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Flavonoids and anti-oxidant activity mediated gastroprotective action of Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. Leaf methanolic extract in rats

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ABSTRACT – **Background** – Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. from family *Combretaceae* is used in Ayurveda and Siddha traditional systems of medicine to heal ulcers. **Objective –** The present study was conducted to assess the gastroprotective effect and understand the fundamental mechanism of action of Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. Leaf Methanolic Extract. **Methods –** The test extract was screened for anti-ulcer activity by Aspirin induced ulcerogenesis in pyloric ligation and ethanol induced gastric ulcers at three doses – 125, 250, and 500 mg/kg, p.o. using Ranitidine 50 mg/kg and Misoprostol 100 µg/kg as standard drug in respective models. Seven parameters were carefully examined, that is, ulcer index, total protein, mucin, catalase, malondialdehyde, and superoxide dismutase levels and histopathology. High Performance Liquid Chromatographic - Ultra Violet profiling and Liquid Chromatography - Mass Spectral analysis of crude *Terminalia coriacea* leaves methanolic extract were carried out as a part of chemical characterization to identify bioactive compounds. **Results –** All the test doses exhibited significant gastroprotective function, particularly the higher doses demonstrated improved action. The results revealed a significant increase in the levels of catalase, superoxide dismutase, and Mucin with reduction in ulcer index, the levels of total protein, and malondialdehyde. Histopathological observations also illustrated the gastroprotective effect of *Terminalia coriacea* leaves methanolic extract. **Conclusion –** *Terminalia coriacea* leaves methanolic extract exhibited strong anti-oxidant and anti-secretory activities mediated gastroprotection besides inducing the gastric mucosal production. The observed pharmacological response can be attributed to the flavonoidal compounds namely – Quercetin-3-O-rutinoside, Luteolin-7-O-glucoside, Myricetin hexoside, Quercetin-3-O-glucoside, Isorhamnetin-3-O-rhamnosylglucoside and Isorhamnetin-3-O-glucoside identified in the extract for the first time with High Performance Liquid Chromatographic - Ultra Violet and Liquid Chromatography - Mass Spectral analysis.

HEADINGS – Gastric Mucosa. *Terminalia*. Plant extracts. Peptic ulcer.

INTRODUCTION

Peptic ulcer disease (PUD) is one of the most prevalent gastrointestinal disorders with high annual incidence and significant mortality rates. Unfortunately, the drugs available for the treatment of PUD confer simple to severe side effects like arrhythmias, gynaecomastia, enterochromaffin like hyperplasia and hematopoietic changes like leucopoenia and thrombocytopenia, limiting drug utility and leading the demand of a safe and effective gastroprotective agents especially for the people of non-industrialized countries[28]. An extensive research is being conducted on herbs to discover and identify remedies and lead compounds for the management of PUD as a consequence of growing interest in natural products and complementary and alternative therapies. In this context, we have also been studying traditional herbs in Ayurveda, Siddha and Unani systems of medicine[1,26-28]. Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. (*Combretaceae*) is a traditional herb used in Siddha for the treatment of ulcers. *Terminalia coriacea* is found in parts of Andhra Pradesh and Tamil Nadu states of India. It is called as "Tani" or "Nalli maddi" by the locals and it is used as cattle feed[13,31]. Our previous studies reveal that *T. coriacea* has anti-convulsant, anti-inflammatory, anti-nociceptive, anti-pyretic and wound healing properties[8,25,29,48,61]. The present study focuses on the assessment of phytochemicals and gastroprotective potential of *Terminalia coriacea* leaves methanolic extract (TCLME) in view of its traditional use.

METHODS

Plant material and the preparation of extract

The fresh leaves of Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. (*Synonyms – T. alata, T. crenulata, T. elliptica*, *T. tomentosa*) belonging to the *Combretaceae* family were collected from Talakonda forest, Tirumala Hills, Tirupathi, Andhra

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Pradesh, India. The plant material was authenticated by a plant taxonomist, Dr. P. V. Prasanna (Scientist-E) at the Botanical Survey of India, Deccan Regional Centre, Hyderabad (establishment under the Ministry of Environment & Forests, Government of India). The specimen deposited in the herbarium was assigned a voucher number BSID 882. After collection, the leaves were shade dried and coarsely powdered. The extraction was carried out in six phases; in each phase approximately 110 gm of the powdered leaves were extracted using methanol AR (SD Fine-Chem Limited) in a soxhlet apparatus in 1:4 ratio. The extract was concentrated under reduced pressure and stored in an airtight container in a refrigerator at the temperature below 10°C. The solution of TCLME was prepared using distilled water for the evaluation of the anti-ulcer activity.

Drugs and chemicals

Chloroform AR, diethyl ether LR, methanol AR, phenolphthalein pH indicator solution, and sodium hydroxide pellets were procured from SD Fine-Chem Limited, Mumbai, while pure aspirin was obtained from Divis Laboratories, Hyderabad. Absolute Ethanol was purchased from Changshu Yangyuan Chem, China, Ranitidine (as Rantac 150 mg) from J.B Chemicals and Pharmaceuticals, Mumbai and Misoprostol (as Misoprost-200) from Cipla Ltd., Goa, while surgical spirit was obtained from Kakatiya Pharma, Hyderabad. Topfer's reagent and distilled water were obtained from Nice Chemicals, China, and Stangen Fine Chemicals, Hyderabad, respectively. All chemicals were used without further purification.

Animals

Adult male Wistar rats weighing 150-200 gm were used for the evaluation of anti-ulcer activity. The animals were maintained under standard laboratory conditions in polypropylene cages under 12 hr light/dark cycle, controlled temperature (24±2°C), fed with commercial pellet diet, and water ad libitum in an animal house approved by the Committee for the Purpose and Supervision of Experiments on Animals (Reg. no. 1534/PO-/a/11/CPCSEA). All the animals were acclimatized to the laboratory environment for 10 days before the initiation of experiments. The protocol (IAEC/AUCP/2011-12/03) was approved by the Institutional Animal Ethical Committee before the commencement of animal experimentation. All measures were taken to ensure that the experiments were conducted in accordance with the instructions of IAEC, Anwarul Uloom College of Pharmacy, New Mallepally, Hyderabad, Andhra Pradesh, India.

