these therapeutic agents are frequently linked to a variety of adverse effects, substantial costs, and challenges regarding their accessibility [5,6]. Rat Wistar albino models mimic human peptic ulcer disease (PUD) by replicating mucosal damage from irritants like NSAIDs (indomethacin) or ethanol, which disrupt the mucus barrier, elevate acid levels, and induce oxidative stress—core factors in human gastric ulcers (prevalence ~3–5%)—thanks to shared gastric physiology including acid secretion and mucosa, though rats heal faster and models lack *H. pylori* dominance seen in humans; ethanol induction best translates acute chemical injury from alcohol/NSAIDs. Wistar rats are preferred for their genetic uniformity, affordability, and reproducible GI responses, enabling reliable screening of extracts before human trials; standard protocols (ethanol/HCl for necrosis, indomethacin for erosion, pylorus ligation for hypersecretion) cover diverse etiologies, are validated against omeprazole, and align with traditional uses of *Ziziphora*-like plants for GI ailments [7]. Therefore, it seems necessary to look for new alternative drugs from natural sources that effectively improve the management of peptic ulcer. Medicinal plants are important sources of chemicals and preparations that have been used since ancient time to to prevent and treat different ailments, including peptic ulcer [8–10].

The *Ziziphora* genus belongs to the *Lamiaceae* family it includes more than 30 species worldwide. [11].

Ziziphora hispanica essential oil from aerial parts, extracted via hydrodistillation/steam distillation (yields ~0.85%, 23–119 components covering 88.7–93%), is dominated by pulegone (78.6–79.5%), with monoterpenes like limonene, menthofuran, piperitenone, trans-isopulegone/isopulegol, and minor sesquiterpenes (δ-cadinene) [12,13]. Polar/methanol extracts contain 53 phenolics, such as caffeoyl/coumarylquinic acid isomers, plus flavonoids/phenylpropanoids. Related *Z. clinopodioides* subsp. bungeana extracts reveal monoterpenoid derivatives (ziziphoric acid, ziziphoroside D, 6′-malonylziziphoroside A), megastigmane glucosides (blumenol C glucoside, 9-O-malonyl derivative), monoterpenoids (7a-hydroxymintlactone, 7-hydroxypiperitone), and polyphenols (pinocembrine-7-O-rutinoside, chrysin-7-O-rutinoside, acacetin-7-O-rutinoside, luteolin-7-O-rutinoside, rutin, rosmarinic acid), contributing to cardiotropic, antioxidant, antibacterial, and gastroprotective effects [14].

It has been traditionally used to deal with various circumstances, among which are cardiovascular disorders, cold and infection. [6,15], headache [11,15] gastrointestinal problems [15–17]. Pharmacological investigations of the genus have also exhibited many activities against different conditions, including antibacterial [18–21], antioxidant [18], cardiovascular [22,23], gastric acid inhibition and anti-inflammatory [24].

In the Maghreb countries, the *Ziziphora* genus is represented by *Ziziphora capitata* L., *Ziziphora tenuior* L. And *Z. Hispanica* L. [25]. The latter species is also found in Spain, and it is used traditionally to deal with digestive problems [26]. In the Maghreb countries, it is also used in folk medicine to manage digestive upsets, as well as in dealing with cold, flu, cough and headache [27]. The pharmacological activities of the species were mainly concerned with the antioxidant antimicrobial and antiviral activities [28,29]. Based on the above-mentioned data, the current study aims to determine and quantify *Z. Hispanica* phytochemical content and evaluate the acute oral toxicity of the plant extract in mice. Moreover, this study assesses its ability to protect the gastric mucosa against ethanol-induced ulceration and investigates some of the possible mechanisms involved.

2. Materials and methods

2.1. Plant material

The fresh aerial parts of *Z. hispanica,* a plant species recognised for its medicinal potential, were carefully collected, and the authentication was performed by Professor Hocine Laouer from the Department of Plant Biology and Ecology, University of Setif 1, Algeria. The collection took place in the ecologically diverse region of Djelfa, Algeria, ensuring the specimens' purity and authenticity. After collection, the plant samples were systematically catalogued and preserved. A voucher specimen, labelled with the identification number 109 ZH/05/20 DEJ/SA/HL, was deposited in the Laboratory of Phytotherapy Applied to Chronic Diseases for subsequent research and reference purposes.

2.2. Animal

The gastroprotective experiment was carried out on female Wistar albino rats (Pasteur Institute, Algiers, Algeria), with body weights ranging from 180 to 220 g. For the acute toxicity assessment, mice weighing between 25 and 30 g were used. This study followed the ethical standards set by the Algerian Association of Experimental Animal Sciences (88–08/1988) and complied with the European Union Guidelines (2010/63/EU). During the first week, the animals were housed in groups in cages with unrestricted access to food and water. Before the experiment, they were fasted for 18 to 20 h, with free access to water until 60 min before the start of the study. To prevent coprophagy during the fasting period, the animals were kept in separate cages with wide-mesh wire flooring. All experiments were conducted in accordance with widely accepted standards for evaluating the efficacy and safety of plant-based medicines [30].

2.3. Plant extraction

Incorporating slight alterations, the extraction methodology was implemented in alignment with the procedure delineated by Benabdallah et al. [31]. Absolute methanol in a 1:10 ratio was utilised to facilitate the extraction from 100 g of *Z. hispanica* dry powder. This process extended over a three-day period at ambient temperature, with regular agitation to ensure uniformity. After the extraction, the mixture was subjected to filtration to separate the liquid phase. Concentration of the filtrate was achieved through evaporation at a controlled temperature of 40 °C. The concentrated extract, referred to as ZME, was subsequently desiccated and stored in a freezing environment to preserve its integrity until further application. The filtered extracts were concentrated using a rotary evaporator under vacuum at 40 °C to obtain the crude extract, which was then freeze-dried to remove any remaining water. The resulting extracts were stored in amber bottles at 4 °C until further analysis.

2.4. Total phenolic content (TPC)

The Folin–Ciocalteu colorimetric assay was employed to quantify the total phenolic content (TPC) of *Z. hispanica*. Each sample was reacted with 500 μL of a tenfold diluted Folin–Ciocalteu reagent. 400 μL of a 7.5% Na_2CO_3 solution was added after 4 min. The completed combination was agitated and allowed to react for 90 min at room temperature in the dark. The reaction mixture's absorbance was then measured at 760 nm. The total quantity of polyphenols in various extracts was calculated using a gallic acid standard curve. For each gram of dried plant extract, the results were represented as mg of gallic acid equivalent (GAE) [32].

2.5. Determination of total flavonoid content

The aluminium chloride assay was used to determine the total

flavonoid concentration[33] [32,33]. A volume of 1 mL from either the extract or quercetin standard was used in the assay. At 430 nm, the absorbance was measured in comparison to a produced blank after 10 min of incubation. Using a quercetin calibration curve, the results were reported as mg of quercetin equivalent per gram of dry plant extract weight (mg QE/g DW).

2.6. Determination of total tannin content

The capacity of the various extracts to precipitate haemoglobin from fresh bovine blood was assessed following the method of Benchikh et al. [33]. In brief, Equal volumes of each plant extract and haemolyzed bovine blood (initial absorbance = 1.6) were combined. Following a 20-min incubation at room temperature, the mixtures were centrifuged at 4000 rpm, and the absorbance of the resulting supernatant was recorded at 576 nm. The outcomes were expressed in terms of tannic acid equivalents (mg TAE/g DW), calculated using a calibration curve prepared with tannic acid.

2.7. HPLC analysis

Extract from *Z. hispanica* underwent HPLC-TOF/MS analysis as prescribed [34]. For the purpose of analysing phenolic acids and flavonoids in plant extracts, an HPLC method was created and validated. To eliminate particles, the extract was first diluted in HPLC-grade MeOH and then filtered through a PTFE (0.45 m) filter using an injector. A ZORBAX SB-C18 (4.6100 mm, 3.5 m) column and a 6210 Time of Flight (TOF) LC/MS detector were connected to an Agilent Technology 1260 Infinity HPLC system. Ultra-pure water containing 0.1% formic acid and acetonitrile was used as mobile phases A and B, respectively. The column temperature was 35 °C, and the flow rate was 0.6 mL min 1. Injection volume was 10 μL. The solvent program was as follow: 0 to 1 min 10% B; 1 to 20 min 50% B; 20 to 23 min 80% B; 23 to 25 min 10% B; 25 to 30 min 10% B. Ionization mode of the HPLC-TOF/ MS instrument was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 4000 V and finally, fragmentor voltage of 175 V. Phenolic compounds were identified by the retention time of sample chromatographic peaks compared with those of authentic standards using the same HPLC operating condition.

2.8. Acute oral toxicity

In accordance with the limit test recommendations outlined by the Organisation for Economic Co-operation and Development (OECD) guideline 423 (2001), the acute oral toxicity of ZME extract was assessed in mice using a limited number of subjects. Two single, high-dose oral administrations of the extract (2000 and 5000 mg/kg) were given, while the control group received 1.5% CMC. Following administration, the animals were deprived of food for three hours. Behavioural and toxicological effects, including agitation, writhing, and lethargy, were closely monitored at regular intervals over 24 h, with special focus on the first 4 h, and subsequently once per day for 14 days. After this period, the mice were euthanised, and gross pathological examinations were conducted on all organs to assess any potential toxic effects.

