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RESEARCH ARTICLE

Innovative approaches for beetroot *(Beta Vulgaris L.)* aqueous extraction by cyclodextrins and its use to alleviate ethanol induced gastric ulcer in rats

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Abstract

One of the most common chronic conditions of peptic ulcer is gastric ulcer (GU), which is recognized as a high-risk illness in the present-day lifestyle. Originating from Asia and Europe, Beetroot (*Beta vulgaris L.*) is packed with high amounts of bioactive compounds like betanin and phenolics. These contribute to its nutritional value and overall health benefits. In this work, varying concentrations (0.5% to 7% w/v) of beta-cyclodextrin (β-CD) and hydroxypropyl-beta-cyclodextrin (HP-β-CD) were used to improve the efficiency of extracting beetroot components with water, using both stirring and ultrasound techniques. The presence of 5% (w/v) HP-β-CD during extraction appeared the maximum values of total phenolic compounds and betanin (3.1 mcg/mL and 3.01 mg/mL, respectively). The extraction recoveries of betanin at 5% (w/v) HP-β-CD emerging with magnetic stirring were 73%, 36% and 50% against water, ethanol and β-CD, respectively. The reproducibility of extraction procedure was found to be 0.86% relative standard deviation (RSD) indicating the highest precision of the beetroot modified HP-β-CD extraction procedure. Then, rats that had been treated with either HP-β-CD-modified aqueous extract of beetroot, omeprazole (20 mg/kg, orally administered) or both were studied to assess whether they helped reduce ethanol-induced GU. The combined treatment of HP-β-CD modified beetroot extract with omeprazole brought a significant decrease in the increased levels of serum oxidative stress malonaldehyde and nitric oxide, inflammatory markers myeloperoxidase, interleukin-10, interleukin-6 and tumor necrosis factor-α. It also restored the decreased levels of antioxidant glutathione and cyclo-protective prostaglandin E2 in comparison to the positive control. Furthermore, the proposed combination of beetroot-modified aqueous extract and omeprazole exhibited less severe histopathological damage in comparison to the positive control. Therefore, a novel synergistic pharmaceutical treatment using HP-β-CD modified aqueous extract of beetroot and omeprazole was presented to enhance GU healing.

Keywords Beetroot *(Beta Vulgaris L.)* · Gastric ulcer · Cyclodextrins · Aqueous extraction · Rats

1 Introduction

Gastric ulcer (GU), also known as stomach ulcer, is a benign lesion that has various causes, which are linked to a lack of balance between gastric protective factors and destructive physical, psychological, or chemical factors on the mucosal epithelium (Li et al. 2014). It is acknowledged that GU is the most common chronic peptic ulcer disease because of the location of the attack (Hamedi et al. 2015) and a large number of people suffer from this disease worldwide. There is equal prevalence of GUs among men and women. Various factors lead to the development of GU, including pepsin, bile acids, *Helicobacter pylori (H. pylori)*, ethanol, and nonsteroidal anti-inflammatory drugs (NSAIDs) that disrupt the stomach's defense mechanisms, e.g. the tight junctions between epithelial cells, bicarbonate secretion, microvascular blood circulation, and prostaglandins and nitric acid production (Kolgazi et al. 2017; Kayali et al. 2018). While various medications exist to treat GUs, for example, H2 receptor antagonists, cytoprotective agents, and proton pump inhibitors, all have limitations and side effects (Armah et al. 2021). For instance, it was found that combining omeprazole and rebamipide enhanced ulcer healing by

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increasing prostaglandin E2 (PGE2) and decreasing interleukin-8 (IL-8) and malondialdehyde (MDA) in the gastric mucosa (Kangwan et al. 2014). Nevertheless, medications often come with unwanted side effects and may not always be as effective as desired. Thus, it is becoming imperative to develop alternative pharmaceutical treatments with antiulcerogenic properties. This has led to the development of new anti-ulcer agents, including herbal medicines, which could potentially lead to the production of novel medications (Chai 2011). Research on traditional medicinal plants has gained momentum worldwide in the past few years, with growing evidence highlighting the fact that medicinal plants being utilized in different traditional treatments have significant potential.

Native to Asia and Europe, beetroot (*Beta vulgaris L.*) is part of the Chenopodiaceae family (Chhikara et al. 2019). Recently, the health beneficial effects of beetroot have attracted scientific attention for its role in preventing and treating different human diseases (Rehman et al. 2021). It offers significant nutritional and health advantages because of its high concentration of bioactive compounds including betalains, carotenoids, polyphenols, and flavonoids (Rehman et al. 2021). In *vitro* and pre-clinical studies have suggested that beetroot has several health benefits, including anti-depressant, anti-oxidative stress, anti-carcinogenic, anti-hypercholesterolemic, anti-hyperglycemic, hematopoietic, anti-inflammatory, anti-bacterial, anti-proliferative, diuretic, hepatoprotective, anti-nephrotoxicity, and immunomodulatory properties (Lu et al. 2009; Chawla et al. 2016; Murthy and Manchali 2013). Beetroot consumption may also offer protection against age-related illnesses (Kapadia et al. 2013). Beetroot's effect on the vascular endothelium of the blood vessels increases the blood flow, and this is largely attributed to its high inorganic nitrate, betaine and NO contents. Beetroot was used to increase exercise stamina and running performance. Also, it was used in management of antiradical, antimicrobial and cytotoxic activities. Therefore, it has hepatoprotective and antidiabetic potential effects (Lu et al. 2009; Chawla et al. 2016; Murthy and Manchali 2013; Kapadia et al. 2013). However, betalains and phenolic compounds are susceptible to rapid degradation under various conditions, such as temperature, pH, light, and oxygen (Khan 2016). Consequently, the processing of beetroot extract faces a significant challenge due to the weak stability of these compounds (Tutunchi et al. 2019).

Traditionally, bioactive compounds were extracted from plant materials through the solid–liquid extraction technique that involved the use of organic solvents and temperature (Albahari et al. 2018). However, this method suffers from several limitations, including extended processing duration, degradation of bioactive compounds throughout processing, and the utilization of hazardous organic solvents (Albahari et al. 2018). Recognizing these challenges, cost-effective and efficient methods for environmentally friendly extraction of bioactive compounds are being developed.

It is possible to use cyclodextrins (CDs) and their derivatives as an alternative to organic solvents as they offer improved solubility and stability for bioactive compounds in aqueous extracts. It was known that CDs are molecules with a cone form having hydrophilic on the cavity's exterior and hydrophobic within. Therefore, CDs can enclose a variety of hydrophobic guest molecules in its non-polar cavity through non-covalent bonds. Several spectroscopic techniques, umbrella sampling/ microsecond timescale molecular dynamics simulations were performed to examine the structural inclusion, the interactions and thermodynamic parameters of inclusion complex formation between bioactive compounds and CDs (Kumar et al. 2023, 2024a, 2024b; Bhardwaj and Purohit 2023; Kumar and Purohit 2024). β-Cyclodextrin (β-CD) is known for its cost-effectiveness. It has a hydrophilic outer surface cavity for several hydroxyl groups, but within the cavity, it is hydrophobic. This makes β-CD water-soluble, which helps capture various hydrophobic guest molecules within its non-polar cavity (Hu et al. 2007). β-CD has found extensive application as a flavor carrier and preservative in different food products, and since 1998, it has been acknowledged as a 'Generally Recognized as Safe (GRAS)' additive (Szente and Szejtli 2004). In addition, in aqueous extractions, β-CD enhances the utilization of natural plant resources by facilitating interaction with guest molecules (bioactive compounds) through non-covalent bonds. In contrast to its naturally occurring parent compound, 2-Hydroxylpropyl-beta-cyclodextrin (HP-β-CD), which is a chemically modified derivative of β-cyclodextrin, offers an enhanced safety profile and cost effectiveness. Thus, it was used to enhance the extraction efficiency of bioactive compounds (Diamanti et al. 2017; Hernández-Sánchez et al. 2017; Albahari et al. 2018). HP-β-CD had the highest binding affinity for phenolic compounds with the most negative complexation energy (Kumar et al. 2024a). The computational analysis was aligned with experimental findings validating HP-β-CD as most effective cavitand molecule for improving the solubility of phenolic compounds (Kumar et al. 2024b). There is widespread use of HP-β-CD as an excipient in pharmaceuticals for inflammation, cardiac dysrhythmia, and fungal diseases (Loftsson et al. 2005). Moreover, it was found recently that the efficiency of extraction using CDs increased by emerging with several advanced technologies.

The methods based on the non-thermal concept are part of emerging technologies, and their goal is to reduce processing time, enhance yield, and decrease energy usage (Deng et al. 2015). Stirring and ultrasound-assisted extraction are the most efficient and promising emerging technologies utilized in extracting beetroot pigments (Maran and Priya 2016). It is believed that these methods are environmentally friendly

and safe because they use very few harmful chemicals, have shorter processing duration, exhibit high energy efficiency, and limited degradation of active compounds. Extraction rates are enhanced by ultrasound-assisted extraction through the cavitation mechanism, which disrupts cell walls within the solid matrix, facilitating solvent penetration and mass transfer (Righi Pessoa da Silva et al. 2018). Ultrasound-assisted extraction techniques have been used in earlier studies for extracting betalains and phenolic compounds from colored quinoa hulls, red beet, and beetroot pomace (Tutunchi et al. 2019). Nevertheless, integrating emerging methods with CDs is capable of further increasing the extraction efficiency of bioactive compounds like betalains and phenolic compounds, without affecting their stability.

Hence, the present study aims to improve the aqueous extraction of red beetroot (Beta vulgaris L.) bioactive compounds through cyclodextrins (β-CD and HP-β-CD) modification. The study examined the potential protective effects of omeprazole, a standard medication, and/or cyclodextrins-modified aqueous extract of beetroot against ethanol-induced gastric mucosal damage in rats. In addition, gastric secretion parameters and histopathological evaluation were carried out on ulcerated rats.

2 Materials and methods

2.1 Chemicals and reagents

Hydroxypropyl-β-cyclodextrin, β-cyclodextrin, and ethanol were procured from Sigma-Aldrich (USA) and beetroot (Beta vulgaris L.) samples were obtained from a local vegetable market in Jeddah, Saudi Arabia. Omeprazole (20 mg capsules under the brand GasecTM, Acino Pharmaceutical Company) were acquired from Nahdi Pharmacy, also located in Jeddah. Commercial diagnostic kits for determining serum were ordered from MyBioSource.com, a commercial trading website.

