Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2015, **6**, 842

Safety and anti-ulcerogenic activity of a novel polyphenol-rich extract of clove buds (*Syzygium aromaticum* L)

Abin Issac,[a] G. Gopakumar,[a] Ramadasan Kuttan,[b] Balu Maliakel[a] and I. M. Krishnakumar*[a]

Received 7th August 2014,
Accepted 12th December 2014

DOI: 10.1039/c4fo00711e

www.rsc.org/foodfunction

Despite the various reports on the pharmacology of Clove bud [*Syzygium aromaticum*]-derived essential oil and its major component eugenol, systematic information on the bioactivity of clove polyphenols is very limited. Clove buds being one of the richest sources of dietary polyphenols with many traditional medicinal uses, the present contribution attempted to derive their standardized polyphenol-rich extracts as a water soluble free flowing powder (Clovinol) suitable for functional food applications, without the issues of its characteristic pungency and aroma. The extract was characterized by electrospray ionization-time of flight mass spectrometry (ESI-TOF-MS), and investigated for *in vivo* antioxidant, anti-inflammatory and anti-ulcerogenic activities. Clovinol showed significant antioxidant and anti-inflammatory effects as measured by cellular antioxidant levels, and the ability to inhibit carrageenan-induced paw swelling in mice. Further investigations revealed its significant anti-ulcerogenic activity (>97% inhibition of ethanol-induced stomach ulcers in Wistar rats when orally administered at 100 mg per kg b.w.) and up regulation of *in vivo* antioxidants such as superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT). Clovinol also reduced the extent of lipid peroxidation among ulcer induced rats, indicating its usefulness in ameliorating oxidative stress and improving gastrointestinal health, especially upon chronic alcohol consumption. The extract was also shown to be safe and suitable for further investigations and development upon acute toxicity studies at 5 g per kg body weight and 28 days of repeated dose toxicity studies at 2.5 g per kg b.w.

1. Introduction

Gastrointestinal health is regarded as one of the primary markers of a healthy life. Various disorders of gastrointestinal functions, such as digestive issues, dyspepsia, gastritis, acidity, constipation and haemorrhage were found to contribute significantly to poor quality of life. Despite significant medical advancements, the incidence of gastric ulcers, a chronic degenerative disease which disrupts the mucosal integrity of the stomach and/or duodenum leading to a local defect and active inflammation, was found to be growing globally.[1,2] Aging, use of non-steroidal anti-inflammatory drugs (NSAID), chronic consumption of alcohol, smoking, and consumption of highly acidic and fat rich food/beverages and malnutrition were identified as the basic reasons for the development of gastric ulcers.[3,4] The pathogenesis of gastric ulcers includes chronic inflammation and subsequent generation of oxygen derived free radicals and lipid peroxidation leading to the elevation of oxidative stress, in turn causing even cancer.[5,6] Though many synthetic drugs are available for the treatment of gastric ulcers, the development of sustainable natural agents exhibiting significant efficacy in preventing and/or relieving the asymptotic complications associated with various ulcerative conditions is of great importance.[7] The situation has thus created growing interest in identifying and developing novel botanical extracts and phytochemicals from edible plants, such as GRAS (Generally Regarded as Safe)-listed vegetables, fruits and spices, as optimal sources for supplementing the human diet to mitigate disease states of inflammation and oxidative stress.[8,9]

Clove buds, a popular class of kitchen spice referred to as 'the champion of spices', have been extensively studied and reported to possess various beneficial health and pharmacological effects, such as antidiabetic, hypolipidemic, gastroprotective and immunomodulatory activities.[10–13] Though the essential oil and its major constituent eugenol have been regarded as the most bioactive aspects, clove buds are also rich

[a]*Akay Flavours & Aromatics Ltd., R&D Centre, Ambunadu, Malayidamthuruthu P.O., Cochin – 683561, India. E-mail: Krishnakumar.IM@akay-group.com; Fax: +91 484 2680891; Tel: +91 484 2686111*
[b]*Amala Cancer Research Centre, Amala Nagar P.O., Trichur – 680555, India*

in non-volatile antioxidants belonging to the group of polyphenols comprising flavonoids, aromatic hydroxy acids, hydrolysable tannins and their glycosylated derivatives.[14,15] Though many studies on the hydro-alcoholic extracts of clove have reported its antioxidant/radical scavenging properties and ability to prevent/control oxidative stress mediated diseases,[11,16–18] no systematic investigations have so far been reported on the production of standardised polyphenol-rich extracts and their easy to use formulations possessing definite bioactivities for functional use. The present contribution describes the selective extraction, characterization and formulation of clove bud polyphenols as a standardized free flowing water soluble powder (*herein after referred to as 'Clovinol'*) as an ingredient suitable for ready use in dietary supplements, functional foods and beverages. High performance liquid chromatography-coupled with electrospray ionization time of flight mass spectrometry (HPLC-ESI-TOF-MS) was exploited to gather comprehensive information about the polyphenols in 'Clovinol' and further demonstrated its safety by sub-acute repeated dose toxicity studies (28 days) employing Wistar rats. Clovinol, having high antioxidant and anti-inflammatory activities was further investigated for its probable effects on gastrointestinal health, particularly upon alcohol induced ulcers and subsequent oxidative stress and inflammation, by analysing its ulcer preventive and/or curative effect *in vivo*. A detailed investigation by monitoring haematology, histology and various other biochemical parameters along with the tissue antioxidant status and lipid peroxidation was carried out to better understand the nexus of 'Clovinol' on ulcerative conditions.

