Original article

Therapeutic strategies of *Moringa oleifera* Lam. (Moringaceae) for stomach and forestomach ulceration induced by HCl/EtOH in rat model

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A B S T R A C T

Background: The drumstick tree *Moringa oleifera* Lam. (Moringaceae), distributed in many parts of the world, is an important food plant with high nutritional value and used in medical applications and pharmaceutical industries. The aim of this study was to highlight the gastroprotective effect of *Moringa oleifera* in hydrochloric acid/Ethanol (HCl/EtOH) in a rat model.
Methods: Moringa phytocompounds were characterized by infrared spectra (FTIR). Rats were induced for gastric ulcer with 150 mmol/L HCl/60% EtOH solution and pretreated orally with the edible infusion extract of the leaves of *Moringa oleifera* at a single dose of 100 mg/kg body weight (bw). Antioxidant parameters and lipid peroxide levels were measured and the pathological damage was histologically analysed.
Results: The FTIR analysis showed the presence of several chemical biocompounds. The methanolic extract is the potent radical-scavengers with an estimated value of 87.54% at the higher concentration used (500 μg/ml) and antibacterial agent. Further, the DPPH inhibition value of the *M. oleifera* infusion was 80.58%. For *in vivo* analysis, mucus was highly produced in gastric mucosa of plant-treated rats, thereby pH were elevated in rats pretreated with *M. oleifera* compared to ulcerated animals. Whereas, lesion index was markedly reduced (79%) in stomach protected with plant. Interestingly, oral administration of *M. oleifera* protected gastric mucosa through decreasing MDA levels as well as increasing antioxidant enzyme activities (CAT, SOD, GPx).
Conclusion: Overall, the therapeutic value against acidified ethanol induced gastric and ulcer ability of *M. oleifera* might be due to its biocompounds.
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1. Introduction

Stomach ulcer is a chronic disease featured with unexpected complications, including bleeding, stenosis and perforation, as well as a high incidence of recurrence (Kangwan et al., 2014). Their etiology is multifactorial and occurs when the balance between offensive and protective factors of the mucosa is disturbed (Serafim et al., 2020). Two major damaging causes implicated in peptic ulcer diseases and ulcer recurrence are infection with *Helicobacter pylori* (*H. pylori*), alcohol intake, stress, indomethacin, bile acids, ische-mia, long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) (Kangwan et al., 2014), aging, gender, smoking, education level, income, obesity and abdominal adiposity, nutrients, blood parameters, and lifestyle (Lee et al., 2018). Already, drug classes such as proton pump inhibitors, histamine (H2) inhibitors, and antacids have been used to treat this disease for the past decades (Armah et al., 2021). The combination of omeprazole and rebamipide accelerated the quality of ulcer healing through an increasing level of prostaglandin E2 (PGE2) and a decreasing level of Interleukin-8 (IL-8) and malondialdehyde (MDA) in the gastric mucosa, but not omeprazole alone (Kangwan et al., 2014). However, the drugs used produce many adverse effects and are less effective than they ought to be. Therefore, there is a growing interest in alternative therapies and the use of natural products (Klein-Junior et al., 2012). There is a revival of interest in herbal products (botanicals) at a global level and the conventional medicine is now beginning to accept the use of botanicals once they are scientifically validated (Gilani and Attar-ur Rahman, 2005). Plants with antiulcerogenic activity were used either as raw materials which

obtained by extraction with solvents or as individual isolated compounds (Awaad et al., 2013). Flavonoids are among the molecules of greatest interest in biological assays due to their anti-inflammatory and antioxidant properties (Serafim et al., 2020).

Moringa oleifera Lam. (Syn *Moringa pterygosperma* Gaertn), a fast-growing drought-resistant, deciduous, perennial softwood tree, is native to the Himalayan foothills (India/Bangladesh) and it become naturalized and widely cultivated in many countries (Adeleye et al., 2021; Paliwal et al., 2011) and is found in the Chinese herbal medicine dictionary in China (Meireles et al., 2020). The trees serve as windbreaks and reduce soil erosion. It is used in lumber production as well as for light construction work in many parts of the developing world. The coarse fiber is often used for the production of ropes or mats. It is also used for contaminant flocculation as well as water purification (Alegbeleye, 2018). All parts of Moringa tree are edible and have long been consumed by humans (Paliwal et al., 2011) and used in Bakery products (Milla et al., 2021).

The variation of Moringa biocompounds depend on climatic conditions. It has abundant deposits of compounds containing simple sugar, rhamnose as well as a somewhat distinctive group of compounds in their radicle and crust called glucosinolates such as 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate and isothiocyanates, including 4- (4′-O-acetyl-a-L-rhamnopyranosyloxy)benzyl isothiocyanate, 4-(a-L-rhamnopyranosyloxy) benzyl isothiocyanate and benzyl isothiocyanate (Alegbeleye, 2018; Bhattacharya et al., 2018). Additionally, its leaves contain high quantities of nutrients: vitamin A, vitamin C, calcium and potassium (Paliwal et al., 2011). Meanwhile, *M. oleifera* has been and continues to be used by folk medicine practitioners to prevent, mitigate, or treat many ailments and to prevent and cure several diseases such as inflammation, ulcer, cardiovascular diseases, diabetes, anemia, stress, skin, arthritis and hypertension (Meireles et al., 2020; Alegbeley, 2018). Further, an herbal mixture formulation containing *Moringa oleifera* possess SARS-CoV-2 inhibitory activity (Adeleye et al., 2021). The natural active compounds, microRNAs (*p*-miRs), present in the plant microvesicles purified from *Moringa oleifera* seeds aqueous extract counteract tumorigenesis (Potestà et al., 2020) and used as antiretroviral therapy, in managing HIV infection and restore in immune system (Minutolo et al., 2021).

To investigate the gastroprotective effect of *M. oleifera*, stomach ulcer was induced by acidified ethanol, lesion index and gastric secretion parameters in ulcerated rats were then evaluated. we measured and determined the levels of lipid peroxides and antioxidant enzymes in the gastric tissues of HCl/EtOH-treated rats.

2. Material and methods

2.1. Reagents

Chlorhydric acid (HCl); Ethanol (EtOH); 4-nitro blue tetrazolium chloride (NBT); sodium carbonate (Na_2CO_3), sodium tartrate ($C_4H_4Na_2O_6$); Copper sulfate ($CuSO_4$); Folin-Ciocalteu (F-C) reagent (Ethylenediaminetetraacetic Acid, Disodium Salt); Na_2EDTA; sodium hydroxide (NaOH); 2-thiobarbituric acid (TBA); sodium nitrite ($NaNO_2$); Tris ((HOCH2)3CNH2); sodium chloride (NaCl); 2,2-Diphenyl-1-picrylhydrazyl (DPPH); Trichloroacetic acid (TCA); ferric chloride ($FeCl_3$); Potassium ferricyanide ($K_3Fe(CN)_6$) (1%); Butylated hydroxytoluene (BHT); ascorbic acid (VIT C); aluminium chloride ($AlCl_3$); hydrogen peroxide (H_2O_2); 5, 5′-dithiobis (2-nitrobenzoic acid (DTNB), gallic acid, quercetin, vanilline, catechin and bacterial growth medium (Mueller Hinton agar) were obtained from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Bovine Serum Albumin (BSA) was purchased from Atlanta Biologicals (Norcross, GA, USA). Omeprazole® Zentiva 40 mg was purchased from Tunisian pharmacy (Tunisia).

2.2. Plant material

The dried leaves of *M. oleifera* (500 mg) were extracted by maceration with methanol and distilled water for 24 h. The extracts were then filtered with wattman paper (11 μm, Merck), centrifuged (4500 g/10 min) and then kept in vials at −20 °C. The yield of methanolic, aqueous and infusion extracts of *M. oleifera* was found to be 14.3, 21 and 28.4%, respectively (Fig. 1**B**).

2.3. Bacteria

The tested bacteria used in this study, *Escherichia coli* (*E. coli* ATCC 35218), *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 27855) and *Staphylococcus aureus* (*S. aureus* ATCC 25923) were purchased from the ATCC.

2.4. Antibiotics

Vancoumycin (VA) (36 μg/μL); Penicillin (P) (10 μg/μL); Bacytracin (B) (10 μg/μL); Ampicillin (AM) (10 μg/μL); Streptomycin (S) (100 μg/μL) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.5. Polysaccharides extraction and FTIR analysis of M. Oleifera

Liposoluble fractions were removed from *M. oleifera* leaf by mixing its powder with EtOH. Afterwards, the sample was soaked in water bath at 80 °C for 3 h, filtered and concentrated to extract polysaccharides. Sevag reagent was used to remove protein fractions from the plant powder. Extracted polysaccharides were then analysed by FTIR spectra that was evaluated in the range 4000–400 cm^{-1} at room temperature with a resolution of 4 cm^{-1} by FTIR spectrometer (Shimadzu 8400 s, France). Experiment has been repeated three times.

