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Gastroprotective and Antiulcer Activity of *Combretum indicum* (Woody Stem) and *Thevetia peruviana* (Flowers): A Comparative Pharmacological, Phytochemical and Mechanistic Study in Wistar Albino Rats

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ABSTRACT

Background: Peptic ulcer disease (PUD) is a global gastrointestinal disorder affecting about 5–10% of the population. It occurs due to an imbalance between aggressive factors such as hydrochloric acid, pepsin, Helicobacter pylori, NSAIDs, and reactive oxygen species, and protective factors such as the mucus-bicarbonate barrier and prostaglandins. Limitations of synthetic antiulcer drugs, including rebound acid hypersecretion, drug resistance, vitamin deficiencies, and infection risk, have increased the need for safer plant-based gastroprotective agents. This study comparatively evaluated the gastroprotective, antiulcer, and antioxidant effects of aqueous and ethanolic extracts of *Combretum indicum* woody stem and *Thevetia peruviana* flowers in Wistar albino rats.

Methodology: Aqueous and ethanolic extracts were prepared through maceration and Soxhlet extraction. Phytochemical screening, total phenolic content, total flavonoid content, and in vitro antioxidant assays were performed. Acute oral toxicity was assessed according to OECD Guideline 423. Antiulcer activity was evaluated in pylorus ligation, ethanol-induced, and cold restraint stress-induced ulcer models. Gastric parameters, ulcer index, percentage protection, mucus content, glycoproteins, antioxidant markers, and histopathology were analysed.

Results: The ethanolic extract of *Combretum indicum* showed the highest phenolic and flavonoid content and strongest antioxidant activity. All extracts were practically non-toxic, with LD_{50} greater than 2000 mg/kg. *Combretum indicum* ethanolic extract at 400 mg/kg produced the highest ulcer protection in all models and showed results comparable to sucralfate. It restored gastric mucus, improved glycoproteins, reduced MDA, and enhanced SOD, CAT, and GSH activities. Histopathology showed near-normal gastric mucosal structure.

Conclusion: Both plant extracts showed multi-mechanistic gastroprotection through acid regulation, mucus protection, cytoprotection, antioxidant defence, and anti-inflammatory effects. *Combretum indicum* ethanolic extract at 400 mg/kg appears to be a promising herbal antiulcer agent.

1. INTRODUCTION

1.1 Global Burden and Epidemiology of Peptic Ulcer Disease

Peptic ulcer disease (PUD) is a persistent, recurring gastrointestinal condition, which involves mucosal ulceration of the stomach and duodenum, caused by the imbalance between the mucosal aggressive and defensive factors [1,2]. Globally, PUD occurs in about 5-10 percent of the population at one point in their life and the Global Burden of Disease 2019 Study estimates that around 8.1 million prevalent cases of PUD exist worldwide [3]. The prevalence rate has decreased to 99 per 100,000 in the year 2019 attributed to improved sanitation, universal eradication therapy of H. pylori and acid suppressive drugs; however, the absolute disease burden is high due to population increase and ageing population [4,5].

The geography of PUD has high heterogeneity in epidemiology. In developing countries in South Asia, sub-Saharan Africa, and Western Pacific, the prevalence is still high because of increased rates of Helicobacter pylori infection estimated at 7090 percent in adults in certain areas - coupled with a lack of access to diagnostics and treatment [6]. However, the situation in developed countries has changed, where NSAID-related ulcers have become the most common etiology due to a decrease in the prevalence of H. pylori [7]. The financial cost of PUD is enormous: the financial loss of healthcare represented by the hospitalization expenses, endoscopic examinations, drug therapy, and the lost productivity are a major health care spending worldwide [8].

1.1 Pathophysiology of Peptic Ulcer Disease

The development of gastric ulcers is since an imbalance between factors of mucosal aggression and cytoprotective mechanisms of the body occurs [9]. Parietal cell secreted hydrochloric acid (HCl) through the H + /K + -ATPase (proton pump), pepsin (activated by pepsinogen at pH<3), the colonization of Helicobacter pylori, non-steroidal anti-inflammatory drugs (NSAIDs), reactive oxygen species (ROS), and the products of lipid peroxidation are The cytoprotective mechanisms include mucus-bicarbonate barrier, surface phospholipids, tight junction epithelial integrity, rapid epithelial restitution, submucosal blood flow, prostaglandins (PGE 2, PGI 2) and nitric oxide, growth factors (EGF, TGF -a) and antioxidant enzyme systems (SOD, CAT, GSH) [12, 13].

Approximately 70-90 percent of duodenal ulcers and 50-70 percent of gastric ulcers are caused by Helicobacter pylori infection that interferes with mucosal defense by various virulence factors: urease-mediated ammonia generation, CagA-mediated intracellular signaling disruption and IL-8 production, VacA-mediated vac. Oxidative stress, increased generation of ROS, lipid peroxidation (elevated MDA), and depletion of antioxidant defenses (reduced SOD, CAT, GSH) has been proven to be a central pathogenic process in all major etiologies of ulcers NSAIDs, H. pylori, stress, ethanol and ischemia-reperfusion injury [16, 17].

1.3 Limitations of Current Pharmacotherapy

Existing pharmacotherapy of PUD comprises of antacids, H 2 receptor antagonists (H 2RAs), proton pump inhibitors (PPIs), prostaglandin analogues, and cytoprotective drugs (sucralfate, bismuth salts), in combination with H. pylori eradication triple/quadruple therapy where necessary [18]. Although effective, both types of drugs have serious

constraints. The most prescribed antiulcer drugs, PPIs, have been linked to such long-term risks as Clostridioides difficile infection, hypomagnesemia, vitamin B12 deficiency, chronic kidney disease, heightened risk of fractures, and rebound acid hypersecretion on withdrawal [19,20]. H2RAs show quick tachyphylaxis in 2 weeks of constant use [21]. Regimens used to eradicate H. pylori are experiencing growing cases of antibiotic resistance (clarithromycin resistance rate over 20% in most areas) and GI side effects, as well as patient non-adherence to multi-drug regimens (complex regimens) [22,23]. Importantly, none of these agents is sufficiently effective to target the oxidative stress part of the PUD pathogenesis, or to supplement the inherent mucosal defense mechanisms [24].

1.4 Phytotherapy as a Complementary Approach

The drug-based constraints of synthetic antiulcer agents, together with the multi-target pathogenesis of PUD, have revived scientific interest in the use of plant-based gastroprotective agents [25,26]. Phytoconstituents flavonoids, phenolics, tannins, saponins, terpenoids, alkaloids have mechanistic benefits: they can simultaneously act on various ulcerogenic pathways, such as the hypersecretion of gastric acid, the damage of the mucosal barrier, oxidative stress, and H. pylori colonization [27,28]. Many Ayurvedic plant species, Traditional Chinese Medicine, Unani, Siddha, and ethnobotanical species have been scientifically proven to have gastroprotective activity: Glycyrrhiza glabra, Aloe vera, Ocimum sanctum, Terminalia chebula, and Moringa oleifera, among others [29,30].

1.5 Botanical Profile and Rationale for Plant Selection

Combretum indicum (L.) DeFilipps (synonym: Quisqualis indica; family: Combretaceae; common name: Rangoon creeper) is a perennial climbing plant that is commonly found in tropical and subtropical Asia. The woody stem of it has a very rich phytochemical composition: polyphenols, condensed tannins, ellagitannins, triterpenes, quercetin, kaempferol and their glycosides, saponins [31,32]. The family members of the Combretaceae are known to be rich in tannins and have been reported to possess anti-inflammatory, antioxidant, and antimicrobial activities [33]. Although the flowers of C. indicum have been reported to have gastroprotective action, systematic pharmacological antiulcer testing of the woody stem that accumulates higher concentrations of polyphenols and tannins with plant maturation has not been previously reported [34].

Thevetia peruviana (Pers.) K. Schum. Family *Apocynaceae* (yellow oleander, family) is an evergreen shrub/small tree indigenous to tropical America but now pan-tropical in distribution. It has a unique set of bioactive secondary metabolites such as cardenolide glycosides (thevetin A, thevetin B, thevetigenin), flavonoids (luteolin, quercetin, apigenin), phenolic acids (caffeic acid, ferulic acid), triterpenoids, and volatile terpenoids [35,36]. The plant holds a significant place in the ethnomedicine in Asia, Africa and Latin America with various disorders such as GI disorders. Although a single study has cited gastroprotective properties of T. peruviana essential oil against gastric lesions induced by ethanol, no systematic comparison of the pharmacological activity of the flowers on multi-validated ulcer models in a comparative manner with a detailed analysis of biochemical endpoints [37].

1.6 Aims and Objectives of the Study

Based on the foregoing review, the present study was designed to: (i) prepare standardized aqueous and ethanolic extracts; (ii) perform qualitative phytochemical screening and quantification of TPC and TFC; (iii) evaluate HPTLC fingerprinting for extract standardization; (iv) assess in vitro antioxidant activity by DPPH, NO, and H_2O_2 radical scavenging assays; (v) determine acute oral toxicity (OECD TG 423); (vi) evaluate antiulcer activity in three validated experimental models pylorus ligation (Shay model), ethanol-induced, and cold restraint stress-induced ulcer models; (vii) assess effects on gastric secretory parameters; (viii) determine effects on gastric mucus content and glycoprotein composition; (ix) evaluate in vivo antioxidant status of gastric tissue; and (x) perform histopathological correlation with pharmacological findings.

2. MATERIAL AND METHODS

2.1 Chemical and Reagents

All reagents and chemicals were analytical grade. DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent, gallic acid, quercetin, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB/Ellman's reagent), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), NADH, bovine serum albumin (BSA), sodium nitroprusside (SNP), Griess reagent, aluminum chloride, sodium carbonate, potassium dichromate, hydrogen peroxide, and curcumin were obtained from Sigma-Aldrich (India). Sucralfate was purchased in the form of a pharmaceutical grade. All the solvents were analytical grade (Merck, India), including absolute ethanol (99.9% v/v). Carboxymethylcellulose (CMC) was of Loba Chemie, India. All the solutions were made in Milli-Q water (Millipore).

2.2 Plant Material Collection, Authentication, and Processing

2.2.1 Collection

Live plant materials of *Combretum indicum* and *Thevetia peruviana* were procured from Indian online nursery suppliers and used as the source material for the study. *Combretum indicum* was provided as a live outdoor plant by Limkrt, whereas *Thevetia peruviana* was supplied as a live yellow oleander plant by Mamta Traders (Amazon.in).

2.2.2 Processing

Plant material collected was washed in a series of steps, first with running tap water, followed by three washes of distilled water to remove surface contaminants. Shade drying in ambient temperature (25–30 °C) in a well-ventilated room with no direct sunlight was done until a constant weight was obtained. This was done using shade drying to reduce the degradation of thermolabile phytoconstituents (especially flavonoids, phenolics and volatile terpenoids) during the drying process. The dry sample was ground in a stainless-steel mechanical grinder and sifted by a 40-mesh filter to produce a uniform particle size distribution, giving the sample the minimal solvent-accessible surface area. Airtight amber glass containers were filled with powdered material stored at room temperature (20-25 °C) without light or moisture and labeled with species name, part of the plant, date of collection and date of processing.