2. Materials and methods

2.1. Preparation of Clovinol

Dried clove buds were received from a selected farm in Indonesia. The samples were identified by an authenticated botanist and a voucher specimen (AK-CLV-011) was deposited at the Herbarium of M/s Akay Flavours & Aromatics Ltd, Cochin, India. 5 kg of clove buds were powdered and extracted in a stainless steel vessel fitted with an agitator and pump to circulate the solvent. The extraction was performed with three to five times excess of an ethanol–water mixture (70 : 30 v/v) for 3 h under ambient conditions. The filtered extract was combined and evaporated below 50 °C under vacuum. The aqueous solution obtained after evaporation of ethanol was further concentrated and kept at 15 °C for 6 h. It was further filtered, homogenized with gum acacia and spray dried to a brownish free flowing Clovinol powder (inlet temperature: 180 °C and outlet temperature: 95 °C) with a polyphenol content of 38.6% gallic acid equivalent (GAE). Total polyphenols were measured by the standard Folin–Ciocalteu test using gallic acid as the standard.[19] HPLC analyses were carried out on a Shimadzu model LC 20 AT, with an M20A photo diode array (PDA) detector (Shimadzu Analytical India Pvt Ltd, Mumbai, India), fitted with a reverse phase C18 column (250 × 4.6 mm, 3 μm) (Phenomenex, Hyderabad, India). Characterization of polyphenols was achieved by 1290 infinity Ultra-performance liquid chromatography (UPLC) system coupled with an Agilent 6530 QTOF instrument having a Jet-Stream source (Agilent India Pvt Ltd, Bangalore, India). Ammonium acetate (10 mM) in water (A) and methanol (B) was employed as the mobile phase with a Zorbax Eclipse Plus C18 (3.0 × 100 mm; 1.8 μm) column at 30 °C and 5 μL injection volume.

2.2. Animals

Wistar rats (150–200 g body weight) were used for toxicological studies and ulcer experiments. Male Swiss albino mice (25–30 g) were used for anti-inflammatory studies. The animals were procured from small animal's breeding station (SABS), Veterinary College, Mannuthy, Kerala, India and were acclimatized for a period of 14 days in ventilated cages and housed in an air-conditioned room at 22 ± 2 °C, and relative humidity of 60 ± 5% with a 12 h light and dark cycle, at the animal house facility of M/s Amala Cancer Research Centre, Kerala, India. All animal experiments were carried out in strict accordance with the ethical norms approved by the Institutional Animal Ethics Committee (IAEC) recognized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (registration no: 149/99/CPCSEA). Animals were provided with a pellet diet (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*.

2.3. Toxicity studies

2.3.1. LD_{50} of 'Clovinol'. An acute toxicity study on Clovinol was carried out according to the method of Lorke,[20] employing adult Wistar rats of both sexes, having an average body weight between 150 and 200 g. Twenty four animals were divided into four groups, each consisting of three male and three female rats as follows. Group I (Normal) was administered with the vehicle (1 mL water) and Groups II, III and IV were orally administered with Clovinol at doses of 1.25, 2.5 and 5 g per kg b.w., respectively. The animals were observed for 24 h for any signs of toxicity, mortality, and adverse reactions.

2.3.2. Sub-acute toxicity (28 days) study of Clovinol. Forty Wistar rats (20 males and 20 females) of average weight between 150 and 200 g were selected by stratified randomization and then divided into four groups as shown below, each consisting of 5 males and 5 females of approximately the same body weight. Clovinol was suspended in distilled water and orally administered to the animals using an oral needle in such a way that all the animals received the same volume of the vehicle.

Group I – Normal (1 mL water)
Group II – Clovinol (2.5 g per kg b.w.)
Group III – Clovinol (1.0 g per kg b.w.)
Group IV – Clovinol (0.5 g per kg b.w.)

The animals were monitored during the study period of 28 days for any type of clinical symptoms, mortality, and adverse reactions of the administered extract. Body weight and

food consumption were determined every 3 days. The food consumption was determined by measuring the left over feed after 24 h and was noted for a single cage of 5 animals. Animals were sacrificed on day 29 by cervical dislocation under ether anesthesia; all the organs were examined visibly for any type of abnormalities in the structure; blood was collected by the direct heart puncture method into EDTA coated and non-EDTA vials for analyzing hematological parameters and serum biochemistry. Red blood cell (RBC) count, total and differential white blood cell (WBC) count, platelet levels and haemoglobin (Hb) content were determined using a haematology analyzer (Model-Diatron, Wein, Austria). Serum was separated from the clotted blood sample by centrifuging at 5000 rpm for 10 min at −4 °C and was stored in a clean sample bottle at −20 °C for the analysis of biochemical parameters such as total protein, albumin, bilirubin, cholesterol, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), serum urea and creatinine following the standard laboratory procedures. The organs (liver, kidney, brain and spleen) were excised, extraneous tissues were trimmed off and the weight was noted. Samples of all organs from each group were taken and fixed in 10% formalin and dehydrated for histopathological examinations. Ultrathin tissue sections were de-waxed by xylene and stained with haematoxylin and eosin, and the histopathological examinations were performed with an optical microscope of 100× magnification (Olympus-Magnus trinocular microscope, Noida, India).

2.4. Anti-inflammatory activity: carrageenan induced paw oedema model

Male Swiss albino adult mice of body weight 25 to 30 g were divided into five groups, each comprising six animals. Acute inflammation was induced by sub-plantar injections of 0.02 mL of a freshly prepared 1% suspension of carrageenan at the right hind paw of animals. Group I was treated with carrageenan alone and Group II was intraperitoneally administered with diclofenac (10 mg per kg b.w.) as a standard reference drug. Groups III, IV and V were orally administered with 25, 50, 100 mg per kg b.w. of Clovinol, respectively. The inflammation was then measured using Vernier calipers one hour before and at different intervals of 1 h followed by the carrageenan injection. The percentage inhibition was then calculated as follows.

$$\%\ \text{Inhibition} = [(V_T - V_0)_{\text{control}} - (V_T - V_0)_{\text{treated group}} / (V_T - V_0)_{\text{control}}] \times 100$$

where, V_T – paw oedema at various time intervals and V_0 – initial paw oedema.

2.5. Antiulcer activity studies

2.5.1. Antiulcer activity with pretreatment of 'Clovinol'. Thirty six Wistar rats were divided into six groups containing six animals each and were treated with various doses of Clovinol and 50 mg per kg b.w. of standard drug Ranitidine through oral gavage using an oral needle (detailed below).