2.9. Ethanol-induced gastric ulceration in rats

In the conducted study, five groups consisting of six Wistar albino rats each were established to evaluate the gastroprotective effects of ZME extract. The experimental setup involved administering 1.5% carboxymethyl cellulose (CMC) to the first group as a negative control and 5 mg/kg of ranitidine to the second group as a positive control. The remaining groups, three through five, received ZME at varying doses of 50, 250, and 500 mg/kg, respectively. Following a 60-min period post-oral administration of these treatments at a volume of 5 mL/kg, ulcer induction was facilitated through the intragastric injection of 70% ethanol (0.5 mL/200 g body weight). After a 30-min interval post-ulcer induction, the animals were euthanised via cervical dislocation. Each stomach was then meticulously washed with saline, incised along the greater curvature for examination, and photographed to assess the presence and extent of linear haemorrhagic lesions in the glandular portion. The total length of these lesions per stomach was measured to calculate the ulcer index (UI), enabling the determination of gastroprotective efficacy through the calculation of percentage inhibition using the formula outlined by [35].

$$\%\text{of inhibition} = \frac{UI\ control \times UI\ treated}{UI\ control} \times 100$$

2.10. Quantification of mucus in the gastric layer

In assessing mucosal adherence, rats were used as model organisms to apply the Alcian blue staining technique, as initially described by Come et al. [36]. This method utilises Alcian blue, a cationic dye that does not permeate mucosal cells but forms stable complexes with soluble mucopolysaccharides and glycoproteins. According to the experimental protocol, the subjects were divided into eleven groups. Each glandular stomach segment was instilled with 10 mL of 0.1% Alcian blue solution (containing 0.16 M sucrose and 0.05 M sodium acetate, pH 5.8). Two hours post-application, the non-adherent dye was thoroughly removed by rinsing twice with 10 mL of 0.25 M sucrose, first for 15 min and then for 45 min. Subsequently, the tissues were immersed in 0.5 M magnesium chloride and agitated for two hours to elute the dye bound to the mucus. To purify the sample for spectrophotometric analysis, 4 mL of the blue solution was vigorously mixed with an equal volume of diethyl ether to remove any particulate matter. The absorbance of the aqueous layer was recorded at a wavelength of 605 nm using a Shimadzu UV/Vis-1601 spectrophotometer (manufactured in Japan). A standard calibration curve was employed to measure the amount of Alcian blue extracted from each gram of wet glandular tissue, with results expressed as micrograms of Alcian blue per gram of tissue based on linear regression analysis.

2.11. Determination of in vitro antioxidant activity

2.11.1 DPPH scavenging capacity

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of the extract was assessed spectrophotometrically using an MRXe tc (DYNEX Technologies GmbH, Denkendorf, Germany) by tracking the reduction of DPPH absorbance at 515 nm, following the method outlined by Benchikh et al. [37]. A volume of 20 μL of ZME extract or ascorbic acid standard in absolute methanol was combined with 180 μL of 0.004% DPPH solution in a 96-well plate. Absolute ethanol served as the blank. The mixtures were incubated for 30 min at room temperature, protected from light. Each experiment was performed in triplicate. The percentage of DPPH free radical scavenging activity was calculated using the following formula:

$$\%\text{inhibition} = \frac{[(\text{Absorbance of control} - \text{Absorbance of test sample})]}{\text{Absorbance of control}} X100$$

To determine the IC_{50} values, a dose-response curve was generated. The IC_{50} is defined as the concentration at which 50% of the maximum scavenging activity is achieved.

2.11.2 ABTS radical scavenging activity assay

The ABTS radical scavenging activity was assessed following the method described by [33] [36]. The ABTS radical stock solution (7 mM in water) was combined with 2.45 mM potassium persulfate and left in the dark at room temperature for 12–16 h. After incubation, the solution

was diluted with methanol to achieve an absorbance of approximately 0.7 at 734 nm. Subsequently, 50 μL of the sample was added to 1 mL of the ABTS solution and incubated in the dark at room temperature for 30 min. The absorbance of the resulting mixture was measured at 734 nm. Trolox served as the positive control. All experiments were conducted in replicates. The scavenging activity of the test compounds was calculated using the following equation:

$$\%\text{inhibition} = \frac{[(\text{Absorbance of control} - \text{Absorbance of test sample})]}{\text{Absorbance of control}} \text{X}100$$

2.11.3 Ferrous ion chelating activity

Reduced glutathione the chelating activity of the extracts on reduced glutathione was determined based on the inhibition of the Fe^{2+} ferrozine complex formation after the samples were treated with Fe^{2+} ions. In brief, 250 μL of the test material or EDTA at varying concentrations was added to 50 μL of $FeCl_2$ (0.6 mM in distilled water) and 450 μL of methanol. After a 5-min incubation, the reaction was initiated by adding 50 μL of 5 mM ferrozine, followed by stirring, and the mixture was allowed to react at room temperature for 10 min. The control contained all reagents except the extract or EDTA. [38]. The absorbance of the Fe^{2+}ferrozine complex was recorded at 562 nm. The chelating activity was calculated as a percentage using the following equation:

$$\text{Chelating activity } (\%) = \frac{[(\text{Absorbance of control} - \text{Absorbance of test sample})]}{\text{Absorbance of control}} X\ 100$$

To determine the IC_{50} values, a dose-response curve was plotted. IC_{50} is defined as the effective concentration of the test material that is required to chelate 50% of iron ions.

2.11.4 Reducing power

The reducing powers of the extracts from *Z. hispanica* L. and BHT were determined according to the method described by Martinez et al. [39]. A 0.1 mL aliquot of each extract and BHT was mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide, followed by incubation at 50 °C for 20 min. To halt the reaction, 0.25 mL of 1% trichloroacetic acid was added, and the mixture was centrifuged at 2790 *g* for 10 min. A 0.25 mL portion of the supernatant was then combined with 0.25 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ solution. The absorbance was subsequently measured at 700 nm. An increase in absorbance indicated greater reducing power of the tested samples.

2.12. In vivo antioxidant parameters of ZME in the rat stomach

2.12.1 Preparation of homogenate

After the sacrifice, the stomach from each animal was removed, opened along the great curvature, washed with ice-cold saline, blotted with filter paper, and the glandular portion was cut, weighed, and homogenized in an ice-cold solution of 50 mM Tris HCl buffer (pH 7.4) to produce A 10% (*w/v*) tissue homogenate was prepared and subsequently centrifuged at 4000 ×*g* for 15 min at 4 °C, and the supernatant was removed and kept at −20 °C for the measurement of the biochemical indicators catalase (CAT), reduced glutathione (GSH), lipid peroxidation (MDA), and superoxide dismutase (SOD).

2.12.2 Quantification of total protein content in gastric tissue

Gastric total protein content was measured using the Biuret method, which utilises a reagent mixture containing potassium iodide, potassium sodium tartrate, copper sulfate, and sodium hydroxide. Gastric total proteins were measured. Proteins react with copper sulphate in an alkaline environment, producing a blue-violet colour. In brief, tissue homogenate or bovine serum albumin standard (25 μL) was mixed with 1 mL of Biuret reagent and incubated at room temperature for 10 min. [40]. The absorbance was then measured at 540 nm, and the total protein content was calculated employing the formula presented below:

$$\text{Total protein}(\text{mg/mL}) = \frac{\text{Abs of sample}}{\text{Abs of standard}} \times nn \text{is standard concentration.}$$

2.12.3 Assessment of reduced glutathione (GSH)

Reduced glutathione (GSH) levels were determined using the method described by Ellman. [41]. The assay relies on the oxidation of GSH by DTNB (Ellman's reagent), 5,5′-dithio-bis (2-nitrobenzoic acid), which produces 2-nitro-5-thiobenzoic acid (TNB), with maximum absorbance measured at 412 nm. In this procedure, 50 μL of the tissue homogenate was diluted in 10 mL of 0.1 M phosphate buffer at pH 8. Then, 20 μL of 0.01 M DTNB was added to 3 mL of the diluted sample, and after a 5-min incubation, the resulting yellow colour was measured at 412 nm. A standard curve of GSH concentrations was used to determine the GSH content, expressed as mmol/g of tissue.

2.12.4 Lipid peroxidation (LPO) estimation

According to the method of Ohkawa [42]malondialdehyde (MDA) production was used to measure the lipid peroxidation of stomach tissue. The basic idea behind this procedure is the formation of a pink MDA-(TBA) complex by the interaction of MDA with thiobarbituric acid (TBA) under acidic conditions and at a higher temperature (100 °C). A volume of 0.5 mL of TCA (20% *w/v*) and 1 mL of TBA (0.67% w/v) were added to 0.5 mL of tissue homogenate. The mixture was incubated for 15 min at 100 °C, then rapidly cooled on ice, followed by the addition of 4 mL of n-butanol and centrifugation at 3000 rpm for 15 min. A blank was used as the reference for spectrophotometric measurement of the absorbance of the clear pink supernatant at 532 nm. The concentration of malondialdehyde (MDA) was calculated using a standard curve of 1,1,3,3-tetraethoxypropane (prepared by serial dilutions from a 10 mM stock solution). The results were expressed as nmol TBA per gram of gastric tissue.