2.6. Phenolic content

The phenolic content was evaluated using Folin-Ciocalteu (FC) reagent. Aliquots of plant samples was mixed with FC (10%) and distilled water. Then, Na_2CO_3 (7.5%) was added. The solution was kept for 1 h until the development of blue color. Gallic acid is the reference standard used to evaluate phenol content. The absorbance was recorded at 725 nm using spectrophotometer (Spectronic 200). Total phenolic values wer expressed as milligram gallic acid equivalent per gram dry weight of plant extract (mg GAE/g DW).

For flavonoid content, aliquots of plant extract was mixed with $NaNO_2$ (5%). Six minutes later, $AlCl_3$ (10%) solution was added. Then, NaOH (1 M) and distilled water were added to the solution. The absorbance was measured after 15 min at 510 nm. The flavonoid content was expressed as milligram quercetin equivalent per gram dry weight of plant extract (mg QE/g DW).

To evaluate plant condensed tannins, aliquots of *M. oleifera* leaf extracts were mixed with vanilline (4%). After 15 min, concentrated HCl was added and the absorbance was read at 500 nm. The condensed tannin content was expressed as milligram catechin equivalent per gram dry weight of plant extract (mg CE/g DW). Experiment have been repeated three times.

2.7. Antioxidant activity

2.7.1. DPPH assay

To estimate the ability of the *M. oleifera* leaf extracts to reduce the DPPH· Effect, we prepared different sample and synthetic antioxidant (ascorbic acid) concentrations (100 to 500 μg/ml). The decrease in the absorbance of DPPH in ethanol at 517 nm
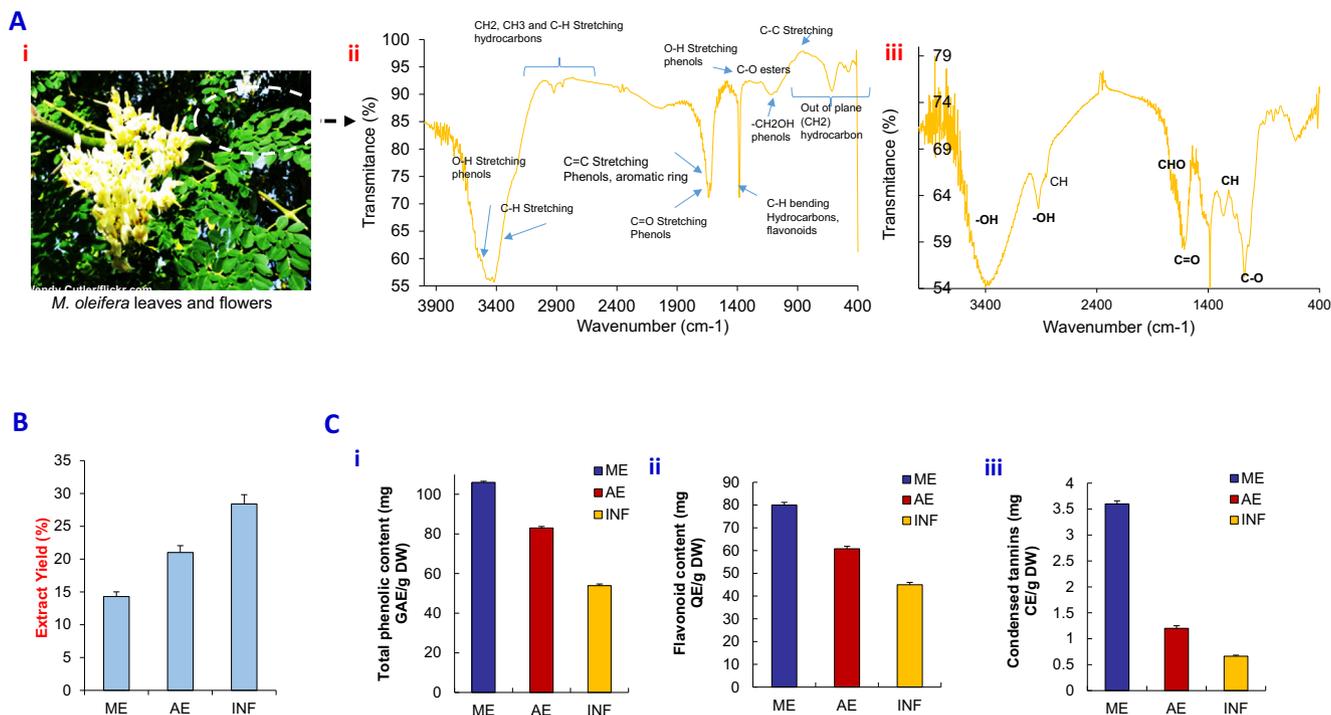
Fig. 1. A. Plant image (i), FTIR bands of biocompounds (ii) and polysaccharides (iii) of *M. oleifera* leaves. **B.** Yield of *M. oleifera* extracts. **C.** Total phenolic content of *M. oleifera* leaf extracts **(i)**. Phenolic content values were expressed as gallic acid equivalents (GAE) mg/g dry weight (DW). Flavonoid content of *M. oleifera* leaf extracts **(ii)**. Flavonoid content values were expressed as quercetin equivalents (QE) mg/g dry dry weight (DW). Condensed tannin content of 3 extracts of *M. oleifera* leaves **(iii)**. Tannin content values were expressed as catechin equivalents (CE) mg/g dry weight (DW). AE: aqueous extract; ME: Methanolic extract; INF: infusion. Each test has been repeated three times.

mediated by radical scavengers was detected after 30 min. Briefly, aliquots with increasing doses of prepared extracts were mixed with DPPH ethanolic solution. For blank, the ethanol was used instead of extracts. Test has been repeated three times.

$$DPPH\ inhibition\ (\%) = A_B - A_S \times 100A_B$$

Where A_B is the absorbance of the blank and A_S is the absorbance in the presence of plant sample.

2.7.2. Ferric reducing antioxidant power

The reducing of Fe^{3+} into Fe^{2+} ions in the presence of *M. oleifera* leaf extracts indicated its potent antioxidant effect. Briefly, aliquots with different doses (100 to 500 µg/ml) of plant extracts or synthetic standard (BHT) were mixed with $K_3Fe(CN)_6$ (1%) and phosphate buffer (0.1 M; pH 6.6) and incubated in water bath (50 °C/20 min). Afterwards, TCA (10%) was added to the solution. The mixture was centrifuged (3000 rpm/10 min) and then mixed with $FeCl^3$ (0.1%) and distilled water. Optic density was measured at 700 nm using spectrophotometr. Increase in absorbance of the reaction mixture shows the reducing power of the samples (Jayaprakasha et al., 2001). Test has been repeated three times.

2.8. In vitro antimicrobial effects of M. Oleifera

The method used in this report to investigate the antibacterial effect of *M. oleifera* is the agar diffusion assay. Brefly, discs (6 mm) of Whatman filter paper N°1 were impregnated with methanolic and aqueous extracts and infusion of Moringa and placed in petri dishes that contain Mueller-Hinton agar and bacteria (10^6 CFU/mL). Antibiotics were considered as positive controls and DMSO were used as negative controls. Plates were incubated (24 h/37 °C) and inhibition diameter (mm) were measured. Experiment has been repeated three times.

2.9. In vivo experimental design

2.9.1. Animal experiment

A total of 30 Sprague –Dawley male rats (8-week-old, 230 ± 3. 6 g) were obtained from the Experimental Animal Laboratory (Sfax, Tunisia), housed in the animal research center of the department of Biology, Faculty of Sciences of Gafsa (FSG) (Gafsa, Tunisia), maintained in propylene cages under normal conditions (12/12 h light/-dark cycles, 25 ± 5 °C, 50% relative humidity) and provided with standard pellets diet (SNA, Sfax) and water. The experimental protocols were approved by Animal Ethics Committee of Gafsa University (G/A/SV; Approval Number GU-2016–001).

2.9.2. Experimental protocol and gastric lesion assessement

After an overnight fasts with food starvation and free access to water, rats were divided into six groups (n = 5) (Table 1);

Group 1 (sham group, water consumption);

Group 2 (ulcerated group) orally treated with 0.1 ml HCl/EtOH (60% ethanol in 40% 150 mmol/L hydrochloric acid) mixture per 10 g body weight (Zhang et al., 2020);

Group 3 (Omeprazole) orally pretreated with the standard anti-stomach ulcer drugs, Omeprazole (20 mg/kg bw);

Table 1
In vivo experimental design.