Group I – Normal (no treatment)

Group II – Control (80% ethanol alone, 5 mL kg^{-1})

Group III – Ranitidine (50 mg kg^{-1}) + 80% ethanol (5 mL kg^{-1})

Group IV – Clovinol (25 mg kg^{-1}) + 80% ethanol (5 mL kg^{-1})

Group V – Clovinol (50 mg kg^{-1}) + 80% ethanol (5 mL kg^{-1})

Group VI – Clovinol (100 mg kg^{-1}) + 80% ethanol (5 mL kg^{-1})

The animals were given 80% (v/v) of ethanol to induce gastric ulcers as described by Lee *et al.*[21] Briefly, the rats were kept deprived of food for 24 h and again deprived of both food and water for another 12 h prior to ethanol administration. 1 h after Clovinol administration, each group of animals except the normal rats of Group I was treated with % ethanol at 5 mL per kg body weight intragastrically to induce ulceration. After 3 h, the animals were sacrificed by cervical dislocation, and the stomach of each animal was excised and washed with ice-cold saline. The ulcer index was then calculated by measuring the severity of the gastric mucosal lesion graded as Erosion grade 1: ≤1 mm mucosal lesion, Erosion grade 2: 2 mm mucosal lesion, Erosion grade 3: ≥2 mm mucosal lesion.

$$\begin{aligned}\text{Ulcer index} = {} & 1 \times [\text{no. of lesions of grade 1}] \\ & + 2 \times [\text{no. of lesions of grade 2}] \\ & + 3 \times [\text{no. of lesions of grade 3}] \ \div \ 10.\end{aligned}$$

A small piece of stomach was taken, washed with saline and kept in 10% formalin for histopathological analysis as mentioned under section 2.3.2. A portion of the stomach mucosa was scraped with a surgical blade and 10% homogenate was prepared with cold Tris HCl buffer (0.1 M, pH 7.4). The homogenate was analysed for both enzymatic and non-enzymatic antioxidant levels. Superoxide dismutase (SOD) activity was measured by the nitro blue tetrazolium (NBT) reduction method of McCord and Fridovich.[22] Catalase activity was estimated by the method of Aebi[23] by measuring the rate of decomposition of hydrogen peroxide. Glutathione (GSH) activity was assayed by the method of Moron *et al.*[24] based on the reaction with dithio nitrobenzoic acid (DTNB). The assay of glutathione peroxidase (GPx) followed the method of Hafeman *et al.*[25] based on the oxidation of GSH in the presence of H_2O_2. The quantitative estimation of lipid peroxidation was done by determining the concentration of thiobarbituric acid reactive substances (TBARS) in gastric mucosa using the method of Ohkawa *et al.*[26] which involved the estimation of the amount of malondialdehyde (MDA). The gastric mucosal content was determined according to the modified method of Corne *et al.*[27] The mucous levels were quantified using a standard curve of Alcian blue at 598 nm and the result was expressed in μg of Alcian blue per g of wet tissue.

2.5.2. Antiulcer activity with simultaneous treatment of 'Clovinol'. Thirty six Wistar rats were divided into six groups containing six animals in each group and treated with various

doses of Clovinol and the standard drug Ranitidine as shown under section 2.5.1. Ulcers were induced with 80% (v/v) of ethanol as described above, except for Groups III, IV and V, in which Clovinol was simultaneously administered at dosages 25, 50 and 100 mg per kg b.w., respectively, along with 80% ethanol. All the animals were sacrificed after 3 h and the stomach of rats were excised and washed with ice-cold saline. The ulcer index, antioxidant status of the mucosa, lipid peroxidation as TBARS and gastric mucosal content were also investigated as described under section 2.5.1.

2.6. Statistical analysis

The values are expressed as mean ± SD. Statistical analyses of the results were performed using one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test. A probability value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Preparation of 'Clovinol'

Matured and dried Indonesian clove buds with an average polyphenol content of 13.5% GAE and 6.75% volatile oil was sampled from a 1000 kg lot and used as the plant material for the present study. A process of hydro-ethanolic extraction followed by purification, evaporation at controlled temperature (<50 °C) and spray drying was developed for the preparation of polyphenol containing water soluble extract (Clovinol) at a yield of 8.8% with respect to the raw-material as a brownish free flowing powder with mild taste and aroma characteristic of clove. The final extract was found to contain 38.6% w/w polyphenols and 5.8% volatile oil. On nutritional analysis, the above extract used in the present study was found to contain 47.31% carbohydrate, 3.51% protein, 2.35% ash and 0.23% fiber. It was found to have 2.2% moisture, 0.38 g per L bulk density, with less than 400 cfu g^{-1} total aerobic plate count and 30 cfu g^{-1} total yeast and mold was obtained. No traces of *E. coli*, *Salmonella* or the like were detected, indicating the adherence to standard microbial specification for food ingredients.

Upon UPLC-ESI-TOF-MS analysis followed by the Molecular Feature Extraction algorithm and the Metlin database to identify the compounds and its empirical formula, 'Clovinol' was found to contain various types of polyphenols. Literature data and reference compound were also used for peak confirmation. Flavonoids, phenolic acids, and hydrolysable tannins and their glycosides were identified as the main groups of polyphenols in Clovinol. When analyzed under positive electrospray ionization, quercetin was observed as glucoside (*m*/*z* 463) and rhamnoside (*m*/*z* 447) as evident from their $[M + H]^+$ peaks. Luteolin glucoside (*m*/*z* 447.9), luteolin rutinoside (*m*/*z* 593.1), kaempferol glucuronide (*m*/*z* 461), apigenin hexosides (*m*/*z* 431.6), and apigenin rutinoside (*m*/*z* 577.4) were also observed along with the free aglycones. Quercetin (*m*/*z* 301), luteolin (*m*/*z* 288), and apigenin (*m*/*z* 271), were characterized by their characteristic fragmentations with respect to the reference compounds. Peaks observed at *m*/*z* 516.4, 396.34, 478.35, 492.38, and 354.3 were characterized as 6-methoxy-2-oxo-2*H*-chromen-7-yl-2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside, ethyl-4-(β-D-glucopyranosyloxy)-1,5-dihydroxy-2-naphthoate, 3,5,8-trihydroxy-2-(4-hydroxyphenyl)-4-oxo-4*H*-chromen-7-yl-β-D-allopyranosiduronic acid, 5,7-dihydroxy-2-(4-hydroxyl-3-methoxyphenyl)-4-oxo-4*H*-chromen-8-yl-β-D-threo-exopyranosiduronic acid, and magnolioside, respectively, using Metlin database. When analysed in the negative ionization mode, a number of phenolic acids and their derivatives were observed. Gallic acid (*m*/*z* 171.1), caffeic acid (*m*/*z* 181.3) chlorogenic acid (*m*/*z* 354.6), ellagic acid (*m*/*z* 301.4), and their derivatives such as the gallic acid ester of caffeic acid (*m*/*z* 333.4) were detected in Clovinol. The identical $[M - H]^+$ molecular ion peaks for quercetin and ellagic acid were distinguished by their molecular ion fragments; quercetin *m*/*z* 301 ion further fragmented to form characteristic *m*/*z* 179 and 151 ions whereas ellagic acid ion yielded ions at *m*/*z* 257 and 229.[28] In addition, molecular ion peaks corresponding to oleanolic acid at *m*/*z* 455.8 and eugenol at *m*/*z* 163.4 were also found in Clovinol. Thus, most of the flavonoids and phenolic acids found in Clovinol have already been shown to be widely present in the plant kingdom and were shown to possess varying beneficial health and pharmacological effects.