2.12.5 Estimation of catalase (CAT) activity

The method for measuring catalase activity was originally described by Clairborn. [43] was slightly modified for this study. The test is based on the decomposition of hydrogen peroxide (H_2O_2) by catalase. Briefly, 50 μL of tissue homogenate was added to a quartz cuvette containing 2.9 mL of 19 mM H_2O_2 solution in 50 mM phosphate buffer (pH 7.4). The enzymatic activity was measured as μM of H_2O_2 decomposed per minute per mg of protein. The rate of H_2O_2 degradation in catalase activity was assessed by measuring absorbance at 240 nm immediately after initiation and at 15-s intervals for 60 s.

2.12.6 Determination of superoxide dismutase (SOD) activity

According to the method of Marklund & Marklund [44], superoxide dismutase (SOD) activity was measured by evaluating the enzyme's ability to inhibit pyrogallol autoxidation. In this assay, 5 μL of supernatant was mixed with 10 μL of 20 mM pyrogallol and 1 mL of 50 mM Tris-HCl buffer (pH 8.2). The increase in absorbance at 420 nm was monitored every 30 s for one minute, compared to a blank. The percentage inhibition of pyrogallol oxidation was calculated using the formula: (Abs control - Abs sample) / Abs control × 100. One unit of SOD activity was defined as the amount of enzyme required to inhibit pyrogallol oxidation by 50% compared to the control, and was determined using the following formula:

$$\text{Enzyme activity} = \frac{\text{Inhibition}}{IC_{50}} \times 10$$

2.13. Statistical data analysis

The results were expressed as means ± standard deviation (SD) and analysed using one-way analysis of variance (ANOVA), followed by Dunnett's test for post-hoc comparisons. A *P*-value of less than 0.05 ($P < 0.05$) was considered statistically significant. The analysis was performed using GraphPad Prism, Version 9.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Polyphenols and flavonoid contents

In this study, we evaluated the phytochemical composition of ZME, focusing on the content of polyphenols, flavonoids, and tannins. The analysis revealed significant quantities of these bioactive compounds, indicative of the extract's potential therapeutic and nutritional benefits. The polyphenol content was measured at 61.62 mg GAE/g of extract, with a standard deviation of ±1.79, highlighting its rich antioxidant profile. Flavonoids, known for their beneficial effects on health, including anti-inflammatory and neuroprotective properties, were present at 21.72 mg QE/g of extract (±0.31 standard deviation). Tannins, another group of compounds with recognised antioxidant and anti-inflammatory activities, were found in considerable amounts, at 42.69 mg TA/g of extract (±1.03 standard deviation). The results presented in Table 1 showed that ZME is rich in phenolic acids and tannins. Phenolic acid amounts were three times higher than the flavonoids and twice the number of tannins.

3.2. HPLC-TOF/MS analysis

The results from the High-Performance Liquid Chromatography-Time of Flight/Mass Spectrometry (HPLC-TOF/MS) analysis indicate the detection of various phenolic acids and flavonoids within ZME. Utilising the applied chromatographic conditions, effective separation of constituents was achieved, as evidenced by distinct peak formation in the chromatogram (Fig. 1). The quantitative assessment revealed that Rosmarinus acid was the predominant compound in the extract, measured at 53.905 mg/kg, with a corresponding retention time (RT) of 11.7 min. Additionally, chlorogenic acid was present in a significant quantity, amounting to 35.86 mg/kg of the extract. Notably, 4-hydroxybenzoic acid and rutin were identified in equal concentrations, further contributing to the phytochemical profile of ZME extract. These findings are succinctly summarised in Table 2, which outlines the concentrations and retention times of the detected compounds.

Table 1
Polyphenols, flavonoids and tannin contents in the methanol extract (ME) of *Z. hispanica.*

Extract	Polyphenol content (mg GAE/g extract)	Flavonoid content (mg QE/g extract)	Tannin content (mg TA/g extract)
ZME	61.62 ± 1.79	21.72 ± 0.31	42.69 ± 1.03

The chromatographic analysis presented in Fig. 1 illustrates the complex composition of ZME, as analysed by High-Performance Liquid Chromatography-Time of Flight/Mass Spectrometry (HPLC-TOF/MS). The conditions optimised for the HPLC analysis resulted in a chromatogram with well-resolved peaks, indicating a successful separation of the extract's constituents. Quantitative analysis of the chromatogram peaks revealed the presence of several phenolic compounds. Notably, rosmarinic acid was found to be the most abundant phenolic acid with a concentration of 53.905 mg/kg of extract and a retention time of 11.7 min. Chlorogenic acid was also present in a substantial concentration of 35.86 mg/kg of extract. Moreover, the chromatogram shows the co-elution of 4-hydroxybenzoic acid and rutin, both present at comparable concentrations. The observed peak patterns and retention times correspond with the known characteristics of these phenolic compounds, allowing for their identification in the extract. The data underscore the rich phytochemical composition of *Z. hispanica,* with its extract demonstrating a diverse spectrum of phenolic acids and flavonoids. The detailed quantitative data, including the retention times and peak areas, are tabulated in Table 2, which complements the visual data presented in Fig. 1. The chromatographic profile of ZME, as presented in Fig. 1, is comprehensively detailed in the accompanying Table 2, which enumerates the various phenolic acids and flavonoids identified. The HPLC-TOF/MS analysis successfully differentiated and quantified multiple compounds, with the retention times and corresponding concentrations in both ppb and mg/kg extract provided. The table highlights rosmarinic acid as the most prominent component, with a retention time of 11.709 min and a concentration of 336.907 ppb, translating to 53.905 mg/kg extract. Chlorogenic acid, with a retention time of 5.852 min, is also notably abundant at 224.184 ppb or 35.869 mg/kg extract. Other identified compounds, such as 4-hydroxybenzoic acid and rutin, were observed at 66.420 ppb (10.627 mg/kg extract) and 66.516 ppb (10.642 mg/kg extract), respectively, with close retention times, indicating a rich and diverse phenolic content. This data signifies the presence of a variety of phenolic constituents in ZME, each contributing to its overall phytochemical profile. The presence of these compounds, particularly in such significant amounts, could imply potential biological activities warranting further investigation. The retention times listed in Table 2 correlate with the peaks observed in the chromatogram, providing a reliable method for the identification and quantification of the individual components within the extract.

3.3. Acute oral toxicity

The in vivo toxicological assessment of the ZME extract was conducted to evaluate its safety profile at various dosages. The treated mice exhibited no adverse reactions in terms of behavioural, motor, and neuronal functions across the administered dosages. Throughout the observation period, there were no mortalities reported, indicating a high degree of tolerability. Further observation of physical and behavioural biomarkers, such as the condition of skin and fur, and ocular health, revealed no deviations from the norm. Similarly, the behavioural pattern activities of mice receiving the plant extract did not differ significantly from those observed in the control group. These observations suggest that the ZME extract does not induce any observable toxicological effects in the parameters assessed. Given these findings, it can be concluded that the oral LD_{50} (lethal dose, 50%), the dose at which half of the test population is expected to die from drug-induced toxicity of ZME extract, is in excess of 5000 mg/kg body weight. This threshold surpasses the commonly referenced benchmark for substances considered non-toxic through oral administration as per OECD guidelines. Therefore, within the scope of this study, the ZME extract demonstrates a favourable safety profile, warranting further exploration into its

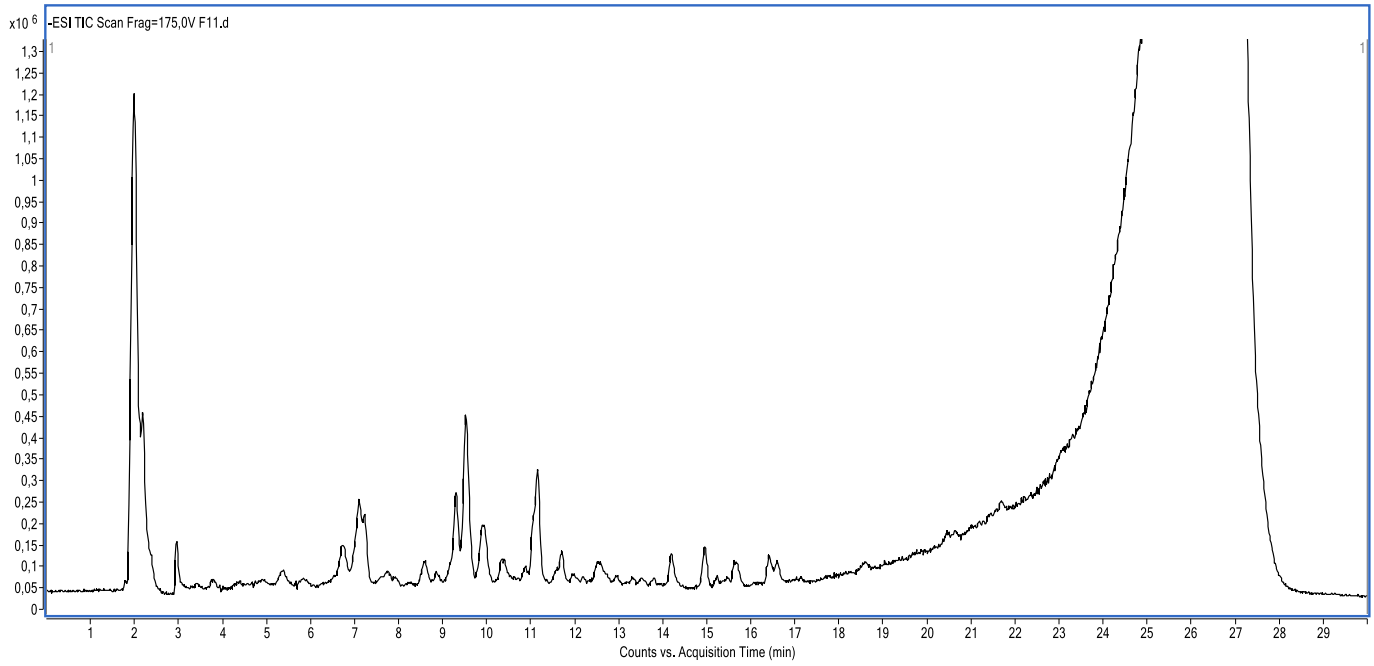

Fig. 1. HPLC chromatogram of flavonoids and phenolic compounds in ZME.