Animal Groups	Treatment
Sham	Distilled water
Ulcer Control	Distilled water, 150 mmol/L HCl in 60% EtOH (0.1 ml/10 g)
Omeprazole	20 mg/kg
Plant extract	100 mg/kg
Plant + HCl/EtOH	Plant, 100 mg/kg + 150 mM HCl in 60% EtOH
Omeprazole + HCl/EtOH	20 mg/kg + 150 mM HCl in 60% EtOH

Group 4 (*M. oleifera*) orally pretreated with *M. oleifera* leaf infusions (100 mg/kg);

Group 5 (*M. oleifera*, 150 mmol/L HCl/60% EtOH) orally pretreated with *M. oleifera* leaf infusions (100 mg/kg bw) 1 h before oral intubation of the mixture of HCl/EtOH to induce gastric mucosa damage;

Group 6 (Omeprazole, 150 mmol/L HCl/60% EtOH) orally pretreated with Omeprazole (20 mg/kg bw) 1 h before oral intubation of the mixture of HCl/EtOH.

For the preparation of *M. oleifera* leaf infusions, dried leave parts (500 mg) were dissolved in distilled water (5 ml) and extracted at the day of experiments.

One hour after treatment with acidified ethanol, all rodents were euthanized using Ether, sacrificed and stomach were collected (Fig. 3**A**), opened along the greater curvature, washed with normal saline solution. Mucus in the stomach mucosa of each rat was scraped with glass slide and transferred to conical tubes and weighed using an electronic balance. Stomach lesions were photographed using LP VEYRON (LP_N-50) digital camera and quantified.

The ulcer index (UI) was measured as follows:

$$UI = \frac{Average\ number\ of\ lesions\ per\ rat\ +\ Average\ number\ of\ severity\ score}{\%\ of\ rats\ with\ ulcers}$$

2.9.3. Microscopic observations

For histopathological investigation, the stomach sections undergoing all administrations were fixed in buffered formalin (10%), embedded in paraffin and thick slices (4 μm) were obtained after section of gastric tissues by Leica microtome. Obtained sections were stained with Hematoxylin and Eosin (H&E) and then observed with light microscopy.

2.9.4. Protein quantification and oxidative stress levels of damaged stomach
2.9.4.1. Gastric sample preparation.
The stomach was excised from all animals and homogenized in potassium phosphate (pH 7.4) buffer solution. Then, the homogenates were centrifuged (3,000 rpm/15 min) and kept at −20 °C until use (antioxidant enzymes and lipid proxides analysis).

2.9.4.2. Protein content.
The quantification of the stomach tissue proteins was performed according to the method of Lowry et al. (1951) using BSA as standard for the calibration curve. Briefly, stomach sample was mixed with distilled water. Afterwards, the mixture ($Na_2CO_3 + C_4H_4Na_2O_6 + CuSO_4$) was added to the diluted homogenate. Then, Folin-Ciocalteu reagent was added to the solu-
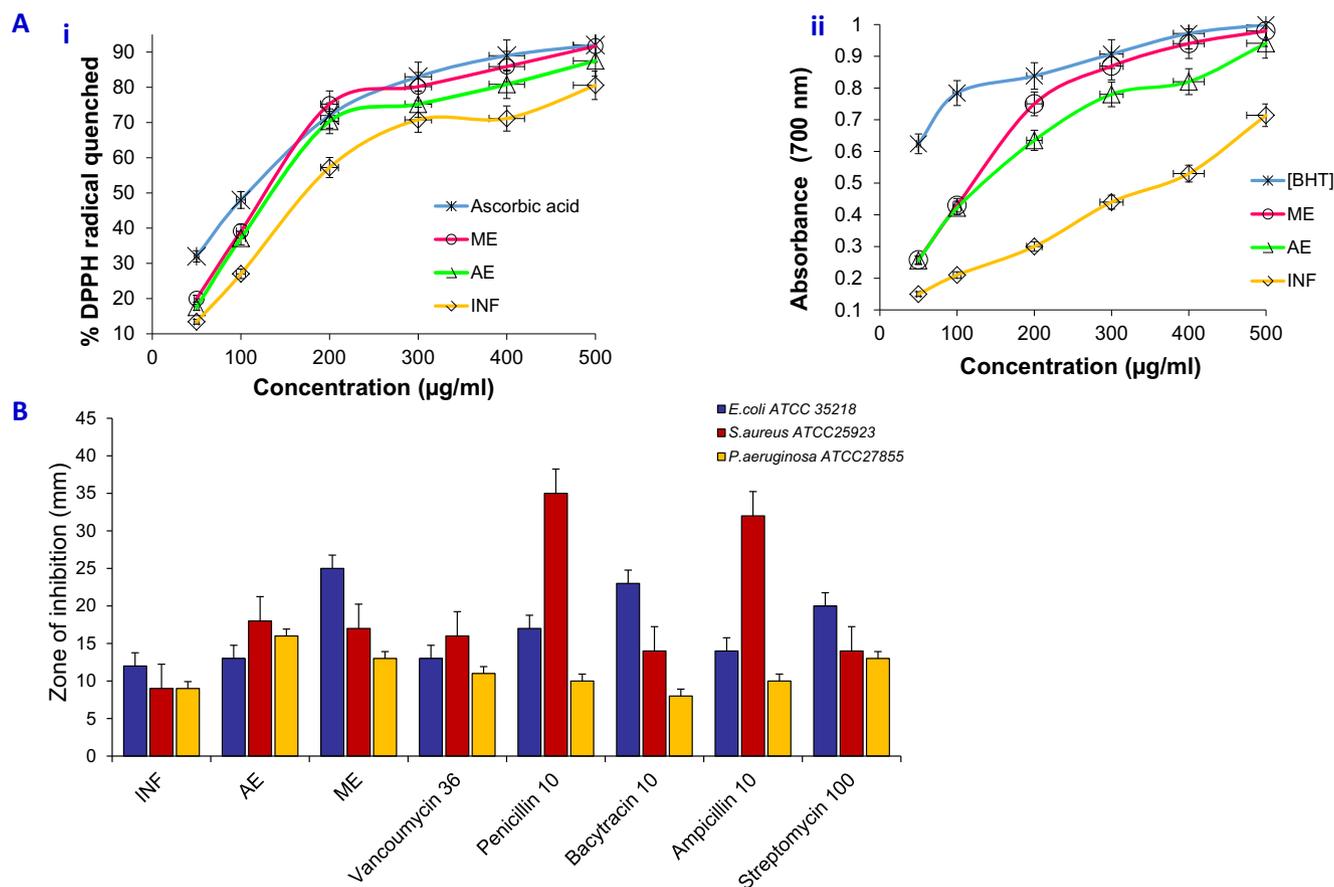
Fig. 2. **A.** DPPH radical scavenging activity (i) and ferric reducing power (ii) of different concentrations (100, 200, 300, 400, 500) of *M. oleifera* leaf extracts. (**B**) Antibacterial effects of *M. oleifera* extracts using disc diffusion assay. Whatman filter disc (6 mm) impregnated with extracts were placed onto the petri dishes seeded with bacteria on the agar Mueller-Hinton. DMSO was used as negative controls and extracts were tested with positive controls (vacoumycin 36, penicillin 10, bacitracin 10, ampicillin 10, and streptomycin 100). The petri dishes were incubated (37 °C/24 h), and inhibition zones (mm) of pathogens in the presence of natural compounds and antibiotics were evaluated. AE: aqueous extract; ME: Methanolic extract; INF: infusion. Each experiment has been repeated three times.
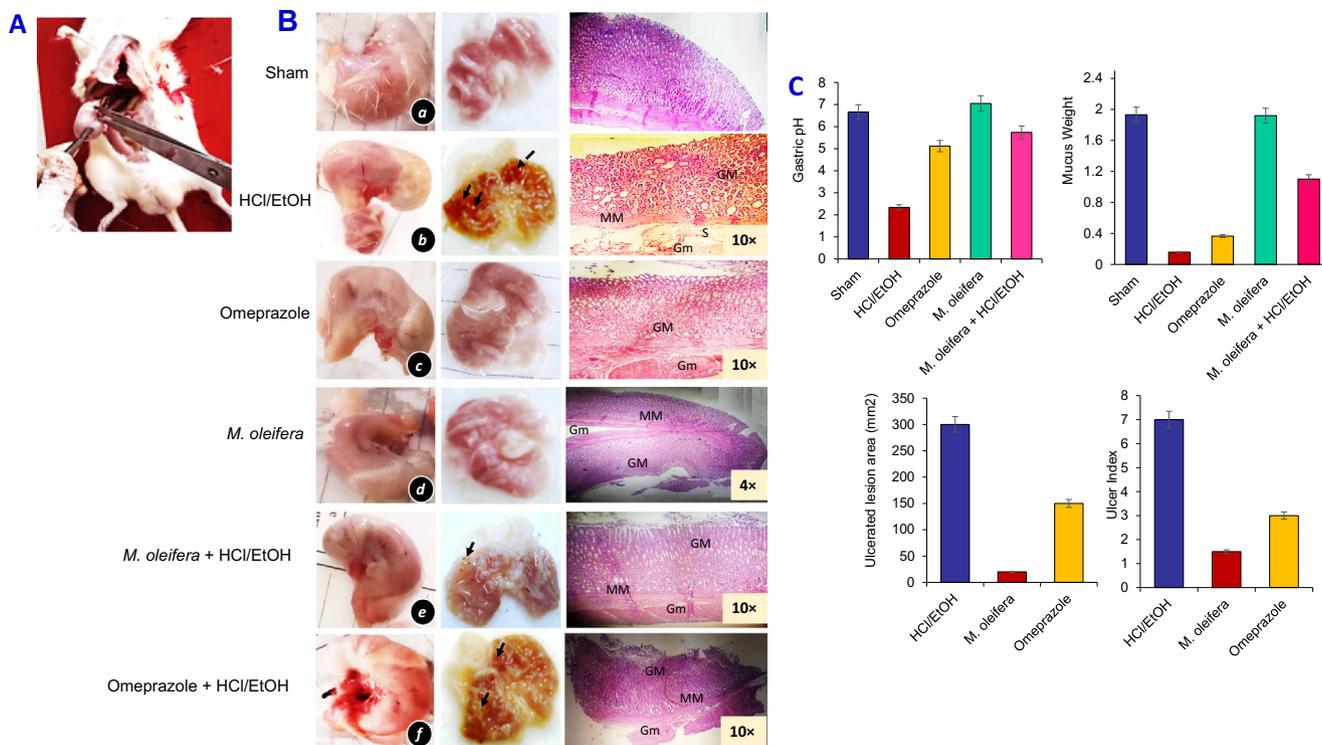
Fig. 3. Macroscopic appearance of dissected and sectioned stomachs from all groups and photomicrograph of gastric mucosa (**A, B**). (*a*) (sham group); (*b*) (Ulcer group); (*c*) (Omeprazole); (*d*) (*M. oleifera*, 500 mg/kg); (*e*) (*M. oleifera* + HCl/EtOH); (*f*) (Omeprazole + HCl/EtOH). GM: gastric mucosa; MM: muscularis mucosa; S: submucosa; Gm: gastric muscle (H&E). There were 5 rats in each group of experiment. **C.** ulcer scoring and gastroprotective effect of *M. oleifera*.