Acute toxicity test and selection of test doses

Three test doses (125, 250, and 500 mg/kg) of TCLME were selected based on our earlier report where maximum safe dose was found to be 2000 mg/kg, p.o.[25]. In order to determine LD_{50}, the method described by Chinedu et al. (2013) was followed with slight modification[14]. Briefly, the study was carried out in eight stages with three test doses given from second to eighth stages. Doses of 50-5000 mg/kg were given in the first four stages (Stage – 1: 50, 200, 400, 800; Stage – 2: 1000, 1500, 2000; Stage – 3: 3000, 4000, 5000). The method was extended and doses from 6000–20,000 mg/kg, p.o. were given in later four stages with an incremental dose of 1000 mg/kg, p.o. to female albino Wistar rats. Each dose was administered to one rat and they were observed for 24 hours for mortality and signs of toxicity. A confirmatory test was performed at the end of each stage by administering the highest dose of each level to one more animal to confirm the lethality. The total number of animals used for acute toxicity study was 33 (inclusive of confirmatory test).

Analytical profile of TCLME

• Confirmatory chemical tests for flavonoids

TCLME was subjected to Alkali reagent, Zinc-Hydrochloric acid and Shinoda tests to reconfirm presence of flavonoids[20,30,32].

• High Performance Liquid Chromatographic (HPLC) and Ultra Violet (UV) spectroscopic analysis

HPLC-UV analysis was carried out at Analytical Development Laboratory, Mylan Laboratories Limited, Bollaram, Hyderabad with the method described earlier by Khan et al.[27]. Briefly, HPLC analysis of TCSBAE was performed by gradient system using Waters 2996 Photo Diode Array HPLC System with Kromasil C18 – (250 X 4.5 mm, 5 µm) column. Two solvents, A (water with 0.1% trifluoroacetic acid) and B (acetonitrile with 0.1% trifluoroacetic acid) were used for elution of constituents. The column was equilibrated in 85% A / 15% B prior to injection of sample and upon injection this composition was then changed to 60% A / 40% B over 30 min utilizing a linear gradient followed by changing to 50% A / 50% B over the next 10 min and then the concentration was returned to 85% A / 15% B over the final 10 min. The flow rate was set to 1 ml/min, injection volume was 10 µL and column temperature was maintained at 30°C. The system was run for 60 minutes. The eluents were monitored at 255 and 350 nm. Further the characteristic A & B band wavelengths in U.V region were recorded, to identify the nature of flavonoids as per the method described by Bohm and Tsimogiannis et al.[9,63]. The peak numbers, retention times, area, percentage area, A & B bands in U.V region were recorded.

• Liquid Chromatography-Mass Spectral (LC-MS) analysis

LC-MS analysis was also carried out at analytical development laboratory, Mylan Laboratories Limited, Bolarum, Hyderabad. The liquid chromatographic analysis of TCLME was performed by gradient system using Waters 2996 PDA HPLC system with Luna C18 - (250 X 4.5 mm, 5 µm) column. Two solvents, A (water with 0.1% trifluoroacetic acid) and B (acetonitrile with 0.1% trifluoroacetic acid) were used for elution of constituents. The column was equilibrated in 85% A / 15% B prior to injection of sample and upon injection this composition was then changed to 75% A / 25% B from 0-22 min. With linear gradient change 85% A / 15% B from 22-35 min. The flow rate was set to 1 mL/min and column temperature was maintained at 30°C. The injection volume was 10 µL. The system was run for 35 minutes and the eluents were monitored at 254 nm, 300 nm and 366 nm. The mass spectral analysis was done by Electro Spray Ionization using a coupled Agilent Ion-Trap Mass Spectrometer in both positive and negative models in the scan range of 100-2000 m/z.

• Evaluation of anti-ulcer activity by *in-vivo* assays

Aspirin induced ulcerogenesis in pylorus ligated rats. Aspirin induced ulcerogenesis in pylorus ligated rats model was used for the evaluation of anti-ulcer activity with slight modification. The animals were divided into five groups (n=6). Group I – served as negative control and received only vehicle. Groups II, III & IV received TCLME at 125, 250 and 500 mg/kg respectively per oral at the volume of 10 ml/kg. Group - V served as standard and was treated with standard drug (Ranitidine 50 mg/kg)[19]. Aspirin suspended in 1% CMC in water was administered orally at a dose of 500 mg/kg in 12 hours fasted rats[24]. The test extract and standard drug treatment was done 30 min prior the administration of

Aspirin. After 30 min, the pyloric ligation surgery was performed as per Shay et al.[60]. Four hours later, the animals were sacrificed by euthanasia.

• Collection and Measurement of Gastric Juice (GJ)

The stomachs were excised carefully keeping the esophagus closed. The stomachs were opened along the greater curvature, removing the luminal contents. The gastric contents were collected and centrifuged at 1000 rpm for 10 minutes. After centrifugation samples were decanted and the volume of gastric juice was noted and is expressed as mL/100 g body weight. The contents were subjected to analysis for free and total acidities.

• Determination of gastric juice pH (pH)

One mL of supernatant liquid was diluted to 10 mL with distilled water. The pH of the solution was recorded with the help of digital pH meter.

• Estimation of Free and Total Acidities (FA & TA)

The above solution was titrated against 0.01 N NaOH using Topfer's reagent as indicator. The end point of the titration was when the solution turns orange in colour. The volume of NaOH was noted which corresponds to the free acidity. Further the titration was continued till the solution regained pink colour. The total volume of NaOH was noted, which corresponds to total acidity.

• Determination of Ulcer Index (UI)

Mean ulcer score for each animal is expressed as Ulcer Index. The stomachs were washed with running water to see the ulcers in the glandular portion of the stomach. The number of ulcers per stomach were noted and the severity of the ulcers was scored microscopically with the help of hand lens (10x) and scoring was done as per Kulkarni[33]. The scoring was based on the following observations and assigned values: 0=Normal Stomach; 0.5=Red Colouration 1=Spot Ulcers; 1.5=Haemorraghic Streaks 2=Ulcers >3 mm but <5 mm; 3=Ulcers >5 mm.