2.3 Preparation of Extracts

2.3.1 Aqueous Extraction (Maceration)

The 100g of powdered plant material were put into a 500 mL of the distilled water in a closed conical flask and left to macerate at room temperature over 24 hours with frequent stirring after every 4 hours. The macerate was filtered using a double-layered muslin cloth and then Whatman No. 1 filter paper. To eliminate the moisture content, the filtrate was concentrated in a rotary vacuum evaporator (Buchi R-210) at 40°C, reduced pressure (100120 mbar), and then the concentrated extract was further lyophilized (freeze-dried at -40 °C, 0.1 mbar; Labconco FreeZone) to obtain a dry, free-flowing powder.

$$Percentage\ yield\ (percent\ of\ weight/weight\ of\ starting\ powder) = (weight\ of\ dry\ extract/weight\ of\ starting\ powder)\ x\ 100.$$

Dried aqueous extracts were kept in airtight amber bottles at 4^{o} C.

2.3.2 Ethanolic Extraction (Soxhlet)

A hundred grams of powdered plant material was placed in a Soxhlet thimble and thoroughly extracted with 500 mL of 95% ethanol in a Soxhlet apparatus over a period of about 18 hours (about 8-10 cycles) to guarantee the extraction of all ethanol-soluble compounds. An ethanolic extract was filtered on Whatman No. 1 filter paper and concentrated at low pressure in a rotary evaporator (500 °C, 60-80 mbar) to obtain a thick concentration. The concentrate was moved to weighed Petri dishes and dried in a hot air oven at 45 °C to a constant weight. Percentage yield was calculated as above. The stored dried ethanolic extracts were in closed amber glass vials at 4o C desiccation.

2.3.3 Preparation of Dosing Suspensions

To conduct all the pharmacological experiments, dried extracts were suspended in 1% w/v carboxymethylcellulose (CMC) sodium solution in normal saline immediately before administration to obtain homogeneous suspensions. All preparations were freshly made on each day of administration. Sucralfate (standard drug) was dissolved in 1% CMC at 100 mg/kg body weight and the volume of administration standardized at 10 mL/kg body weight given by oral gavage.

2.4 Physiochemical Parameters

All dried extracts were determined in triplicate on quality control parameters: (i) Loss on drying (LOD) 105 °C to constant weight; (ii) total ash content by incineration at 600 °C; (iii) acid-insoluble ash (HCl-insoluble residue); (iv) water-soluble ash (total ash minus water-soluble ash); (Mean ± SD of all the results.

2.5 Qualitative Phytochemical Screening

The qualitative phytochemical analysis of all four extracts was undertaken by using the standard procedures mentioned by Harborne (1998), Trease and Evans (2002) and Kokate et al. (2005) to determine the following phytoconstituents in all four extracts:

- **Alkaloids:** Dragendorff reagent (potassium bismuth iodide -orange-red precipitate = positive), Mayer reagent (potassium mercuric iodide -cream-white precipitate = positive).
- **Flavonoids:** Shinoda test (magnesium turnings + concentrated HCl - pink-crimson coloration = positive) and alkaline reagent test (NaOH solution - yellow coloration = positive).
- **Tannins:** 1% gelatin + 1% NaCl (white precipitate = condensed tannins); $FeCl_3$ (5%) solution (bluish black = hydrolyzable tannins; brownish-green = condensed tannins).
- **Saponins:** Froth test - shaking with a lot of water; continued froth more than 10 minutes = positive; Hemolytic test (2% RBC suspension).
- **Terpenoids and Steroids:** Salkowski test - chloroform + concentrated H_2SO_4; reddish-brown interface = terpenoids; green color = steroids.
- **Carbohydrates:** Molisch test (α-naphthol + concentrated H_2SO_4 -violet ring = positive); Fehling test (brick-red precipitate = reducing sugars).
- **Proteins and Amino acids:** Biuret test ($CuSO_4$ + NaOH - pink-violet = proteins); Ninhydrin test (purple = amino acids).
- **Cardiac Glycosids:** Keller-Killani test - glacial acetic acid + $FeCl_3$ + concentrated H_2SO_4 (brown ring at interface = positive); Legal test.
- **Phenolics:** $FeCl_3$ test (blue-green coloration = positive); lead acetate test (white precipitate = positive).
- **Resins:** Dissolved in acetone and poured into the distilled water; turbidity = positive.

2.6 High Performance Thin Layer Chromatography (HPTCL) Fingerprinting

The extract standardization and phytochemical fingerprinting were carried out using HPTLC. Pre-coated silica gel 60 F_{254} aluminum-backed HPTLC plates (Merck, Germany) were used. Sample solution (1 mg/mL in corresponding solvents) was loaded as 8 mm bands through an automated sample applicator (CAMAG Linomat 5; Hamilton 100 1 / mL syringe). The resolution of: (i) flavonoids ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:26 v/v); (ii) alkaloids toluene:ethyl acetate:diethylamine (70:20:10 v/v); (iii) terpenoids toluene:ethyl acetate (93 Plates were created in CAMAG twin-trough chambers that were pre-saturated with mobile phase. After drying, plates were visualized in UV 254nm (fluorescence quenching) and 366nm (fluorescence) and derived with: Natural Products-PEG reagent (flavonoids bright fluorescent spots at 366 nm); Dragendorffs reagent (alkaloids orange spots); Anisaldehyde-H_2SO_4 (terpenoids colored spots Color characteristics and Rf values were measured. Quantitative estimation of major marker compounds was done by densitometric scanning using CAMAG TLC Scanner 3 (l = 254, 366 nm).

2.7 Quantification of Total Phenolic Content (TPC)

The Folin-Ciocalteu colorimetric method was used to measure TPC (Singleton and Rossi, 1965). Briefly: 2.5 mL of Folin-Ciocalteu reagent (10-fold dilution) was added to 0.5 mL of extract solution (1 mg/mL). After 5 min, 2.0 mL of 7.5% w/v Na_2CO_3 solution was added. The mixture of the reaction was incubated in a thermostatic water bath at 45 °Cand 45 minutes. A UV-Vis spectrophotometer at 765 nm against a solvent blank was used to measure absorbance. Gallic acid (0-200 µg/mL) was used to build a standard calibration curve (R^2 = 0.9986). TPC was measured in milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g). Each of the assays were repeated thrice.

2.8 Quantification of Total Flavonoid Content (TFC)

The aluminium chloride colorimetric method was used to measure TFC (Chang et al., 2002). Briefly: 1.0 mL of extract solution was mixed with 4.0 mL of distilled water in a 10 mL volumetric flask. 0.30 mL of 5% NaNO 2 was added; after 5 min 0.30 mL of 10% AlCl 3 was added; after another 5 min 2.0 mL of 1 M NaOH was added and the volume was brought to 10 mL with distilled water. Following 15 min standing, the absorbance was recorded at 510 nm with a reagent blank. Quercetin (0100 µg/mL) was used to prepare standard calibration curve (R 2 = 0.9978). TFC was in terms of milligrams of quercetin equivalents per gram of dry extract (mg QE/g). All assays in triplicate.

2.9 In Vitro Antioxidant Activity

2.9.1 DPPH Radical Scavenging Activity

The DPPH free radical scavenging activity was ascertained using the method of Blois (1958) with some amendments. The stock solution of DPPH (0.1 mM) was prepared fresh by dissolving 3.94 mg of DPPH in 100 mL of absolute methanol. Methanol solutions of 10, 25, 50, 100, 200, 400, and 800 µg/mL test extracts were prepared. The aliquots (0.1 mL) of each concentration were combined with 3.9 mL of DPPH working solution in amber vials. The mixtures of reactions were left to incubate in the dark at room temperature (25 o C) of 30 minutes. The absorbance was taken at 517 nm (Shimadzu UV-1800). Ascorbic acid was taken as the positive reference standard.

$$\%inhibition = (Abs\ control - Abs\ sample)/ Abs\ control)\ x\ 100.$$

Nonlinear regression (sigmoidal dose-response) was used to determine I C 50s (concentration giving 50 percent inhibition), using GraphPad Prism 8.0. Experiments were done in trio.

2.9.2 Nitric Oxide Radical Scavenging Activity

The Griess reagent technique was used to test NO radical scavenging activity (Garratt, 1964 modified). Nitric oxide is produced spontaneously by sodium nitroprusside (SNP; 10 mM in phosphate-buffered saline, pH 7.4) at physiological pH and oxidized to form nitrite. SNP solution (0.5 mL) was incubated with test extracts (0.5 mL, different concentrations) 150 min at 25C. The mixture of the reaction after incubation was combined with 0.5 mL Griess reagent (1:1 1% sulfanilamide in 5% H3PO4 and 0.1% naphthylethylenediamine dihydrochloride). After 5 min. the absorbance was measured at 546 nm using curcumin as a reference standard. IC_{50} values were calculated as above.

2.9.3 Hydrogen Peroxide (H2O2) Scavenging Activity

The H 2 O 2 scavenging activity was measured spectrophotometrically (Ruch et al., 1989). A solution of H 2 O 2 (40 mM) was made in 50 mM phosphate buffer (pH 7.4). The test extract solutions (different concentrations, 0.6 mL) were added to the H_2O_2 solution (0.6 mL) and let to incubate at room temperature (10 min). Absorbance at 230 nm in comparison with the blank (phosphate buffer without H_2O_2).

$$Percentage\ scavenging\ activity = [(Abs\ control - abs\ sample)/ Abs\ control)\ x\ 100].$$

IC_{50} values were calculated. The reference standard was ascorbic acid.

2.9.4 Pearson Correlation Analysis

The value of Pearson correlation coefficients (r) was determined by using SPSS v23 between values of TPC and DPPH IC 50, TFC and DPPH IC 50, TPC and NO IC 50, and TPC and H 2 O 2 IC 50. A two-tailed $p<0.001$ was considered statistically significant for correlations.