2.2 Preparation of beetroot extracts

After washing and slicing the beetroot with a slicer, the slices were dried for one week at 25 ºC. Subsequently, the dried beet slices were powdered using a mill and preserved at 4 ºC until required.

For the preparation of ethanolic and aqueous beetroot extracts, 0.5 g of beetroot powder was dissolved in 125 mL of ethanol and water, respectively. The solutions were then stirred for 3 h at 800 rpm or sonicated for 15 min at a temperature of 25 °C. To remove any residue from the extracts, common filter paper was used. The filtrate was dried and/or stored at −20 ºC so that it could be analyzed subsequently.

For the preparation of CDs modified aqueous beetroot extract, 0.5 g of beetroot powder was dissolved in 125 mL of water containing various amounts of CDs (0.62, 1.25, 3.75, 6.25, and 8.75 g) to prepare multiple β-CD and HP-β-CD modified beetroot extracts. These solutions were subjected to stirring or sonication under identical conditions as the net-modified solutions. The resulting filtrates were dried and/or stored at −20 ºC for subsequent evaluation.

2.3 Determination of total phenolic content (TPC) and betanin concentration

A Thermo Scientific HPLC-LTQ-XL linear ion trap mass spectrometer was used along with an Accela autosampler and Accela pump (San Jose, CA, USA) to measure the total phenolic content (TPC) and betanin amount. Electrospray ionization (ESI) was utilized as the ion source. Xcalibur® Thermo Fisher Scientific Inc, version 2.07 SP1 was used to regulate the system. The parameters optimized included a spray voltage of 5.0 kV, sheath gas flow rate of 42 mL/min, auxiliary gas flow rate of 10 mL/min, capillary voltage of 60 V, and capillary temperature of 325 °C. The collision energy was set at 35 V. Chromatographic separation was carried out using an Eclipse Plus C18 column 3.5 µm with dimensions of 4.6×100 mm (Agilent, Palo Alto, USA). The column oven temperature was maintained at 40 ± 3 °C, while the tray temperature was set to 20 °C. The ion trap-mass spectrometer (IT-MS) detector was configured to monitor ions in positive scan mode within the range of 100—1200 m/z and in dependent auto-fragmentation mode. For ions exceeding a mass count of 1000, MS spectra were created. To enable subsequent analysis of the data, it was saved in raw file format. This would be done using Processing Setup, ThermoXcalibur 4.5.474.0, and FreeStyle™ 1.8 SP2, Modern Data Visualization Software, Version 1.8.63.0, with a build date of Friday, July 30, 2021, copyrighted by Thermo Fisher Scientific Inc. The NIST Mass Spectral Search Program, Version 2.4, built Mar 25, 2020, was employed, supplemented by the online MassBank of North America (MoNA) database available at https://mona.fiehnlab.ucdavis.edu.

2.4 In vivoexperimental design

2.4.1 Experimental animals

The study employed 50 male albino Wistar rats (150 – 200 g), which were obtained from the Mansour Scientific Foundation for Research and Development (MSF) in Jeddah, Saudi Arabia. The rats were kept in plastic cages, with seven rats per cage, for one week before commencing the experiments. This period allowed them to adapt to the standard laboratory conditions, including a temperature of 20 °C,

humidity ranging from 55 to 65%, and a light–dark cycle of 12 h each. The rats had unrestricted access to both water and standard rodent feed during this period. The Research Ethics Committee (REC) of the Faculty of Medicine at King Abdul Aziz University approved the study protocol (Reference No: p453-2020).

2.4.2 Experimental protocol

Following overnight fasts and food starvation, but with unlimited access to water, the rats were randomly allocated into five groups, each consisting of 10 rats.

Group 1 (Normal group): Normal diet was administered to healthy rats, along with 1 mL/kg body weight phosphate-buffered saline.
Group 2 (Ulcerated group): Gastric ulcers were induced in rats by a single gavage of absolute ethanol (1 mL/kg b.w./rat) following 24 h of fasting (Dalhoumi et al. 2022).
Group 3 (Beetroot, EtOH): 0.5 mg/kg b.w./day of optimized beetroot extract was used for pretreating ethanol-ulcerated rats. The beetroot extract was prepared by dissolving 0.5 g of beetroot sample in 125 mL water containing HP-β-CD at a concentration of 50 g/L. The solution was stirred for 3 h at 800 rpm, was filtrated and then was dried with rotatory evaporator under nitrogen atmosphere.
Group 4 (Omeprazole, EtOH): Ethanol-induced ulcerated rats were orally pretreated with the standard anti-stomach ulcer drug, omeprazole, at a dose of 20 mg/kg b.w./day.
Group 5 (Beetroot, Omeprazole, EtOH): Pretreatment of ethanol-induced ulcerated rats was carried out with the optimized simultaneous mixture of beetroot extract and omeprazole containing the same amounts as described above.

The rats were orally administered optimized beetroot extract, omeprazole, and combination treatments for 10 days before ulcer induction. For 24 h before ulcer induction, only water was given to Groups 2, 3, 4, and 5. A digital scale was used to measure the body weight of the animals sat the start and the end of the experiments (Elnaga et al. 2016).

2.4.3 Blood samples collection

Animals fasted for 1 h when the experimental period ended, after which they were anaesthetized. Retro-orbital venous plexus was used to obtain the blood samples. A single portion of each blood sample was accumulated in a dry centrifuge tube, allowed to clot, and subsequently centrifuged at 3000xg for 15 min at a temperature of 40 ℃ using the Heraeus Multifuge X3R centrifuge, resulting in the isolation of clear serum. The sera were aliquoted and stored at −20 ℃ for subsequent analyses.

2.4.4 Determination of malondialdehyde (MDA)

The competitive enzyme immunoassay approach was used by the MDA ELISA kit by employing a polyclonal anti-MDA antibody and an MDA-HRP conjugate. Following incubation of the assay sample and buffer with the MDA-HRP conjugate in a pre-coated plate for one hour, the wells were decanted and washed five times. Subsequently, a substrate for the HRP enzyme was used to incubate the wells, resulting in the formation of a blue-colored complex through the enzyme–substrate reaction. A stop solution was ultimately ended to bring an end to the reaction, causing the solution to turn yellow. The color intensity was spectrophotometrically measured in a microplate reader at 450 nm. The MDA from samples and MDA-HRP conjugate fight for the anti-MDA antibody binding site was found to have an inverse relationship with the MDA concentration. A standard curve that linked the color intensity with the concentration of standards was plotted. Subsequently, this standard curve was interpolated to determine the MDA concentration in each sample.

2.4.5 Determination of nitric oxide (NO) and glutathione (GSH)

The double-sandwich ELISA approach was utilized in these experiments; making use of pre-coated Rat NO and Rat GSH monoclonal antibodies as well as biotin-labeled polyclonal antibodies as detecting antibodies. Samples and biotin-labeled antibodies were introduced into the ELISA plate wells and subsequently washed with either Phosphate Buffered Saline (PBS) or Tris–HCl Buffered Saline (TBS). Following this, Avidin-peroxidase conjugates were included in the ELISA wells. After thorough washing of reactants with buffers, 3,3', 5,5' tetramethylbenzidine dihydrochloride (TMB) substrate was utilized for coloring. In peroxidase catalysis, TMB turns blue and under the action of acid, it changes to yellow.

2.4.6 Determination of myeloperoxidase (MPO)

Using the quantitative sandwich enzyme immunoassay approach, an antibody specific to MPO was pre-coated over a microplate. After this, standards and samples were pipetted inside the wells, where any MPO available was bound by the immobilized antibody. After eliminating any unbound substances, a biotin-conjugated antibody specific to MPO was introduced into the wells. The antibody was then washed, after which avidin-conjugated HRP was included in the wells. A substrate solution was included in the wells

after another wash to eliminate any unbound avidin-enzyme reagent, resulting in color development which was linked to the amount of MPO bound during the foremost step. The process of color development was then halted, and the color intensity was ascertained.

2.4.7 Determination of tumor necrosis factor-α (TNF-α)

A sandwich enzyme-linked immunosorbent assay (ELISA) technology was the basis of the kit. The 96-well plates were pre-coated with the anti-TNF-α polyclonal antibody, and the biotin-conjugated anti-TNFα polyclonal antibody served as detection antibodies. After adding the standards, test samples, and biotin-conjugated detection antibody into the wells, they were washed using wash buffer. Avidin–Biotin-Peroxidase Complex was introduced, followed by washing away unbound conjugates using a washing buffer. To visualize the Horseradish Peroxidase (HRP) enzymatic reaction, 3,3', 5,5' tetramethylbenzidine dihydrochloride (TMB) substrates were employed. HRP was used for catalyzing TMB to create a blue-colored product, which transformed to yellow after an acidic stop solution was added. There was a direct relationship between the density of yellow and the amount of TNFα captured from the sample on the plate. A microplate reader was used to determine the optical density (O.D.) absorbance using a microplate reader, following which the concentration of TNF-α was computed.

2.4.8 Determination of interleukin −6 (IL-6)

The competitive enzyme immunoassay method was used by the IL-6 ELISA kit that employed an anti-IL-6 antibody and an IL-6-HRP conjugate. Co-incubation of the assay sample and buffer was carried out with the IL-6-HRP conjugate on a pre-coated plate for one hour. The wells were decanted and washed five times following the incubation period. Subsequently, a substrate for the HRP enzyme was used to incubate the wells, leading to the formation of a blue-colored complex as a product of the enzyme–substrate reaction. A stop solution was finally included to end the reaction, resulting in the solution turning yellow. Spectrophotometric measurement of the color intensity was carried out using a microplate reader at 450 nm. There was an inverse relationship between this intensity and the IL-6 concentration as IL-6 from samples competed with IL-6-HRP conjugate for the anti-IL-6 antibody binding site. Due to the limited number of binding sites, IL-6 from the sample occupied a greater number of sites, which reduced the availability of sites to bind IL-6-HRP conjugate. A standard curve was constructed that linked the intensity of color with the concentration of standards. This standard curve was interpolated to determine the IL-6 concentration in each sample.