tion and the mixture were kept in the dark at ambient temperature/30 min. Optic density (OD) was measured at 490 nm using spectrophotometer. Assays were carried out in triplicate.

2.9.4.3. Assessment of antioxidant enzymes. To assess the SOD enzyme level in stomach mucosa of all animals, the supernatant was mixed with Na_2EDTA-Methionine and phosphate buffer (7.8, 50 mM). Then, NBT and riboflavin were added to the solution and kept for 20 min in the light, except for the blanck, which was maintained in the darck. The absorbance was assayed at 580 nm. SOD enzyme level was determined as follows:

$$SOD\,activity\,(Y) = \%\,Inhibition/mg\,protein$$
$$= \frac{OD\,Sample - OD\,Blank}{OD\,Blank}100\,x\,1/Protein\,mg/ml\,x\,FD$$

SOD Unit correspond to the quantity of protein that induce 50 % inhibition:

$$SOD\,specific\,activity = Y/50\,Unit\,SOD/mg\,protein$$

CAT activity (IU) $= \Delta$ OD / ε L. X. F_d

For CAT assay, tissue homogenate was mixed with phosphate buffer (pH 7, 100 mM) and H_2O_2. Absorbance was measured at 240 nm using spectrophotometer. The enzyme activity was evaluated by reading the change in absorbance between the fifteenth and sixty second [**17**].

With CAT activity (IU): Catalase activity expressed in International Unit (µmoles H_2O_2/min/mg protein); Δ OD: variation of the optic density per min (A_1-A_2); ε: Molar extinction Coefficient (0.043 mM^{-1}. cm^{-1}); L: Length of optic curve = 1 cm or 0.776 cm; X: concentration of protein in the homgenate (mg. Ml^{-1}); F_d: dilution factor (0.02).

For the estimation of GPx activity, homogenate was mixed with reduced GSH (0.1 mM) and phosphate buffer (pH 7,8). The reaction

mixture was incubated at 25 °C/5 min. Then, H_2O_2 (1,3 mM/l) was added to initiate the reaction and kept to react for 10 min. The reaction was stopped by the addition of TCA (1%) (30 min in the ice). Afterwards, we centrifuged the mixture at 3000 rpm/min for 10 min. The supernatant was mixed with Na_2HPO_4 (320 mM) and DTNB (1 mM). OD was recorded at 412 nm over an interval of 5 min. One unit of GPx activity was defined as a decrease of 1 µmol/L of [GSH] (37 °C; pH 6.5). GPx activity was evaluated as follows:

$$GPx\,activity\,(Y) = \frac{[\frac{(OD_S - OD_B)}{OD_B}x0.04x5x1000]}{10\,min\,x\,X(mg/ml)}$$

With GPx activity: Glutathion peroxydase activity expressed in µmoles of consumed or oxydized GSH /min / g protein; OD_S: optic density of the sample; OD_B: optic density of the blanck; X: concentration of protein in the homgenate (mg. Ml^{-1}).

2.9.4.4. TBARS level in ulcerated mucosa. Stomach mucosal tissue MDA was assessed by the reaction with TBA. Briefly, gastric homogenate was mixed with TCA-BHT and TBS (Tris + NaCl) (pH 7.4) and then centrifuged for 10 min. Afterwards, hydrochloric acid and Tris-TBA were added to the solution and the mixture was incubated in water bath (80 °C/10 min). OD was measured at 530 nm. The [TBARS] was expressed as nmol MDA/mg protein and calculated as follows:

$$[TBARS] = OD10^6/\varepsilon.L.X.F_d$$

With ε: Molar extinction Coefficient of MDA (ε MDA = 1,56 10^5 M^{-1}. cm^{-1}); L: Length of optic curve = 0,779 cm; X: concentration of protein in the homogenate (mg. Ml^{-1}); F_d: dilution factor.

2.10. Statistical analysis

Data were analysed by GraphPad Prism 4.02. The values were reported as mean ± S.E.M and One-Way ANOVA and Tukey's test were used to compare the groups. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. FTIR analysis of M. Oleifera

As seen in Fig. 1**A**, FTIR chromatogram of *M. oleifera* (Fig. 1**Ai**) leaf extract revealed the presence of several bands ((Fig. 1**Aii**). The bands at 3447 cm^{-1} and at 3417 cm^{-1} would be due to O-H bending vibration. The bands at 2970 cm^{-1}, 2921 cm^{-1}, 2847 cm^{-1} and 2752 cm^{-1} correspond to CH_2, CH_3 and C-H stretching vibrations. The bands at 1674 cm^{-1}, 1635 cm^{-1} and 1618 cm^{-1} would be related to C = C and C = O stretching vibrations. The bands at 1379 cm^{-1} and 1314 cm^{-1} were assigned to C-H stretching and to O-H bending vibrations, respectively. The band in the region 1074 cm^{-1} would be ralated to $–CH_2OH$ and CO bending vibration and the band at 861 cm^{-1} would be due to C-C stretching vibrations. Fig. 1**Aiii** showed the FT-IR spectrum for polysaccharides present in *M. oleifera* leaves. Bands observed in 3400 and 2800 cm^{-1} are attributed to the group –OH and C-H stretching that correspond to asymmetric aliphatic of polysaccharides (methyl and methylene). The bands at 1500 cm^{-1}, 1600 cm^{-1} and 1700 cm^{-1} would be due to the tannic compounds, the carbony group (C = O) and aliphatic aldehydes (CHO).

3.2. Total phenolic content

Phenols are plant active phytocompounds and act as antiradical scavengers. The total phenolic content (phenolic acids, flavonoids, condensed tannins) of *M. oleifera* leaf extracts were evaluated in the Fig. 1**C**. The results presented in Fig. 1**Ci** showed that *M. oleifera* methanolic extract (ME) displayed higher total phenolic content, amounting to 106 ± 0.56 mg GAE/g dry weight of extract. For phenolic content in the infusion, value is in the range of 53.9 ± 0.82 mg GAE/g dry weight. From Fig. 1**Cii**, *M. oleifera* leaf infusion (INF) contain less amount of flavonoids and the value is about 45 ± 1.02 mg QE/g DW, whereas, the higher content of flavonoid was detected in the ME (80 ± 1.16 mg QE/g DW).