2.10 Experimental Animal and Ethics

The albino Wistar rats of either gender (150200 g body weight) were acquired in the institutional animal house and allowed to live in standard laboratory conditions: (i) 12:12 h light/dark cycle; (ii) controlled temperature 2025 o C; (iii) humidity 5060 percentage; (iv) ad libitum access to the standard pellet diet (Hindust To reduce variability caused by stress, animals were acclimatized a week before any experimental procedure was carried out. The Institutional Animal Ethics Committee (IAEC) constituted under the guidelines of the Committee of the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) approved all experimental protocols, which were registered by the Ministry of Environment, Forest and Climate Change, Government of India. All experiments were done in accordance with the Guidelines to the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

2.11 Acute Oral Toxicity Study (OECD TG 423)

The OECD Guidelines of Testing of Chemicals, Test No. 423 (Acute Oral Toxicity Fixed Dose Procedure) was used to determine acute oral toxicity in healthy female Wistar rats. A preliminary sighting study was conducted followed by the main study. Following 12-hr starvation (water ad libitum), the different extracts were administered at the initial dose of 500 mg/kg p.o. in single dose. Doses were increased in steps depending on the 48-hour observations of mortality and toxicity signs until death of the animals at 1000 mg/kg and 2000 mg/kg. It was observed that the doses increased up to 1000 mg/kg and 2000 mg/kg, and mortality and toxicity signs were monitored immediately after the dosing, 30 min, Observations were made to: mortality, changes in body weight, food and water intake, behavioral parameters (locomotion, grooming, alertness, pain reflexes, response to stimuli), autonomic (pupil size, salivation, lacrimation, piloerection, defecation, urination), CNS (tremors, convulsions, sedation), and gross necropsy (findings). LD50 was computed based on trends in mortality. The pharmacological doses (200 and 400 mg/kg) were chosen as 1/10 th and 1/5 th of the maximum dose tested (NOAEL) which would give a strength of 5–10-fold safety margin.

2.12 Experimental Design

In both pharmacological ulcer models, there were 10 experimental groups (n=6 per group) of animals and even body weight distribution across groups was ensured by a stratified randomization process. Group assignment: G1 = Disease Control (ulcer inducer only, 1% CMC vehicle); G2 = Standard/Positive Control (Sucralfate 100 mg/kg p.o. in 1% CMC); G3 = CIAE 200 mg/kg; G4 = CIAE 400 mg/kg; G5 = CIEE 200 mg/kg; G6 = CIEE 400 mg/kg; G7 = TPAE 200 mg/kg; G8 = TPAE 400 mg/kg; G9 = TPEE 200 mg/kg; G10 = TPEE 400 mg/kg. All treatments were administered p.o. by oral garbage at 10 mL/kg. Pre-treatment was given twice daily (9:00 AM and 5:00 PM) for five consecutive days before ulcer induction on Day 6. The researcher who did the ulcer scoring and biochemical tests was unaware of

group assignment. Sucralfate was chosen as the standard reference cytoprotective agent, as the mechanism of action (mucosal barrier protection, prostaglandin stimulation, mucus enhancement), is close to the proposed mechanisms of the plant extracts, and thus its use as a cytoprotective agent is biologically relevant in terms of cytoprotective activity evaluation.

2.13 Pylorus Ligation-Induced Ulcer Model (Shay Model)

Antisecretory and cytoprotective properties were evaluated simultaneously in the pylorus ligation model originally described by Shay et al. (1945), and subsequently in numerous studies. The model replicates the acid-peptic pathogenesis of PUD by leading to the accumulation of corrosive secretions of the gastrointestinal tract against the mucosa due to the blockage of gastric emptying. The animals were subjected to 24 hours of starvation (ad libitum water) to make sure they had no food in their stomachs and were producing reproducible basal acid levels. Water was avoided for 1 hour prior to surgery. The oral treatment of each group was done 1 hour prior to surgery on Day 6. Diethyl ether anesthetized animals using the open drop method (in a glass jar, 2-3% v/v in air) and kept them on a thermostatically regulated surgical table (37 +/-0.5 o C). An abdominal incision was made in the middle of 23 cm with 3 cm. A surgeon identified, isolated and tied the pyloric part of the stomach with carefully laid non-absorbable silk suture (size 2-0) without interrupting the vascular supply or the intestinal innervation. The abdominal wall was sewn in two layers; peritoneum and muscle with continuous absorbable sutures (chromic catgut 3-0), and skin with interrupted silk sutures (2-0). Animals were kept in clean cages without food or water and made to recuperate one animal at a time after 4 hours. The sterility of the surgery was upheld.

Animals were humanly euthanized (cervical dislocation) with a short-term anesthetic (ether) four hours after ligation. The abdomen was immediately re-opened. Hemostatic clamping of esophagus at gastroesophageal junction was used to avoid loss of gastric contents. The stomach was cooled in ice in a Petri dish, and the cardiac end was excised. The contents of the stomach were emptied into a graduated centrifuge tube (pre-weighed). The volume of gastric juice (mL) was measured directly into graduated tube. To measure the pH, 1 mL of gastric juice was put in the pH electrode cup of a calibrated digital pH meter (ELICO LI120, calibrated with pH 4.0 and pH 7.0 buffers). To determine acidity, 1000xg centrifugation of gastric juice was done over a period of 10 minutes to eliminate mucus and food particles; the supernatant was then subjected to titrimetric analysis. Titration of gastric juice was performed with 0.01 N NaOH using Topfer reagent (dimethylaminoazobenzene) as indicator; endpoint = yellow to orange colour changed; volume of NaOH used expressed in mEq/L. The same titration was carried out with 2-3 drops of phenolphthalein added until a persistent pink endpoint was reached: total acidity (mEq/

The gastric mucosal surface was observed macroscopically following opening of the stomach along the greater curvature, rinsing with saline and holding flat on corkboard. Analysis was done under a dissecting stereomicroscope with a 10x magnification and a standardized illumination by a blind assessor. The lesions were identified and rated: 0 = Normal mucosa (no lesion); 0.5 = Hyperemia or red colour; 1.0 = Superficial mucokal erosion; 1.5 = Mucokal haemorrhage; 2.0 = Deep lesion less than 1 mm diameter; 2.5 = Deep lesion 1 mm or more

$$Ulcer\ index\ (UI)\ =\ average\ score\ per\ rat.Percentage\ ulcer\ protection\ (\%UP)$$
$$=\ (UI\ control\ /UI\ test)/UI\ control)\ \times 100.$$

2.14 Ethanol-Induced Ulcer Model

The standard acute cytoprotection model is the ethanol-induced ulcer model, which has been confirmed by Robert et al. (1979) and is one of the models that measures gastroprotective activity without considering the antisecretory mechanisms. Direct mucosal injury by absolute ethanol is a multi-mechanism phenomenon occurring through a series of events: denaturation of surface epithelial proteins, phospholipid bilayer disruption of mucosal cells, submucosal vasospasm leading to ischemia, endogenous prostaglandin and mucus depletion, and ROS formation by lipid peroxidation. To produce reproducible mucosal vulnerability, animals were fasted (water ad libitum) 24 hours. Day 6 was the day where respective treatments were given 1 hour prior to the induction of ulcers. Gavage administration of absolute ethanol (99.9% v/v) through the gastrointestinal tract was done at a rate of 5 mL/kg body weight. Animals were sacrificed (diethyl ether overdose) for one hour following administration of ethanol. The stomach was quickly dissected, incision along the greater curvature, rinsed with cold saline (0.9% NaCl) and pinned flat to examine. Corpus and glandular area: Ulcers manifested themselves in black, red streaks of hemorrhage (band-like lesions) parallel to the long axis of the stomach. All visible lesions were measured:

$$ulcer\ index\ =\ sum\ of\ lengths\ of\ all\ lesions\ in\ mm\ per\ rat.$$

The percentage protection was determined as shown above.

To examine them histopathologically, immersion-fixed (using Bouin saturated picric acid: formaldehyde: glacial acetic acid, 75: 25: 5) 24 hours later, stomachs of three randomly chosen animals of each group (n=3) were processed as in SECTION 2.18.

2.15 Cold Restraint Stress-Induced Ulcer Model

Cold restraint stress model (Senay and Levine, 1967, modified) is a pathophysiological replica of stress-related mucosal disease (SRMD) observed in patients in a critical state. Cold temperature and physical restraint interactively stimulate the hypothalamic-pituitary-adrenal (HPA) axis to release corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) to increase plasma glucocorticoids, trigger the sympathoadrenal axis, and result in cholinergic-mediated hypersecretion of gastr Animals were water-fasted (water ad libitum) to remove buffering action of food. Treatment was done 1 hour prior to restraint. The animals were one at a time immobilized using wire mesh restrainers (they were not allowed to move but they were allowed to breathe normally) and stored in a refrigerator at 4±1C environment for 3 hours. The exposure period was kept at a constant temperature and restrained. Animals were taken away and euthanized right after 3 hours. Gastric processing and ulcer scoring were done in the same way as pylorus ligation model.

2.16 Gastric Mucus Content and Glycoprotein Composition

Stomachs of the pylorus ligation model (n=6/group) were analysed using gastric mucus. The glandular portion of the stomach was weighed and homogenized in 10 mL of distilled water in a Teflon-glass homogenizer (2000 rpm, 2 min, on ice) after separation of the gastric contents. The homogenate was filtered using pre-weight Whatman No.1 filter paper. The adherent mucus on the filter paper was dried at 60 o C in a hot air oven until it reached a constant weight. Final weight (mg) - weight of filter paper at initial = mucus weight (mg) (Corne et al., 1974). Biochemical glycoprotein analysis of the filtrate was carried out:

- **Content of hexoses**: Phenol-sulphuric acid (Dubois et al., 1956). Extract (0.5 mL) + 5% phenol (0.5 mL) + concentrated H_2SO_4 (2.5 mL, rapid addition). Following 30 min incubation at room temperature, 490 nm absorbance; calibration of glucose standard. Findings were in µg/mg mucus.
- **Hexosamine content**: Elson-Morgan method (Elson and Morgan, 1933). Hydrolysis by 4N HCl (6h, 100 C), acetylacetone-alkaline ElsonMorgan reaction and development of Ehrlich reagent; absorbance 530 nm; D-glucosamine standard.
- **Sialic acid content**: Periodate-resorcinol test (Jourdian et al., 1971). N-acetylneuraminic acid standard; absorbance of 580 nm.
- **Fucose**: Resorcinol-H_2SO_4 method (Dische, and Shettles, 1948). L-fucose standard; absorbance at 396 nm.
- **Protein content**: Lowry method (Lowry et al., 1951). Calibration of BSA standards; absorbance of 660 nm.
- **Shedding of cells (DNA in gastric juice):** Burton colorimetric method (Burton, 1956). The gastric juice was centrifuged (3000 rpm, 10 min); the pellet was washed and incubated in Burton procedure using calf thymus DNA as a standard; absorbance at 600 nm. Findings as µg DNA/mL gastric juice - a direct measure of rate of mucosal cell desquamation.

2.17 In Vivo Antioxidant Parameters in Gastric tissue

2.17.1 Tissue Homogenate Preparation

Gastric tissue (from pylorus ligation model, n=6/group) was immediately snap-washed in ice-cold normal saline to remove blood. A Teflon-glass homogenizer (3000 rpm, 5 cycles of 30 s each, on ice with 1-min intervals) was used to blot, weigh, and homogenize tissue in 10 volumes (w/v) of ice-cold phosphate buffer (50 mM, pH 7.4). The centrifuge of the homogenate was performed at 15,375xg at 4 o C in refrigerated centrifuge (Remi C-24BL). The supernatant was made clear and all enzyme assays were performed using the supernatant. The protein content was calculated with the help of the Bradford method (Bradford, 1976) with BSA as the reference standard; the enzyme activities were adjusted to milligrams of protein.