• Determination of Gastric Mucin Content (GMC)

Mucin content was estimated by the method described by Corne et al.[16] with some modifications. The glandular segments of the stomachs of rats subjected to the ethanol induced ulcers model were isolated and weighed. Each glandular segment was immediately immersed in 10 mL of 0.1% alcian blue solution (0.16 M sucrose/0.05 M sodium acetate, pH 5.8). After immersion for 2 h, excess dye was removed by two successive rinses with 10 mL of 0.25 M sucrose, first for 15 min and then for 45 min. The stomachs were all sequentially transferred to a 0.5 M magnesium chloride and shaken for 2 h. Four mL of the blue extract was then shaken vigorously with an equal volume of ether. The resulting emulsion was centrifuged at 3600 g-force and the absorbance of the aqueous layer was read at 580 nm. The amount of alcian blue extracted per gram of wet glandular tissue was then calculated.

• Estimation of Catalase (CAT)

Catalase activity in stomach tissue was determined according to the method of Leyck and Parnham[36]. The stomach tissue was scraped and homogenized in ice cold saline medium with the help of a homogenizer. The solution was centrifuged for 10 minutes at 3000 *g*-force and collected for the experiment. 100 L of the supernatant was added to a solution of 3 L of H_2O_2, phosphate buffer mixture (50 mM phosphate buffer, pH 7.0, and 30% H_2O_2). The change in optical density at 240 nm per unit time was measured.

• Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase activity in stomach tissue was determined according to the method of Fridovich[18]. The stomach tissue was scrapped and homogenized in ice cold normal saline medium with the help of a homogenizer. Then, the tissue homogenate was centrifuged for 10 minutes at 3000 g force and the supernatant was collected and used for the estimation of SOD activity. 10 mL of the solution was taken in a test tube and mixed with 0.5 mL of 50 mM phosphate buffer (pH 7.8), 0.1 mM of EDTA, 0.05 mM xanthine, and 0.01 mM cytochrome c, and then, 100 mL of 2.5 mM of xanthine oxidase was added to start the reaction, and the absorbance was measured at 550 nm.

• Determination of Malondialdehyde (MDA)

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour absorbing light maximally at 535 nm. One g of tissue sample with 10 mL of 0.2 M Tris HCl buffer (pH 7.2) was taken in a tissue homogenizer to get a 10% homogenate. 500 µL of supernatant from the homogenate, 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid were taken in a tightly stoppered tube. The tube was heated to boiling temperature for 45 min. After cooling the tube, the contents were centrifuged. The supernatant was read at 532 nm against blank. The concentration of test samples was obtained using molar extinction coefficient of MDA. The amount of MDA is expressed as number of moles of MDA / mg of tissue[64].

• Estimation of Total Protein content (TP)

Total protein content was estimated by the method of Lowry et al.[39]. The dissolved proteins in gastric juice were estimated in the alcoholic precipitate obtained by adding 90% alcohol with gastric juice in a 9:1 ratio, respectively. Then, 0.1 mL of alcoholic precipitate of gastric juice was dissolved in 1 mL of 0.1 N NaOH. From this, 0.05 mL was taken in another test tube and 4mL of alkaline mixture was added and allowed to stand. After 10 min, 0.4 mL of phenol reagent was added and again allowed to stand for 10 min for the development of colour. Reading was taken against a blank prepared with distilled water at 610 nm. The protein content was calculated from the standard curve prepared with bovine albumin and has been expressed in terms of µg/ mL of gastric juice.

• Acute Ethanol induced gastric lesions

All the animals (n=6) in each group were fasted for 36 hours before the administration of ethanol. The standard drug (Misoprostol 100 g/kg, p.o.) or the test extract was administered one hour before ethanol administration. Ethanol (90%) was administered to all animals at a dose of 1 mL/200 gm. After one hour all animals were sacrificed, and stomachs were isolated as per Salim et al.[57]. Lesion severity was determined by measuring ulcer index.

Histopathological Studies

The isolated stomachs were preserved in 15% formalin solution and were sent to the pathologist for histopathological examination by staining with haematoxylin and eosin. The morphological changes were observed and recorded with 100x lenses[1].

Statistical analysis

Data obtained was analyzed by One way ANOVA followed by Dunnett's multiple comparisons *post-hoc* test using Graphpad Prism version 5.0, 32 bit for windows, Graphpad software, San Diego, California, USA (http://www.graphpad.com/). The values are expressed as Mean ± standard error of mean (SEM). <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results of extraction and acute toxicity testing

The dried mass of crude *Terminalia coriacea* leaves methanolic extract (TCLME) was found to be 24.54% w/w with respect to the powdered leaves. There were no mortalities or signs of toxicity up to the dose of 20,000 mg/kg, p.o, therefore further dosing was discontinued.

Results of Analytical Profile of TCLME

• Confirmatory chemical tests for flavonoids

All the chemicals tests confirmed the presence of flavonoids.

• Results of HPLC-UV analysis

On HPLC analysis of TCLME, 25 peaks were recorded. Peaks 8, 12, 13, 14, 15, 17, 21, and 22 had noteworthy area and percentage area. The peak numbers and their respective retention times are shown in Table 1 and Figure 1. Further, the UV spectrum plot indicates, that peaks 8-15 gave characteristic band A & B wavelengths demonstrating flavonoidal nature of compounds. The six peaks 8-10 and 13-15 gave typical band A & B wavelengths of flavonols while peaks 11 and 12 were confirmed as flavones. The HPLC-UV spectrum plot results are summarized in Table 1.