Table 2
Phenolic acids and flavonoids determined by HPLC-TOF/MS in *Z. hispanica.* ME.

Compound	Retention time (min)	ppb value	mg/kg extract
Gallic acid	2209	6.117	0.978
Gentisic acid	4.424	15.438	2.47
Chlorogenic acid	5.852	224.184	35.869
4-hydroxy benzoic acid	6.606	66.420	10.627
Caffeic acid	7.617	48.862	7.817
Rutin	9.350	66.516	10.642
Chloric acid	9.253	9.430	1.508
p-Coumaric acid	9.895	13.557	2.169
Rosmarinic acid	11.709	336.907	53.905
Quercetine	16.330	5.770	0.923

Table 3
In vitro antioxidant activities of methanol extract (ME) from *Z. hispanica* L.

	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (µg/mL)	Iron chelation IC_{50} (µg/mL)	Reducing power (EC_{50} (mg/mL)
ZME	0.086 ± 0.006	0.047 ± 0.005	0.35 ± 0.008	0.407 ± 0.003
Trolox	/	0.0031 ± 0.01	/	/
EDTA	/	/	0.020.28 ± 0.0002	/
BHT	0.014 ± 001	/		0.074 ± 0.001

therapeutic potential.

3.4. In vitro antioxidant activities

In the investigation of the antioxidant potential of the ZME extract, a series of assays were employed, including DPPH and ABTS radical scavenging tests, iron chelating, and reducing power assays. The results of these tests are summarised in Table 3. The data demonstrate a significant antioxidant capacity of the ZME extract, as evidenced by the low IC_{50} values in both the DPPH and ABTS assays. Specifically, the ZME extract exhibited a potent radical scavenging activity with an IC_{50} of 0.047 ± 0.005 mg/mL for DPPH and 0.086 ± 0.006 mg/mL for ABTS, indicating strong antioxidant activity. In terms of iron chelating capacity, the ZME extract showed moderate efficacy with an IC_{50} value of 0.35 ± 0.008 mg/mL. Similarly, the reducing power assay revealed an IC_{50} of 0.407 ± 0.003 mg/mL, further suggesting moderate antioxidant activity. These findings indicate that while the ZME extract has a particularly strong radical scavenging ability, its capacity to chelate metal ions and to act in a reductive capacity is somewhat less pronounced, yet still significant. The observed antioxidant activities are indicative of the ZME's potential to neutralise reactive oxygen species and possibly contribute to mitigating oxidative stress-related damage. This is consistent with the known properties of the phenolic compounds identified within the extract, which are recognised for their electron donation abilities and consequent antioxidant effects.

3.5. Assessment of ZME extract's impact on ethanol-induced gastric mucosal damage in rats: A macroscopic and histopathological study

The macroscopic evaluation of rat gastric mucosa following the administration of the ZME extract provided significant observations on its gastroprotective effects. Fig. 2 displays the comparative results between the gastric tissues of control and ZME-treated groups post-ethanol exposure. Upon treatment with 70% ethanol, the control group exhibited substantial gastric mucosal damage. This was macroscopically evident through the alteration of the mucosal colour, disruption of mucus integrity, and the occurrence of petechiae. Furthermore, haemorrhage and oedema were prominently visible, indicating severe mucosal injury. In contrast, the groups pre-treated with ZME extract showed a noticeable reduction in ethanol-induced gastric damage. The severity of gastric lesions was markedly diminished, as reflected by the preservation of mucosal colour and integrity. Additionally, there was a significant decrease in the appearance of petechiae, haemorrhage, and oedema. The degree of attenuation of these macroscopic damages correlated with the administered dosages of the ZME extract, suggesting a dose-dependent protective effect. These results suggest that the ZME extract has therapeutic potential in reducing ethanol-induced gastric mucosal injury. The observed protective effects may be attributed to the phytochemical constituents of the extract, which could be linked to the strengthening of mucosal defence mechanisms or to anti-inflammatory

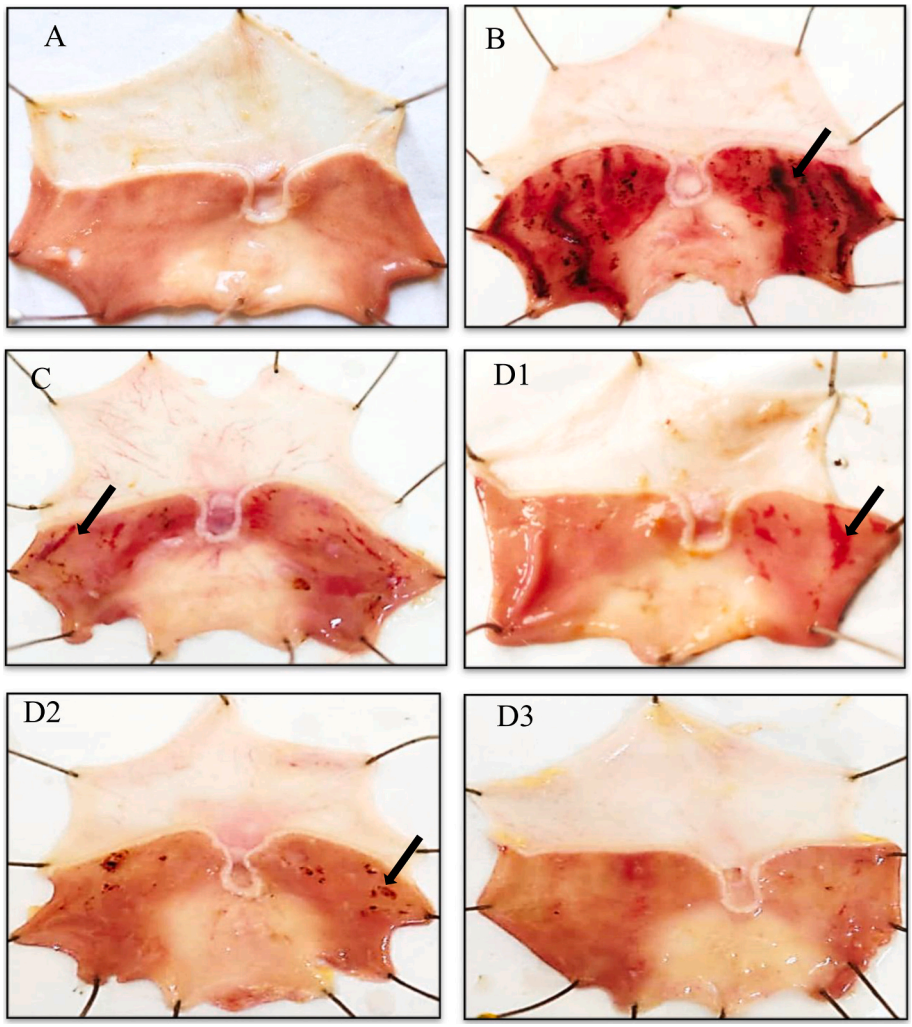

Fig. 2. Macroscopic evaluation of gastric mucosa following treatment with ZME extract in ethanol-induced gastric lesion model in rats. (A) Normal group with no treatment. (B) Ulcer control group administered ethanol and pre-treated with CMC vehicle (negative control). (C) Group pre-treated with ranitidine (5 mg/kg), serving as the positive control. (D1, D2, D3) Groups pre-treated with ZME extract at doses of 50, 250, and 500 mg/kg, respectively, before ethanol administration.

and antioxidative properties. The histological assessment of gastric tissues treated with ZME extract reveals its potent gastroprotective effect against ethanol-induced damage Fig. 3. In the ZME-treated groups (D1, D2, D3), a dose-dependent improvement was evident, with the highest dose (500 mg/kg) markedly preserving gastric mucosal integrity. Inflammatory cell infiltration (blue arrow) and vascular congestion (green arrow) were notably reduced relative to the control. Notably, this high dose of ZME showed greater protection than ranitidine (C), suggesting a promising therapeutic potential for ZME in treating gastric ulcers.