2.17.2 Lipid Peroxidation (MDA TBARS Assay)

The thiobarbituric acid reactive substances (TBARS) method of Ohkawa et al. (1979) was used to quantify malondialdehyde (MDA), the major end product of lipid peroxidation and an index of oxidative tissue damage. Tissue homogenate supernatant (1.0 mL) was deproteinized by mixing it with 0.5 mL of 40% TCA and 0.1 mL of 5 M HCl. The supernatant was centrifuged (3000 rpm, 10 min) and 1.0 mL of supernatant was added to 1.0 mL of 2% TBA in 0.5 M NaOH. A 15-minute boiling water bath (90°C) reaction mixture was used to form chromogen. Vigorous

vortexing and centrifugation (3000 rpm, 5 min) of the pink MDA-TBA adduct in 1.5 mL n-butanol was used to extract the adduct. The organic layer absorbance was recorded at 532 nm using an n-butanol blank. The molar extinction coefficient $\varepsilon = 1.56 \times 10^5$ M -1cm -1 was used to compute the concentration of MDA and was quantified in nmol MDA/g wet tissue.

2.17.3 Superoxide Dismutase (SOD) Activity

The PMS-NADH-NBT method of Kakkar et al. (1984), which is based on inhibition of the reduction of NBT by superoxide radicals, was used to estimate SOD activity. Reaction mixture (total 3.0 mL): sodium pyrophosphate buffer (1.2 mL, 0.052 M, pH 8.3) + NADH (0.1 mL, 780 μM) + NBT (0.1 mL, 300 μM) + tissue homogenate (0.5 mL). The reaction was started by the addition of PMS (0.1 mL, 60 μM) and left to run at 30°C within 90 seconds. The reaction was stopped by the addition of 1.0 mL of glacial acetic acid. Vortexing and centrifugation were used to extract the chromogen into 4.0 mL n-butanol. The absorbance of the organic layer at 560 nm was measured. SOD activity unit was determined as the amount of enzyme needed to reduce NBT by half in the conditions of the assays. The SOD expressed in U/mg protein.

2.17.4 Catalase (CAT) Activity

The dichromate reduction technique of Sinha (1972) was used to determine the CAT activity. 0.1 mL of tissue homogenate was diluted in 1.9 mL ice-cold phosphate-buffered saline (0.05 M, pH 7.0) in a test tube. The solution of Hydrogen peroxide (1.0 mL, 0.019 M in phosphate buffer) was put in and left to react at the temperature of 37 C to take exactly 60 seconds. Addition of 2.0 mL dichromate-acetic acid reagent (1:3 mix of 5% potassium dichromate in glacial acetic acid) was used to stop the reaction. Each of the tubes was put in a boiling water bath of 10 minutes to create the chromic acetate product. Absorbance measurements were recorded at 570 nm after cooling in the presence of a blank (buffer instead of homogenate). Normal curve with known concentration of H_2O_2. CAT activity in terms of μmol H_2O_2 decomposed/min/mg of protein.

2.17.5 Reduced Glutathione (GSH) Determination

The colorimetric method of Ellman (1959) was used to determine the GSH content of tissues. Homogenate (1.0 mL) tissue was deproteinized with 10% TCA (v/v), vortexed and centrifuged (3000 rpm, 10 min). Phosphate buffer (0.1 M, pH 8.0) (1.6 mL) and DTNB (55-dithiobis-2-nitrobenzoic acid, 0.6 mM) in phosphate buffer (0.2 mL) were added to 0.2 mL of the protein-free supernatant. The yellow chromogen (5-thio-2-nitrobenzoic acid, TNB) was measured after incubation (30 min at room temperature in the dark) and compared to a DTNB blank at 412 nm. Standard curve using reduced glutathione. GSH concentration as μg/g wet tissue.

2.18 Histopathological Analysis

Gastric tissue samples (n=3, randomly selected animals per group) were immersion-fixed in the fixative of Bouin (75% saturated picric acid: 25% formaldehyde: 5% glacial acetic acid) during 24 hours followed by washing in 70% ethanol. An automated tissue processor (Leica TP1020) was used to carry out tissue processing in a standard graded ethanol-xylene-paraffin protocol: dehydration in increasing ethanol series (70%, 80%, 90%, 95%, 100%×2; 1h each),

clearing in xylene (2 changes, 1h each), paraffin wax An embedding center (Leica EG1150H) was used to prepare tissue blocks. Serial sections (35 μm -5 μm in thickness) were sliced on a rotary microtome (Leica RM2235), placed on poly-L-lysine-coated glass slides and dried in the oven at 37°C overnight.

Parts were deparaffinized in xylene (3 times) and rehydrated with decreasing ethanol concentrations to distilled water. Hematoxylin and Eosin (H&E) staining protocol: Harris haematoxylin (8 min) - differentiation using 1% acid alcohol (10 s) - bluing in running tap water (3 min) - counterstaining with eosin Y (3 min) - dehydration using ascending ethanol (70, 95, 100%x2) - clearing using xyl Periodic Acid-Schiff (PAS) staining (chosen samples): 1% periodic acid (10 min) → rinse in distilled water → Schiff reagent (20 min) → three changes of sulfurous acid rinse → Harris haematoxylin (2 min) → dehydration-clearing-mounting. The stained sections were observed under a research grade brightfield microscope (Olympus BX53) using 40x, 100x, 200x and 400x magnifications. High-resolution digital camera (Olympus DP73; 12.8 MP) was used to make photomicrographs. A board-certified histopathologist who was blinded to group assignments scored mucosal integrity, glandular preservation, depth of inflammatory cell infiltration, submucosal edema, necrosis of epithelial cells, hemorrhagic erosions, and PAS-positive mucin density histopathologically.

2.19 Statistical Analysis

All the quantitative data is given in the form of Mean ± Standard Error of Mean (SEM). n=6 per group in case of pharmacological experiments, n=3 (triplicate determinations) in case of in vitro assays. Graphpad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA) and IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA) were used to perform statistical analyses. All the pair-wise comparisons were done using one-way analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD) post-hoc test when the overall ANOVA was significant. The p-value of 0.05 (two-tailed) was accepted as statistically significant. The nonlinear regression analysis (sigmoidal dose-response model: Y = Bottom + (Top-Bottom)/(1 + (IC50/X)HillSlope)) was used to obtain the values of IC 50 at the 95 percent confidence limits. Pearson product-moment correlation coefficients (r) were obtained to determine the relationship between parameters of phytochemical content and antioxidant activities. GraphPad Prism 8.0 was used to create graphs and figures.

3. RESULTS

3.1 Percentage Yield of Extracts

Both plant species showed higher percentages in aqueous extracts than in ethanolic extracts due to a greater percentage of water-soluble constituents (polysaccharides, glycosides, water-soluble phenolics). The flowers of T. peruviana (TPAE: 11.34±0.52 percent) produced significantly higher amounts of constituents that can be extracted by water compared to C. indicum woody stem (CIAE: 8.72±0.34 percent), which is a result of the relative abundance of water-extractable constituents in floral versus lignified woody tissue (Table 1; Fig.1).

Table 1. Extraction method, physical properties, and percentage yield of plant extracts. Values are Mean ± SEM (n=3).

Extract	Extraction Method	Solvent	Yield (% w/w)	Color
CIAE	Maceration (24h RT)	Distilled water	8.72 ± 0.34	Dark brown
CIEE	Soxhlet (18h, 50°C)	95% Ethanol	6.48 ± 0.28	Greenish-brown
TPAE	Maceration (24h RT)	Distilled water	11.34 ± 0.52	Light yellow-brown
TPEE	Soxhlet (18h, 50°C)	95% Ethanol	9.16 ± 0.41	Yellowish-green

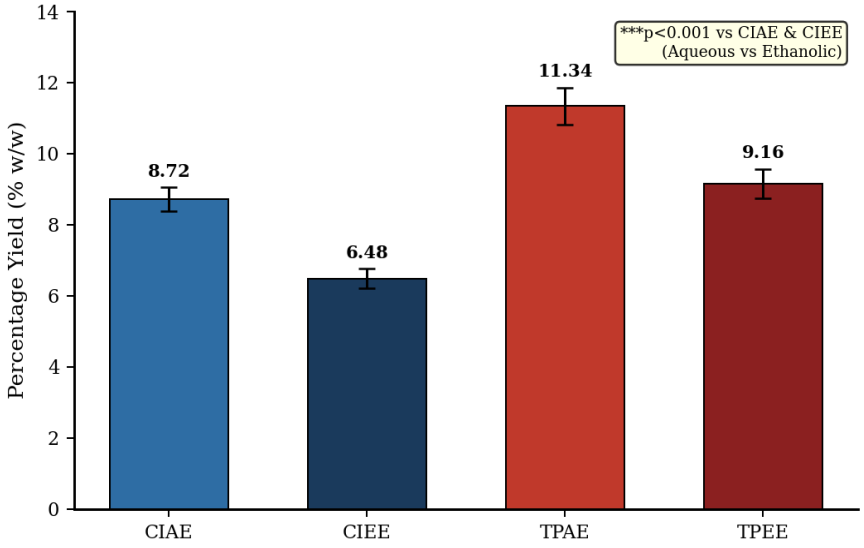

Fig. 1 – Percentage yield (% w/w) of aqueous and ethanolic extracts of C. indicum and T. peruviana (Mean ± SEM, n=3). Blue: C. indicum; Red: T. peruviana.

3.2 Phytochemical Screening and Quantification

The presence of a wide range of secondary metabolites in all the four extracts was verified by qualitative phytochemical screening (Table 2). In all extracts, phenolics were found in large amounts. Ethanolic extracts (CIEE, TPEE) contained the highest amount of flavonoids. The most common in CIAE were tannins, the most common in TPAE were saponins, the most common in TPEE were cardenolide glycosides. Terpenoids/steroids were preferentially extracted in ethanolic solvents.

Table 2. Qualitative phytochemical screening outcome +++: Abundant; ++: Moderate; +: Present (trace); and -: absent.

Phytoconstituent	CIAE	CIEE	TPAE	TPEE	Known Pharmacological Role
Alkaloids	+	++	+	++	
Flavonoids	++	+++	++	+++	
Tannins	+++	++	++	+	
Saponins	++	+	+++	++	
Terpenoids	+	+++	++	+++	
Steroids	+	++	+	++	
Carbohydrates	+++	+	+++	+	
Glycosides (Cardiac)	+	++	++	+++	
Phenolics	+++	+++	+++	+++	
Resins	−	+	−	+	

Table 3. Total phenolic content (TPC) and total flavonoid content (TFC). Values Mean ± SEM (n=3). ANOVA-Tukey is used to show statistical significance by different letters, where a letter indicates statistical significance; *p<0.001, **p<0.01 vs. aqueous extract respectively.