3.2. Toxicity studies

High dose administration of Clovinol (1.25 to 5 g per kg b.w.) did not produce any mortality, changes in clinical and/or behavior signs or adverse reactions during the lethal dose study. Body weight and food consumption of the animals also remained unchanged. Sub-acute toxicity studies of Clovinol at doses of 0.5, 1.0 and 2.5 g per kg b.w., also did not induce any mortality, abnormal clinical and/or behavioral signs or change in body weight as compared to that of the control group of animals, indicating its safety even upon continuous administration for 28 days, irrespective of the sex of animals.

During the 28 day study the body weight (g) of normal male animals increased from 157.20 ± 14.34 to 215.18 ± 12.67 with a growth rate of 2.07 ± 0.12 per day and that of female increased from 146.40 ± 8.02 to 205.58 ± 9.41 with a growth rate of 2.11 ± 0.12, respectively. There was no marked difference in weight gain and growth rate observed in male and female animals administered with Clovinol (0.5, 1.0 and 2.5 g per kg b.w.) when compared to the normal. The body weight (g) of male rats administered with high dose (2.5 g per kg b.w.) Clovinol increased from 164.26 ± 7.91 to 220.72 ± 9.87 with a growth rate of 2.02 ± 0.14 per day and that of female rats increased from 163.20 ± 8.76 to 224.84 ± 8.08 with a growth rate of 2.20 ± 0.17 per day, respectively; likewise for the Clovinol lower doses (0.5 and 1.0 g per kg b.w.). The relative weight (g) of different organs was found to be 3.51 ± 0.27, 0.44 ± 0.07, 0.70 ± 0.05 and 0.64 ± 0.09 for liver, spleen, kidney and brain respectively in normal male rats; and 3.56 ± 1.4, 0.43 ± 0.13, 0.76 ± 0.32 and 0.88 ± 0.28 for liver, spleen, kidney and brain respectively in normal female rats. The values were found to be similar in

the case of Clovinol administered animals also. In male rats administered with Clovinol (2.5 g per kg b.w.), the relative organ weight noted was 3.26 ± 0.30, 0.52 ± 0.20, 0.64 ± 0.21 and 0.68 ± 0.09 for liver, spleen, kidney and brain, respectively; and 3.53 ± 0.23, 0.53 ± 0.13, 0.75 ± 0.12 and 0.82 ± 0.23 for liver, spleen, kidney and brain respectively in normal female rats. There was no wide deviation even in the values in the case of animals administered with lower doses of Clovinol (0.5 and 1.0 g per kg b.w.).

Also Clovinol did not produce any significant ($p > 0.05$) changes in haematological and biochemical parameters. Haemoglobin, WBC, RBC, platelet counts and differential counts (*i.e.* lymphocyte, eosinophil and neutrophils) of treated animals remained in the normal range, when compared to the normal group (Table 1). Biochemical parameters related to hepatic and renal function have also showed normal values. Renal profile, liver function markers, creatinine, urea or the serum electrolyte levels in both male and female rats were comparable to the normal animals after 28 days of supplementation. The lipid profile remained unchanged with no significant ($p > 0.05$) variation in cholesterol, HDL, LDL and VLDL levels of both male and female rats, and was comparable to that of the normal group (Table 1).

Necropsy of the treated animals showed normal appearance for various organs and tissues. The relative weight of organs from male and female rats (liver, spleen, kidney and brain), administered with Clovinol did not show any significant ($p > 0.05$) difference when compared with the normal animals. The histopathological examination of various organs of animals treated with 2.5 g per kg b.w. Clovinol showed normal cellular architecture similar to the normal group (Fig. 1). The liver section of Clovinol treated animals showed normal portal triads and central venous system; normal hepatocytes were arranged in cords with Kupffer cells and showed normal sinusoidal spaces, which were identical with those from the normal animals. The tissue sections of spleen from Clovinol treated animals showed normal lymphoid follicles with areas prominent in germinal centers. The medullary region showed sinusoidal congestion, lymphostasis and histiocytic proliferation with cellular architecture and morphology similar to those of spleen from normal animals. The kidney tissues of Clovinol treated animals showed normal glomeruli with Bowman's capsule and renal tubules. The interstitial tissues appeared normal with no apparent abnormalities when compared with the tissues of the normal group of animals. The section of brain tissue of Clovinol administered rats showed hyperplasia of the astrocytes with pleomorphism. There were occasional mitotic cells, normal glial cells and the interstitial tissues. The cerebellum also appeared normal. Overall cellular architecture and morphology of the Clovinol treated animals were similar to the brain from normal animals.