I. *Macroscopic examination*

II. *Histopathological examination*

3.6. Effect of methanol extract ME on ethanol-induced gastric ulcer in rats

The investigation into the gastroprotective effects of *Z. hispanica* methanol extract (ME) on ethanol-induced gastric ulcers in rats yielded noteworthy results. Acute oral administration of ethanol to the rats resulted in pronounced gastric mucosal damage, manifested as mucosal oedema, congestion, and an inflammatory response characterised by neutrophil infiltration. The administration of ME at varying doses of 50, 250, and 500 mg/kg was observed to significantly mitigate the mucosal injuries caused by ethanol. As demonstrated in Fig. 4, the therapeutic efficacy of ME increased in a dose-dependent manner. At the highest tested dose of 500 mg/kg, ZME conferred a remarkable level of protection, showing a 97.18% ulcer protection rate. This protection rate notably exceeds that of the positive control, ranitidine, which offered a 66.95% protection rate. This result is graphically represented in Fig. 4, which illustrates the percentage of ulcer protection afforded by the different treatments. The bar chart indicates a nonsignificant change in ulcer protection at the lower doses of ZME (50 and 250 mg/kg) when compared to the positive control, indicating a highly significant difference with a p-value ≤ 0.01. The values presented in the bar chart are expressed as means $\pm$ SEM (standard error of mean) for a sample size of six ($n = 6$), reinforcing the statistical robustness of the observed protective effect at higher doses of ZME against ethanol-induced gastric ulceration.

3.7. Effect of ZME on gastric mucus content in rats

The quantification of gastric mucus content in rats, an important defensive factor against gastric lesions, revealed the beneficial effects of the ZME extract. As depicted in Fig. 5, ZME treatment resulted in a significant increase in mucus production when compared to the vehicle-treated group. Statistical analysis confirms the significant enhancement of gastric mucus content with ZME administration. The highest dose of

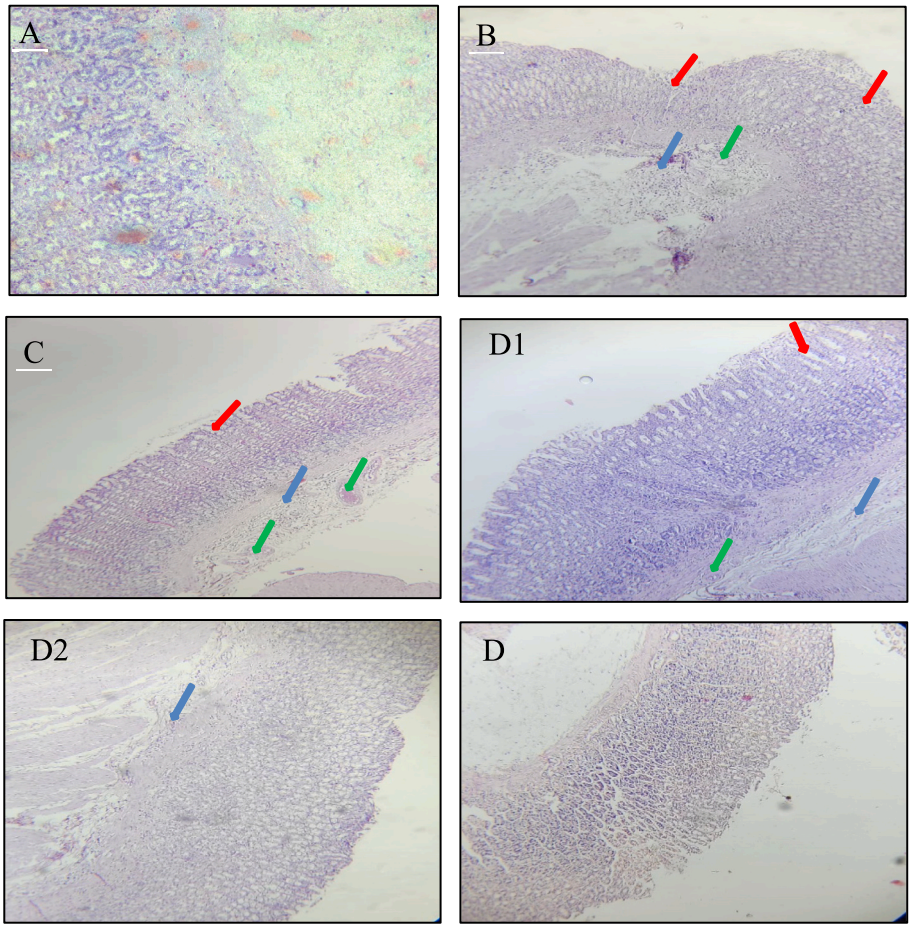

Fig. 3. Effects of ZME extract on the macroscopic appearance of the gastric mucosa in ethanol-induced gastric lesions in rats are as follows: (A) Normal control group showing intact gastric mucosa. (B) Ethanol ulcer control group pre-treated with CMC vehicle, serving as the negative control. (C) Group pre-treated with ranitidine (5 mg/kg) was used as the positive control. (D1, D2, D3) Groups pre-treated with ZME extract at doses of 50, 250, and 500 mg/kg, respectively.

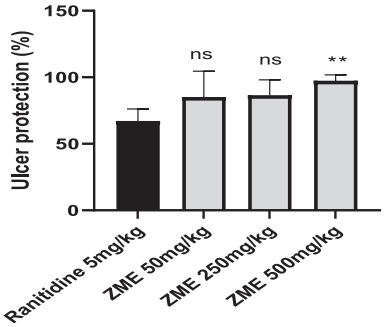

Fig. 4. Effect of ZME extract on gastric ulcer in rats. The values of the bar chart are expressed as means ± SEM ($n = 6$). **$P \leq 0.01$; ns: not significant vs ranitidine as positive control.

ZME (500 mg/kg) was particularly effective, yielding an average of 282.20 ± 9.36 μg of Alcian blue bound per gram of wet gastric tissue. This is a noteworthy increase compared to the positive control group treated with ranitidine, which exhibited a mucus content of 235.66 ± 21.44 μg Alcian blue/g wet tissue. The *P*-value of ≤0.0001 indicates a highly significant effect of ZME on mucus production. These results suggest that the ZME not only has protective effects against gastric ulcers, as shown in previous figures, but also promotes the activity of one of the stomach's primary protective mechanisms by augmenting mucus content. This increase in mucus production may contribute to a protective barrier against the corrosive action of substances such as ethanol.

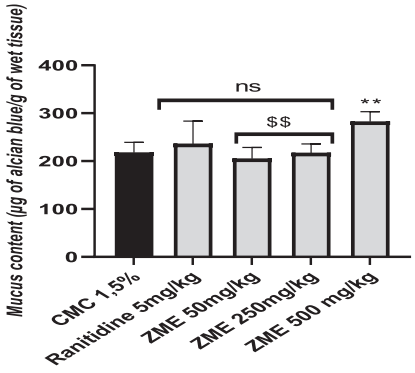

Fig. 5. Effect of ZME extract on gastric mucus content in rats. The values of the bar chart are expressed as means ± SEM (n = 6). ns: not significant; (**P ≤ 0.01) vs ranitidine as positive control.

In Fig. 5, the bar chart clearly demonstrates the mean gastric mucus content for each group, with error bars representing the standard error of the mean (SEM) for the six animals in each group (n = 6). The level of significance compared to ranitidine, the positive control, is indicated by asterisks, underscoring the statistical relevance of the findings.

3.8. *In vivo antioxidant parameters of ZME in the rat stomach*

The investigation into the in vivo antioxidative potential of ZME

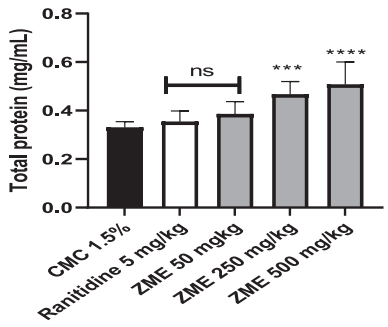

Fig. 6. Effect of ZME extract on total protein level in rat stomach tissue. The values of the bar chart are expressed as means ± SEM ($n = 6$). ((****$P \leq 0.0001$; ***$P \leq 0.001$; ns: not significant) vs vehicle (CMC 1.5% p.o.).

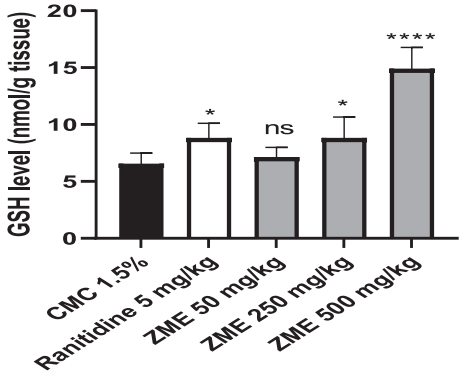

Fig. 7. Effect of ZME extract on GSH level in rat stomach tissue. The values of the bar chart are expressed as means ± SEM ($n = 6$). (****$P \leq 0.0001$; *$P \leq 0.05$; ns: not significant) vs vehicle (CMC 1.5% p.o.).