2.4.9 Determination of interleukin −10 (IL-10)

Rat IL-10 was quantitatively measured in serum using the Rat IL-10 ELISA kit that included an in vitro enzyme-associated immunosorbent assay. An antibody specific to Rat IL-10 that was coated on a 96-well plate was used in this assay. After pipetting the standards and samples into the wells, IL-10 present in the sample was attached to the wells using the immobilized antibody. A biotinylated anti-Rat IL-10 antibody was introduced after washing the wells. Once the unbound biotinylated antibody was washed away, HRP-conjugated streptavidin was added to the wells. Another washing step was performed, followed by the addition of a TMB substrate solution, resulting in the transformation of color from blue to yellow relative to the quantity of IL-10 bound. The color intensity was then determined at 450 nm.

2.4.10 Determination of prostaglandin E2 (PGE2)

The competitive enzyme immunoassay method was employed by the PGE2 ELISA kit, which included a polyclonal anti-PGE2 antibody and a PGE2-HRP conjugate. Co-incubation of the sample and buffer was carried out with the PGE2-HRP conjugate on a pre-coated plate for an hour. The wells were decanted and washed five times following the incubation period. Subsequently, a substrate for the HRP enzyme was used for incubating the wells, which formed a blue-colored complex as a product of the enzyme–substrate reaction. A stop solution was ultimately included to end the reaction, causing the solution to turn yellow. Spectrophotometric measurement of the color intensity was then carried out using a microplate reader at 450 nm. There was an inverse relationship between the color intensity and the PGE2 concentration as PGE2 from samples competed with PGE2-HRP conjugate for the anti-PGE2 antibody binding site. Due to the limited number of binding sites, PGE2 from the sample occupied a greater number of sites, which reduced the availability of sites to bind PGE2-HRP conjugate. A standard curve was constructed to link the intensity of color with the concentration of standards, and this curve was interpolated to determine the PGE2 concentration in each sample.

2.5 Histopathological investigation

The animals were quickly sacrificed following blood collection; the stomach of every animal was extracted through dissection. They were then rinsed with ice-cold isotonic saline solution and gently dried between two filter papers. To perform histopathological examination, sections of the stomach subjected to all administrations were preserved in buffered formalin (10%), and implanted in wax blocks, and a Leica microtome was used to obtain thick sections of gastric

Table 1 Histopathological scoring criteria

Pathological state	Score	
Gastric mucosa injury	0	Intact
	1	Desquamation of epithelial lamina
	2	Desquamation of superficial lamina propria or 1/3 reduction of gastric glands
Leucocytes infiltration	0	Absent
	1	2–10/HPF
	2	11–20/HPF
	3	21–30/HPF
	4	> 31/HPF
Gastric hemorrhage	0	Absent
	1	< 10% of total area/LPF
	2	11%–20% of total area/LPF
	4	21%–30% of total area/LPF
	5	> 30%

tissues (4 µm). These obtained sections were stained with Hematoxylin and Eosin (H&E) and subsequently examined under light microscopy. Samples were scored under light microscopy using a scoring system (Table 1). This score comprises the graded evaluation of gastric mucosa damage and infiltration of immune cells. A scale of 0–4 was utilized (Liu et al. 2016).

2.6 Statistical analysis

The Statistical Package for the Social Sciences (SPSS), version 22 (IBM SPSS, IBM Corp., Armonk, N.Y., USA) was used to carry out data analysis. The parametric values were presented as mean ± standard error of the mean (SEM). The normality of data distributions was assessed using the Shapiro–Wilk Test. As the parametric data exhibited normal distribution, analysis was performed using One-Way ANOVA, after which Tukey's test was carried out for group comparisons. Statistical significance was set at $P < 0.05$.

3 Results

3.1 Enhancing the aqueous extraction of bioactive compounds from red beetroot

Solvent extraction involved the use of various concentrations of β-CD and HP-β-CD dissolved in water, ranging from 0.62 g to 8.75 g (0.5–7%, w/v), using both magnetic stirrer (conventional technology) and ultrasound (developing technology). The reproducibility of the extraction procedure was checked by repeating the procedure three times within three days ($n = 9$) and measured the betanin concentration every time; the relative standard deviation (RSD) was found to be 0.98% & 0.75 with β-CD emerging with magnetic stirring and sonication and 0.86% & 0.65 with HP-β-CD emerging with magnetic stirring and sonication, respectively. The obtained data indicated the acceptable reproducibility of CD-modified extraction procedure.

3.1.1 Determination of the betanin content of beetroot extracts

The current study examined the trend in betanin concentration across all beetroot samples in the presence of β-CD and HP-β-CD. The UV–Vis spectrophotometer spectrum of beetroot extract is shown in Fig. 1, where two strong absorbance peaks at 478 and 538 nm were observed and attributed to betaxanthin and betanin, respectively (Righi Pessoa da Silva et al. 2018). Therefore, all spectrophotometric measurements of betanin were fixed at 538 nm. Table 2 and Fig. 2 show the findings of the betanin content of beetroot extracts altered by β-CD. In the presence of a magnetic stirrer, the trend in betanin concentration across all beetroot samples demonstrated the following order: EtOH > β-CD3 > β-CD2 > β-CD1 > β-CD4 > β-CD5 > W. On the other hand, the order shifted to β-CD2 > β-CD3 > β-CD1 > β-CD4 = β-CD5 = W > EtOH in the presence of ultrasound. In addition, Table 3 and Fig. 3 show the outcomes of the betanin concentrations of extracts altered by HP-β-CD. In the presence of a magnetic stirrer, the order observed was: HP-β-CD4 > HP-β-CD5 > HP-β-CD1 > HP-β-CD3 > HP-β-CD2 > EtOH > W. In contrast, the order shifted to HP-β-CD4 > HP-β-CD3 > HP-β-CD2 > HP

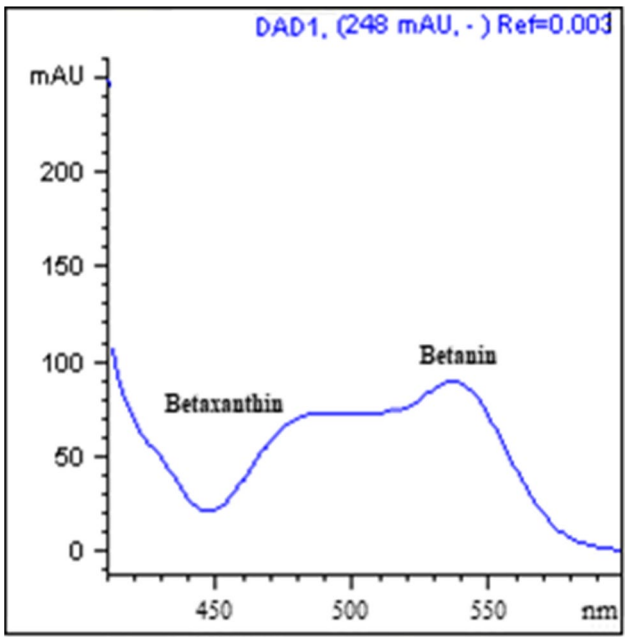


Fig. 1 UV–Vis spectrum of the extracted beetroot samples

Table 2 Total betanin concentration (±SD, $n=3$) of beetroot samples in the presence of β-CD with magnetic stirring and sonication

Sample	β-CD concentration, %, w/w	Total Betanin concentration with magnetic stirring, mg/mL ± SD ($n=3$)	Total Betanin concentration with sonication, mg/mL ± SD ($n=3$)
EtOH*	0.00	1.92 ± 0.11	0.96 ± 0.14
W*	0.00	0.82 ± 0.09	1.16 ± 0.08
β-CD1	0.50	1.81 ± 0.03	1.45 ± 0.05
β-CD2	1.00	1.85 ± 0.06	2.47 ± 0.02
β-CD3	3.00	1.88 ± 0.04	2.44 ± 0.08
β-CD4	5.00	1.53 ± 0.03	1.16 ± 0.01
β-CD5	7.00	0.95 ± 0.05	1.16 ± 0.09

*W water, *EtOH* Ethanol, *β-CD* beta-cyclodextrin

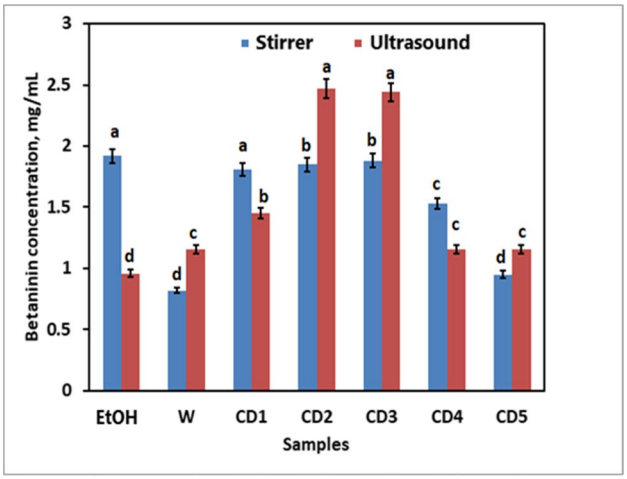


Fig. 2 Total betanin concentration of beetroot samples in β-CD presence with magnetic stirring and sonication. Data expressed as mean ± standard deviation ($n=3$) and different letters show significant variation at 5% level in Duncan's test ($p<0.05$). W: Water, EtOH: Ethanol, CD1: 0.5% (w/v) β-CD, CD2: 1.0% (w/v) β-CD, CD3: 3.0% (w/v) β-CD, CD4: 5.0% (w/v) β-CD and CD5: 7.0% (w/v) β- CD

-β-CD1 > HP-β-CD5 > W > EtOH in the presence of ultrasound. As it was observed in above results, the extraction recovery (complexation efficiency) of betanin content has influenced by varying β-CD and HP-β-CD concentrations. In Table 3, the extraction recovery (complexation efficiency) of betanin has increased from 69% with stirring & 41% with sonication at 0.5% (w/v) HP-β-CD to 73% with stirring & 56% with sonication at 5% (w/v) HP-β-CD followed by decreasing to 72% with stirring & 27% with sonication at 7% (w/v) HP-β-CD. The highest extraction recovery of betanin was found in the presence of 5% (w/v) HP-β-CD with magnetic stirring against water and ethanol alone with recovery 73% and 36%, respectively. These results of extraction methods with HP-β-CD compare to those without CDs have clarified HP-β-CD significantly enhanced betanin content recovery beyond baseline extraction.