3.3. Anti-inflammatory activity: carrageenan induced paw oedema model

Carrageenan-induced paw oedema in Swiss albino mice with reference to the standard drug diclofenac was followed to assess the anti-inflammatory activity of 'Clovinol' upon oral administration. Various doses of Clovinol (25, 50 and 100 mg per kg b.w.) were used for the study. The sub-plantar injection of carrageenan was found to produce severe oedema in mice paws, which reached a maximum at 3 h post-injection. The inhibition of oedema followed by Clovinol and diclofenac administration was compared at different time intervals for up to 5 h and also for 24 h of post-injection (Fig. 2). It was observed that the oral administration of Clovinol resulted in a dose-related reduction in the volume of carrageenan-induced paw oedema than in the untreated control, with a significant ($p < 0.05$) inhibition of 13.41, 21.95 and 47.34% respectively for 25, 50 and 100 mg per kg b.w. doses of Clovinol, when the oedema was measured after 3 h of injection. On the other hand, the standard drug diclofenac exhibited a relative inhibition of 52.70% upon intraperitoneal administration at (10 mg per kg b.w.).

3.4. Anti-ulcerogenic activity of Clovinol

3.4.1. Effect on ethanol-induced gastric ulcer. Oral administration of ethanol caused severe necrotic lesions throughout the glandular portion of rat stomach with an ulcer index of 7.88 ± 1.0. Pretreatment with Clovinol, significantly inhibited the formation of ethanol-induced gastric ulcers in a dose dependent manner. While the administration of 25 mg per kg b.w. Clovinol reduced the ulcer index to 6.41 ± 0.47 ($p < 0.01$) with 22.08% inhibition, the ulcer index followed by 50 mg per kg b.w. was 4.68 ± 0.76 ($p < 0.01$), with an average 40.6% inhibition and that at 100 mg per kg b.w. oral administration was only 1.82 ± 0.40 ($p < 0.01$) with an average ulcer inhibition of 78.9%. The observed ulcer inhibition with the standard drug ranitidine (50 mg per kg b.w.), was only 54.31% with an ulcer index of 3.60 ± 0.80 ($p < 0.01$).

The morphology of the stomach of normal animals in comparison with that of alcohol-induced ulcer bearing animals and Clovinol treated animals is depicted in Fig. 3A. Histopathological analysis of stomach tissues revealed that the mucosa of the alcohol treated control group of animals have haemorrhagic erosion, discontinuity in the lining of epithelium cells and significant damage in sub and muscularis mucosa. The standard drug ranitidine administration protected the stomach to a greater extent, but with mucosal disruption in some places. Small atrophic glands and mild hyperplasia without oedema were observed in these animals. Similarly, mucosa of animals administered with 25, 50 and 100 mg per kg b.w. of Clovinol showed significant protection in a concentration dependent manner. Administration of Clovinol at 100 mg per kg b.w., on the other hand, significantly reduced the gastric lesion formation and sub-mucosal oedema better than the standard drug ranitidine (Fig. 3B).

Measurements of the mucosal volume, mucosal antioxidant status and extent of mucosal lipid peroxidation upon ethanol administration and Clovinol treatment were also carried out in the present study to better understand the physiological actions of Clovinol upon chronic alcohol consumption. There was a significant dose dependent enhancement in the rat

Table 1 Effect of administration of Clovinol (28 days) on hematological and biochemical parameters in Wistar rats (male and female)[a]