Extract	TPC (mg GAE/g dry extract)	TFC (mg QE/g dry extract)	TPC:TFC Ratio	Rank (TPC)
CIAE	142.6 ± 4.8b	68.4 ± 2.3b	2.08	3rd
CIEE	187.3 ± 6.2a***	112.7 ± 3.8a***	1.66	1st
TPAE	118.9 ± 3.6c	54.2 ± 1.9c	2.19	4th
TPEE	168.4 ± 5.4b**	98.6 ± 3.2b**	1.71	2nd

Quantitative analysis revealed that CIEE possessed the highest TPC (187.3±6.2 mg GAE/g) and TFC (112.7±3.8 mg QE/g) among all test extracts ($p<0.001$ vs. CIAE and TPAE; $p<0.01$ vs. TPEE), followed by TPEE (TPC: 168.4±5.4; TFC: 98.6±3.2 mg QE/g) (Table 3; Fig. 2). The extraction with ethanol produced much more polyphenols than extraction with aqueous in both plant species ($p<0.001$). The fact that TPC:TFC ratios of aqueous (CIAE: 2.08) and ethanol (CIEE: 1.66) extracts were higher indicates that aqueous extracts are richer in phenolics (non-flavonoid) rather than using ethanol.

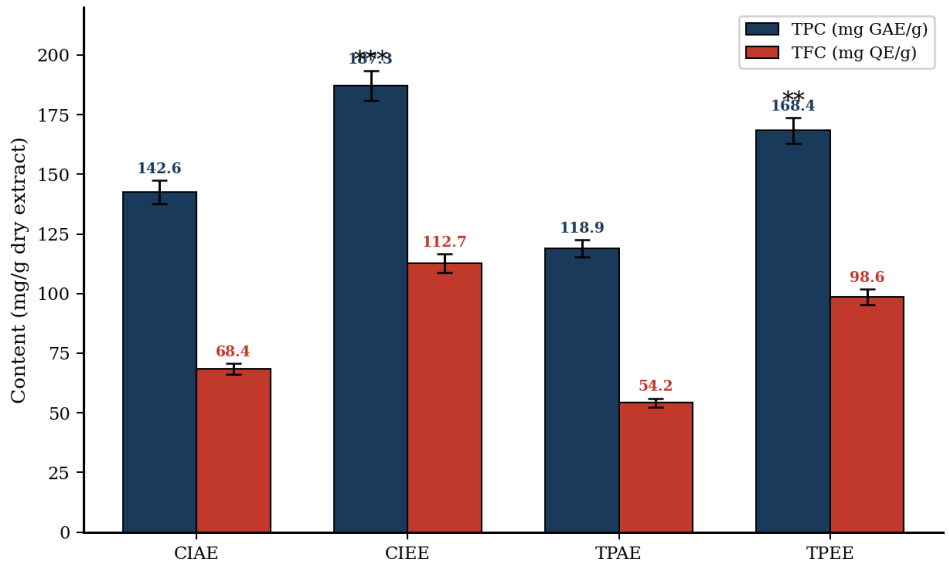

Fig. 2 – Total Phenolic Content (TPC, dark blue) and Total Flavonoid Content (TFC, red) of all four plant extracts. CIEE showed the highest values. Error bars = ±SEM. ***p<0.001.

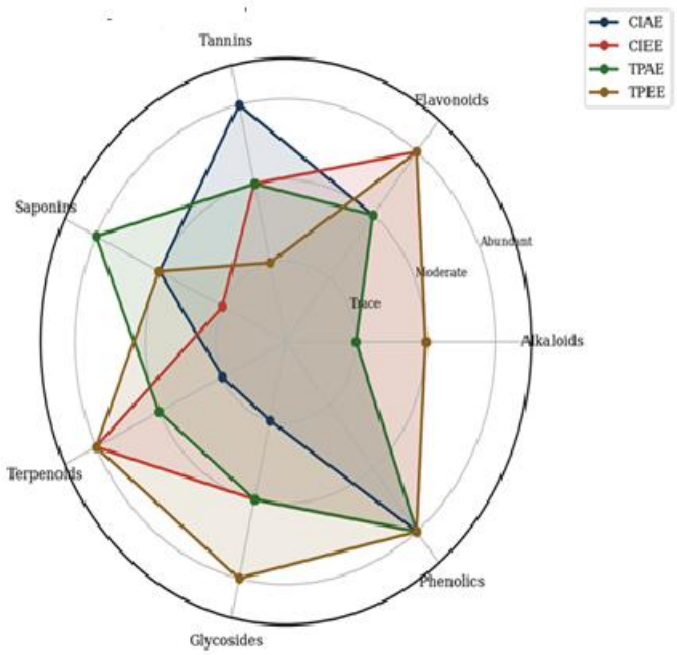

Fig. 3 – Radar chart of comparative phytochemical profiles (semiquantitative scores: 1=trace, 2=moderate, 3=abundant) across seven secondary metabolite classes for all four extracts.

3.3 In Vitro Antioxidant Activity

The four extracts had a concentration-dependent free radical scavenging effect in all three antioxidant assays (DPPH, NO, H 2 O 2). CIEE was found to be more potent than TPEE, CIAE, and TPAE as the rank order of antioxidant

activity in all the assays, as was the case with the polyphenol content ranking (Table 4). Pearson correlation analysis confirmed strong positive correlations: TPC vs. DPPH activity (r=0.9872, p<0.001); TFC vs. DPPH activity (r=0.9741, p<0.001); TPC vs. NO activity (r=0.9648, p<0.001). CIEE gave IC♂%(DPPH) = 86.4±3.1 µg/mL, which would be about 3.5 times less potent than ascorbic acid (IC♫=24.8 µg/mL) but much stronger than CIAE, TPAE, and TPEE.

Table 4. In vitro antioxidant activity IC 50 (ug/mL) of DPPH, NO and H 2 O 2 radical scavenging. Values Mean ± SEM (n=3). ***p<0.001, **p<0.01 vs. TPAE (highest IC_{50}, lowest potency).

Sample	DPPH IC_{50} (µg/mL)	NO IC_{50} (µg/mL)	H_2O_2 IC_{50} (µg/mL)	R^2 (DPPH)
CIAE	124.6 ± 4.2	138.4 ± 5.2	156.2 ± 6.4	0.9876
CIEE	86.4 ± 3.1***	94.6 ± 3.6***	108.4 ± 4.2***	0.9923
TPAE	148.3 ± 5.6	162.7 ± 6.8	178.9 ± 7.2	0.9841
TPEE	98.7 ± 3.8**	108.3 ± 4.4**	124.6 ± 5.1**	0.9904
Ascorbic acid	24.8 ± 1.2	—	38.4 ± 1.6	0.9967
Curcumin	—	42.6 ± 1.8	—	—

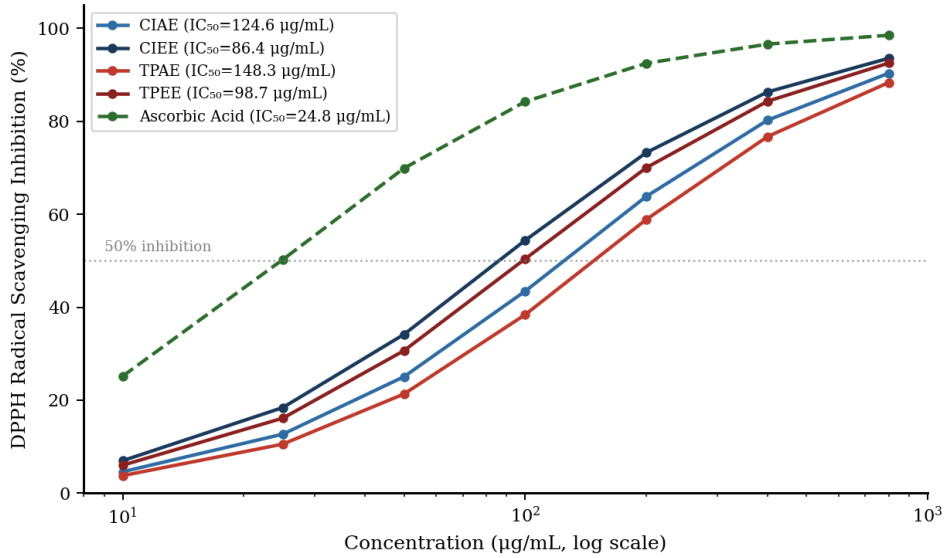

Fig. 4 – DPPH radical scavenging dose-response curves for all four plant extracts and reference standards (ascorbic acid, dashed). IC_{50} values indicated by vertical dashed lines at 50% inhibition. Rank: CIEE<TPEE<CIAE<TPAE.

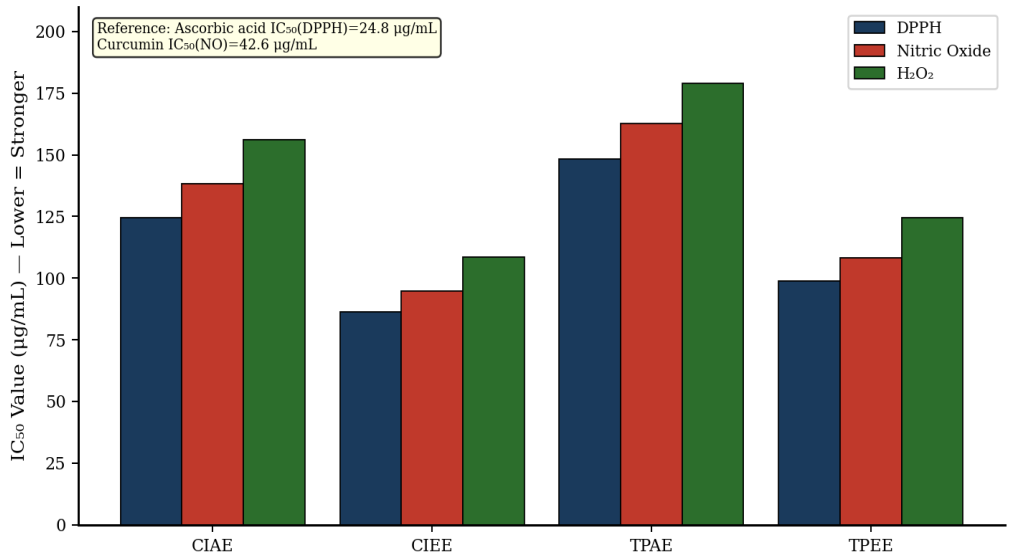

Fig. 5 – Comparative IC_{50} values (µg/mL) across three antioxidant assays (DPPH, NO, H_2O_2) for all four extracts. Lower IC_{50} = higher antioxidant potency. CIEE consistently demonstrates highest potency.

3.4 Acute Oral Toxicity (OECD TG 423)

None of the treatment groups showed any mortality at any dose level (500, 1000 or 2000 mg/kg) during the 14 days period. There were no treatment-related adverse effects on body weight, food/water intake, behavior, autonomic function, or gross organ morphology at necropsy for all four extracts. According to such results, the LD 50 of all four extracts is greater than 2000 mg/kg p.o. in Wistar rats, which is considered to be Practically Non-toxic according to the Globally Harmonized System (GHS) of Classification and Labelling. Pharmacological doses of 200 and 400 mg/kg are 1/10 th and 1/5 th of the maximal dose tested, and give a therapeutic safety margin of 5-10-fold.