Regarding the condensed tannins levels, the ME of *M. oleifera* demonstrated the highest level of condensed tannins content with a value of 3.6 ± 0.06 mg CE/g dry weight of extract, followed by the aqueous extract (AE) and the infusion (1.2 ± 0.05 and 0.662 ± 0.02 mg CE/g DW, respectively) (Fig. 1**Ciii**).

3.3. DPPH· Reducing ability

Radical scavengers are popular because they scavenge free radicals that cause oxidative stress, cell damage and inflammation (Milla et al., 2021). Exposure to antioxidants that donate electron or hydrogen atom reduce the DPPH free radical with deep violet color into its reduced product (DPPH-H) with yellowish color. The DPPH radical scavenging activity of the 3 *M. oleifera* extracts at various tested concentrations are summarized in Fig. 2**Ai**. The highest dose (500 µg/ml) of the ME markedly exhibit the greatest DPPH reducing capacity with an estimated value of 91.69%, followed by AE (87.54%), and INF (80.58%). Therefore, the synthetic antioxidant ascorbic acid used for the preparation of calibration graphs showed similar activity with the methanolic extract.

The order of reducing activity was: ascorbic acid $\approx$ ME > AE > INF.

These data suggest that the potent DPPH scavenging activity may be related to plant-derived biocompounds and the position of hydroxy group in its structures.

3.4. M. Oleifera ferric-reducing power evaluation

Ferric reducing antioxidant power is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm (Bhalodia et al., 2013). The best reducing power effect was detected with ME (Fig. 2**Aii**). In fact, the reduction of a colorless Fe^{3+}/ferricyanide ($Fe(CN)_6^{3-}$) complex into blue-colored Fe^{2+}/ferrous ($Fe(CN)_6^{4-}$) complex that binds the ferricyanide is dose-dependent manner.

3.5. Antibacterial activity

Phenolic compounds have been associated with the antimicrobial and antifungal activities of *Moringa oleifera* extracts (Milla et al., 2021). The methanolic and aqueous extracts and infusion of *M. oleifera* mediate growth inhibition of bacteria (Fig. 2**B**), with an inhibition diameter between 13 and 25 mm, detected in *E. coli* strains and between 8 and 16 mm for *P. aeruginosa*. Our data suggested that the infusion of *M. oleifera* leaves revealed a moderate potency on all bacterial strains.

3.6. Ulceroprotective effect of M. Oleifera

Macroscopic observation of the gastric damage induced by HCl/EtOH was shown in the left panel of Fig. 3**B**. Administration of acidified ethanol to animals induced high stomach mucosal damage (red bands), whereas, oral gavage of the *M. oleifera* leaf infusion (500 mg/kg bw) reduced the gastric lesions to a markedly extent (79%) and protected the rats from ulceration compared to omeprazole-treated rats. Gastric gross appearance of vehicle-administered sham rats was normal compared to acidified ethanol group.

As seen in the right panel of Fig. 3**B**, the histological gastric injuries induced by HCl/EtOH were reversed by the *M. oleifera* pretreatment. Ulcerated mucosal layer of plant-pretreated animals showed less neutrophil infiltration, mild edema in the submucosa and markedly regeneration of the stomach mucosa, submucosa and glandular epithelium width and highly presence of collagen in the damaged tissue, whereas sham group showed normal mucosa and no histopathological alterations were observed, even at high magnification the cells of the stomach are intact with a normal architecture in the case of the normal group and that treated with *M. oleifera* (Fig. 4,**5**). The microscopic analysis of the forestomach Fig. 5.revealed the ulceration of the mucosal layer (Fig. 6).

The present work demonstrated that stomach content acidity was markedly reduced in ulcerated tissue of rats pretreated with *M. oleifera* leaf infusion (Fig. 3**C**) compared to that of the HCl/EtOH -treated rats ($P < 0.05$). Whereby, rats pretreated with plant showed a stomach wall mucus weight 8 fold greater when compared to the ulcer control group. The same value was detected to those of rats in the sham group.

3.7. Effect of M. Oleifera on antioxidant status and MDA levels into ulcerated tissues

As shown in Fig. 7, oral treatment of animals with acidified ethanol attenuated antioxidant enzyme levels and increased MDA (21.54 ± 0.105 mmol MDA/mg protein, $P < 0.05$) in gastric tissue; while *M. oleifera* oral administered markedly reduced ulceration by abolishing lipid peroxidation (7.54 ± 0.112 mmol MDA/
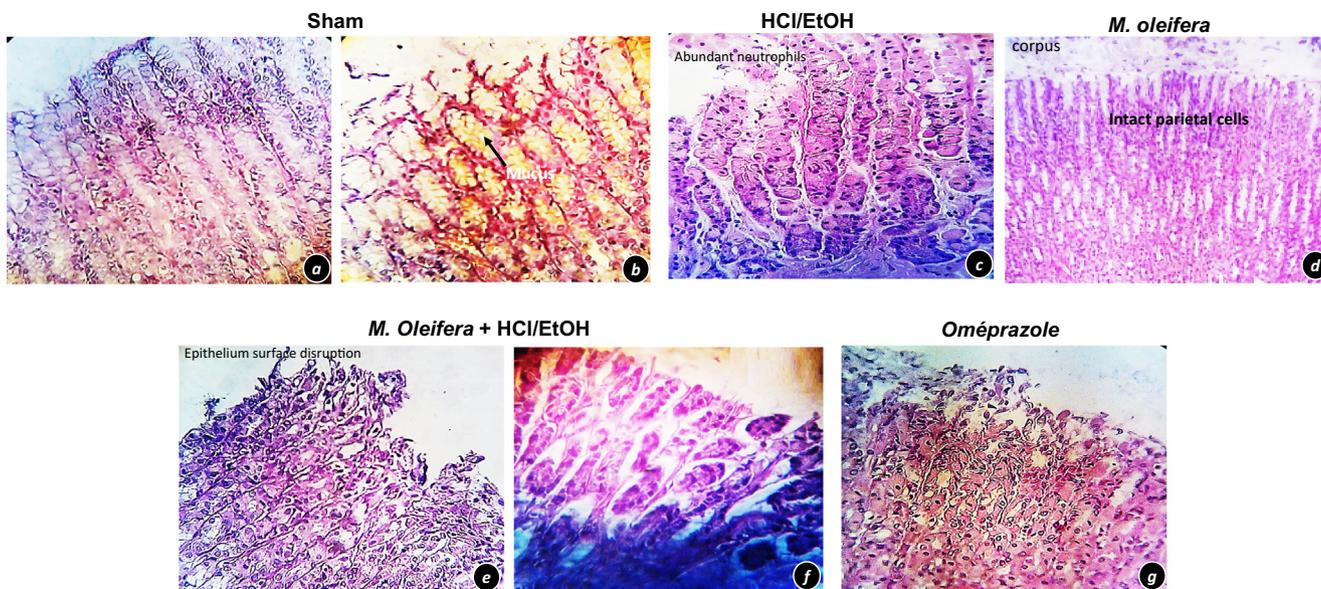
Fig. 4. Histological evaluation of the HCl/EtOH-induced gastric mucosal damage in rats. *a,b.* The normal control group showing normal gastric mucosa (arrow) and submucosa (arrow head). *c.* Rats treated with HCl/EtOH (ulcer control). There is abundant neutrophils and severe disruption to the surface epithelium. *d.* Rats treated with *M. oleifera* showing intact parietal cells and normal epithelium surface. *e,f.* Rats pre-treated with *M. oleifera* showing epithelium surface disruption. *g.* Rats pre-treated with omeprazole (20 mg/kg) showing mild epithelium surface disruption. (H & E stain *a,b,c,e,f,g*: 40×; *d*: 10 ×).
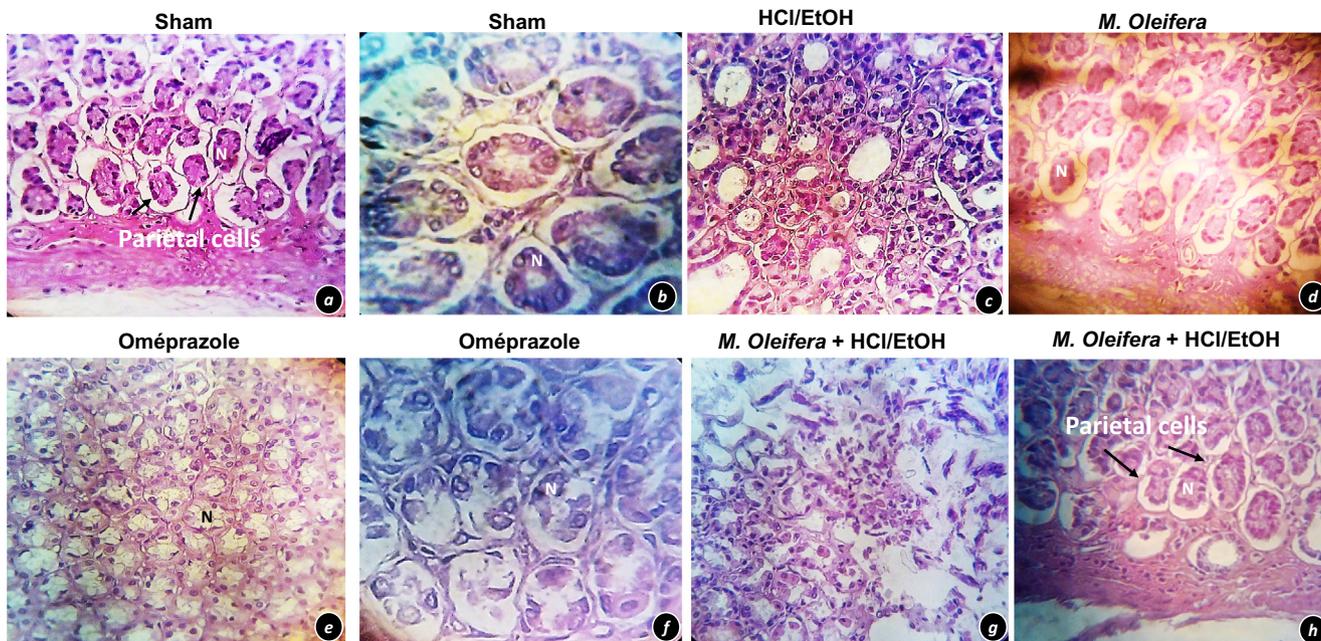