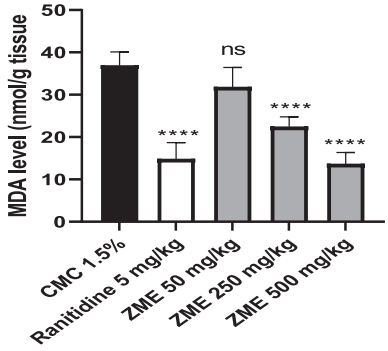

Fig. 8. Effect of ZME extract on MDA level on gastric tissues of rats. The values of the bar chart are expressed as means ± SEM ($n = 6$). ****P ≤ 0.0001; ns: not significant) vs vehicle (CMC 1.5% p.o.).

extract in rat stomach tissues provided significant insights into its antiulcerogenic mechanism. Oxidative stress plays a pivotal role in gastric ulcer pathogenesis, where an imbalance between oxidative and antioxidative processes leads to tissue damage (Salim, 2012). The assessment of oxidative stress biomarkers in the gastric tissue of rats revealed a marked alteration in these parameters upon gastric ulcer induction. The levels of lipid hydroperoxides (LOOH), which are products of lipid peroxidation and indicators of oxidative stress, were elevated in the gastric tissues of the ulcer-induced group. Conversely, the activities of critical antioxidative enzymes—catalase, glutathione (GSH), and superoxide dismutase (SOD)—were significantly reduced. These changes, showcased in Figs. 6–10, were observed by comparing the values with those from vehicle-treated animals, indicating a clear

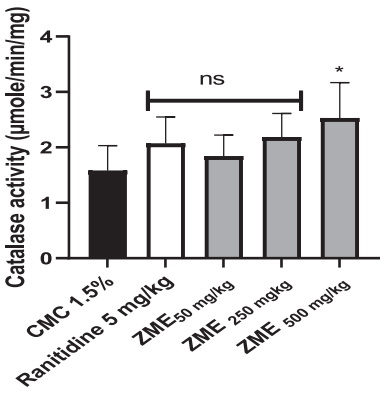

Fig. 9. Effect of ZME extract on catalase activity in rat stomach tissue. The values of the bars chart are expressed as means ± SEM ($n = 6$); *$P \leq 0.05$; ns: not significant) vs vehicle (CMC 1.5% p.o.).

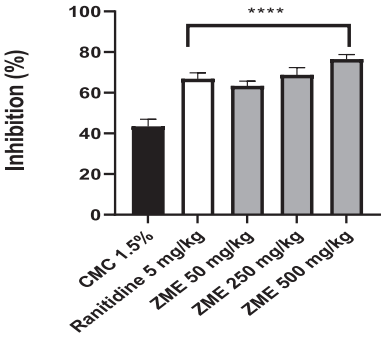

Fig. 10. Effect of ZME extract on SOD activity in the gastric tissues of rats, and gastric mucus content in rats. The values of the bar chart are expressed as means ± SEM (n = 6). (****$P \leq 0.0001$); ns: not significant vs vehicle (CMC 1.5).

oxidative stress profile associated with gastric ulceration. Pretreatment with ZME extracts demonstrated a significant reversal of these stress markers. LOOH levels decreased upon treatment with ZME, suggesting a reduction in lipid peroxidation processes within the gastric tissue. Furthermore, the activities of catalase, GSH, and SOD were restored to levels that were comparable to the positive control group that received ranitidine, a known antiulcer agent with antioxidative properties (Bhattacharya et al., 2011). These findings underscore the antioxidative efficacy of ZME, positing that its antiulcerogenic effects are mediated through the modulation of oxidative and antioxidative parameters in the stomach. The ZME extract appears to confer protection against oxidative damage by enhancing the endogenous antioxidative defence system, thereby mitigating the harmful effects of reactive oxygen species (ROS) involved in gastric ulcer development.

3.8.1. *Effect of ZME on total protein level*

The administration of ZME extract in rats demonstrated a significant impact on the total protein levels in the gastric tissues. As indicated in Fig. 5, there was a notable increase in protein concentrations when ZME was administered at doses of 250 and 500 mg/kg compared to the vehicle-treated control group. The quantitative analysis revealed that the total protein levels in the ZME-treated groups were significantly higher, with a *P*-value of ≤0.0001, suggesting a strong effect of the treatment. Notably, the increase in protein levels was dose-dependent, with the higher dose of 500 mg/kg resulting in a greater enhancement of total protein content than observed in the positive control group treated with ranitidine. This suggests that the ZME not only has a protective effect against gastric ulcers but may also promote the restoration or maintenance of gastric tissue integrity, as indicated by the increased

total protein levels. Proteins play a vital role in cell structure and function, and their levels in gastric tissues can be reflective of the regenerative processes following injury. In summary, the results indicate that ZME treatment leads to a significant elevation in total protein levels in rat gastric tissue, especially at higher doses, surpassing the effects of ranitidine, which is widely used for gastric protection.

3.8.2. Effect of ZME on GSH level

Analysis of the impact of ZME extract on glutathione (GSH) levels in rat gastric tissue revealed notable results, as depicted in Fig. 6. GSH, a crucial antioxidant found within cells, plays a significant role in cellular defence against oxidative damage. The ZME was administered to rats at various doses, with a particular focus on the 250 and 500 mg/kg concentrations. The vehicle-treated control group presented a baseline GSH level of 6.52 ± 0.96 nmol/g tissue. In comparison, the groups treated with ZME showed a dose-dependent elevation in GSH levels. At the highest administered dose of 500 mg/kg, there was a pronounced increase in GSH levels, reaching 14.88 ± 1.88 nmol/g tissue. This enhancement was not only significant when compared to the vehicle control but also when compared to the positive control, which recorded a GSH level of 8.79 ± 1.31 nmol/g tissue. The statistical significance of this increase was confirmed with a *P*-value of ≤0.0001. These findings demonstrate the potent effect of ZME on the antioxidative capacity of gastric tissue, as evidenced by the substantial increase in GSH levels. This effect was most marked at the highest tested dose, suggesting a potential dose-response relationship in the efficacy of ME treatment.

3.8.3. Effect of ZME on lipid peroxidation (LOOH)

In evaluating the effect of ZME extract on lipid peroxidation within the gastric tissues of rats, as presented in Fig. 7, a notable modulation was observed. Lipid peroxidation, measured by malondialdehyde (MDA) levels a byproduct indicative of oxidative stress was significantly influenced by the treatment with ZME. Following the induction of gastric ulcers using ethanol, the control group of normal rats displayed a considerable increase in MDA content, with a mean value of 36.89 ± 3.19 nmol/g tissue. In contrast, the administration of ME extracts resulted in a significant, dose-dependent decrease in MDA levels. Notably, the highest dose of ZME (500 mg/kg) was effective in reducing MDA levels to 13.38 ± 2.7 nmol/g tissue, which was not only a statistically significant reduction ($P \leq 0.0001$) from the ethanol-induced elevation but also slightly lower than the reduction achieved by the positive control, ranitidine, which showed an MDA content of 14.83 ± 3.85 nmol/g tissue. These results indicate that ZME possesses potent properties that counteract lipid peroxidation, a key process in the pathogenesis of ethanol-induced gastric damage, and that its antioxidative efficacy may exceed that of ranitidine at this high dosage.

3.8.4. Effect of ZME on catalase activity

The administration of ZME extract in rats has been shown to affect the activity of catalase, an enzyme integral to the breakdown of hydrogen peroxide, a reactive oxygen species. As illustrated in Fig. 8, a dose-dependent response was observed in catalase activity levels. While there was an overall increase in catalase activity following ME treatment, the elevation was statistically significant only at the highest tested dose. The catalase activity measured at this dose was 2.52 ± 0.64 μmoL/min/mg protein, which is a marked increase from the activity levels in the vehicle-treated negative control group. Furthermore, comparison with the positive control, ranitidine, revealed that the ZME at this dosage had a greater impact, enhancing the catalase activity beyond the level of the positive control, which stood at 2.07 ± 0.47 μmoL/min/mg protein. These results indicate that ZME has the potential to boost antioxidant defenses in gastric tissue, particularly at higher doses, by increasing the activity of enzymes that combat oxidative stress.

3.8.5. Effect of ZME on superoxide dismutase (SOD) activity

The administration of ZME extract was observed to enhance superoxide dismutase (SOD) activity in rat gastric tissue, a pivotal antioxidant defence against the superoxide radical. The results, as demonstrated in Fig. 9, indicated a significant enhancement of SOD activity in a dose-dependent manner following oral treatment with ZME. The vehicle-treated control group displayed a baseline SOD activity level of 43.37 ± 3.55%. In comparison, ME treatment at increasing concentrations led to an upsurge in SOD activity, with the highest dose of the extract showing an inhibition capacity of 76.42 ± 2.35% against pyrogallol autoxidation. This level of SOD activity not only represents a significant increase from the control group but also exceeds the activity observed in the positive control group, which was at 66.75 ± 3.06%. These findings illustrate that ME has a potent effect on the enzymatic activity of SOD, suggesting that the extract may play a role in mitigating oxidative stress by enhancing the biological systems responsible for neutralising reactive oxygen species.