3.1.2 Determination of total phenolic content (TPC) of beetroot aqueous extracts

Table 4 and Fig. 4 show the TPC values of beetroot extracts. After the addition of 1% and 3% β-CD to the extraction solvent, there were significant improvements in the TPC values ($P<0.05$) to 2.5 and 2.4 mcg/mL, respectively. Similarly, the addition of 5% and 7% HP-β-CD (Table 5 and Fig. 5) into the extraction solvent brought an increase in the TPC of the sample extracted with water and

Table 3 Total betanin concentration (±SD) of beetroot samples in the presence of HP-β-CD with magnetic stirring and sonication

Sample	HP-β-CD concentration, %, w/w	Total Betanin concentration with magnetic stirring, mg/mL ± SD ($n=3$)	Total Betanin concentration with sonication, mg/mL ± SD ($n=3$)
EtOH*	0.00	1.92 ± 0.09	0.96 ± 0.12
W*	0.00	0.82 ± 0.11	1.16 ± 0.09
HP-β-CD1	0.50	2.66 ± 0.04	1.95 ± 0.07
HP-β-CD2	1.00	2.23 ± 0.03	2.26 ± 0.05
HP-β-CD3	3.00	2.52 ± 0.07	2.28 ± 0.02
HP-β-CD4	5.00	3.01 ± 0.04	2.61 ± 0.04
HP-β-CD5	7.00	2.93 ± 0.03	1.59 ± 0.07

*W water, *EtOH* Ethanol, *HP-β-CD* hydroxypropyl-beta-cyclodextrin

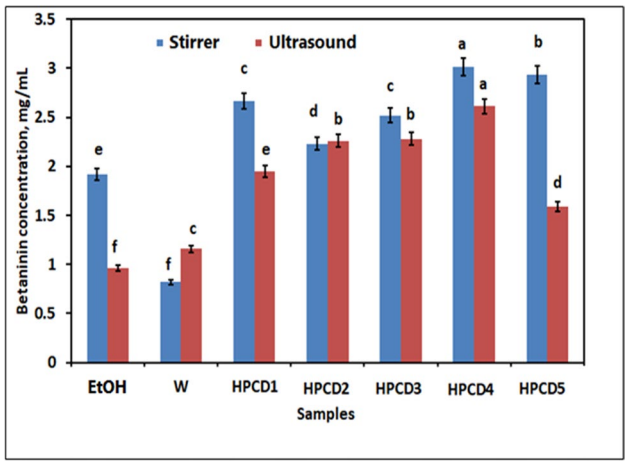


Fig. 3 Total betanin concentration of beetroot samples in the presence of HP-β-CD with magnetic stirring and sonication. Data are expressed as mean ± standard deviation ($n = 3$) and different letters show significant difference at the 5% level in Duncan's test ($p < 0.05$). W: Water, EtOH: Ethanol, HPCD1: 0.5% (w/v) HP-β-CD, HPCD2: 1.0% (w/v) HP-β-CD, HPCD3: 3.0% (w/v) HP-β-CD, HPCD4: 5.0% (w/v) HP-β-CD and HPCD5: 7.0% (w/v) HP-β-CD

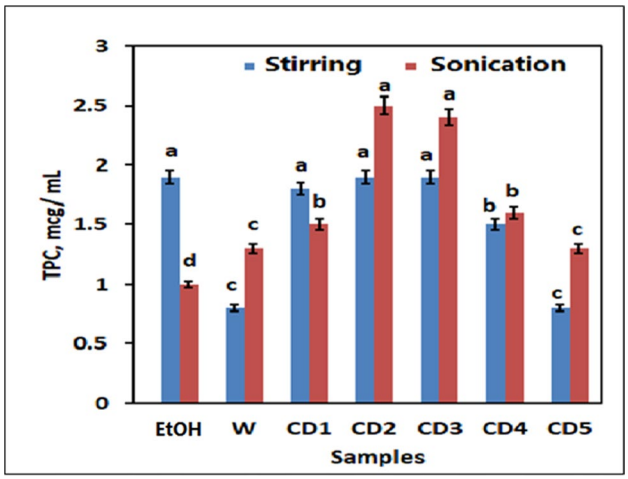


Fig. 4 Total phenolic content (TPC) of beetroot samples in the presence of β-CD with magnetic stirring and sonication. Data are expressed as mean ± standard deviation ($n = 3$) and different letters show significant difference at the 5% level in Duncan's test ($p < 0.05$). Others as mentioned in Fig. 2

Table 4 Total phenolic content (TPC ± SD) of beetroot samples in the presence of β-CD with magnetic stirring and sonication

Sample	β-CD concentration, %, w/w	TPC with magnetic stirring, mcg/mL ± SD ($n = 3$)	TPC with sonication, mcg/mL ± SD ($n = 3$)
EtOH*	0.0	1.9 ± 0.06	1.**0** ± 0.11
W*	0.0	0.8 ± 0.03	1.3 ± 0.08
β-CD1	0.5	1.8 ± 0.05	1.5 ± 0.06
β-CD2	1.0	1.9 ± 0.08	2.5 ± 0.03
β-CD3	3.0	1.9 ± 0.11	2.4 ± 0.09
β-CD4	5.0	1.5 ± 0.03	1.6 ± 0.08
β-CD5	7.0	0.8 ± 0.06	1.3 ± 0.01

*W water, *EtOH* Ethanol, *β-CD* beta-cyclodextrin

stirred during the process to 3.1 and 2.9 mcg/mL, respectively, representing a significant ($p < 0.05$) increase from the original value of 0.8 mcg/mL. As it was observed in the above results, the enhancement percent (complexation efficiency) of TPC has influenced by varying β-CD and HP-β-CD concentrations. In Table 5, the values have increased from 70% with stirring & 43% with sonication at 0.5% (w/v) HP-β-CD to 74% with stirring & 50% with sonication at 5% (w/v) HP-β-CD followed by decreasing to 72% with stirring & 19% with sonication at 7% (w/v) HP-β-CD. The highest enhancement was found in the presence of 5% (w/v) HP-β-CD with magnetic stirring against water and ethanol alone with recovery 74% and 39%, respectively. Based on these findings, the addition of 5% (w/v) HP-β-CD to water yielded the largest TPC value in comparison to other methods which have clarified HP-β-CD significantly enhanced betanin and phenolic content recovery (complexation efficiency) beyond baseline extraction.

3.2 The prophylactic impacts of beetroot cyclodextrin modified aqueous extract and/or omeprazole on ethanol ulcerated rats

The findings of the present study show how ethanol oral administration leads to gastric ulcers and the prophylactic effects of beetroot cyclodextrin-modified aqueous extract and/or omeprazole as a reference drug.

The statistical analysis of results involved comparing the mean values of various experimental groups with those of individual normal groups. The results are presented as mean ± SEM. To assess the significant differences between the various experimental groups, One-Way ANOVA and post-hoc least significance difference (LSD) tests were employed. Tables 6 and 7 present a summary of these findings, along with the percentage change of the aforementioned parameters in distinct groups relative to the ethanol group.

According to the data outcomes, oral administration of absolute ethanol (1 mL/kg body weight) caused gastric ulcers in rats and brought a substantial increase in the serum levels of MDA (a marker of membrane lipid peroxidation) and NO (a marker of nitrosative stress), accompanied by a decrease in concentration of serum GSH (an antioxidant marker) in comparison to normal animals ($P < 0.0001$), as demonstrated in Figs. S1, S2, and S3 (supplementary materials). Ulcerated rats were made to undergo prophylactic

Table 5 Total phenolic content (TPC ± SD) of beetroot samples in the presence of HP-β-CD with magnetic stirring and sonication

Sample	HP-β-CD concentration, %, w/w	TPC with magnetic stirring, mcg/mL ± SD ($n=3$)	TPC with sonication, mcg/mL ± SD ($n=3$)
EtOH*	0.0	1.9 ± 0.11	1.**0** ± 0.06
W*	0.0	0.8 ± 0.08	1.3 ± 0.03
HP-β -CD1	0.5	2.7 ± 0.06	2.3 ± 0.08
HP-β-CD2	1.0	2.2 ± 0.05	1.9 ± 0.11
HP-β-CD3	3.0	2.5 ± 0.03	2.3 ± 0.05
HP-β-CD4	5.0	3.1 ± 0.06	2.6 ± 0.01
HP-β-CD5	7.0	2.9 ± 0.08	1.6 ± 0.03

*W water, *EtOH* Ethanol, *HP-β-CD* Hydroxypropyl-beta-cyclodextrin

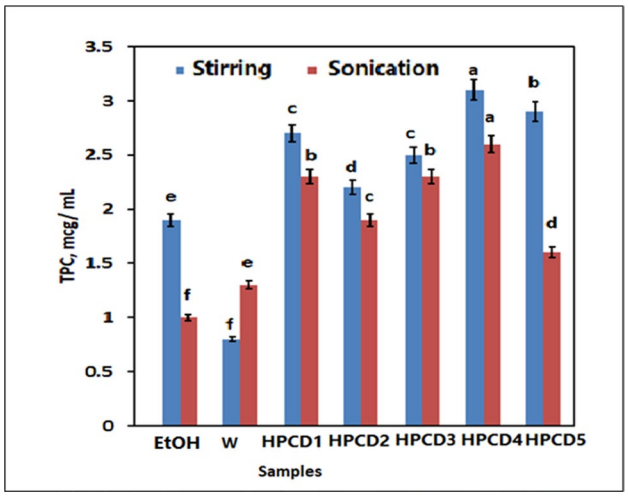


Fig. 5 Total phenolic content (TPC) of beetroot samples in the presence of HP-β-CD with magnetic stirring and sonication. Data are expressed as mean ± standard deviation ($n=3$) and different letters show significant difference at the 5% level in Duncan's test ($p<0.05$). W: Water, EtOH: Ethanol, Others as mentioned in Fig. 3

treatment with beetroot extract (0.5 mg/kg body weight) and/or omeprazole (20 mg/kg body weight/day) every day for ten days before ethanol administration, which successfully normalized the increased levels of MDA and NO. When the rats were treated with beetroot extract alone, serum GSH levels increased significantly compared to untreated ethanol-ulcerated rats ($P<0.0001$). In contrast, the decline in serum GSH levels in ethanol-ulcerated rats was effectively normalized following pre-treatment with the reference drug (omeprazole) or the combination of both agents (beetroot extract and omeprazole). Furthermore, oral administration of absolute ethanol to rats was lead to a substantial serum MPO activity (an inflammatory and oxidative stress marker) in ethanol-ulcerated rats as compared to normal ones ($P<0.0001$) (Fig. S4, supplementary material). Serum MPO activity levels decreased significantly when oral pre-treatment of ethanol-ulcerated rats was carried out with beetroot extract alone compared to untreated ethanol-ulcerated rats ($P<0.0001$). Nevertheless, the serum MPO activity in ethanol-ulcerated rats was successfully normalized following pre-treatment with omeprazole alone or in combination with beetroot extract. The findings also indicated that following oral administration of ethanol to rats, TNF-α, IL-6, and IL-10 serum levels increased significantly in ethanol-ulcerated rats compared to normal ones ($P<0.0001$), as depicted in Figs. S5, S6, and S7 (supplementary materials). When ethanol-ulcerated rats were made to undergo oral pre-treatment with beetroot extract and/or omeprazole, there was a significant decline in the increases of TNF-α and IL-10, and their normal levels were restored. Pre-treatment with