Fig. 5. Photomicrograph of the stomachs of different groups that demonstrate superficial cells of the epithelium with protectif mucus in small vacuoles. Stomachs in the sham, *M. oleifera* and omeprazole groups demonstrate clear intact mucous secreting cells with located nuclei. In the ulcerated group gastric mucosa showed vacuolated secreting cells, neutrophil infiltration (H & E stain *a, c, d, e, g, h*: ×40; *b, f*: ×100). N: nucleus.

mg protein, $P < 0.05$) and exhibited highly synthesis of SOD (3.54 ± 0.115 U/mg protein, $P < 0.05$), CAT (7.54 ± 0.122 µmoles H_2O_2 consumed/min/mg protein, $P < 0.05$) and GPx (2.45 ± 0.112 µmoles of consumed or oxydized GSH/min/g protein, $P < 0.05$) into ulcerated mucosa tissue. Indeed, proton pump inhibitor, omeprazole, slowly reduced lipid peroxides (12.55 ± 0.124 mmol MDA/mg protein) and highly elevated the level of CAT (4.58 ± 0.172 µmoles H_2O_2 consumed/min/mg protein), SOD (2.58 ± 0.111 U/mg protein) and GPx (1.74 ± 0.012 µmoles of consumed or oxydized GSH/min/g protein) in the stomach mucosa of ulcerated rats as compared to sham group.

4. Discussion

Gastric ulcer affect many people in Tunisia, and this may be due to the traditional Tunisian diet, because of lack of natural healthy diet, including vegetables and fruits with high level of carbohydrates, phenols and fibers (pomegranate, apple, curcumin, green tea, grape). Natural diets have antacid and anti-secretory effects mediated by reducing H^+, K^+-ATPase activity and antihistaminic function, along with the enhancement of mucosal defensive agents (mucin and hexosamine) (Farzaei et al., 2015). In view of previous reports regarding minerals and antioxidant compounds found in *M.*
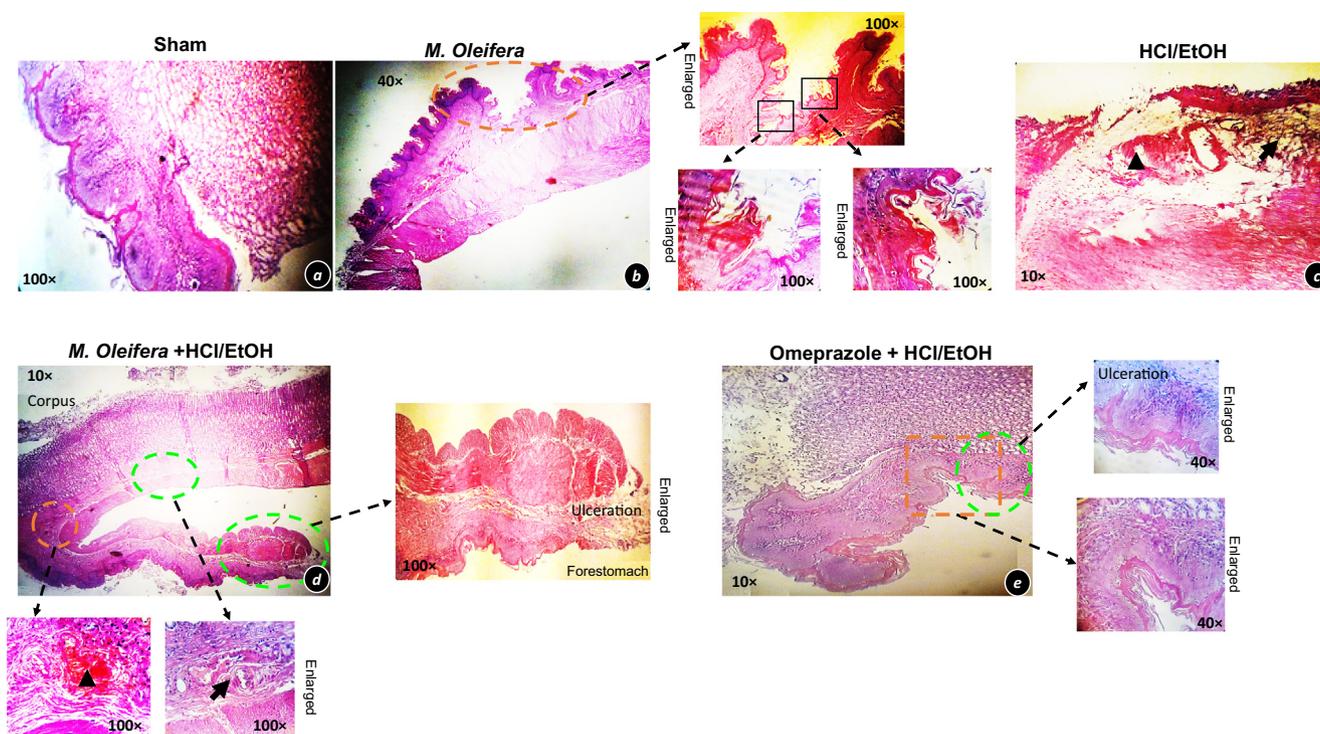
Fig. 6. Forestomach ulceration (H & E stain); *a*: sham group with normal forestomac architecture; *b*: *M. oleifera* treated group with normal forestomach; *c*: Forestomach of ulcerated group with absence of a portion of the epithelium, loss of epithelial cell layers of the mucosa extending through to the submucosa, submucosal edema (arrow) and congested mucosal blood vessel (arrow head); *d*: Stomach and forestomach of *M. oleifera*-pretreated rat with mild ulceration, congestion of the blood capillaries (arrow head) and submucosal edema (arrow); *e*: Mild-ulceration of the forestomach of omeprazole-pretreated rats.

oleifera, many researchers noted that this specie serves as an extremely valuable food source of highly digestible protein, calcium, iron, fiber, lipids, carbohydrates, fatty acids, potassium, vitamins (A, B1, B2, B3, C, E), magnesium, sodium, phosphorus, sulfur, zinc, copper, manganese, iron, selenium, amino acids (arginine and histidine−2), antioxidants (phenols, flavonols), and carotenoids suitable for combating malnutrition in many developing nations of the world where malnourishment is a major concern (Alegbeley, 2018). It is considered one of the world's most useful trees, as almost every part of the Moringa tree can be used for food or has some other beneficial property (Paliwal et al., 2011).