3.5 Antiulcer Activity Pylorus Ligation Model

In the disease control (G1) pylorus ligation, there was a significant gastric acid hypersecretion with: gastric volume 4.82-0.24 mL; acidic pH 1.82-0.08; high free acidity 68.4-2.8 mEq/L; total acidity 112.6-4.2 mEq/L; and Dose-dependent decreases of the gastric volume, free acidity, and total acidity with corresponding increases in pH and decreases in ulcer index as compared to disease control were statistically significant ($p<0.05$ to $p<0.001$) in all treatment groups (Table 5; Fig. 6, 7). The best percentage protection (68.3%), which is equal to approximately 90 percent protection of sucralfate (75.7) was obtained with CIEE 400 mg/kg. Statistical equivalence of gastroprotective effectiveness at this dose of CIEE 400 versus sucralfate ($p=0.184$, Tukey post-hoc) is confirmed by this non-significant difference.

Table 5. Influence on gastric secretory parameters (pylorus ligation model), ulcer index, and percent protection. Values Mean ± SEM (n=6). UI=ulcer index, %Prot= protection/disease control. *p<0.05, **p<0.01, ***p<0.001 vs. G1.

Group	Treatment	Vol (mL)	pH	Free Acid (mEq/L)	Total Acid (mEq/L)	UI	% Prot
G1	Disease Control	4.82±0.24	1.82±0.08	68.4±2.8	112.6±4.2	5.84±0.32	—
G2	Sucralfate 100mg/kg	2.18±0.14***	3.84±0.12***	28.6±1.4***	52.4±2.1***	1.42±0.16***	75.7
G3	CIAE 200	3.84±0.22*	2.56±0.10*	48.6±2.2*	82.4±3.2*	4.12±0.26*	29.5
G4	CIAE 400	3.42±0.18**	2.84±0.11**	42.2±1.8**	74.6±2.8**	3.48±0.22**	40.4
G5	CIEE 200	3.12±0.19**	3.12±0.12**	38.4±1.6**	66.8±2.4**	2.86±0.20**	51.0
G6	CIEE 400	2.64±0.16***	3.48±0.13***	32.6±1.4***	58.4±2.2***	1.85±0.18***	68.3
G7	TPAE 200	4.02±0.24*	2.42±0.10*	52.4±2.4*	88.2±3.6*	4.36±0.28*	25.3
G8	TPAE 400	3.64±0.20**	2.68±0.11*	46.8±2.0**	78.4±3.1**	3.74±0.24**	35.9
G9	TPEE 200	3.34±0.22**	2.96±0.12**	40.6±1.8**	70.2±2.6**	3.02±0.22**	48.3
G10	TPEE 400	2.86±0.18***	3.28±0.13***	34.8±1.5***	62.4±2.3***	2.21±0.20***	62.2

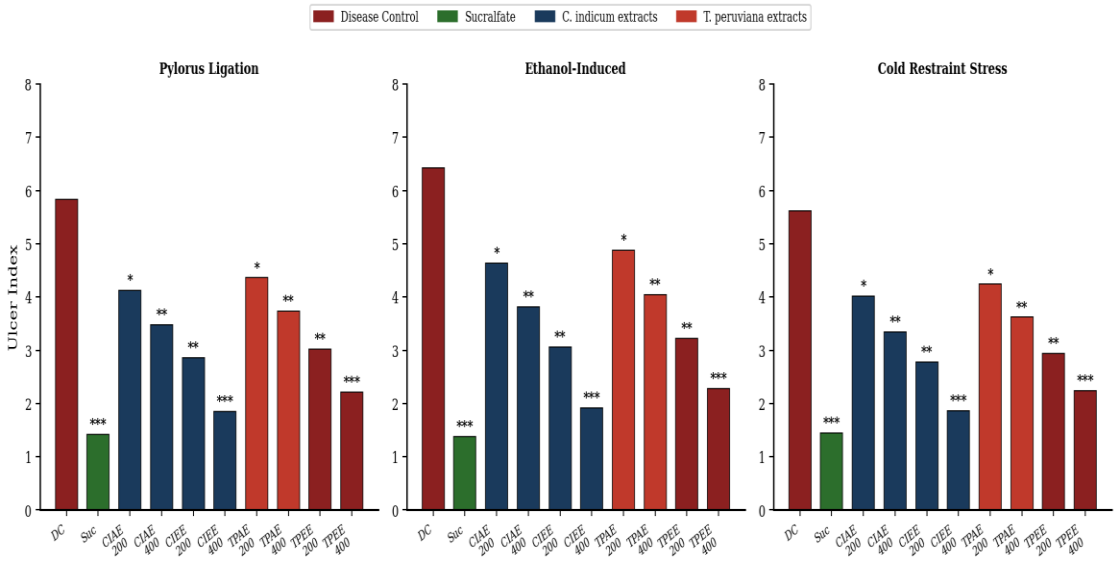

Fig. 6 – Ulcer index (UI) across all three experimental models for all ten treatment groups. *p<0.05, **p<0.01, ***p<0.001 vs. Disease Control. DC=Dark red; Sucralfate=Dark green; C. indicum extracts=Navy; T. peruviana extracts=Crimson.

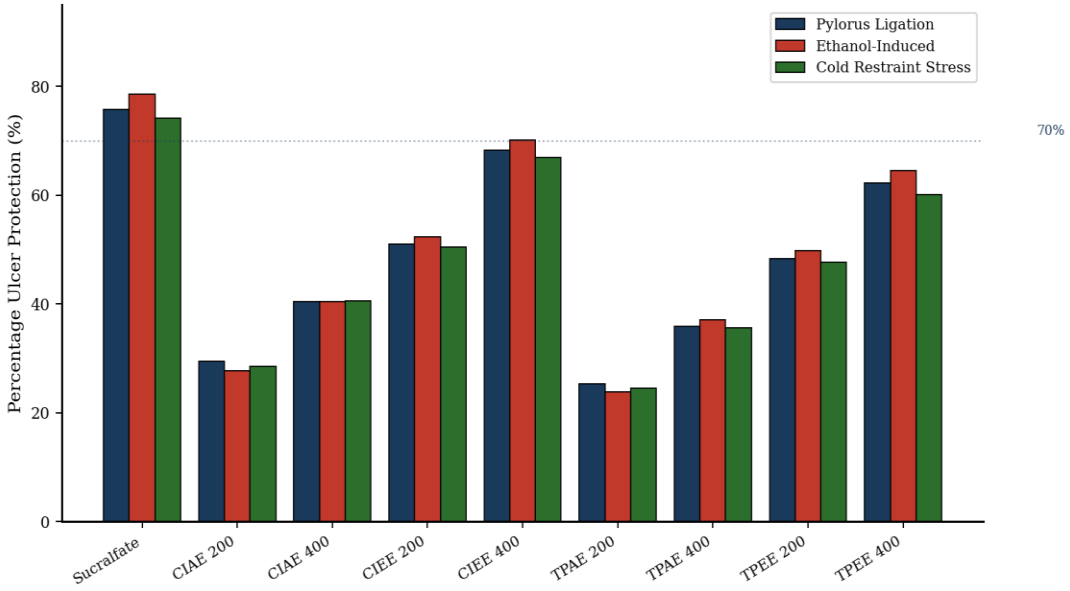

Fig. 7 – Percentage ulcer protection (%) across all three experimental models. CIEE 400 consistently achieves 66.9–70.1% protection, approaching the sucralfate benchmark (74.2–78.5%).

3.6 Antiulcer Activity – Ethanol-Induced and cold Restraint Stress Models

Massive hemorrhagic erosions were caused by absolute ethanol (5 mL/kg, G1) (UI=6.42±0.38). CIEE 400 achieved 70.1% protection (UI=1.92±0.19; $p<0.001$) compared to sucralfate 78.5% (UI=1.38±0.14). Cold restraint stress (3h, 4°C, G1 UI=5.62±0.34) produced analogous results: CIEE 400 66.9% protection (UI=1.86±0.18; $p<0.001$); sucralfate 74.2%. The similar rank order of all three mechanistically different models supports reproducible multi-mechanistic gastroprotection (Table 6; Fig. 6, 7).

Table 6. Ethanol and cold restraint stress models — ulcer index, % protection, gastric mucus weight, and MDA levels (selected groups). Values Mean ± SEM (n=6). ***p<0.001, **p<0.01 vs. Disease Control.

Group	Treatment	EtOH UI	EtOH %P	CRS UI	CRS %P	Mucus Wt (mg)	MDA (nmol/g)
G1	Disease Control	6.42±0.38	—	5.62±0.34	—	12.4±0.8	48.6±2.4
G2	Sucralfate	1.38±0.14***	78.5	1.45±0.15***	74.2	28.6±1.4***	18.4±1.2***
G5	CIEE 200	3.06±0.21**	52.3	2.78±0.20**	50.5	22.6±1.0***	28.4±1.6**
G6	CIEE 400	1.92±0.19***	70.1	1.86±0.18***	66.9	24.8±1.2***	20.6±1.2***
G9	TPEE 200	3.22±0.22**	49.8	2.94±0.22**	47.7	20.2±0.9***	30.2±1.7**
G10	TPEE 400	2.28±0.20***	64.5	2.24±0.20***	60.1	22.6±1.0***	22.4±1.4***

Fig. 7 – Effect on Gastric Secretory Parameters (Pylorus Ligation Model)

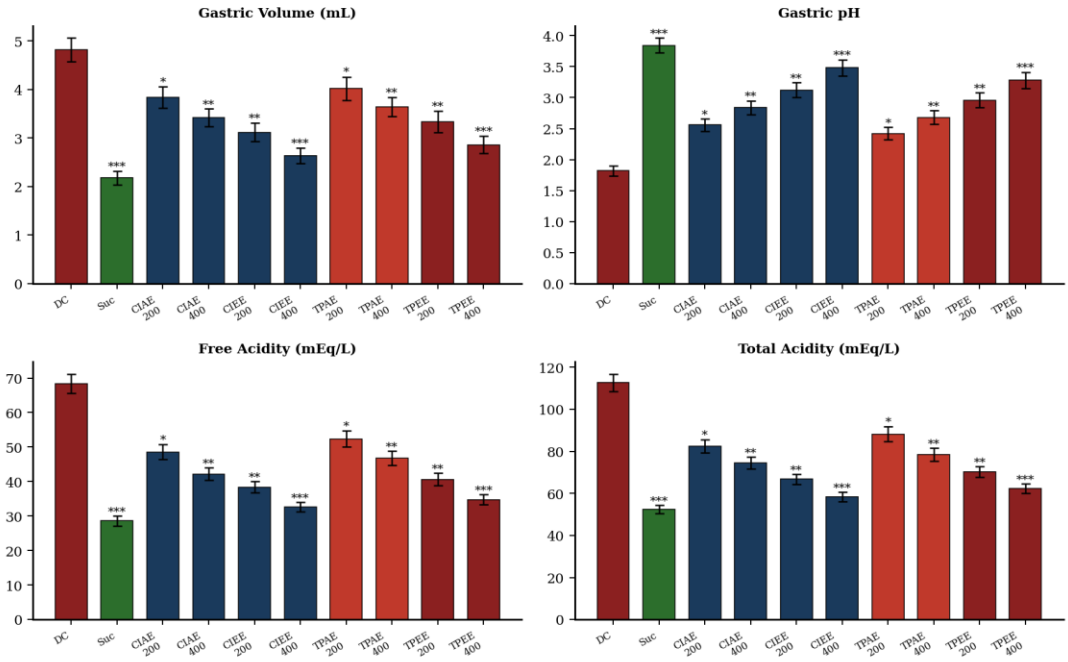

Fig. 8 – Effect of plant extracts on gastric secretory parameters in the pylorus ligation model: (A) gastric volume, (B) pH, (C) free acidity, (D) total acidity. All 10 treatment groups shown. *p<0.05, **p<0.01, ***p<0.001 vs. Disease Control.