Table 6 Influence of beetroot extract and/or omeprazole on the serum levels of different parameters in ethanol ulcerated rats

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
MDA	0.42 ± 0.1	$1.38 \pm 0.19^{a***}$	$0.67 \pm 0.11^{b***}$	$0.48 \pm 0.03^{b***}$	$0.34 \pm .02^{b***}$
NO	23.7 ± 1.22	$72.4 \pm 8.69^{a***}$	$27.2 \pm .86^{b***}$	$21.4 \pm 2.15^{b***}$	$19.68 \pm .49^{b***}$
GSH	18.8 ± .75	$3.3 \pm .38^{a***}$	$11.98 \pm .93^{a*b***}$	$16.76 \pm 1.36^{b***c*}$	$17.74 \pm 1.53^{b***c*}$
MPO	3.66 ± .48	$12.5 \pm .59^{a***}$	$6.5 \pm .58^{a*b***}$	$4.04 \pm .53^{b***c*}$	$3.78 \pm .41^{b***c*}$
TNF-α	12.4 ± .33	$48.2 \pm 4.20^{a***}$	$16.7 \pm 1.57^{b***}$	$14.92 \pm .35^{b***}$	$12.1 \pm .46^{b***}$
IL-6	4.74 ± .23	$17.94 \pm 0.52^{a***}$	$7.26 \pm 1.57^{a*b***}$	$4.4 \pm .34^{b***c*}$	$5.88 \pm .57^{b***c*}$
IL-10	4.98 ± .45	$10.57 \pm .63^{a***}$	$4.58 \pm .29^{b***}$	$4.20 \pm .4^{b***}$	$5.14 \pm 0.36^{b***}$
PGE2	188.0 ± 5.01	$76.0 \pm 6.9^{a***}$	$157.20 \pm 4.43^{a*b***}$	$186.8 \pm 7.87^{b***c*}$	$187.00 \pm 6.63^{b***c*}$

MDA Malondialdehyde, *NO* Nitric Oxide, *GSH* Glutathione (reduced form), *TNF-α* Tumor necrosis factor-α, *IL-6* Interleukin- 6, *IL-10* Interleukin- 10, *MPO* Myeloperoxidase, *PGE2* prostaglandin E2

Table 7 Percentage change of serum diferenent parameters in different groups with respect to ethanol group

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
MDA	−68.42%	-	−51.44%	−65.21%	−74.4%
NO	−67.26%	-	−62.4%	−70.44%	−72.82%
GSH	+468.27%	-	+263.03%	+407.87%	+437.57%
TNF-α	−74.27%	-	−65.35%	−69.04%	−74.90%
IL-6	−73.57%	-	−59.86%	−75.50%	-−67.22%
IL-10	−52.88%	-	−56.66%	−60.30%	−51.40%
MPO	−70.72%	-	−48%	−67.68%	−69.76%
PGE2	+147.36%	-	+106.84%	+145.78 v	+146.1%

MDA Malondialdehyde, *NO* Nitric Oxide, *GSH* Glutathione (reduced form), *TNF-α* Tumor necrosis factor-α, *IL-6* Interleukin- 6, *IL-10* Interleukin- 10, *MPO* Myeloperoxidase, *PGE2* prostaglandin E2. a: significance versus normal group; b: significance versus ethanol group; c: significance versus ethanol + beetroot. ***$P < 0.0001$, *$P < 0.050$

beetroot extract alone significantly reduced serum IL-6 levels compared to ethanol-ulcerated rats ($P < 0.0001$). However, the elevation in serum IL-6 levels was effectively normalized when the ulcerated rats were pre-treated with the reference drug (omeprazole) or a combination of both agents (beetroot extract and omeprazole). It was also noted that serum levels of PGE2 reduced substantially following ethanol ingestion in ethanol-ulcerated rats in comparison to normal rats ($P < 0.0001$), as depicted in Fig. S8 (supplementary material). When ulcerated rats were made to undergo oral pretreatment with beetroot extract alone, there was a significant increase in serum PGE2 levels compared to ethanol-ulcerated rats ($P < 0.0001$). On the contrary, PGE2 levels were restored to normal following pre-treatment of ulcerated rats with omeprazole alone or in combination with beetroot extract.

3.3 Histopathological examination

Figure 6 demonstrates the protective effects of beetroot extract and/or omeprazole on gastric ulcer histopathological modifications caused by absolute ethanol administration. The results of histological examination of the gastric tissue section from a normal control rat (Fig. 6A), which was smeared with hematoxylin and eosin (H&E), showed the presence of gastric mucosa and submucosa. The mucosa exhibited normal invaginator surface columnar epithelia known as foveolar cells, along with normal gastric glands. A thin layer of smooth muscle (muscularis mucosa) was seen in addition to the gastric mucosa, displaying normal characteristics. This was followed by a submucosa that had a normal appearance. Various changes and significant injury were observed in the gastric tissue section of the ethanol-ulcerated rat. These alterations were characterized by erosion of the mucosal surface epithelia, infiltration of inflammatory immune cells, hemorrhagic necrosis of the gastric mucosa, and edema in the submucosa (Fig. 6B). An improvement in gastric tissue architecture was observed in the gastric tissue section from the ethanol-ulcerated rat that had been orally pretreated with beetroot extract. This was characterized by the absence of mucosal hemorrhage and a reduction in the infiltration of inflammatory immune cells. However, there was still evidence of erosion of the mucosal surface epithelia (Fig. 6C). Significant improvement in gastric tissue architecture was exhibited by the gastric tissue section in ethanol-ulcerated rats that were orally pretreated with omeprazole. This was indicated by a reduction in necrotic cells and damage to gastric mucosa (Fig. 6D). Almost normal gastric tissue architecture was observed in the gastric tissue section in ethanol-ulcerated rats that were orally pretreated with a combination of beetroot extract and omeprazole (Fig. 6E). These microscopic observations agreed with the quantitative analysis of gastric histopathological images using the scoring system (Table 1). In relation to ulcerative rat group, marked reduction in histopathological changes in rats treated with beetroot HP-β-CD modified aqueous extract and/or omeprazole ($P < 0.0001$/each) was recorded. With respect to normal rats, marked variation in histopathological scoring in ulcerative rats treated with beetroot extract only ($P < 0.0001$) or omeprazole only ($P = 0.012$) was observed. However, the treatment of ulcerative rats with the combination of the two agents (beetroot HP-β-CD modified aqueous extract and omeprazole) normalized the histopathological scoring of gastric tissue ($P = 0.61$ vs. normal group) as shown in (Fig. 6F).

4 Discussion

4.1 Enhancing the extraction of bioactive compounds from beetroot

Improving the extraction efficiency and stability of beetroot compounds has gained the attention of researchers due to their high nutritional and health benefits. In previous studies, betalains were extracted by using traditional solid–liquid

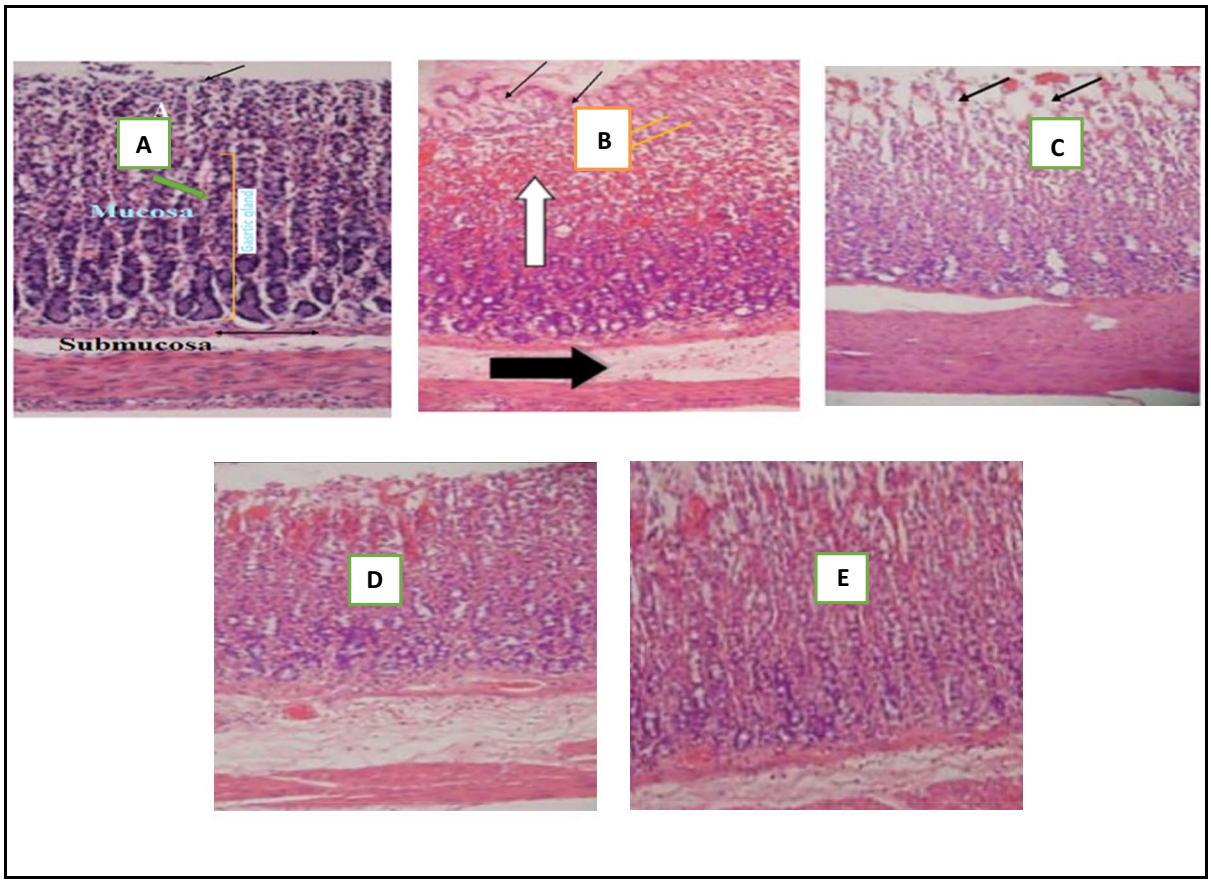
extraction technique that involved the use of organic solvents and high temperature (Albahari et al. 2018). However, this method suffers from several limitations, including extended processing duration, instability of betalains, and the utilization of hazardous organic solvents (Albahari et al. 2018). Recognizing these challenges, cost-effective and efficient methods for environmentally friendly extraction of bioactive compounds are being developed.