TABLE 1. Nature of compounds confirmed by characteristic bands A & B in U.V region with respective retention time and peak numbers on HPLC analysis

Peak no.	HPLC retention time in min.	Wavelength of bands A & B in UV region in nm	Nature of compound
8	9.653	354.0 & 253.7	Flavonol
9	9.890	356.1 & 253.7	Flavonol
10	10.679	353.7 & 263.1	Flavonol
11	11.162	347.7 & 265.5	Flavone
12	11.523	338.1 & 269.1	Flavone
13	12.118	357.2 & 254.9	Flavonol
14	12.325	354.9 & 256.0	Flavonol
15	12.594	354.9 & 256.0	Flavonol

HPLC: High Performance Liquid Chromatographic.

• Results of LC-MS analysis

The LC-MS analysis of crude TCLME lead to the identification of seven flavonoids by the recorded m/z values of molecular ions and their fragment ions via reported fragmentation patterns. The compounds could mostly be Rutin (Quercetin-3-O-rutinoside), Luteolin-7-O-glucoside, Myricetin hexoside, Apigenin-6-C-glucoside, Quercetin-3-O-glucoside, Isorhamnetin-3-O-rhamnosylglucoside, and Isorhamnetin-3-O-glucoside. An intense peak with m/z 633.1 was recorded between 7.7-7.9 min in positive mode ESI-MS. Rutin is reported to be characterized by the presence of sodium adduct (m/z 633). The molecular ion $[MH]^+$ with m/z 611 is almost absent and presence of aglycone residue $[aglycone+H]^+$ of m/z 302 is rare indicating hindrance in the removal of glucose[50]. Therefore, the observed peak could be due to Rutin. The retention time 8.1-8.4 min showed molecular ion peaks m/z 449.2 $[M+H]^+$ and 447.0 $[M-H]^-$ in both ESI positive and negative modes respectively and the various reported fragments - 413.2, 329.1, and 299.1 of Luteolin-7-O-glucoside in ESI positive mode as described by Colombo et al.[15]. The retention time 8.9-9.2 min revealed molecular ion m/z of Myricetin hexoside 479.0 $[M-H]^-$ & 316 $[Aglycone-H]^-$. The recorded m/z values were in conformance with the plant metabolomics standards of Institute of Food Research, Norwich Research Park, UK[52].

During the retention time 10.6-10.8 min, in the positive and negative mode ESI-MS, molecular ion m/z of 433.2 $[M+H]^+$, 431.1 $[M-H]^-$ and the standard fragments 397.2, 367.1, 313.2 were observed in + ESI-MS. These facts establish identity of compound as Apigenin-6-C-glucoside as mentioned by Correia et al.[17]. The same report evidenced the character of next compound as Quercetin-3-O-glucoside that appeared between the retention time 11.1-11.3 min. The observed sodium adduct, molecular ion, and aglycone fragments in +ESI-MS m/z were at 487.1 $[M+Na]^+$, 465.1 $[M+H]^+$, 303.1 $[Aglycone+H]^+$ while deprotonated dimer, glycoside, and aglycone were recorded in -ESI-MS as 927.1 $[Dimer-H]^-$, 463.1 $[M-H]^-$, 301 $[Aglycone-H]^-$. The molecular ions and fragments of Isorhamnetin-3-O-rhamnosylglucoside 663.2

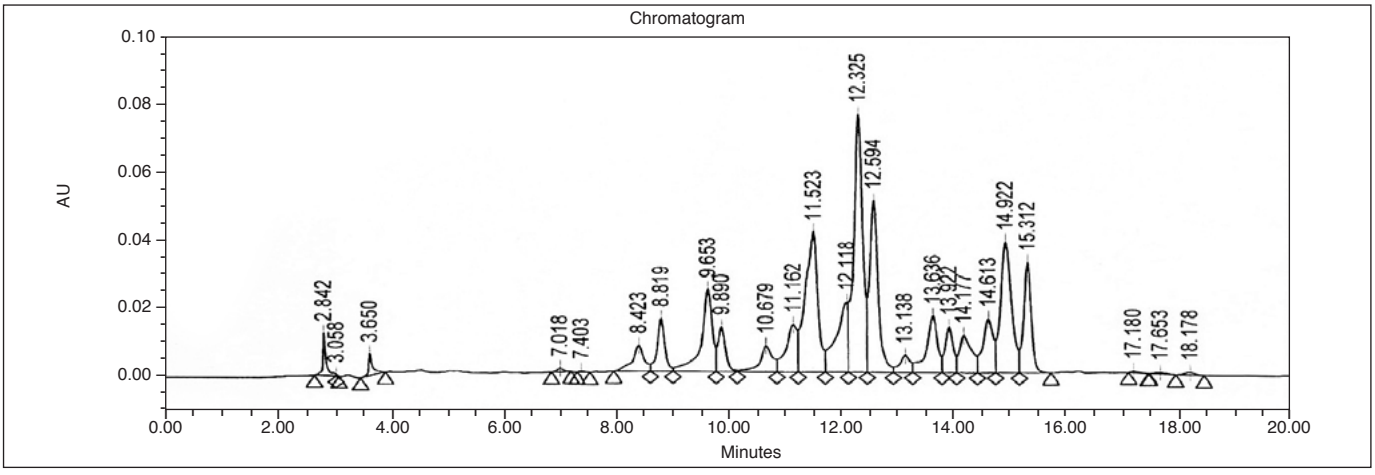

FIGURE 1. HPLC chromatogram of *T. coriacea* leaf methanolic extract.

$[M+K]^+$, 647.2 $[M+Na]^+$, 625.2 $[M+H]^+$, and 623.1 $[M-H]^-$ and Isorhamnetin-3-O-glucoside 501.1 $[M+Na]^+$, 479.1 $[M+H]^+$, 317.1 [Aglycone+H]$^+$ & 477.1 $[M-H]^-$ were observed between 12.5-12.8 and 13.8-14.1 min respectively. The identity of former was established by the plant metabolomics standard MS data by IFR, UK and later compound was recognized with the reports of Makris and Kefalas; Scheiber et al.[40,59]. Beside these compounds, the spectra also revealed presence of Kaempferol and its fragments. The observed characteristic m/z values and corresponding fragments were 121 [0, 2 B^+ ring fragment], 133 [1,3B^+ ring fragment], 213 [M+H-H2O-2CO]$^+$, 231 [M+H-2CO]$^+$, 259 [M+H-CO]$^+$, 287 $[M+H]^+$, and 309 $[M+Na]^+$ (data not shown) Tsimogiannis et al.[63]. The LC-MS analysis results are summarized in the Table 2.