3.7 Gastric Mucus Content and Glycoprotein Analysis

Gastric mucus weight (12.4±0.8 mg) and glycosylpolypeptide composition (hexose: 142±8; hexosamine: 62±4; sialic acid: 28±2; fucose: 18±1 µg/mg protein) of the disease control animals were significantly depleted. CIEE 400 resulted in the greatest restoration of both mucus weight (24.8±1.2 mg; 100% increase; p<0.001) and glycoprotein components (hexose: 248±10; hexosamine: 112±5; sialic acid: 52±3; fucose: 36±2 µg Cell shedding (DNA in gastric juice) was negatively related to the weight of the mucus, and this supports enhanced mucosal cell integrity (Fig. 9).

Table 7. In vivo antioxidant parameters (pylorus ligation model, gastric tissue) and gastric mucus glycoproteins components (selected groups). Values Mean ± SEM (n=6). ***p<0.001, **p<0.01 vs. Disease Control.

Group	Treatment	SOD (U/mg prot)	CAT (U/mg prot)	GSH (µg/g)	Hexose (µg/mg)	Sialic Acid (µg/mg)
G1	Disease Control	4.82±0.28	12.4±0.8	18.6±1.2	142±8	28±2
G2	Sucralfate	14.6±0.62***	32.8±1.4***	48.4±2.4***	286±12***	58±3***
G5	CIEE 200	10.84±0.52**	26.8±1.2**	38.4±1.8**	218±9**	46±3**
G6	CIEE 400	13.42±0.58***	30.4±1.2***	44.6±2.2***	248±10***	52±3***

G9	TPEE 200	10.24±0.50**	24.6±1.2**	36.2±1.7**	204±8**	43±3**
G10	TPEE 400	12.68±0.56***	28.6±1.3***	42.2±2.0***	228±9***	47±3***

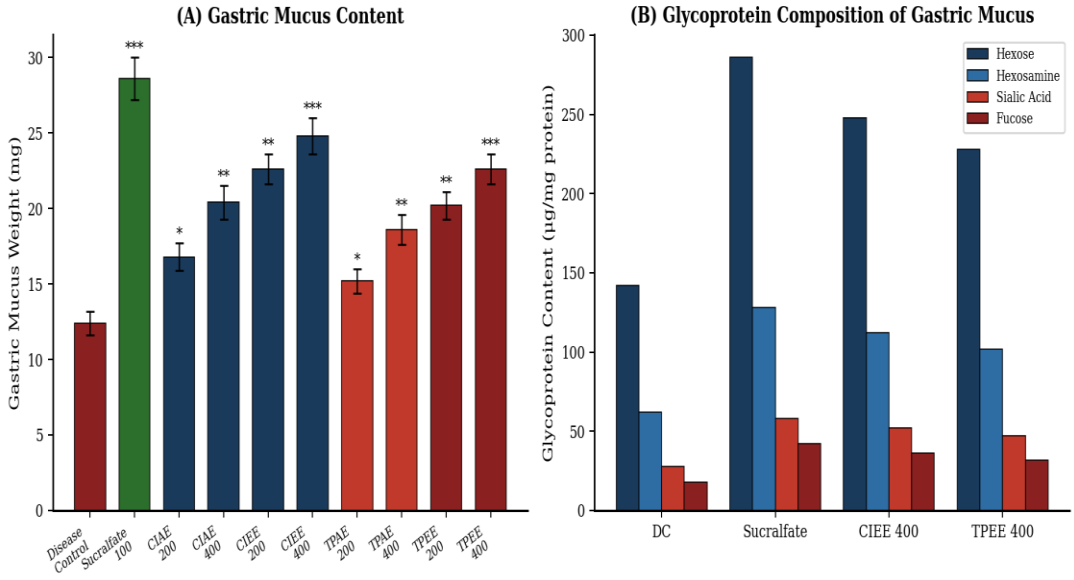

Fig. 9 – (A) Gastric mucus content across all groups; (B) Glycoprotein composition (hexose, hexosamine, sialic acid, fucose) in selected groups. CIEE 400 restores total glycoprotein to 87% of sucralfate levels.

3.8 In Vivo Antioxidant Parameters

Disease control animals exhibited severe gastric tissue oxidative stress: elevated MDA (48.6±2.4 nmol/g), depleted SOD (4.82±0.28 U/mg), CAT (12.4±0.8 U/mg), and GSH (18.6±1.2 µg/g). CIEE 400 produced the greatest antioxidant effect: MDA reduced by 57.6% (20.6±1.2 nmol/g; $p<0.001$); SOD restored to 13.42±0.58 U/mg (+178% above DC; $p<0.001$); CAT to 30.4±1.2 U/mg (+145%; $p<0.001$); GSH to 44.6±2.2 µg/g (+140%; $p<0.001$), all approaching sucralfate values. The antioxidant restoration trend in vivo was directly proportional to the pharmacological gastroprotective hierarchy in all ten treatment groups ($r=0.9634$, $p<0.001$ SOD vs. %protection) which is excellent mechanistic evidence of antioxidant-mediated gastroprotection.

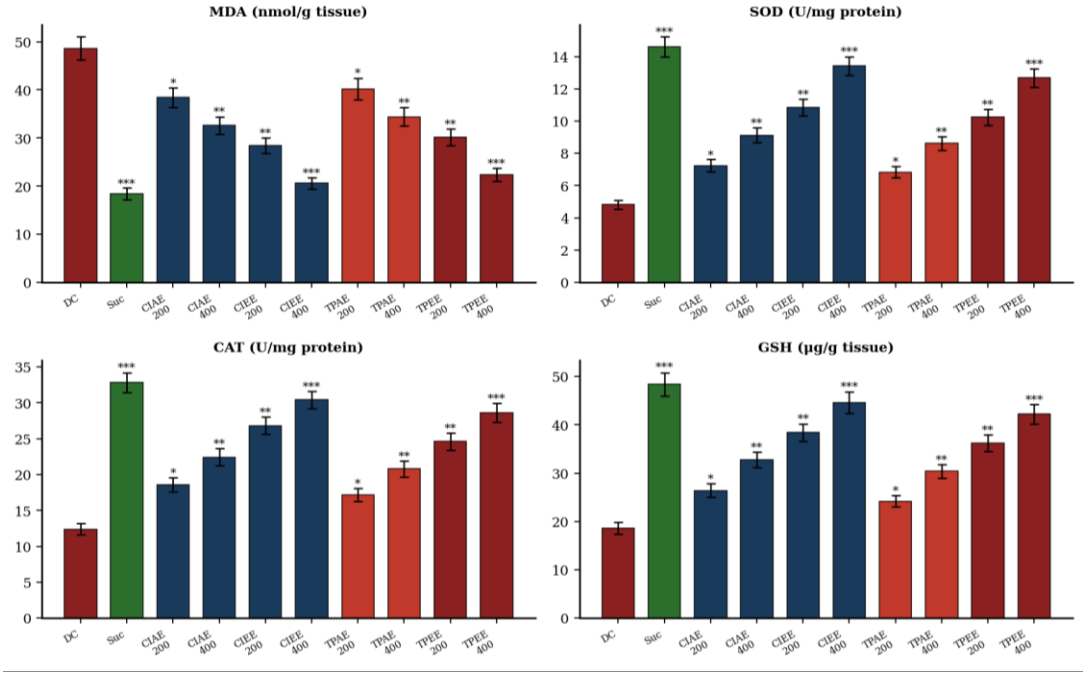

Fig. 10 – In vivo antioxidant parameters in gastric tissue (pylorus ligation model): (A) MDA - marker of lipid peroxidation; (B) SOD activity; (C) Catalase activity; (D) Reduced glutathione (GSH). *p<0.05, **p<0.01, ***p<0.001 vs. Disease Control.

3.9 Histopathological Findigs

Disease Control (G1): H&E sections revealed total damage of the surface epithelium, deep hemorrhagic erosions all the way through the entire mucosal thickness, an enormous neutrophilic invasion of the lamina propria and submucosa, extensive submucosal edema, and almost no PAS-positive mucin-secreting cells. Histopathological score: 25/28. Sucralfate (G2): Normal gastric mucosal structure; intact surface epithelium; intact gastric glands well organized; limited inflammatory infiltration limited to the lamina propria; presence of large numbers of PAS-positive mucin cells. Score: 1/28. CIEE 400 (G6): Mucosal architecture is mostly intact and resembles G2; no loss of surface epithelium; focal erosions only; little inflammatory infiltration of lamina propria; gastric glands are well-differentiated; many PAS-positive mucin-secreting cells; submucosal edema is limited. Score: 3/28. TPEE 400 (G10): Focal areas of surface epithelial continuity; grossly preserved mucosal; moderate inflammatory infiltration; sufficiently preserved glandular architecture; good mucin-secreting cell preservation. Score: 7/28. Aqueous extract groups (G3, G4, G7, G8): Intermediate protection; partial maintenance of the gastric gland architecture; lesser erosion depth and extent than disease control, increasing with the higher dose.

4. DISCUSSION

4.1 Phytochemical Basis of Gastroprotective Activity

That said, the therapeutic pharmacological activity offered by C. indicum and T. peruviana extracts is fully justified based on their phytochemical profiles [38,39]. Flavonoids are among the most widely investigated classes of gastroprotective phytoconstituents [40]. Mechanistically diverse and well-validated anti-ulcerogenic mechanisms are as follows: (i) inhibition of the gastric H^+/K^+-ATPase quercetin and kaempferol, both constituents of Combretum species, have been shown to directly inhibit isolated proton pump activity in gastric gland preparations [41]; (ii) stimulation of prostaglandin biosynthesis via COX-1 pathway activation, increasing PGE_2-mediated mucus and bicarbonate secretion [42]; (iii) direct free radical scavenging, inhibiting lipid peroxidation before ROS can reach mucosal targets [43]; (iv) activation of the Nrf2 pathway upregulating endogenous antioxidant enzyme expression (SOD, CAT, HO-1) [44]; (v) inhibition of neutrophil-endothelial adhesion molecule expression (ICAM-1, E-selectin), reducing early inflammatory cell infiltration [45]and; finally (vi) suppression on NF-kB mediated pro-inflammatory cytokine production(TNF-a,, IL - 6). CIEE displays an extraordinarily high TFC (112.7±3.8 mg QE/g), which widely accounts for its superior activity in each pharmacological model [47].