The current study demonstrates that the methanolic extract *M. oleifera* possessed relatively the highest phenolic (106 mg GAE/g DW), flavonoids (80 mg QE/g DW), and condensed tannins (3.6 mg CE/g DW) values along with the strongest antiradical activity, followed by aqueous extract and leaf infusion; as evidenced by the FTIR biocompound profiling that indicate the presence of phenols, polysaccharides, hydrocarbon and esters. Interestingly, the greater antiradical scavenging activity of *M. oleifera* was investigated. Among various methods of extraction, extracts obtained by maceration of the dried leaves with ethanol (70%) provided the highest yield (40.50%, w/w) with the important phenolics (13.23 g CAE/100 g exract) and flavonoids content (6.20 g IQE/100 g extract) and displayed high antioxidant potential using DPPH assay (EC50 62.94 g/mL) and ferric reducing power (FRAP) value (51.50 mmol FeSO4 equivalent/100 g extract) (Vongsak et al., 2013). Additionally, the dried leaves of *M. oleifera* have at least 60% of antiradical effects compared to fresh leaves (Wangcharoen and Gomolmanee, 2013). Further, the extract of leaves of *M. oleifera* promoted the highest DPPH radical scavenging and FRAP total reducing power activities with inhibitory concentration at 50 percent (IC_{50}) values of 1.02 ± 0.13 mg/mL and 0.99 ± 0.06 mM Fe^{2+}/g, respectively (Xu et al., 2019).

This report investigates also the antibacterial effect of *M. oleifera* that penetrates deeply into the body's tissues and particularly into the bone marrow itself cleaning all impurities, toxins, parasites and metabolic wastes (Meireles et al., 2020). Moreover, Alegbeley (2018) revealed that *M. oleifera* extracts may replace chemicals and antibiotics for waste treatment and disease prevention and control, since it is an abundant source of antioxidants, and coagulating substances. In addition, *M. oleifera* seed and leaf extracts have exhibited antimicrobial activity against human pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *E. coli*, *Salmonella typhi*, and *V. cholerae*. Ethanolic extracts of bark and Root of the *Moringa* species possessed antifungal activity against Microsporum gypseum, Aspergillus niger, Rhizopus stolonifer and Neurospora crassa and also promoted inhibitory effect against Leishmania donovani (Bhattacharya et al., 2018).

This report indicates that *M. oleifera* infusion was efficient in protecting stomach mucosa of rats against oxidation induced by acidified ethanol and the secretion of mucus; however omeprazole was somewhat less effective in healing ulcerated area. Other studies have demonstrated that Omeprazole was effective in alleviating oxidative stress in the HCl/Ethanol model (Guesmi et al., 2014). The comparative drug, omeprazole (20 mg/kg) and Kaempferol (40, 80 and 160 mg/kg, p.o.) protects stomach against ulcers mediated by ethanol in mice, suppressing neutrophils accumulation myeloperoxidase activity and decreasing the levels of pro-inflammatory cytokines (TNF-α, interleukin-6 (IL-6), IL-1β), improving gastric mucus and NO (Serafim et al., 2020). Interestingly, the main factor implicated in the development and progression of peptic ulceration was an hypersecretory acidic environment and together with dietary factors and/or stress was thought to cause most of peptic ulcer disease (Périco et al., 2020). The mucus and the bicarbonate are the first line of defense against acid because they cover the
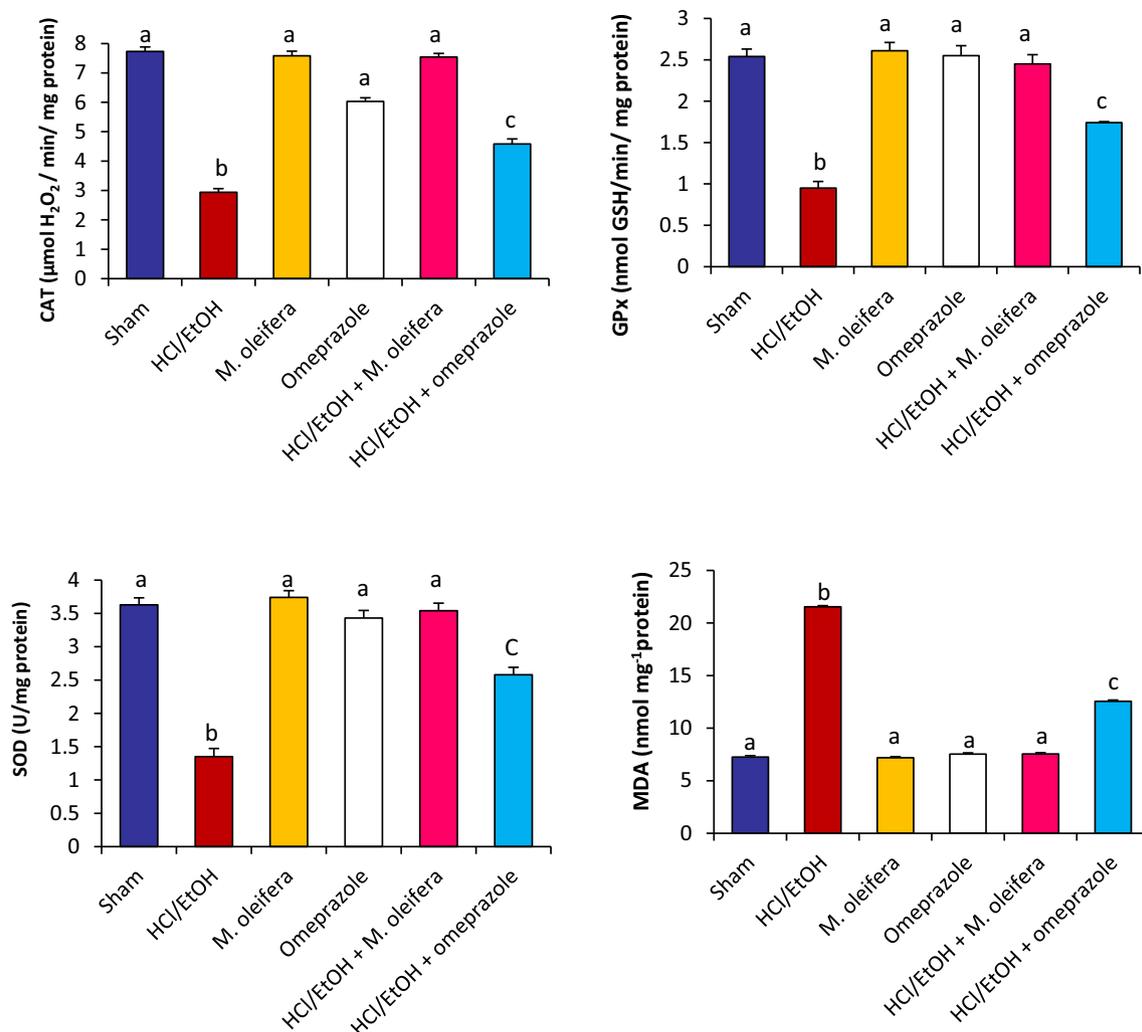
Fig. 7. Effect of *Moringa oleifera* and omeprazole treatments on the activities of catalase (CAT), glutathione peroxidase and superoxide dismutase (SOD) and the gastric level of malondialdehyde (MDA) in acidified ethanol ulcerated rats. Values are expressed as means ± SD (n = 5). Means marked with different letters are significantly different ($P < 0,05$); **a**: significatif difference between sham group and the other groups ($P < 0,05$); **b**: significatif difference between ulcer group and treated groups ($P < 0,05$); **c**: significatif difference between group pre-treated with omeprazole and other groups ($P < 0,05$).

entire stomach mucosa and protects against the colonization of bacteria and mechanical forces of proteolytic digestion (Caldas et al., 2014). Other studies have shown the ulceroprotective effect of *M. oleifera*. The ethanolic extract of *M. oleifera* root-bark markedly decreased the free acidity, ulcer index and the total acidity and increased the gastric content pH compared with the control group (Choudhary et al., 2013).

The results of the present study are similar to the finding of Devaraj et al. (2007) who found that extracts of *M. oleifera* prepared with acetone and methanol reduced the secretion of gastric acid showing their antisecretory activity. This study is in accordance with recent studies (Almuzafar, 2018), demonstrated that *M. oleifera* ethanolic leaf extract possesses significant gastroprotective effect by reducing ulcer index compared to control ($P < 0.01$), and this effect may be due to its direct action on the mucus secretion or by increasing prostaglandins. Furthermore, *M. oleifera* leaf extracts reduced ulcer index in ibuprofen-induced gastric ulcer model and in pyloric ligation test and a significant reduction in cysteamine-induced duodenal ulcers and stress ulcers was also observed (Bhattacharya et al., 2018). Oral treatment with HCl/EtOH induced a significant increase of the lesion numbers in the gastric mucosa, gastric juice volume, acidity, reduced the pepsin activity, increased the level of lipid peroxides and diminished tissue antiox-

idant enzymes (Ganesan et al., 2010). In another report, oral administration of acidified ethanol significantly increase the nuclear translocation factor (NF-κB), also it elevate pro-inflammatory cytokine mRNA (IL-1β and TNF-α), and reduce IκB-α protein expression (Shin et al., 2020).