• Results of Aspirin induced ulcerogenesis in pylorus ligated rats model

The results are shown in Table 3.

• Effect of TCLME on Volume of gastric juice

TCLME 250 & 500 mg/kg and standard drug exhibited potent anti-secretory effect. All the above treatments decreased the volume of gastric juice by $P<0.001$ while the decrease with TCLME 150 mg/kg was found to be $P<0.01$.

• Effect of TCLME on pH of gastric juice

Ranitidine elevated the pH of gastric juice with $P<0.001$ while all the doses of test extract raised pH with $P<0.05$.

• Effect of TCLME on free and total acidities

Ranitidine 50 mg/kg reduced the free and total acidity with $P<0.001$ and $P<0.01$ respectively. TCLME 500 mg/kg was the only dose that reduced both free and total acidities ($P<0.01$ and $P<0.05$ respectively). TCLME 125 mg/kg did not show any significant reduction in free and total acidities whereas TCLME 250 mg/kg reduced only free acidity by $P<0.01$.

TABLE 2. Identification of flavonoids present in *Terminalia coriacea l*eaf methanolic extract by LC-MS analysis

LC-MS Retention Time in min.	Identified Compounds & m/z values	ESI +/- Mode Mass Fragments	% Area
7.7 – 7.9	Quercetin-3-O-rutinoside [610]	633.1 $[M+Na]^+$	0.7469
8.1 – 8.4	Luteolin-7-O-glucoside [448]	449.2 $[M+H]^+$, 413.2, 329.1, 299.1 447.0 $[M-H]^-$	1.7445
8.9 – 9.2	Myricetin hexoside [480]	479.0 $[M-H]^-$ & 316 [Aglycone-H]$^-$	4.8938
10.6 – 10.8	Apigenin-6-C-glucoside [432]	433.2 $[M+H]^+$, 397.2, 367.1, 313.2 431.1 $[M-H]^-$	11.7753
11.1 – 11.3	Quercetin-3-O-glucoside [464]	487.1 $[M+Na]^+$, 465.1 $[M+H]^+$, 303.1 [Aglycone+H]$^+$ 927.1 [Dimer-H]$^-$, 463.1 $[M-H]^-$, 301 [Aglycone-H]$^-$	15.3687
12.5 – 12.8	Isorhamnetin-3-O-rhamnosylglucoside [624]	663.2 $[M+K]^+$, 647.2 $[M+Na]^+$, 625.2 $[M+H]^+$ 623.1 $[M-H]^-$	2.3087
13.8 - 14.1	Isorhamnetin-3-O-glucoside [478]	501.1 $[M+Na]^+$, 479.1 $[M+H]^+$, 317.1 [Aglycone+H]$^+$ 477.1 $[M-H]^-$	5.3556

TABLE 3. Results of Aspirin induced ulcerogenesis in pylorus ligated rats model

Parameters	Negative Control	Standard Drug	Test-I TCLME 125 mg/kg	Test-II TCLME 250 mg/kg	Test-III TCLME 500 mg/kg
Volume of Gastric Juice (mL / 100 g)	4.51±0.26	1.90±0.27^c	3.10±0.22^b	2.81±0.22^c	1.86±0.22^c
pH of Gastric Juice	2.93±0.24	4.46±0.16^c	3.68±0.13^a	3.73±0.10^a	3.70±0.17^a
Free Acidity (mEq / L / 100 g)	39.67±1.20	22.00±1.88^c	41.67±1.62^{ns}	32.17±1.10^b	31.33±0.91^b
Total Acidity (mEq / L / 100 g)	90.50±3.06	78.00±0.96^b	83.33±1.64^{ns}	84.17±2.12^{ns}	80.17±1.53^a
Ulcer Index	12.75±1.03	2.75±0.38^c	6.16±0.44^c	4.58±1.04^c	1.16±0.24^c
Mucin Content (µg / g tissue)	0.49±0.02	0.82±0.07^a	1.02±0.04^c	1.30±0.05^c	1.62±0.11^c
Catalase (µMol H_2O_2 utilized / min / mg tissue)	3.56±0.14	8.59±0.14^c	4.20±0.11^b	4.32±0.05^b	6.06±0.12^c
Superoxide dismutase (µMol H_2O_2 utilized / min / mg tissue)	3.25±0.11	5.83±0.27^c	4.27±0.11^b	4.55±0.16^c	4.57±0.21^c
Malondialdehyde (no. moles / mg tissue)	8.01±0.08	7.28±0.06^b	6.99±0.10^c	6.43±0.16^c	6.08±0.14^c
Total Protein (µg / mL gastric juice)	341±4.31	321.3±4.68^a	314.8±5.04^b	312.5±3.0^c	308.7±3.52^c

Sample size (n=6) rats per group. Data is expressed as Mean ± Standard Error of Mean. ^{a} <0.05, ^{b} <0.01, ^{c} <0.001 and ^{ns} non-significant versus Negative Control (on statistical analysis with ANOVA, followed by Dunnett's multiple comparison *post-hoc* test). Ranitidine 50 mg/kg was the standard drug. TCLME stands for *Terminalia coriacea* leaf methanolic extract.

• Effect of TCLME on ulcer index

All the doses of test extract and the standard drug displayed strong anti-ulcer effect as all the treatments lead to decrease in ulcer index by $P<0.001$ when compared to the negative control (as shown in Figure 2).