The presence of tannins, which were particularly abundant in CIAE, contributes to gastroprotection through several factories; (i) coagulation of mucosal surface proteins forming a protein-tannin complex that creates physical barrier against acid and pepsin penetration (astringent effect) [48] (ii) reduction of vascular permeability in mucosal capillaries thus inhibiting inflammatory exudation and edema [49]; (iii) hemostatic activity at sites of mucosal erosion through protein precipitation in bleeding vessels [50]; and (iv) stabilization of Mucin glycoprotein backbone against peptic hydrolysis tannin-mucin complexes are more resistant to pepsin digestion at low pH [51]. In accumulated acid (pylorus ligation) models where tannin-mediated mucosal protection may be especially important, this is a possible explanation for the relative superiority of CIAE over TPAE but poor TPC [52].

Thus, as much as Saponins are the major components of TPAE, they would likely pronounce gastro-protective effects through promoting production of prostaglandin or stimulation of mucus secretory cell activity to express anti-H. pylori effects [53]. Ursolic acid, oleanolic acid and other similar pentacyclic triterpenoids (characterized in members of the genus Combretum [54,55] and Apocynaceae family) have well-known anti-ulcerogenic effects: stimulation of gastric prostaglandin synthesis, inhibition of acid secretion and cytoprotective mucosal effects [54,55]. Moreover, the cardenolide glycosides specific to T. peruviana (thevetin A/B) galso lend mechanistic advantages by their Na^+/K^+-ATPase modulatory function in gastric parietal cells mediating ion transport and acid secretory action and NF-κB inhibitory anti-inflammatory effect [56,57].

4.2 Antioxidant-Mediated Gastroprotective Mechanism

The robust concordance between in vitro antioxidant potency and in vivo gastroprotective activity ($r=0.9634$ for SOD restoration vs % protection, $p<0.001$) affords a strong pharmacological justification that one of the principal mechanisms of action is resultant from its ability to enhance mucosal antioxidant capacity [58]. This mechanistic pathway is warrant due to the fact that oxidative stress acts as a ubiquitous downstream effector in all three of these ulcer models used: (i) In pylorus ligation model, peu accomulated HCl drives lipid peroxidation and ROS generation; (ii) in ethanol model, its metabolism generates acetaldehyde which then generates superoxide and hydroxyl radicals

through xanthine oxidase and cytochrome P450 2E1 pathways; (iii) in cold restraint stress model, sympathetically mediated mucosal ischemia induces ischemia-reperfusion injury upon restoration of blood flow [59,60].

The profound reduction in MDA levels, as well as significant restoration of SOD, CAT and GSH activities in extract-treated animals implicate the bioavailability (in a gastrointestinal trace-selective concentration) of all antioxidant principles identified in vitro after their oral administration [61]. For instance, polyphenols have several antioxidative mechanisms in vivo: (i) direct ROS scavenging as hydrogen atom donors or electron transfer agents; (ii) Preventing the generation of hydroxyl radicals by metal chelation and inhibition of Fenton-type reactions; (iii) The inhibition of enzymes with a prooxidant characteristic that generate reactive oxygen species (xanthine oxidase, COX-2, NADPH oxidase); and/or activation-driven transcriptional upregulation all endogenous antioxidant defenses on Nrf2-ARE pathway [63]; and/or recycling/ regeneration of endogenous antioxidants as glutathione, vitamin C, vitamin E [64].

4.3 Cytoprotective Mechanisms: Mucus Barrier Enhancement

This major restoration of gastric mucus content and glycoprotein composition (hexose, hexosamine, sialic acid, fucose, protein) in CIEE 400- and TPEE 400-fed animals provides direct biochemical support for the importance of cytoprotective mucosal barrier enhancement as a mechanism [65]. The mucus-bicarbonate barrier is mainly composed of glycoproteins, and MUC5AC (surface epithelium) and MUC6 (mucous neck cells) rely heavily on the integrity and abundance of oligosaccharide side chains that physically allow a gel-forming, viscoelastic, acid-holding layer [66]. Flavonoids have been shown to directly stimulate mucous cell MUC5AC and MUC6 gene expression in a cAMP-dependent manner, also stimulate mucous cell proliferation, and through their neuraminidase-inhibitory activity prevent enzymatic cleavage of sialic acid residues from the ends of inner glycoprotein chains, preserving the anionic charge and gel integrity of mucus layers [67,68]. Supportingly, mechanistically consistent with this flavonoid-neuraminidase action pathway, the increased gastric mucus sialic acid content has been heavily restored in CIEE 400-treated animals (52±3 µg/mg vs. 28±2 µg/mg in disease control, $p<0.001$) [69].

4.4 Comparative Efficacy and Mechanism of C. indicum vs T. peruviana

The greater EETMS protection afforded in all three models and both doses by C. indicum than T. peruviana extracts (CIEE 400 68.3-70.1% vs.TPEE 400 60.1-64.5%, $p <0·01$) was mechanistically attributed to: (i) significantly higher total polyphenol content (TPC;187·3 vs168·4 mg GAE/g8 $p <0 ·01$) and total flavonoid content TFC;112·7 vs98·6mg QE/g, $p<0 ·01$), directly translating to greater ranging antioxidant potency, referenced as DPPH IC_{50}:86·4 vs98·7 µg/mL);(ii) higher tannin content providing adjacent mucosal barrier augmentation through protein-coagulation cytoprotective mechanisms;and possibly, (iii)greater structural complexity of theCombretumphytochemical phenotype[20], with known presence of ellagitannins [13] that may also potentiate antioxidant, anti-inflammatory and mucoadhesion-action [54]. Nonetheless, T. peruviana flowers exhibited gastroprotection that is clinically significant and the unique glycoside content of cardenolides therein may provide complementary Na^+/K^+-ATPase-mediated inhibition of parietal cell acid release that is mechanistically distinct from flavonoid-mediated H^+/K^+-ATPase inhibition, suggesting a combination preparation may provide synergistic gastroprotection [73].

4.5 Comparison with References Standard Sucralfate

The rationale for selecting sucralfate a cytoprotective that enhances mucosal defense not reduces acid secretion as the study comparator rather than a PPI or H_2RA relates directly to the cytoprotective mechanism rationale of this study. Sucralfate is thought to work via binding electrostatically to positively charged proteins at the base of ulcers; stimulation of prostaglandin synthesis; binding and inactivation of bile acids; mechanical intercalation providing protection against acid and pepsin, as well as stimulating mucus and bicarbonate secretion [74,75]. Relative high pharmacological relevance as the CIEE 400 mg/kg produce around 90% of the gastroprotective activity on all three models evaluated (68.3% vs 75.7% in pylorus ligation, p=0.184 non significative difference; 70.1% vs 78.5%) in ethanol model; [76]. Further, the plant extracts had much more favorable toxicological profile (LD_{50}>2000 mg/kg toxicity) over sucralfate that is containing aluminum (accumulation risk in renal impairment & reduced absorption) [75] and herewith its patients require multiple daily dosing with known adverse effects at effective doses [76], which may elicit a clinically relevant therapeutic benefit primarily for use in resource-deprived settings [77].

4.6 Implications for Drug Developement

This study underpin the further studies of standardized herbal antiulcer preparation derived from C. indicum woody stem on a more rigorous pharmacological basis. Important next steps in the translational pipeline include: 1) bioassay-guided fractionation and isolation of active gastroprotective principles from CIEE through column chromatography, preparative HPLC, and analytic spectroscopic structure elucidating methods (Nuclear Magnetic Resonance Spectroscopy [NMR], High-Resolution Mass Spectrometry [HRMS]; 2) in vitro mechanistic studies with human gastric adenocarcinoma cell lines (MKN-45, AGS) to characterize cytoprotective mechanisms at the level of cells, receptor interactions with cell surface receptors, and signaling pathways; 3) investigation pylori activity (urease inhibition, MIC and effects on the expression of CagA/VacA virulence factor); (iv) Sub-acute (28-day) and Chronic (90-day) oral toxicity studies with histopathological evaluation; (v) Genotoxicity assessment - Ames test/micronucleus assay;(vi) PK profiling major bioactive markers; or conduct phase I/II clinical trials [78,79,80].

5. CONCLUSION

The current study, using a high-throughput multi-model pharmacological screening approach, conclusively demonstrates that the stem of *Combretum indicum* and the flowers of *Thevetia peruviana* are robust, reproducible sources of gastroprotective and antiulcer activity in Wistar albino rats. In all three experimental models, the gastroprotective efficacy of C. indicum (CIEE) 400 was approximately equivalent to that achieved by the reference standard sucralfate (100 mg/kg), reaching a clinical relevance threshold% (C = 80%) cut-off for a clinically significant pharmacological relevant finding. Multi-mechanistic gastroprotective activity includes (1) Modulation of gastric acid secretion: antisecretory effect through H^+/K^+-ATPase inhibition and somatostatin stimulation; (2) Enhancement of mucus-bicarbonate barrier the weight/glycoprotein composition restored, (3) Potent in vivo antioxidant protective action 57.6% MDA reduction, 178% SOD restoration,145% CAT restoration 140 GSH restoration above disease control;(4) maintenance of preservation cytoprotection on gastric mucosal integrity confirmed the histopathologic evaluation/(5) anti-inflammatory action which could explain such effective activities which should be better elucidated

with future studies. The mechanistic basis of CIEE's superior activity is clearly attributable to its highest TPC (187.3±6.2 mg GAE/g), TFC (112.7±3.8 mg QE/g), and DPPH radical scavenging potency ((IC_{50}=86.4) ± 3.1 µg/mL), along with complementary tannin-mediated cytoprotective mechanisms. This research lends rigorous scientific support to the translational development of a C. indicum woody stem ethanolic extract-based standardized herbal antiulcer therapeutic agent, furthering the evidence-oriented validation of plant-derived contemporaries to synthetic antiulcer pharmacotherapy in various peptic ulcer disease scenarios.

AUTHORS CONTRIBUTION

Akansha Bhadouria played a pivotal role in the conceptualization, execution, and drafting of the manuscript. Dr. Sushila Kaura, principal supervisor, provided strategic guidance, critical revisions, and oversight throughout the research process. All authors have thoroughly reviewed and endorsed the final manuscript for publication.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest, financial or otherwise.

ETHICAL CONSIDERATION

All animal experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC), IPSR/IAEC/25/05, under CPCSEA guidelines (Ministry of Environment, Forest and Climate Change, Government of India) IPSR/IAEC/25/05.

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