Ethanol destroys cells by causing mucosal disturbances in the microcirculation of free radicals in the mucosa, increased lipid peroxidation, decreased non-protein sulfhydryl groups (GSH) and mucus production and inhibition of gastric prostaglandins (Caldas et al., 2014). Consumption of alcohol may interfere with metabolism and gastric motility (Shin et al., 2020). It occurs throughout the gastrointestinal tract. After absorption, the alcohol dehydrogenase enzyme transform ethanol to acetaldehyde and then to acetic acid, which is cytotoxic to gastric cells (Serafim et al., 2020). Ethanol directly penetrates the mucosa of the stomach, damaging the membrane, exfoliating the cells, and leading to the erosion of tissues via mechanisms particularly the reactive oxygen species (ROS) formation, a decrease in the SH concentrations, increase in the secretion of gastric acid, rupture of the mucus, and mucosal damage due to hemorrhagic lesions, lipid peroxidation induction, cellular apoptosis, and GSH decrease (Périco et al., 2020). In addition, polymorphonuclear cell infiltration that release ROS, increase the pro-oxidative substances and

pro-inflammatory molecules formation was mediated after the contact of the gastric mucosa with ethanol (Serafim et al., 2020).

As expected, *M. oleifera* leaf infusion pretreatment protected gastric mucosal layers of rats from ulceration induced by HCl/EtOH. In a study previously reported by Ijioma et al. (2018), the level of protection of stomach against aspirin-induced ulcer was sufficiently increased in animals treated with 800 mg/kg of Moringa extract as there was increased protection of surface epithelium with more mucus globules. Other reports showed a significant reduction in ulcer index and increase in regenerated glandular epithelium width and collagen content after treatment with *M. oleifera* flower and leaf extracts when compared with ethanol-treated animals (Patel and Lariya, 2019; Devaraj et al., 2007).

When gastric mucosa is exposed to damaging agents, it encompasses the disruption of the unstirred mucus/bicarbonate/phospholipid layer, exfoliation of the surface epithelium with loss of its barrier and the deeper gastric mucosal layers, including microvascular endothelial cells, progenitor, parietal and chief cells (Kangwan et al., 2014).

M. oleifera protects the gastric mucosa against oxidative stress through the increase of the antioxidant enzyme activities (SOD, CAT, GPx) and decrease the MDA level. Lipid peroxidation occurs when ROS attack cell membranes, allowing them to enter intracellular structures (Caldas et al., 2014). Our findings are consistent with the data obtained by Ganesan et al. (2010) indicated that HCl/EtOH reduce the levels of GSH dependent antioxidant enzymes (GPx and GST), GSH and antiperoxidative enzymes, such as SOD and CAT in ulcerated rats. Recently, the work done by Kim et al. (2020), demonstrated that the GSH levels and SOD activities were markedly elevated in the stomach tissues of HCl/EtOH-ulcerated rats. Further, it was observed that in acidified EtOH- administered rats there was increased ROS generation assessed by increased level of TBARS and attenuated levels of CAT, SOD, GSH and GPx activities and GST along with decreased mucus secretion (Guesmi et al., 2014). According to Shin et al. (2020), gastric cells promote mediation of several antioxidant enzymes to maintain the homeostasis of gastrointestinal tract through the ROS scavenging. The antioxidant system depletion enhances the susceptibility of the gastric mucosal cells to oxygen metabolites and acid mediated cell damage (Ganesan et al., 2010). ROS overproduction under oxidative stress results in stomach cellular damage (Shin et al., 2020). Myeloperoxidase (MPO), as the main markers to investigate antioxidant mechanisms, is an enzyme present in the malondialdehyde (MDA), a final product from the reaction between ROS and polyunsaturated fatty acids from cell membranes and neutrophils (Périco et al., 2020). The lipid peroxidation marker in water immersion restraint stress-induced gastritis *in vivo* (Kangwan et al., 2014).

Taking into account the pharmacological effeciency *M. oleifera* leaf infusion, it is important to note that the ulceroprotective effect of Moringa leaf extract may be attributed to several active phytocompounds ubiquitously detected in different parts of *M. oleifera*, including terpenoids, phenols, flavonoids, tannins, sterols, alkaloids and glycosides. The leaves of *M. oleifera* contain also phytosterols such as β-sitosterol, carotenoids (E-lutein, β-carotene), flavonoids, and pro-vitamin A. The predominant flavonols in *M. oleifera* leaves are quercetin and kaempferol, in their 3-O-glycoside forms. Other phytochemicals detected in all parts of this specie, including moringine, 5-O-acetyl-thio-octyl, vanillin, Chlorogenic acid, moringinine, β-sitosterone, gamma-sitosterol, alkaloids, 4-hydroxymellin, β-sitosterol, pterygospermin, pregna-7- diene-3-ol-20-one, niazimicin, octacosanoic acid and niaziminin (Alegbeleye, 2018; Bhattacharya et al., 2018). Thus stomach muco-

sal layer protection against oxidative stress by active components of *M. oleifera* is studied by many researchers. Currently, flavonoids such as nobiletin, kaempferol, baicalein, hesperidin, and diosmin reduced ethanol-induced ulcerations in rats, markedly protecting the gastric mucosa, inferring that these phytochemicals have a gastroprotective effect (Serafim et al., 2020). In addition, an enriched flavonoid fraction obtained from leaves extract of *Alchornea castaneaefolia* (Bonpl. ex Willd.) A. Juss. (Euphorbiaceae) reduced gastric lesions induced by HCl/ethanol and indomethacin/bethanechol in mice at a dose of 100 mg/kg (Awaad et al., 2013). Results obtained from *in vivo* experiments that involved quercetin, as a gastroprotective agent, inhibit the production of ROS and act as an anti-apoptotic during gastric injury (Brito et al., 2018) and suggest a blockage of the synthesis and secretion of matrix metalloproteinase 9 (MMP9), as well as, of the oxidative damage and infiltration of inflammatory cells, regulation of apoptosis and the cyclooxygenase (COX) and nitric oxide synthase (NOS) activity, increase of antioxidant enzyme activities (SOD, GPx) and the nuclear translocation of the nuclear factor related to erythroid 2 (Nrf$_2$), inhibition of nuclear factor kappa B (NF-kB), inflammatory cytokines tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1 β) reduction (Serafim et al., 2020).

Phenols are among the other phytocompounds that possess protective and therapeutic potential in peptic ulcer mediated by: re-epithelialization, neovascularization; down-regulating anti-angiogenic factors; improving cytoprotection and angiogenesis; up-regulating tissue growth factors and prostaglandins; enhancing endothelial nitric oxide synthase derived NO; suppressing oxidative mucosal damage; amplifying antioxidant performance, antacid, and antisecretory activity; increasing endogenous mucosal defensive agents; and blocking *Helicobacter pylori* colonization associated gastric morphological changes and gastroduodenal inflammation and ulceration. Polyphenolic compounds, including flavonoids are widely distributed in nature and recognized as the pigments responsible for the colors of the leaves (Serafim et al., 2020). The phenolic compounds allylpyrocatechol demonstrated a protective effect on mucosa and submucosa through improvement of prostaglandin expression and activation, which is mediated by the stimulation of COX-1 (Farzaei et al., 2015). Further, 200 mg/kg, p.o. of rutin demonstrated gastroprotective activity against gastric lesions mediated with indomethacin in rats, suppression of the generation of oxidative stress (increasing GSH and SOD and reducing MPO), and inhibition of neutrophil infiltration (Serafim et al., 2020).

5. Conclusion

In conclusion, the present observations indicate the potent antiulcerogenic effects of *M. oleifera* in rat models. The overall cytoprotective effect of *M. oleifera* may be due to its hydrochloric acid secretion neutralization. In addition, it maintains the antioxidant enzyme levels under the normal conditions and diminish lipid peroxide levels induced by acidified ethanol-treated rats. Hence, the importance of *M. oleifera* requires other studies to explore the principle that act as gastroprotectif agent and its mechanism of action. Further, why not to develop ulceroprotectif drugs, food diet formulations and to apply the tree in clinical trials.

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.

Acknowledgments

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