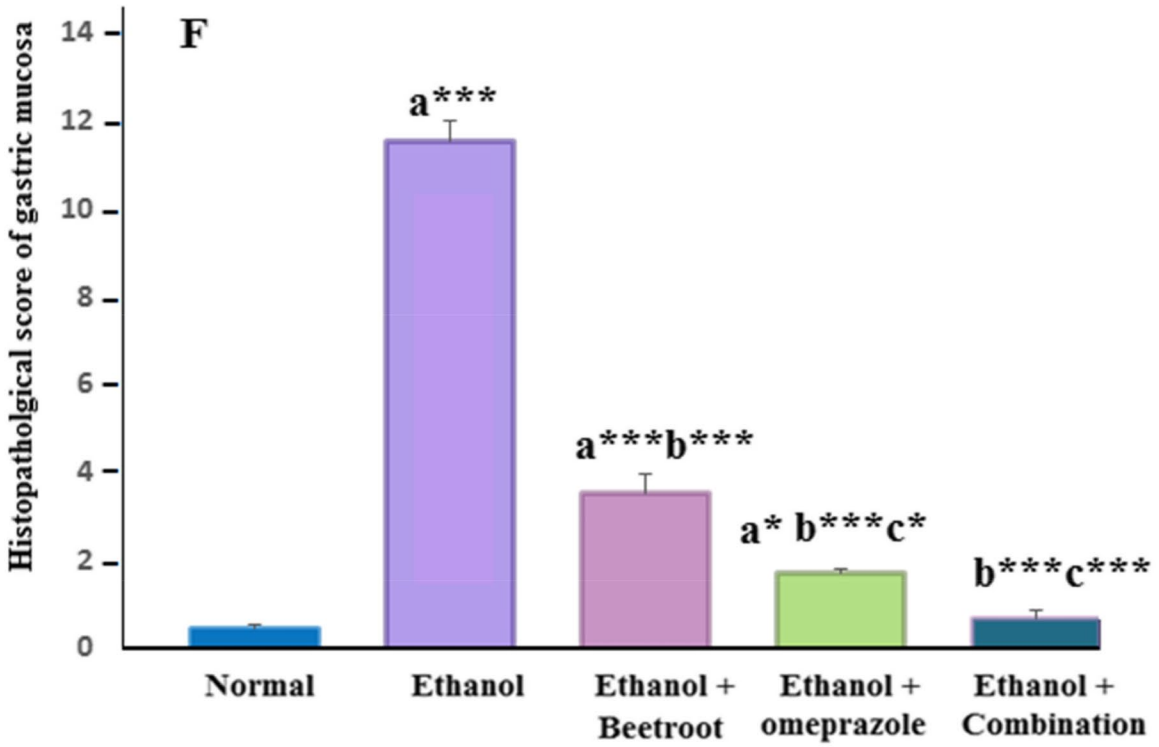
Cyclodextrins (CDs) and their derivatives are good alternatives to organic solvents since they improve solubility and stability for bioactive compounds in aqueous extracts. There is a widespread interaction of CDs with various bioactive components (hydrophobic as well as hydrophilic). For example, betanin could adhere to the exterior of β-CD through hydrogen bonding or interactions among the carboxylate groups of betanin and the OH groups of HP-β-CD (Tutunchi et al. 2019). The high polarity and hydrophilicity of the betanin compound make it interacted with hydrophilic environments, which possibly explained the lesser concentration of betanin in samples using ethanol alone (Roriz et al. 2017). The findings also demonstrated that the highest total betanin concentration was exhibited by 5% (w/v) HP-β-CD. Albahari et al. (2018) presented similar findings previously, showing that CDs increased the extractability of polyphenols. It was also found that extraction by CD aqueous solutions increased the recovery of epicatechin and catechin from red grape pomace.

To determine the extraction efficiency of phenolic component in the presence of CDs, the TPC values of beetroot extract samples were also examined. The results indicated that recovery of phenolic compounds improved when the CDs content was increased. This improvement can be attributed to the potential of CDs to form inclusion complexes with phenolic compounds, thereby increasing their solubility and stability during the extraction process and facilitating their recovery (Kushwaha et al. 2018; Roriz et al. 2017). It was known that CDs are molecules with a cone form having hydrophilic on the cavity's exterior and hydrophobic within. Therefore, CDs can enclose a variety of hydrophobic guest molecules in its non-polar cavity through non-covalent bonds. Several molecular dynamics simulations were used to examine the structural inclusion complex formation between phenolic compounds and CDs (Kumar et al. 2023, 2024a, 2024b; Bhardwaj and Purohit 2023; Kumar and Purohit 2024). In another investigation, aqueous β-CD was used for the extraction of red beet extract, and it revealed the highest content of betanin, total phenolic compounds, and DPPH inhibition activity (Tutunchi et al. 2019). The complexation of betanin with β-CD significantly enhanced its stability and antiradical activity. This has been confirmed by several spectroscopic techniques such as FTIR and DSC which indicated that certain groups of betalains such as -OH, -COOH and -NH, are capable of forming hydrogen bonds with the -OH groups of CDs (Tutunchi et al. 2019). Furthermore, the conventional (magnetic stirring) and developing (ultrasound) extraction techniques could be emerged with CDs in order to enhance the extraction efficiency (complexation efficiency). The advantage of ultrasound over magnetic stirring is mass transfer enhancement of bioactive compounds (Maran and Priya 2016). However, magnetic stirring is less cost, less consumed energy and more available in laboratories. Both of them could be used to enhance the diffusion of solvent inside the plant cell wall, and then the cell's contents seep out.

In the current study, for the first time, aqueous HP-β-CD, which is a chemically modified derivative of β-CD, offered a markedly enhancement in the extraction efficiency with a simple and cost effectiveness stirring emerging technology. The reasons could be attributed to the formation of hydrogen bonding via certain groups such as -OH, -COOH and -NH with the -OH groups of HP-β-CD (Tutunchi et al. 2019). As well, interactions among the carboxylate groups of betanin and the OH groups of HP-β-CD could also be suggested. In essence, the pigment that is attached to the cell membrane is released into the solvent with the assistance of an external mechanical force (Deng et al. 2015). HP-β-CD had the highest binding affinity for phenolic compounds with the most negative complexation energy (Kumar et al. 2024a). The computational analysis aligns with experimental findings validating HP-β-CD as most effective cavitand molecule for improving the solubility of phenolic compounds (Kumar et al. 2024b). Therefore, the main factors for enhancement of betanin and phenolic extraction are: the creation of betanin-HP-β-CD complexation, the development of betanin solubility by HP-β-CD-based emerged technology and the improvement of betanin stability in an aqueous environment.

Previous studies have highlighted the significant potential of CD aqueous solution in recovering phenolic compounds from various sources such as whole pomegranate fruit (Diamanti et al. 2017), Sideritis scardica (Diamanti et al. 2017), and peach pomace (El Darra et al. 2018). Betalains are essentially removed from beetroot by macerating or crushing the root into a solid–liquid state (Nirmal et al. 2021). With the help of an external mechanical force, the pigment bound to the cell membrane gets released into the solvent (Deng et al. 2015). The conventional method usually used for beetroot pigment extraction involves solvent extraction with stirring. Ethanol, methanol, or the addition of citric or ascorbic acid is commonly employed to help extract pigment; however, water can also be used to extract beetroot pigments (Nirmal et al. 2021). Three conventional extraction techniques (infusion, decoction, and maceration) can be used for recovering betalains pigment from beetroot peel powder. It is determined that the highest betalains content was present in the infusion extract followed by stirring. Sturzoiu et al. (2011) also demonstrated that water containing 0.2% citric acid and 0.1% ascorbic acid, or 20% ethanol and 0.5%


A
Mucosa
Submucosa
B
C
D
E


F
Histopathological score of gastric mucosa
14
12
10
8
6
4
2
0
a***
a***b***
a* b***c*
b***c***
Normal
Ethanol
Ethanol + Beetroot
Ethanol + omeprazole
Ethanol + Combination

◂**Fig. 6** Light microscopic feature of gastric mucosa in different experimental gastric ulcer rat groups: Illustrative photomicrographs of gastric wall sections taken one hour after ethanol administration (H&E, X 400). **A** Gastric mucosa from normal rat showing normal surface columnar epithelia (black arrow), normal gastric gland (right bracket) with normal parietal cells (green arrow). The muscularis mucosa (double black arrow) appears normal, followed by submucosa with normal feature. **B** Gastric mucosa from ethanol ulcerated rat showing erosion of surface epithelia (black thin arrows), hemorrhagic necrosis of the gastric mucosa (white thick arrow), infiltration of inflammatory immune cells (orang arrows) and edema in submucosa (black thick arrow). **C** Gastric mucosa from ulcerated rat pretreated with beetroot extract showing slight improvement in histological appearance of gastric architecture in ethanol. The surface epithelia are still appeared with erosion (arrows) with greatly decrease in infiltration of inflammatory immune cells. **D** Gastric mucosa from ulcerated rat pretreated with omeprazole showing great improvement in gastric tissue architecture as shown by decrease in necrotic cells and gastric mucosa damage. **E** Gastric mucosa from ulcerated rat pretreated with the combination of beetroot extract and omeprazole showing more or less normal gastric tissue architecture. **F** Quantitative statistical analysis of histopathological HE staining. Values are expressed as mean ± SE of 5 rats. a: significance versus normal group; b: significance versus ethanol group; c: significance versus ethanol + beetroot, ***$P < 0.0001$, *$P < 0.01$

ascorbic acid, extracted higher betanin from dried beetroot under specific conditions, such as a sample/solvent ratio of 1:5 at 25 °C for three minutes. Extensive applications in the domains of food- and pharmaceutical-controlled release, separation, and adsorption have been published elsewhere (Hu et al. 2007; Szente and Szejtli 2004; Diamanti et al. 2017; Hernández-Sánchez et al. 2017; Albahari et al. 2018).