	Normal		Clovinol 0.5 g kg^{-1}		Clovinol 1.0 g kg^{-1}		Clovinol 2.5 g kg^{-1}	
Parameters	Male	Female	Male	Female	Male	Female	Male	Female
Hematology								
Hb (g dL^{-1})	15.36 ± 0.84	14.28 ± 0.77	14.58 ± 1.84	14.9 ± 0.56	15.4 ± 0.96	14.92 ± 0.19	15.6 ± 1.39	14.38 ± 1.36
WBC (mm^3)	9140 ± 2059	9380 ± 2190	9760 ± 1348	9078 ± 3192	8920 ± 1590	8100 ± 1465	8780 ± 1295	8020 ± 2021
RBC × 10^6 $(mm^3)^{-1}$	7.6 ± 0.39	7.20 ± 0.35	7.24 ± 1.05	7.54 ± 0.22	7.54 ± 0.37	7.35 ± 0.32	7.85 ± 0.76	7.02 ± 0.60
Platelet × 10^5 $(mm^3)^{-1}$	8.05 ± 1.64	8.11 ± 1.24	6.38 ± 1.95	8.82 ± 0.69	6.39 ± 0.76	8.81 ± 0.55	5.97 ± 0.58	7.79 ± 0.53
Lymphocyte (mm^3)	4045 ± 900	3046 ± 1040	4186 ± 376	4035 ± 534	4116 ± 482	4026 ± 391	4553 ± 836	4265 ± 741
Eosinophils (mm^3)	879 ± 354	620 ± 223	512 ± 145	523 ± 219	728 ± 129	745 ± 197	561 ± 111	574 ± 132
Neutrophils (mm^3)	1716 ± 836	1540 ± 501	1532 ± 378	1425 ± 339	1623 ± 256	1527 ± 983	1490 ± 570	1627 ± 324
Biochemical								
SGPT (U L^{-1})	60.40 ± 4.56	59.00 ± 2.91	69.20 ± 4.43	49.80± 2.91	72.60 ± 2.88*	62.00 ± 11.14	57.60 ± 4.77	50.80 ± 9.77
SGOT (U L^{-1})	136.00 ± 15.86	148.00 ± 15.07	148.00 ± 15.86	157.80 ± 46.90	133.20 ± 17.61	138.80 ± 22.46	140.00 ± 18.44	148.60 ± 33.47
ALP (U L^{-1})	347.00 ± 36.20	176.00 ± 60.20	353.80 ± 38.03	288.40 ± 22.23**	335.20 ± 21.18	336.20 ± 26.31**	387.20 ± 22.02	415.40 ± 15.45**
Bilirubin (mg dL^{-1})	0.42 ± 0.07	0.30 ± 0.05	0.42 ± 0.08	0.42 ± 0.09	0.38 ± 0.08	0.32 ± 0.08	0.44 ± 0.05	0.36 ± 0.05
A/G ratio	1.16 : 1 ± 0.67	1.40 : 1 ± 1.04	1.49 : 1 ± 0.39	0.62 : 1 ± 0.34	1.26 : 1 ± 0.23	1.01 : 1 ± 0.24	1.02 : 1 ± 0.23	1.31 : 1 ± 0.86
Albumin (g dL^{-1})	2.38 ± 0.54	2.72 ± 0.58	3.08 ± 0.16	2.34 ± 0.61	3.02 ± 0.25	3.06 ± 0.79	2.96 ± 0.32	2.96 ± 0.47
Globulin (g dL^{-1})	2.44 ± 1.07	2.90 ± 1.78	2.20 ± 0.67	4.46 ± 1.80	2.44 ± 0.34	3.06 ± 1.70	2.98 ± 0.61	3.01 ± 1.70
Cholesterol (mg dL^{-1})	66.60 ± 4.39	76.40 ± 7.33	74.80 ± 4.02	69.80 ± 8.70	79.40 ± 5.36	74.80 ± 8.13	74.20 ± 3.89	77.40 ± 11.63
Triglycerides (mg dL^{-1})	71.40 ± 6.80	92.80 ± 17.12	99.40 ± 7.63	90.20 ± 13.18	97.40 ± 5.50	98.80 ± 7.15	86.40 ± 11.84	88.00 ± 11.70
HDL (mg dL^{-1})	25.40 ± 1.51	22.40 ± 2.60	24.00 ± 2.00	24.00 ± 1.58	23.60 ± 1.14	22.00 ± 1.00	22.80 ± 0.83	22.60 ± 1.81
LDL (mg dL^{-1})	33.00 ± 4.95	29.00 ± 12.02	31.00 ± 4.95	30.80 ± 6.22	36.20 ± 6.38	32.20 ± 6.38	34.20 ± 4.38	27.20 ± 6.69
VLDL (mg dL^{-1})	13.20 ± 1.64	25.00 ± 7.51	19.20 ± 2.04	15.80 ± 4.86	19.80 ± 1.30	29.20 ± 1.64	16.30 ± 1.43	37.60 ± 7.63
Urea (mg dL^{-1})	42.60 ± 2.07	41.20 ± 4.14	37.80 ± 1.64	36.50 ± 2.82	37.60 ± 5.59	37.60 ± 0.89	38.00 ± 3.14	38.40 ± 5.41
Creatinine (mg dL^{-1})	0.84 ± 0.20	0.70 ± 0.12	0.70 ± 0.12	0.66 ± 0.16	0.78 ± 0.08	0.56 ± 0.05	0.70 ± 0.18	0.60 ± 0.07
Na^+ (mmol L^{-1})	139.50 ± 7.95	135.80 ± 1.68	140.24 ± 1.35	138.58 ± 1.57	138.42 ± 2.73	138.26 ± 2.43	136.88 ± 2.50	137.96 ± 1.73
K^+ (mmol L^{-1})	4.74 ± 0.09	5.63 ± 0.92	5.17 ± 0.90	4.67 ± 0.31	4.76 ± 0.91	5.30 ± 1.12	5.50 ± 1.13	4.55 ± 0.75
Cl^+ (mmol L^{-1})	94.74 ± 0.91	96.02 ± 1.43	95.50 ± 2.18	96.82 ± 0.70	96.76 ± 2.94	97.48 ± 0.89	96.74 ± 1.02	96.58 ± 0.99
HCO_3^+ (mmol L^{-1})	22.60 ± 1.14	22.80 ± 1.30	22.60 ± 1.51	24.00 ± 1.22	23.80 ± 1.48	24.40 ± 2.07	23.40 ± 1.51	23.40 ± 1.81

[a] The values are expressed as mean ± SD for 5 animals in each group, where * denotes $p < 0.05$ and ** denotes $p < 0.01$ when compared with the normal.

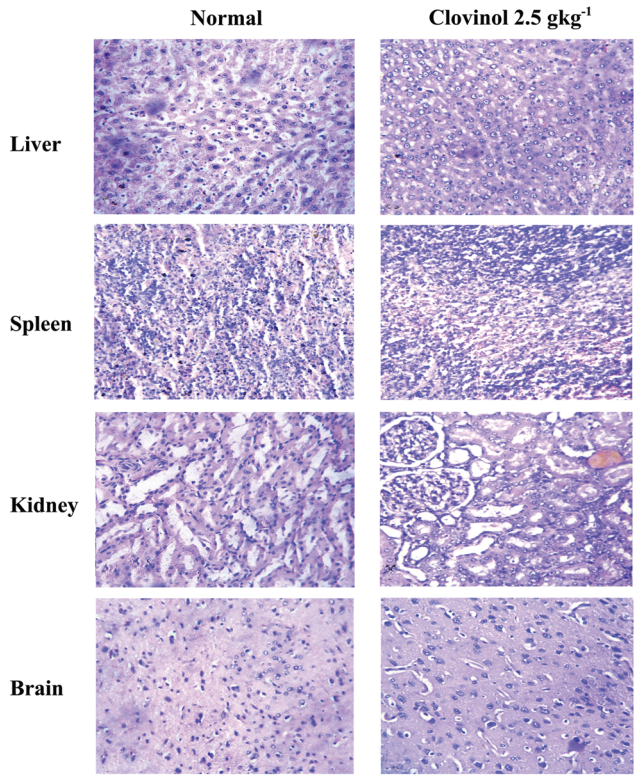

Fig. 1 Histological sections of various organs of normal animals and those treated with Clovinol for 28 days at 2.5 g per kg b.w.