4. Discussion

4.1. Polyphenols and flavonoid contents

The findings of this study contribute to the growing body of evidence supporting the health benefits of plant-derived extracts, particularly those rich in polyphenols, flavonoids, and tannins. The high polyphenol content observed in the ZME extract suggests a potent antioxidant capacity, which could be harnessed in various pharmacological and nutraceutical applications. Antioxidants are crucial for neutralising free radicals, thereby preventing cellular damage and reducing the risk of chronic diseases such as cancer, diabetes, and heart disease [45–47]. The flavonoid content, although lower than that of polyphenols, is significant and indicates the extract's potential for combating inflammation and protecting against neurodegenerative diseases. Flavonoids have been extensively studied for their role in modulating signalling pathways involved in inflammation and cell survival. Tannins, with their notable presence in the extract, are known for their astringent properties and their ability to precipitate proteins. This characteristic could underlie potential health benefits, such as reducing blood pressure and combating heart disease [48]. Moreover, tannins have been shown to possess antimicrobial properties, suggesting the extract could also be valuable in preventing and treating infections. Comparatively, the ZME extract's phytochemical profile is consistent with or exceeds those reported in similar studies, underscoring its potential as a superior source of natural antioxidants [49]. The variation in compound concentrations, as reflected by the standard deviations, suggests a degree of variability that could be attributed to factors such as plant source, extraction methods, and environmental conditions. Overall, this study reinforces the importance of plant extracts in the search for natural compounds with health-promoting properties. The ZME extract, with its rich composition of polyphenols, flavonoids, and tannins, holds promise for various applications in the fields of medicine and nutrition. The methanol extract analysed in this study is a potent source of polyphenols, flavonoids, and tannins, suggesting its considerable antioxidant and health-promoting potential [50,51].

4.2. HPLC analysis

The analysis presented in Fig. 1 and Table 2 reveals a rich profile of phenolic compounds in the ZME extract, consistent with the phytochemical properties reported in related literature. Phenolic acids and flavonoids, as identified in our study, are widely recognised for their antioxidant activities, contributing to the pharmacological potential of plant extracts[52,53]. Rosmarinic acid, the predominant compound detected with a retention time of 11.709 min and concentration of 336.907 ppb (53.905 mg/kg extract), is known for its anti-inflammatory and neuroprotective effects. The high concentration observed here aligns with the findings of [54], who reported rosmarinic acid as a major constituent in various medicinal herbs. Chlorogenic acid, identified with

a retention time of 5.852 min at 224.184 ppb (35.869 mg/kg extract), has been reported to exhibit antidiabetic and antihypertensive properties. Chlorogenic acid (CGA) is a powerful antioxidant polyphenol molecule found in many diets and liquid beverages, playing a preventive and therapeutic role in various diseases caused by oxidative stress and inflammation [55]. The amount of chlorogenic acid detected in our study is consistent with its reported abundance in certain medicinal plants, as supported. [56]. The presence of other phenolics, such as 4-hydroxybenzoic acid and rutin, in comparable concentrations (66.420 ppb and 66.516 ppb, respectively), suggests a complex interplay of antioxidants in ZME extract. The biological significance of these compounds has been highlighted in several studies, where they are noted for their cardioprotective and vasodilatory effects [57,58]. Interestingly, the co-elution of some compounds, as indicated by the closely related retention times, for example, caffeic acid at 7.617 min and rutin at 9.350 min, may suggest similar structural properties that influence their chromatographic behaviour, as discussed by [59]. The observed concentrations and retention times not only provide insights into the phytochemical composition of ZME but also corroborate with previous research, thereby supporting the reliability of our analytical approach. It is crucial, however, to consider the limitations of the current study, such as potential matrix effects or the influence of extraction methods on phytochemical yield, as noted by Brown et al. [60]. Further studies should aim to isolate individual compounds and investigate their specific biological activities. Additionally, exploring the synergistic effects of these compounds could uncover potential therapeutic applications of ZME extract, building upon the foundation laid by the present analysis.

4.3. Acute oral toxicity

The findings from the acute toxicity study of ZME extract suggest a high margin of safety for oral administration in mice. Throughout the dosing regimen, the maintenance of normal behavioural, motor, and neuronal functions in the treated mice is indicative of the extract's non-toxic nature. No mortality or signs of acute toxicity were observed, even at the highest dose tested, which is consistent with the traditional use of *Z. hispanica* as a medicinal plant with no reported adverse effects. The absence of observable changes in critical indicators such as skin and fur condition, eyes, and behavioural patterns aligns with the findings of previous studies that have reported a similar lack of toxicity in plant extracts with comparable phytochemical profiles [61]. These results are encouraging and support the hypothesis that the presence of phenolic compounds, identified in our chromatographic analysis, may contribute to the extract's non-toxic profile [62]. The estimated oral LD_{50} value exceeding 5000 mg/kg body weight situates the ZME extract well within the “practically non-toxic” category as per OECD Guidelines for the Testing of Chemicals. This threshold is several-fold higher than the concentrations at which pharmacological effects are anticipated, offering a substantial safety buffer [30]. However, it is imperative to consider that the LD_{50} is not an absolute measure of a substance's safety and does not account for potential chronic toxicity or idiosyncratic reactions. Therefore, while acute toxicity did not manifest at high doses, comprehensive evaluations, including sub-chronic and chronic toxicity studies, are recommended to fully understand the safety profile [63]. Furthermore, as metabolic pathways in mice are not identical to those in humans, there is a need for careful extrapolation of these results to potential human therapeutic use. Species-specific variations in metabolism can significantly affect the pharmacokinetics and toxicological outcomes of phytochemicals [64]. The present study lays a foundational understanding of the acute toxicity of ZME extract, suggesting its potential for safe consumption. However, further research involving long-term dosing, a broader range of biological markers, and eventually human trials, is essential to translate these findings into clinical recommendations.

4.4. In vitro antioxidant activities

The in vitro antioxidant activities of the ZME extract, as elucidated through various assays, provide valuable insights into its potential therapeutic applications. The extract exhibited potent radical scavenging activities in the DPPH and ABTS assays, which is indicative of a strong ability to donate electrons and neutralise free radicals [65]. These results are in line with previous studies that have associated high phenolic and flavonoid contents with robust antioxidant activity [66]. Comparatively, the extract demonstrated moderate activity in the iron chelating and reducing power assays. While the chelating ability is less than the radical scavenging potential, it remains a crucial aspect of the overall antioxidant capacity of the extract. Chelating agents can attenuate oxidative stress by binding to pro-oxidant metals, preventing these metals from catalysing the production of reactive oxygen species [67]. The moderate reducing power of the extract suggests its capability to exert antioxidant effects via electron transfer to stabilise and deactivate free radicals. The reducing capacity of a compound is associated with the presence of reductions, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [68]. These findings are substantial as they suggest the methanol extract of *Z. hispanica* may serve as a potential source of natural antioxidants. The strong DPPH and ABTS scavenging activities correlate with the presence of compounds such as rosmarinic acid and chlorogenic acid, previously identified in the chromatographic analysis of the extract. These compounds are well-documented for their antioxidant properties, which are likely contributing to the observed activities [69]. It is essential to note that while the in vitro assays indicate antioxidant potential, the in vivo relevance of these activities must be considered with caution. The bioavailability, metabolism, and interaction with biological targets of the extract's constituents can influence their antioxidant efficacy in a physiological context [70]. In conclusion, the *Z. hispanica* methanol extract possesses significant in vitro antioxidant activities, warranting further studies to explore its effects in vivo and its possible health benefits.

4.5. Macroscopic and histopathological examination of ME effect on ethanol-induced gastric mucosa damage in rats

In analysing the results delineated in Fig. 2, the study presents clear visual evidence of the deleterious effects of ethanol on gastric tissue. When administered 70% ethanol, the specimens exhibited substantial macroscopic damage. This was primarily characterised by a discernible alteration in the gastric mucosa's colouration and texture, with a noticeable depletion of the protective mucus layer. Moreover, there were observable instances of petechiae and haemorrhage, alongside evidence of oedema within the gastric tissues, which are indicative of underlying tissue trauma and inflammation [71]. Interestingly, the severity of these ethanol-induced gastric lesions appears to be modulated by the administration of ZME. The protective effect of ZME, manifested in a graded response, indicates its potential therapeutic properties, possibly attributed to its antioxidant and anti-inflammatory effects [72,73]. The mitigation of damage ranged from partial to significant, suggesting a dose-dependent efficacy of ZME in preserving the integrity of the gastric mucosa. The mechanism underlying the gastroprotective action of ZME may involve the enhancement of mucosal defence factors or inhibition of the inflammatory cascade often triggered by ethanol exposure. These findings are in congruence with the growing body of research which posits the gastroprotective effects of plant-derived compounds, highlighting their therapeutic potential in the treatment of alcohol-induced gastric lesions [46,73]. Upon close examination of the histological images presented in Fig. 3, a comparative analysis across the different groups unveils the gastroprotective potential of ZME (methanol extract) against ethanol-induced gastric lesions. Image (A) displays the gastric mucosa of the normal control group, which remains intact, with no signs of damage or inflammation. In stark

contrast, Image (B), representing the ethanol ulcer control group, displays extensive damage to the surface epithelium and haemorrhagic necrosis that deeply penetrates the gastric mucosa (red arrow), along with significant inflammatory cell infiltration (blue arrow) and vascular congestion (green arrow), corroborating the findings of [74] regarding the characteristic pathologies induced by ethanol. Image (C) illustrates the gastric mucosa in the group pretreated with ranitidine (5 mg/kg), a known anti-ulcer agent serving as a positive control. Here, the mucosal integrity appears markedly preserved compared to the ethanol group, with reduced signs of haemorrhagic necrosis and less pronounced inflammatory cell infiltration and vascular congestion, aligning with the therapeutic efficacy reported in an earlier study [75]. The subsequent images (D1, D2, and D3) depict the gastric tissue of groups pre-treated with varying dosages of ZME (50, 250, and 500 mg/kg, respectively). There is a dose-dependent amelioration of ethanol-induced damage observable. With increasing doses of ZME, there is a successive decrease in the depth and severity of epithelial damage, inflammation, and vascular congestion. The highest dose (Image D3) presents a mucosal appearance that is close to the normal control group, underscoring the extract's capacity to significantly counteract the harmful effects of ethanol on gastric tissue. These findings are reflective of ZME's constituents' antioxidant and cytoprotective properties, which likely contribute to its efficacy in preventing gastric mucosal damage [76]. The reduction in inflammatory cell infiltration (blue arrow) observed at higher doses of ZME may be indicative of its anti-inflammatory effects, possibly through the inhibition of inflammatory mediators as described by [77]. In conclusion, the histopathological evidence strongly suggests that ZME extract possesses considerable gastroprotective effects, which could be attributed to its anti-inflammatory, antioxidant, and mucosal protective properties. Further studies to isolate specific active compounds within ZME and elucidate their mechanisms could pave the way for novel therapeutic agents against ethanol-induced gastric damage.