4.2 The prophylactic impacts of beetroot cyclodextrin modified aqueous extract and omeprazole on ethanol ulcerated rats

Peptic ulcers occur in the stomach's epithelial lining when pepsin levels and acid secretion increase, which decreases the mucus layer. Gastric ulcers arise when there is an imbalance between the protective and invasive factors of the stomach mucosa. These factors collaborate to compromise the mucosal protective barrier, leading to ulcer formation. Proton pump inhibitors (PPIs), antihistaminic drugs, and antacids continue to be the primary clinical interventions available. However, these medications exhibit limited efficacy and are associated with several adverse effects, like altered absorption of micronutrients, liver disorders, dementia, and gastric neoplasia (Fossmark et al. 2019). Consequently, exploring safe, effective, and non-toxic alternative therapies to avoid damage to gastric mucosa is imperative. Researchers have recognized the significance of evaluating the antiulcer properties of plant-derived compounds as possible novel therapeutics (Liu et al. 2012).

Rats were administered oral doses of absolute ethanol (1 ml/kg body weight) in this study to induce mucosal damage in the stomach. Three hours following ethanol administration, the rats were euthanized. According to the results, ethanol consumption may induce oxidative stress in the rats, as shown by significantly high levels of blood MDA and NO, in comparison to control rats. Furthermore, rats exposed to ethanol exhibited increased serum MPO activity and decreased levels of the non-enzymatic antioxidant GSH in comparison to normal rats. These findings are consistent with previous research demonstrating that ethanol consumption in experimental mice leads to increased production of MDA and NO, along with increased MPO activity in serum and stomach mucosa and a decrease in GSH levels (Gilani et al. 2022; Li et al. 2021; Shin et al. 2020). The rise in serum MDA levels can be attributed to the production of free radicals by oxygen reacting with cell membrane lipids to generate MDA, resulting in lipid peroxidation and subsequent cell damage. Some researchers suggested that the influx of inflammatory immune cells to the site of gastric injury essentially leads to the overproduction of reactive oxygen species (ROS) and inflammatory mediators in the gastric mucosa, and this causes oxidative damage (Kadasah et al. 2021). Superoxide radical anions ($O_2\bullet -$) are produced by inflammatory immune cells, particularly neutrophils, that react with lipids, particularly polyunsaturated fatty acids, resulting in lipid peroxidation (Kwiecień et al. 2002). While cells may repair mild disruptions, cell death may be caused by severe oxidative stress. Low levels of oxidative stress can trigger apoptosis, while more severe stress, cytotoxicity, and carcinogenicity can lead to tissue necrosis (Esatbeyoglu et al. 2015).

The ethanol's ability to induce nitric oxide synthase (NOS) may explain the increase in serum NO levels following ethanol administration, as observed in the results of this study, which leads to increased oxidative and nitrosative stress within the stomach. NOS enzymes, including constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS), are involved in NO production. It is possible for cNOS to steadily release low NO levels within the physiological standard level, whereas iNOS can generate higher levels of NO when activated by inflammatory cytokines, contributing to vascular microcirculation disturbances and gastric mucosal damage (Wallace et al. 2017). The increased NO production by iNOS may play a part in the pathogenesis of various gastroduodenal disorders, such as peptic ulcers. Studies have indicated that ulcer development may be caused by the production of cytotoxic peroxide free radicals by NO resulting from iNOS activation (Cho 2001). When superoxide radical ($O2\bullet -$) combines with excess NO, it generates powerful secondary oxidizing species like peroxynitrite ($ONOO-$) that are capable of oxidizing cellular structure and inflicting direct damage to stomach cells. $ONOO-$ exhibits greater reactivity than NO or $O2\bullet -$ in biological systems, leading to a range of chemical reactions

like lipid peroxidation induction, nitration of protein tyrosine residues, oxidation of low molecular weight sulfhydryl (e.g. glutathione and cysteine), and degradation of vital macromolecules such as DNA, enzymes, membrane phospholipids, polysaccharides, and structural proteins.

Serum MPO activity in rats exposed to ethanol ingestion increases, which may suggest the infiltration of inflammatory immune cells. MPO, a peroxidase enzyme, serves as a key marker of neutrophil infiltration in injuries leading to ulcer formation. There is high expression of this enzyme in neutrophils (Khan et al. 2014), which is released into the extracellular space as a result of oxidative stress and inflammatory factors. Chlorinative stress is caused following its release, as MPO catalyzes the reaction between chloride (Cl^-) and Hydrogen peroxide (H_2O_2) to produce a potent oxidant referred to as hypochlorous acid (HOCl). Being a vital cytotoxic agent, HOCl interacts with proteins, lipids, and DNA within cells, thereby inducing MPO-regulated oxidative tissue damage (Pattison et al. 2003). In addition, myeloperoxidase-derived HOCl indirectly inactivates various cytoplasmic enzymes, including glyceraldehyde-3-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, and creatine kinase, because of its high chemical reactivity,

The elevated levels of ROS formed within the gastric tissue may lead to a significant decrease in the serum levels of the non-enzymatic antioxidant glutathione (GSH) in rats exposed to ethanol ingestion, which plays a crucial part in the depletion of non-enzymatic antioxidants (Sanpinit et al. 2021). GSH, which is a tripeptide containing a free sulfhydryl group, plays a vital role in combating cellular damage caused by oxidative stress and achieving normal intracellular redox balance (Dey and Cederbaum 2006). Its synthesis involves the reduction of oxidized glutathione (GSSG) by glutathione reductase, utilizing NADPH generated from glucose 6-phosphate dehydrogenase within the pentose phosphate pathway. It has been suggested in a few studies that ethanol may potentially inhibit glucose 6-phosphate dehydrogenase in the gastric mucosa, which decreases NADPH levels, thus impairing the production of GSH. The decrease in GSH makes the cell more likely to experience oxidative damage as crucial GSH-related processes are compromised (Masella et al. 2005). Enzymes such as glutathione peroxidase (GPx) and glutathione transferase, which play a role in detoxification and protection against oxidative stress, rely on GSH as a cofactor. GPx, for example, catalyzes the detoxification of hydrogen peroxide and lipid peroxides by directly scavenging hydroxyl radicals and singlet oxygen. In addition, GSH plays a role in replenishing essential non-enzymatic antioxidant vitamins such as vitamins C and E (Valko et al. 2007).

Compared to rats exposed to ethanol alone, the increase in serum levels of oxidative stress markers (MDA, MPO, and NO) was effectively mitigated by prophylactic administration of beetroot HP-β-CD modified extract (0.5 mg/kg body weight/day) and/or omeprazole (20 mg/kg body weight/day) for ten consecutive days before ethanol ingestion in rats, which also restored the antioxidant index (GSH). This shows that both beetroot HP-β-CD modified extract and omeprazole have potential antioxidant effects. When ethanol-ingested rats were pre-treated with omeprazole alone or in combination with beetroot extract, alterations in all markers (MDA, NO, MPO, and GSH) were regulated to levels comparable to those of normal rats. Beetroot HP-β-CD modified extract may exhibit protective action against oxidative stress in ethanol-induced ulcers possibly because of the antioxidant properties of its bioactive components. Beetroot includes phenolic compounds, ascorbic acid, carotenoids, flavonoids, vitamin E, and betanin (also referred to as betalains) which possess potent antioxidant capabilities (Vulić et al. 2014). The phenolic hydroxy groups present in betanin contribute to its robust free radical scavenging ability and high antioxidant activity, which have been demonstrated in cultured cells (Costa et al. 2017). Betanin can scavenge hydroxyl and superoxide radicals and protect against DNA damage caused by hydrogen peroxide. It has been shown that pretreatment with beetroot juice reduced lipid peroxidation and restored the antioxidant defense system compromised by oxidative stress in experimental models. Betanin in beetroot also suppresses the oxidative metabolism of neutrophils in humans, and in liver injury, it regulates MDA and GSH levels (Han et al. 2014). Betanin in beetroot may also protect cells against nitrative stress by inhibiting peroxy nitrate nitration regulated by tyrosine in vitro. Furthermore, some studies have found that lipid peroxidation and MPO of ulcerated gastric tissue and nephrotoxicity in experimental animal models can be improved when treated with betanin and beetroot extract (Karampour et al. 2019). Betanin was also found to strengthen the endogenous antioxidant network by increasing the activity of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that transcribes various endogenous antioxidant enzymes. Studies have also shown the anti-oxidative stress of omeprazole in experimental gastric ulcer rat models (Raish et al. 2021). Pharmacologically, the presence of plant extract in CD modified form may enhance its powerful functionalization capacity via increasing its stability, bioavailability, delivery and cellular uptake. CDs and their derivatives have been shown to enhance drug permeability across different biological barriers (Chen et al. 2023). A previous study revealed that the complexation of beetroot extract with β-CD significantly enhanced the antiradical activity of betanin and phenolic (Tutunchi et al. 2019). Similarly β-CD-aided polyphenol extraction from *Mentha piperita* provided extracts with enriched polyphenolic composition and improved antioxidant characteristics (Athanasiadis et al. 2022). Gastric oxidative stress can also be inhibited by omeprazole, which suppressed lipid peroxidation (MDA)

and MPO activity and restored depleted GSH levels. The potential protective mechanism exhibited by omeprazole against oxidative stress involved scavenging hydroxyl radical-mediated membrane lipid peroxidation, GSH depletion, and upregulation of Nrf2 and heme oxygenase-1 (HO-1), which played crucial roles in the expression of ROS detoxifying agents (Raish et al. 2021).