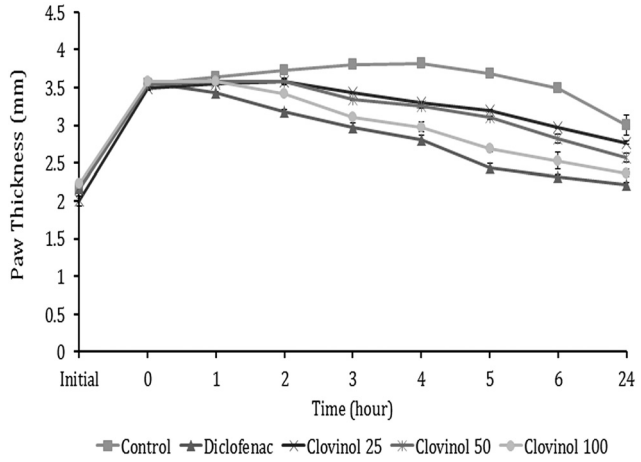

Fig. 2 Effect of Clovinol on carrageenan induced paw oedema in Swiss albino mice. The values are expressed as mean ± SD for six animals in each group.

mucosal volume upon Clovinol treatment. The ethanol administration steeply reduced the mucosal content from 234.17 ± 12.42 μg Alcian blue per g wet tissue (normal) to 157.83 ± 13.47 μg Alcian blue per g wet tissue (control). The pre-administration of Clovinol at 50 and 100 mg per kg b.w. significantly ($p < 0.01$) improved the mucosal volume from 157.83 ± 13.47 μg Alcian blue per g wet tissue (control) to 201.67 ± 9.81 μg Alcian blue per g wet tissue and 221.50 ± 9.54 μg Alcian blue per g wet tissue respectively.

Though a significant ($p < 0.05$) decrease in cellular antioxidants and a steep increase in lipid oxidation were observed upon ethanol-induced ulcer formation, the pre-treatment with Clovinol produced a significant ($p < 0.05$) increase in cellular antioxidant enzymes as measured by the levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the non-protein thiol, glutathione (GSH), as depicted in Fig. 4A. Ulcer induction was found to reduce the SOD activity to 3.60 ± 0.89 U per mg protein as compared to the normal group of animals (8.43 ± 0.86). Clovinol administration produced a dose dependent increase in SOD activities to 4.88 ± 1.04 at 25 mg kg^{-1} ($p < 0.05$), to 6.4 ± 1.36 at 50 mg kg^{-1} ($p < 0.05$) and to a maximum of 7.42 ± 0.51 at 100 mg per kg b.w. ($p < 0.01$). The standard drug ranitidine at a dose of 50 mg kg^{-1} also increased the SOD level to 6.31 ± 0.88 ($p < 0.05$), which was similar to Clovinol at 50 mg per kg dosage. The catalase activity was reduced (3.87 ± 1.25 U per mg protein) in comparison with that of the normal group of animals (8.21 ± 1.34) upon alcohol administration, and the treatment with Clovinol elevated the reduced catalase activity in a dose dependent manner; to 4.13 ± 1.36 at 25 mg kg^{-1}, to 5.79 ± 1.02 ($p < 0.05$) at 50 mg kg^{-1} and to 6.89 ± 1.29 ($p < 0.01$) at 100 mg per kg b.w. A similar reduction in both GPx activity (22.19 ± 3.33 U per mg protein) and GSH levels (20.89 ± 2.59 nmol per mg protein) ($p < 0.05$) was also observed upon induction of gastric ulcers as compared to that of the normal group of animals. However, administration of Clovinol significantly increased these enzyme levels in a dose dependent manner. GPx activity was raised to 25.23 ± 3.25, 31.31 ± 1.80 ($p < 0.05$), 34.55 ± 1.69 U per mg protein ($p < 0.01$) and GSH levels were enhanced to 25.28 ± 4.19, 28.56 ± 4.57 ($p < 0.05$) nmol per mg protein and 31.63 ± 3.68 nmol per mg protein ($p < 0.01$), when supplemented with 25, 50 and 100 mg per kg b.w. of Clovinol respectively. The extent of lipid peroxidation was found to be significantly ($p < 0.05$) reduced upon Clovinol treatment, as evident from the TBARS values (Fig. 4B). Ulcer induction led to a significant ($p < 0.01$) elevation of lipid peroxidation levels in the stomach tissues of ethanol treated control animals and reached up to 7.71 ± 1.38 nmol MDA per mg protein from the normal range of 1.11 ± 0.21 nmol MDA per mg protein. The pre-administration of Clovinol at 25, 50 and 100 mg per kg b.w. significantly ($p < 0.01$) inhibited the lipid oxidation and brought down the levels to 4.69 ± 0.72 ($p < 0.05$), 3.06 ± 0.45 ($p < 0.05$) and 1.69 ± 0.98 nmol MDA per mg protein ($p < 0.01$), respectively, in a dose dependent manner, while the standard drug ranitidine (50 mg per kg b.w.) treated group showed a TBARS value of 2.93 ± 0.86 nmol MDA per mg protein. Thus the pre-treatment with Clovinol at 100 mg per kg b.w. demonstrated better efficacy than the standard drug ranitidine in ethanol induced ulcer models.

3.4.2. Effect of Clovinol on simultaneous administration with ethanol. Simultaneous treatment with Clovinol at 25, 50 and 100 mg per kg b.w. was found to be very significant