4.6. Effect of ZME extract on ethanol-induced gastric ulcer in rats

The gastroprotective effect of the ZME extract depicted in Fig. 4 underscores its potential as an effective natural remedy against ethanol-induced gastric ulcers. This suggests not only the efficacy of the ZME in ameliorating gastric damage but also its superiority over ranitidine at this dosage. The absence of significant protective effects at lower doses of ZME (50 and 250 mg/kg) may indicate a threshold effect, where the concentration of active constituents within the extract becomes therapeutically relevant. This observation is consistent with the dose-dependent nature of many plant-derived compounds, where a minimum threshold concentration is often required to exert a biological effect [8,46]. The high level of ulcer protection by the ZME could be attributed to the antioxidant compounds identified within the extract, such as rosmarinic acid and chlorogenic acid. These compounds are known for their ability to scavenge free radicals and strengthen mucosal defence mechanisms, which can prevent the oxidative damage and inflammation typically induced by ethanol [78]. Additionally, the anti-inflammatory properties of ZME, likely contributed to the reduction of neutrophil infiltration in the gastric mucosa, a key marker of inflammation. While the results are promising, the mechanism by which ZME exerts its protective effect remains to be fully elucidated. It is hypothesized that the ZME may act by enhancing the gastric mucosal barrier, increasing mucus production, or modulating the activity of endogenous protective enzymes such as superoxide dismutase and glutathione peroxidase [79]. Given the complexity of polyphenolic interactions and the potential for synergistic effects, it is plausible that the combined action of multiple compounds within the ZME contributes to its overall efficacy. Isolating the individual components may not provide the same degree of protection, emphasizing the importance of the extract's holistic composition. Furthermore, the comparison to ranitidine, a well-established pharmaceutical agent for gastric ulcers, highlights the potential of ZME as an alternative or adjunct therapy. However, caution must be exercised in extrapolating these results to clinical practice. It is imperative to consider the pharmacokinetics and potential for adverse effects in human populations, which may differ from the rat model. In conclusion, the methanol extract of *Z. hispanica* exhibits potent gastroprotective properties against ethanol-induced ulcers in rats, especially at higher doses. The findings from this study warrant further investigation into its therapeutic mechanisms and the potential translation into clinical applications for the management of gastric ulcers.

4.7. Effect of ZME on gastric mucus content in rats

The significant increase in gastric mucus content following the administration of ZME extract, as reported in Fig. 5, is a remarkable finding that highlights the extract's potential gastroprotective mechanisms. The production of gastric mucus is a primary defence mechanism against the corrosive properties of gastric acid and other injurious agents like ethanol [80]. The observed increase in mucus production with ZME treatment suggests that one of the protective actions of ZME against gastric ulceration could be the strengthening of this mucosal barrier. The enhancement of mucus content was dose-dependent, with the highest ZME dose (500 mg/kg) surpassing the protective effects of ranitidine, a commonly used anti-ulcer medication. Ranitidine works primarily by reducing gastric acid secretion and has lesser-known effects on mucus production [81] **.** The superior mucus augmentation by ZME implies that it might offer additional protective effects beyond those provided by ranitidine, which could be beneficial in clinical settings where mucus depletion plays a role in ulcer pathogenesis. The active constituents within the ZME, possibly phenolic acids and flavonoids identified in previous analyses, might be responsible for the observed increase in mucus production. These compounds have been shown to exert mucogenic effects in gastric tissues, which might be attributed to their influence on mucosal prostaglandin E2 levels or direct action on mucus-secreting cells [82]. The exact biochemical pathways through which ZME induces this increase in mucus production remain to be elucidated. Further studies are warranted to identify the specific components of ZME responsible for this effect and to determine whether these components act synergistically or independently.

While the current findings are promising, it is essential to note that the clinical translation of these results requires careful consideration of human pharmacokinetics and possible long-term effects. Moreover, the relationship between increased mucus production and overall ulcer healing and prevention needs to be further explored in chronic ulcer models. In conclusion, the methanol extract of *Z. hispanica* shows a dose-dependent increase in gastric mucus content, suggesting a potential therapeutic advantage in the management of gastric ulcers. The findings from this study will pave the way for the development of novel ulcer treatments that harness the natural protective mechanisms of the gastrointestinal mucosa.

4.8. In vivo antioxidant parameters of ZME in the rat stomach

The investigation into the in vivo antioxidative potential of ZME extract in rat stomach tissues provided significant insights into its antiulcerogenic mechanism. Oxidative stress plays a pivotal role in gastric ulcer pathogenesis, where an imbalance between oxidative and antioxidative processes leads to tissue damage [83]. The assessment of oxidative stress biomarkers in the gastric tissue of rats revealed a marked alteration in these parameters upon gastric ulcer induction. The levels of lipid hydroperoxides (LOOH), which are products of lipid peroxidation and indicators of oxidative stress, were elevated in the gastric tissues of the ulcer-induced group. Conversely, the activities of critical antioxidative enzymes, catalase, glutathione (GSH), and superoxide dismutase (SOD) were significantly reduced. These changes, showcased in Figs. 6–10, were observed by comparing the values with those from vehicle-treated animals, indicating a clear oxidative stress profile associated with gastric ulceration. Pretreatment with ZME extracts

demonstrated a significant reversal of these stress markers. LOOH levels decreased upon treatment with ZME, suggesting a reduction in lipid peroxidation processes within the gastric tissue. Furthermore, the activities of catalase, GSH, and SOD were restored to levels that were comparable to the positive control group that received ranitidine, a known antiulcer agent with antioxidative properties [84]. These findings underscore the antioxidative efficacy of ZME, positing that its antiulcerogenic effects are mediated through the modulation of oxidative and antioxidative parameters in the stomach. The ZME appears to confer protection against oxidative damage by enhancing the endogenous antioxidative defence system, thereby mitigating the harmful effects of reactive oxygen species (ROS) involved in gastric ulcer development.

5. Conclusion

The findings of this study underscore the potent gastroprotective and antioxidative properties of *Ziziphora hispanica* methanol extract against ethanol-induced gastric ulcers in rats. By systematically evaluating the effects of ZME on critical biochemical markers and gastric mucosal integrity, the research highlights the extract's capacity to significantly mitigate gastric tissue damage through several mechanisms. Key observations include the dose-dependent enhancement of antioxidative enzyme activities, such as catalase and superoxide dismutase, alongside the reduction of lipid peroxidation markers. Additionally, the extract's ability to significantly elevate gastric mucus content, compared to both the vehicle control and ranitidine, points to its protective role in reinforcing the gastric mucosal barrier. The superiority of ZME over ranitidine, a well-established anti-ulcer medication, in certain experimental parameters, notably in reducing malondialdehyde levels and increasing superoxide dismutase activity, suggests that the extract could offer a novel and effective alternative for gastric ulcer treatment. These findings, particularly the significant antioxidative action and enhancement of gastric mucosal defenses, align with the traditional use of *Ziziphora hispanica* in herbal medicine and support its therapeutic potential. This study contributes to the growing body of evidence on the health benefits of phytochemical-rich plant extracts and underscores the importance of exploring traditional medicinal plants for developing new therapeutic agents.

This study is limited by the use of an acute ethanol-induced gastric ulcer model in rats, which does not fully mimic human peptic ulcer disease. Only one ulcer model, a restricted dose range, and an acute setting were evaluated, and the crude extract was tested without isolating or characterising the active constituents or assessing their pharmacokinetics. Therefore, the findings cannot be directly extrapolated to humans and require confirmation in additional preclinical models and future clinical studies.

CRediT authorship contribution statement

Hind Amira: Writing – original draft, Methodology, Investigation. **Walid Mamache:** Writing – original draft, Validation. **Fatima Benchikh:** Conceptualization. **Hassiba Benabdallah:** Investigation. **Asma Ouaret:** Methodology, Formal analysis. **Islam Amira:** Investigation. **Hocine Laouer:** Investigation. **Mostefa Dahia:** Resources, Data curation. **Aya Amira:** Visualization, Conceptualization. **Ibrahim Demirtach:** Validation. **Smain Amira:** Software. **Hamdi Bendif:** Writing – review & editing, Supervision, Funding acquisition. **Sulaiman A. Alsalamah:** Project administration. **Tarek H. Taha:** Project administration. **Elfalleh Walid:** Funding acquisition. **Stefania Garzoli:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All the data in the article are available from the corresponding author upon reasonable request.

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