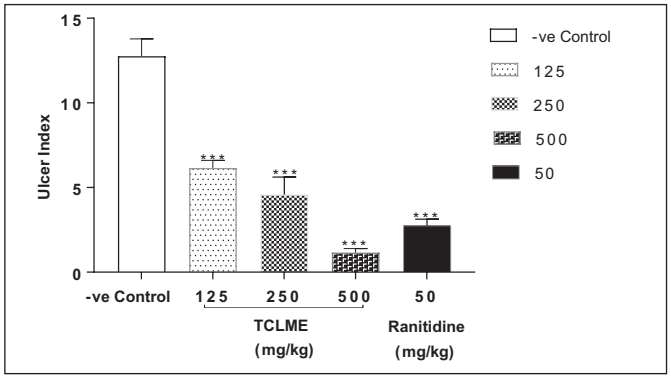

FIGURE 2. Ulcer index in Aspirin induced ulcerogenesis in pylorus ligated rats model.

• Effect of TCLME on gastric mucin content

TCLME 125-500 mg/kg enhanced the production of gastric Mucin with $P<0.001$. Though Ranitidine showed a significant effect ($P<0.05$) but it not as effective as the test extracts (as shown in Figure 3).

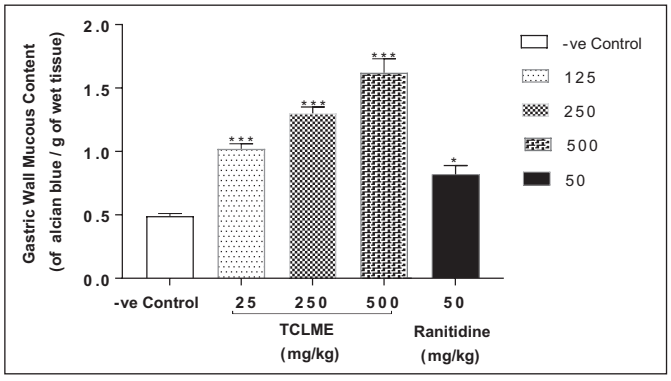

FIGURE 3. Gastric wall mucous content in Aspirin induced ulcerogenesis in pylorus ligated rats model.

• Effect of TCLME on Total Protein (TP)

Although significant inhibition but of varying levels ($P<0.01$ - $P<0.001$) was seen with the treatment of TCLME on depletion of proteins from gastric tissue. In all the test extract treated groups the effect was better than the standard that showed $P<0.05$ (as shown in Table 3).

• Effect of TCLME on Malondialdehyde (MDA)

All the test extracts demonstrated greater degree of inhibition on lipid peroxidation ($P<0.001$) while the standard suppressed level of MDA by $P<0.01$ (as shown in Table 3).

• Effect of TCLME on Catalase (CAT)

Ranitidine 50 mg/kg and TCLME 500 mg/kg increased the levels of Catalase with $P<0.001$ whereas TCLME 125 & 250 mg/kg improved the levels by $P<0.01$ (as shown in Table 3).

• Effect of TCLME on Superoxide dismutase (SOD)

Ranitidine and the higher test dose (250 & 500 mg/kg) of TCLME upgraded the level of Superoxide dismutase by $P<0.001$. TCLME 125 mg/kg promoted SOD with $P<0.01$ (as shown in Table 3).

Results of Acute ethanol induced gastric lesions model

• Effect of TCLME on Ulcer Index of acute ethanol induced gastric lesions

The gastroprotective action of TCLME was also evident in ethanol induced gastric lesions model. TCLME at all the test doses diminished the ulcer index like standard with $P<0.001$ v*s.* Control Table 4 and Figure 4. The macroscopic images of stomachs of rats subjected to this model are shown in Figure 5.

TABLE 4. Result of Ulcer Index in Ethanol-induced gastric lesions

Parameters	Negative Control	Standard Drug	Test-I TCLME 125 mg/kg	Test-II TCLME 250 mg/kg	Test-III TCLME 500 mg/kg
Ulcer Index†	10.17±0.24	8.08±0.30*	8.16±0.24*	5.91±0.35*	4.66±0.16*

Sample size (n=6) rats per group. Data is expressed as Mean ± Standard Error of Mean. * <0.001 versus Negative Control (on statistical analysis with ANOVA, followed by Dunnett's multiple comparison *post-hoc* test). Misoprostol 100 µg/kg was the standard drug.

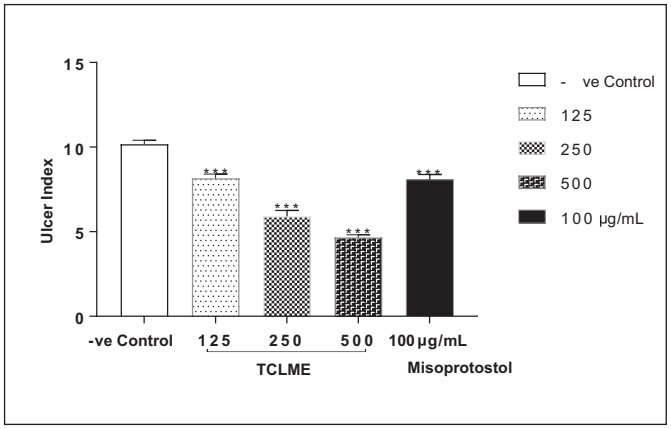

FIGURE 4. Ulcer Index in Ethanol induced gastric lesions model.

• Effect of TCLME on histology of gastric tissues

(A) *Negative Control.* The stomachs of negative control group showed degenerated epithelial cells, severe haemorrhage, necrosis, vascular congestion and marked inflammatory infiltration.

(B) *Standard Misoprostol (100 µg/kg).* Grossly, the mucosa of standard group showed intact epithelial lining. The sub-mucosa was found to be mostly normal, however mild haemorrhage was seen in few stomachs.

(C) *TCLME (125 mg/kg).* The stomachs of rats treated with TCLME 125 mg/kg revealed moderate haemorrhage, mild oedema, and few scattered inflammatory cells.

(D) *TCLME (250 mg/kg).* Mild inflammatory infiltration and congestion of vascular spaces was noticed in stomachs of group that received TCLME 250 mg/kg.