Another critical factor in the pathogenesis of gastric mucosal damage caused by ethanol is inflammation (Salga et al. 2012). It was revealed in this study that ethanol-induced destruction of gastric mucosa caused the serum levels of inflammatory cytokines, including TNF-α and IL-6, to increase significantly, along with an increase in the anti-inflammatory cytokine IL-10, in rats with ethanol-induced gastric ulcers in comparison to a negative control group. Previous research has also indicated that alcohol consumption disrupts the gastric mucosal barrier, causing an increase in inflammation through the formation of inflammatory cytokines such as IL-6 and TNF-α. Infiltration of neutrophils can be triggered by the production of these cytokines, leading to the generation of further inflammatory cytokines and subsequent gastric tissue damage (Li et al. 2014). In addition, oxidative stress in the stomach tissue may be caused by oxidative pathways and the generation of ROS by inflammatory cytokines, which may lead to gastric ulcer development (Albaayit et al. 2016). TNF-α, a major inflammatory cytokine, is released by macrophages, which plays a vital role in causing gastric mucosal damage. TNF-α intensifies the inflammatory response, thus reducing microcirculation around gastric ulcers and delaying ulcer healing (Abdelwahab 2013). In addition, ethanol induces gastric epithelial cell apoptosis, which increases the value of mucosal TNF-α. In contrast, neutrophils, lymphocytes, and monocytes/macrophages are activated by the increase in IL-6 levels at the site of inflammation, leading to the release of ROS, toxic metabolites, and lysosomal enzymes, all of which contribute to local tissue damage in peptic ulcer disease. Thus, the downregulation of inflammatory cytokines may lead to the mitigation of inflammation and the formation of ROS in the gastric mucosa, ultimately reducing gastric tissue damage. Rats that were administered ethanol exhibited increased serum levels of the anti-inflammatory cytokine IL-10, potentially serving as a compensatory process to mitigate tissue damage. IL-10, known for its role in conserving mucosal homeostasis, has been associated with higher production in patients with gastritis, ulcerative colitis, and inflammatory bowel disease. This suggests that the apparent increase in IL-10 levels in active diseases may be caused by immunological compensatory mechanisms aimed at decreasing local inflammation.

Pre-administration of beetroot HP-β-CD modified extract and/or omeprazole to rats exposed to ethanol significantly decreased the increases in TNF-α, IL-6, and IL-10 in contrast to rats not treated with ethanol. Administration of the reference drug (omeprazole) and the combined treatment of beetroot extract and omeprazole effectively normalized the elevated serum concentrations of these inflammatory cytokines, suggesting that both beetroot extract and omeprazole had a robust synergistic anti-inflammatory impact. Pietrzkowski et al. (2010) presented similar findings for the two agents, demonstrating that therapeutic oral intake of capsules containing betanin suppressed the production of TNF-α and IL-6 and thus reduced pain and inflammation in osteoarthritic patients. The anti-inflammatory effects of beetroot extract may be attributed to its ability to directly inhibit the flow of nuclear factor-kappa B (NF-κB), which plays a vital role in the transcription and activation of various target genes involved in the regulation and amplification of inflammatory cytokines. In addition, omeprazole reduces TNF-α and IL-6 levels to suppress gastric inflammation (Raish et al. 2021). The anti-inflammatory effects of beetroot HP-β-CD modified aqueous extract and omeprazole may be attributed to their ability to directly inhibit the flow of nuclear factor-kappa B (NF-κB), which plays a vital role in the transcription and activation of various target genes involved in the regulation and amplification of inflammatory cytokines. The beneficial anti-inflammatory impact of beetroot extract may attribute to its bioactive constituents like betanin. Besides, the presence of plant extract in HP-β-CD modified form may enhance its anti-inflammatory properties. This suggestion is supported by previous investigation found that cyclodextrin complex of curcumin is more potent than free curcumin in suppressing TNF-induced NF-κB activation due to increased bioavailability delivery and cellular uptake.

Prostaglandins are significant factors that protect against gastric mucosal injury caused by different risk factors, in particular prostaglandin E2 (PGE2). The main mechanisms by which PGE2 safeguards the stomach mucosa are by stimulating the secretion of gastric mucus and bicarbonate, enhancing mucosal blood flow, preventing leukocyte aggregation, decreasing acid secretion, stabilizing mast cell membranes, and supporting tissue healing processes. Thus, PGE2 may play a pivotal role in inhibiting and healing ulcers caused by harmful compounds (Hwang and Jeong 2015). Consistent with previous research, it was demonstrated in the current study that there was a significant decrease in serum PGE2 levels in rats exposed to ethanol compared to normal rats, which indicates that ethanol inactivates prostaglandin synthase, resulting in reduced prostaglandin biosynthesis. This decrease in PGE2 levels is a major cause of gastric ulceration and can exacerbate already existing gastric ulcers. It has been found that administration of exogenous prostaglandins before ethanol exposure can prevent hemorrhagic gastric mucosal damage.

Oral administration of beetroot HP-β-CD modified aqueous extract and/or omeprazole to ethanol ingested rats

significantly increased serum PGE2 levels compared to untreated rats. When ethanol ingestion rates were pre-treated with omeprazole alone or in combination with beetroot extract, PGE2 levels increased significantly to reach normal levels, which showed their beneficial antiulcer effects. This suggests that both beetroot extract and omeprazole may have a gastro-protective effect against ethanol-induced gastric ulcers by stimulating PGE2 production, a major factor that protects against gastric mucosal damage. Beetroot extract may have a beneficial impact on PGE2 levels because of its phenolic compounds. These findings are aligned with earlier studies that demonstrate that phenolic compounds mitigate hemorrhagic gastric mucosal damage by promoting PGE2 synthesis, thus preventing the buildup of inflammatory cells and increasing antioxidant enzyme activity in gastric ulcers caused by ethanol in rats. Previous research has also shown that pretreatment with omeprazole increases PGE2 levels to a significant extent in ethanol-induced ulcerated rats (Raish et al. 2021).

4.3 Histopathological examination

In this study, histopathological examination of gastric tissue confirmed gastric mucosal injury caused by orally administering absolute ethanol. The gastric tissue section from normal control rats exhibited standard architecture with normal gastric mucosa and submucosa, characterized by normal surface epithelial cells and gastric glands. Both the muscularis mucosa and submucosa appeared normal as well. However, gastric tissue sections from rats administered ethanol displayed serious cytomorphological damage in the gastric mucosa in contrast to normal rats. These histopathological changes included erosion of the mucosal surface epithelia, infiltration of inflammatory immune cells, hemorrhagic necrosis of the gastric mucosa, and submucosal edema, all of which are indicative of ulceration of the gastric mucosa due to absolute ethanol ingestion. These findings are consistent with previous investigations (Raish et al. 2021) and suggest that oxidative stress, inflammation, and the depletion of cytoprotective factors such as PGE2 contribute to ethanol-induced gastric ulcers in rats. When ethanol-ulcerated rats were pre-treated with beetroot extract, there were modest improvements in gastric tissue architecture, characterized by a lack of mucosal hemorrhage and a reduction in inflammatory immune cell infiltration. However, erosion of the mucosal surface epithelia was still evident. Gastric tissue architecture exhibited significant improvement following pre-treatment with omeprazole as indicated by a decline in necrotic cells and gastric mucosa damage. The combination of beetroot HP-β-CD modified aqueous extract and omeprazole exhibited the most effective protection against ethanol-induced gastric ulceration in rats, with gastric tissue architecture appearing mostly normal. This result is further supported by the quantitative analysis of histopathological scoring of gastric tissue which confirmed that a marked improvement in ulcerative rats treated with beetroot extract and/or omeprazole with relation to ulcerative untreated ones. Relative to normal rats, a significant variation in histopathological score in ulcerative rats treated with beetroot extract or omeprazole each alone; however, treatment with the combination of the two agents has kept the score of gastric histological structure close to normal. The combination of beetroot extract and omeprazole exhibited the most effective protection against ethanol-induced gastric ulceration in rats, with gastric tissue architecture appearing mostly normal. The protective effects of beetroot extract and omeprazole combination may be attributed to their antioxidant and anti-inflammatory properties, as well as their ability to regulate the level of PGE2, a cytoprotective agent against gastric ulceration. Previous studies have investigated the cytoprotective effects of both agents against histopathological tissue damage in experimental models. For instance, Dhananjayan et al. (2017) found that significant protection against diabetes-induced histopathological lesions in the liver and pancreas was offered when diabetic rats were treated with betanin, the active compound in beetroot. Similarly, it has been confirmed that omeprazole has a protective effect on gastric mucosa histopathological lesions (Sanpinit et al. 2021).

5 Conclusion

To sum up, the study findings showed, for the first time, that modified beetroot aqueous extract exhibited powerful antiulcerogenic effects in rat models. The use of an aqueous solution containing HP-β-CD at a concentration of 50 g/L to extract beetroot was a novel approach that was found to offer significantly better extraction efficacy. The beetroot aqueous extract obtained was combined with omeprazole, which exhibited anti-oxidative stress and anti-inflammatory properties that function as effective preventive measures against ethanol-induced stomach ulcers. In addition, this combined therapy demonstrated the potential to protect stomach tissue from damage. From these investigations, it can be proposed that the prophylactic mechanisms of beetroot HP-β-CD modified aqueous extract and omeprazole against ethanol induced gastric ulcer were via reducing the oxidative stress markers (MDA, NO and MPO) and inflammatory markers (TNF-α and IL-6), and increasing the antioxidant marker (GSH) and up-modulating the level of PGE2 as a cyto-protective factor against gastric ulceration. The combination of the plant extract with omeprazole exhibited the most effective protection by modulating the deviation in MPO, GSH, IL-6 and histopathological scoring and image of gastric tissue. The use of this pharmaceutical combination could

decrease the cost incurred in treating stomach ulcers and promote preventive care for the broader population. Till now no previous clinical studies on the HP-β-CD-modified beetroot aqueous extract have been carried out, and also no study on its dosage optimization and bioavailability testing has been carried out. In our expectations, it could be existence of broader therapeutic potential of the HP-β-CD-modified beetroot aqueous extract beyond gastric ulcers. This could include the improvement in the protection against age-related illnesses, the increase of blood flow, and the increase exercise stamina and running performance. It could also use in management of antiradical, antimicrobial and cytotoxic activities. Future studies for clinical trials, dosage optimization, and bioavailability would strengthen the translational impact of the current findings.

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Rasha M.A. Mousa: The corresponding author designed experiments, involved in the experiments, wrote the manuscript and revised the manuscript

Hanaa A.S. Alghamdi: Co-author performed the experiments and assisted with writing the text.

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Data availability All data are available in the manuscript and in the supplementary material.

Declarations

Ethics declaration For using experimental animals in the current study, The Research Ethics Committee (REC) of the Faculty of Medicine at King Abdul Aziz University-Saudi Arabia approved the study protocol (Reference No: p453-2020).

Competing interests The authors declare that they have no competing interests.

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