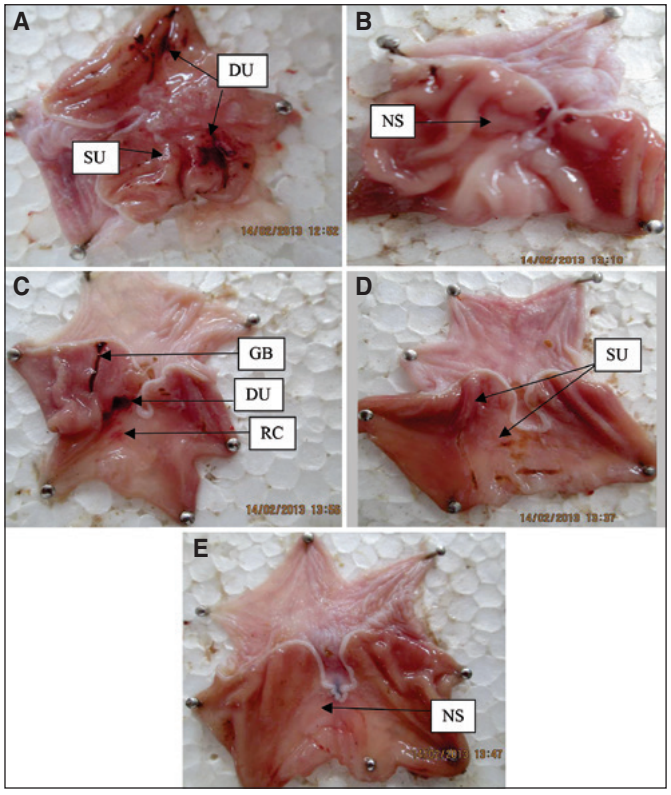

FIGURE 5. Photographs of stomachs subjected to Ethanol induced gastric ulcers. **(A)** Negative Control; **(B)** Standard (Misoprostol 100 g/kg); **(C)** Test-I (TCLME 125 mg/kg); **(D)** Test-II (TCLME 250 mg/kg) and **(E)** Test-III (TCLME 500 mg/kg). Where **DU** - Deep Ulcer, **GB** - Gastric Bleeding, **NS** - Normal Stomach, **RC** - Red Coloration, **SU** - Spot Ulcer and **TCLME** - *Terminalia coriacea* leaf methanolic extract.

(E) *TCLME (500 mg/kg)*. The treatment of TCLME 500 mg/kg prevented gastric ulceration. On examination the stomachs were found to be normal with intact epithelial lining and no signs of injury. Histopathological slides of rat's stomachs are shown in Figure 6.

All these flavonoids are reported in the allied species. Quercetin-3-O-glucoside was isolated from aerial parts of *T. muelleri*[55] while Quercetin-3-O-rutinoside (Rutin) is reported in *T. catappa*[38,44]. Luteolin-7-O-glucoside (Cynaroside) is reported to be present in *T. arjuna*[49]. Isorhamnetin glycosides was isolated from *T. chebula* leaves whereas Apigenin-6-C-glucoside (Isovitexin), Kaempferol and Quercetin glycosides are reported in *T. arjuna*, *T. catappa*[2,38,44]. Myricetin hexoside is present in *T. ferdinandiana*[45]. Flavonoids are being extensively studied for their beneficial effects on human health and literature reveals that they produce no or very little toxicity[21,41]. Moreover, they are considered to be responsible for the therapeutic effects of many traditional herbs. The gastroprotective role of flavonoids is well documented[47,58,62,65]. More than 95 flavonoids have been studied for their effectiveness in PUD. About 42 flavonoids were found to be inactive. The inactivity varies broadly with the experimental model, animal used in the study, route of administration and dose[46]. Flavonoids exhibit anti-secretory[10], anti-spasmodic[37], anti-ulcer[12], and anti-diarrhoeal[10] properties. The other actions like anti-inflammatory and anti-platelet effects are the secondary actions or alternative mechanisms that support therapeutic actions of flavonoids on gastrointestinal tract[11]. The biochemical and pharmacological actions of flavonoids are attributed to their strong antioxidant potential[34]. The antioxidant mechanism of action of flavonoids, especially rutin and quercetin, is mainly due to the presence of an O-dihydroxy in the B ring (catechol), and additionally a 2, 3 double bond in conjugation with a 4-oxo function, as well as the presence of hydroxyl groups in positions 3, 5 and 7 in their structures[21,51,56]. These compounds cover a full range of activity from weak to strong. Apigenin is inactive[4] while Kaempferol exhibited gastroprotection at doses 50 & 100 mg/kg[22]. Luteolin-7-O-glucoside (Cynaroside) at 47.4 mg/kg and Myricetin at a dose of 0.05 mL/g were found to be active anti-ulcer compounds in mice and rats on screening with reserpine induced gastric ulcers model[4,6,53].

Rutin prevents gastric mucosal ulceration in wide-range of *in-vivo* models like absolute ethanol, acidified-ethanol, and reserpine induced gastric ulcers[3,12,22]. The cytoprotective effect is mediated by dose dependent inhibition of mucosal platelet-activating factor (PAF) and anti-oxidant mechanism[22]. In a study, Rutin at a dose of 200 mg/kg, reduced level of lipid peroxides and increased the activity of anti-oxidant enzymes like glutathione peroxidase. The beneficial effects of Rutin do not involve effects on endogenous prostaglandins and non-protein sulfhydryls[12]. Quercetin is one of the most studied flavonoids that protects gastrointestinal mucosa from acute lesions induced by various methods like absolute ethanol[23,42,54], acidified-ethanol[22], aspirin[54], indomethacin, pyloric ligation[43], reserpine[3-6] and restraint stress[22] induced gastric ulcers.

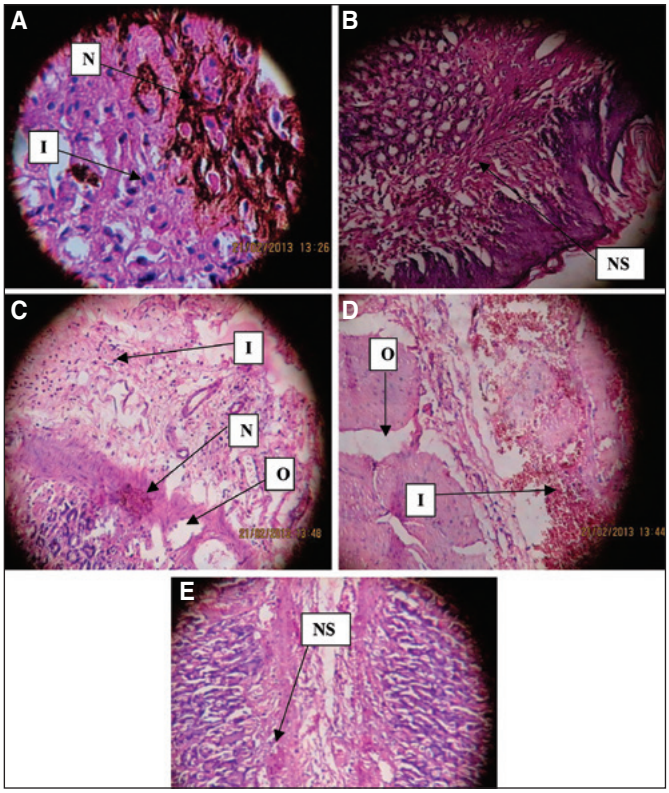

FIGURE 6. Histopathological slides of rat's stomachs subjected to Ethanol induced gastric ulcers. **(A)** Negative Control; **(B)** Standard (Misoprostol 100 g/kg); **(C)** Test-I (TCLME 125 mg/kg); **(D)** Test-II (TCLME 250 mg/kg) and **(E)** Test-III (TCLME 500 mg/kg) where **I** - Inflammatory infiltration, **N** - Necrosis, **NS** - Normal Stomach, **O** - Oedema and **TCLME** - *Terminalia coriacea* leaf methanolic extract.

Quercetin (3, 5, 7, 3', 4' - pentahydroxy-flavonol) is also one of the compounds that shows diverse mechanisms of action like increase in mucous production[42], enhancement of mucosal sulfhydryl[23] and anti-oxidant mechanism (increase in superoxide dismutase)[35], anti-histaminic properties like decrease in histamine level, reduction in number of mast cells, PAF[22], inhibition of *H. pylori* growth, lipid peroxidation[23,42] and formation of acid by parietal cells[7].

CONCLUSION

It is concluded that Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. possesses strong dose-dependent gastroprotective action and it can be mainly attributed to the flavonoids present in it. The identified flavonoids Kaempferol, Luteolin, Myricetin, Quercetin and Rutin possess weak to strong anti-ulcer activity. The present study highlights that TCLME exhibits gastroprotective action predominantly due to its anti-secretory, anti-ulcer, and anti-oxidant effects besides prevention of lipid peroxidation and induction of mucous secretion.

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Authors' contributions

Dr. Mohammed Safwan Ali Khan designed the study, performed the *in-vivo* experiments, histopathology, statistical analysis and drafted the manuscript. Miss. Shaaz Nazan performed the HPLC-UV, LC-MS analysis and took part in biochemical studies. Prof. Dr. Abdul Manan Mat Jais assisted the whole project and helped in the preparation of manuscript. All authors read and approved the final manuscript.

Ali Khan MS, Nazan S, Mat Jais AM. A ação gastroprotetora antioxidante e flavonoide de extrato metanólico de folhas de Leathery Murdah, *Terminalia coracea* (Roxb.) Wight & Arn. em ratos. Arq Gastroenterol. 2017;54(3):183-91.

RESUMO – **Contexto** – Leathery Murdah, *Terminalia coriacea* (Roxb.) Wight & Arn. da família *Combretaceae* é usada nos tradicionais sistemas da medicina Ayurveda e Siddha para cicatrização de úlceras. **Objetivos** – O presente estudo foi realizado para avaliar o efeito gastroprotetor e para esclarecer o mecanismo fundamental da ação do extrato metanólico de folhas de Leathery Murdah, *Terminalia coracea* (Roxb.) Wight & Arn. **Métodos** – O extrato teste foi testado para ação antiulcerogênica induzida pela Aspirina através da ligação pilórica e úlceras gástricas induzidas por etanol em três doses – 125, 250 e 500 mg/kg, via oral, utilizando-se Ranitidina 50 mg/kg e Misoprostol 100 µg/kg como drogas padrão nos respectivos modelos. Sete parâmetros foram cuidadosamente analisados tais como índice ulcerogênico, níveis de proteínas totais, de mucina, de catalase, de malondialdeído e de superoxido dismutase, além da histopatologia. A análise do perfil espectroscópico pela Cromatografia Líquida de Alta Eficiência - Ultravioleta e análise crua pela Cromatografia Líquida - Espectrometria de Massas foram realizadas como parte da caracterização química para identificar os componentes bioativos. **Resultados** – Todas as doses utilizadas exibiram função gastroprotetora, em particular as doses mais elevadas. Os testes revelaram aumentos significantes de catalase, superóxido dismutase e mucina, com diminuição do índice ulcerogênico, dos níveis de proteínas totais, e de malondialdeído. As observações histopatológicas também ilustraram o efeito gastroprotetor do extrato metanólico de folhas de *Terminalia coracea*. **Conclusão** – O extrato metanólico de folhas de *Terminalia coracea* mostrou forte atividade antioxidante e antissecretória além de induzir a produção de mucosa gástrica. A resposta farmacológica observada pode ser atribuída aos compostos flavonoides denominados Quercetin-3-O-rutinosideo, Luteolin-7-O-glucosideo, Myricetin hexosideo, Quercetin-3-O-glucosideo, Isorhamnetin-3-O-rhamnosylglucosideo e Isorhamnetin-3-O-glucosideo, identificados no extrato pela primeira vez pelas análises de Cromatografia Líquida de Alta Eficiência - Ultravioleta e Cromatografia Líquida - Espectrometria de Massas.

DESCRITORES – Mucosa gástrica. *Terminalia*. Extratos vegetais. Úlcera péptica.

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