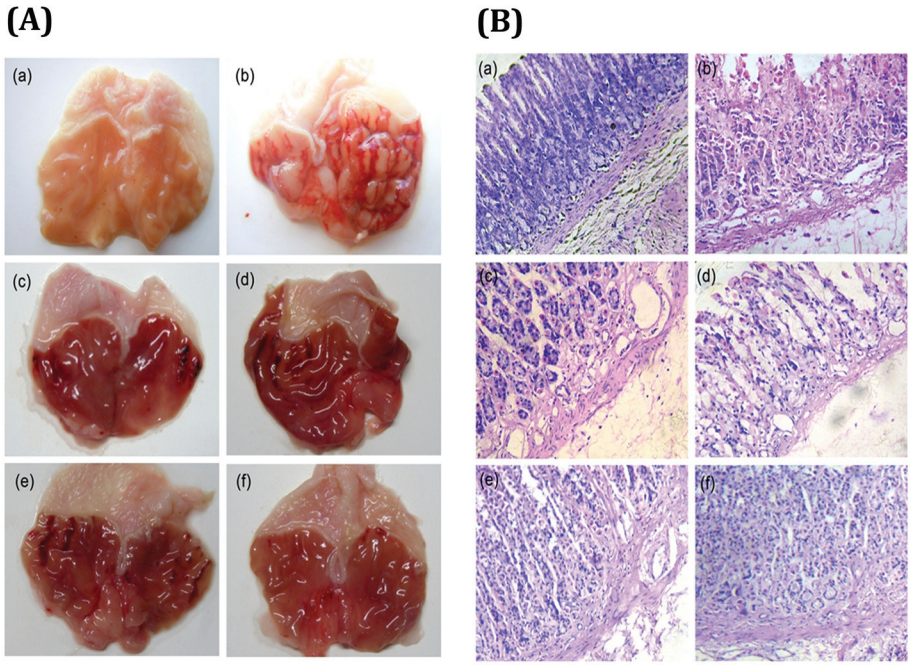

Fig. 3 (A) Morphology of stomach and (B) histopathology of stomach tissues from ethanol treated Wistar rats in comparison with normal, ranitidine and Clovinol treated groups of animals; (a) normal, (b) control, (c) ranitidine, (d) Clovinol – 25 mg kg^{-1} treated, (e) Clovinol – 50 mg kg^{-1} treated, (f) Clovinol – 100 mg kg^{-1} treated animals.

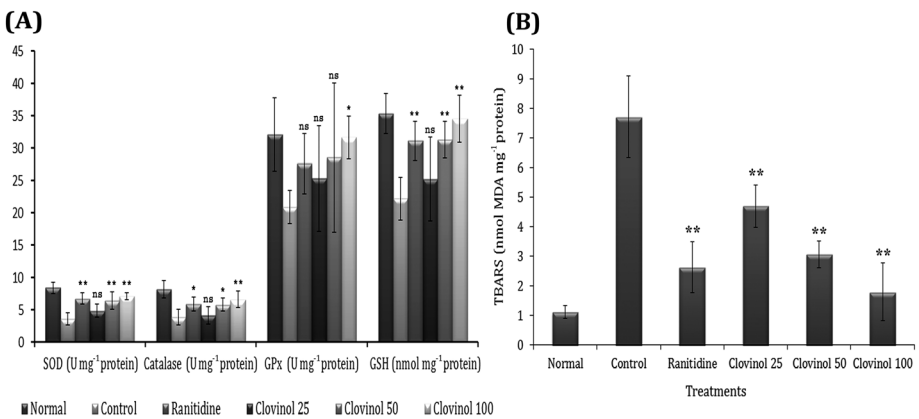

Fig. 4 Effect of Clovinol pre-treatment on (A) the gastric mucosal antioxidant levels, and (B) the gastric mucosal lipid peroxidation in animals administered with 80% ethanol. Values expressed as mean ± SD, where ** denotes $p < 0.01$; * denotes $p < 0.05$ and 'ns' denotes $p > 0.05$, when compared with the control.

($p < 0.01$) in inhibiting the formation of ethanol-induced gastric ulcers, as compared to the control group of animals who had severe necrotic lesions throughout the glandular portion of the stomach with an ulcer index of 6.95 ± 0.57 when administered with ethanol. Between the doses of Clovinol, 100 mg kg^{-1} administrations produced an ulcer index of only 0.07 ± 0.16 with 98.99% inhibition. The lower doses 25 and 50 mg per kg b.w., showed 68.77 and 93.1% inhibition respectively. A detailed investigation on the mucosal content, mucosal antioxidant status and extent of mucosal lipid peroxidation on ethanol-administered control group animals and simultaneously Clovinol-treated animals was carried out to better understand the pharmacological effects. The mucosal content was significantly ($p < 0.01$) enhanced upon Clovinol administration and was found to be 228.33 ± 16.28 and 246.50 ± 13.68 μg Alcian blue per g wet tissue for 50 and 100 mg per kg b.w. respectively. When supplemented with Clovinol at 100 mg per kg b.w., the cellular antioxidant levels showed a significant increase in the depleted values as compared to ethanol alone treated control animals, and the enhanced levels were comparable to the values from the normal group of animals (Fig. 5A). The extent of lipid peroxidation was also lowered to levels almost similar to those of the normal group of animals, though a significant rise was observed when the ulcer was

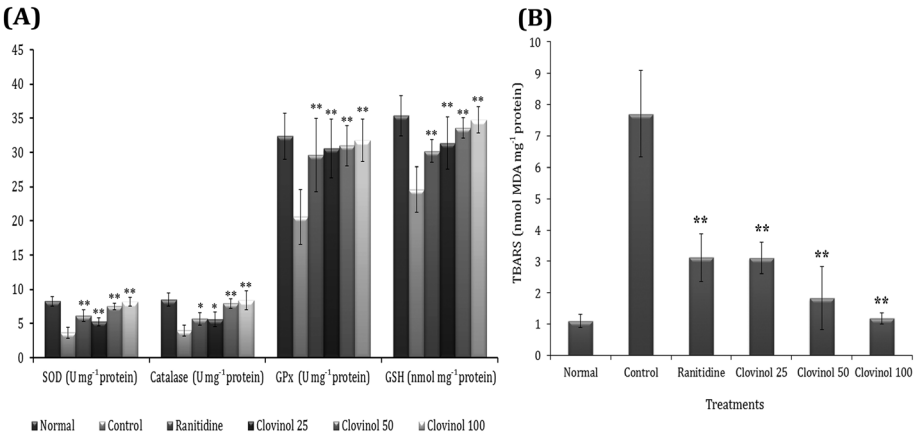

Fig. 5 Effect of Clovinol simultaneous-treatment on (A) the gastric mucosal antioxidant levels, and (B) the gastric mucosal lipid peroxidation in the animals administered with 80% ethanol. Values expressed as mean ± SD, where ** denotes $p < 0.01$ and * denotes $p < 0.05$, when compared with the control.

induced by ethanol administration (Fig. 5B). The results demonstrated the absorption and cellular availability of Clovinol to prevent and/or control gastric ulcers and to regulate the oxidative stress and inflammation induced upon chronic alcohol consumption.

4. Discussion

Besides the wide array of flavour applications, aromatic clove buds rich in volatile oil and non-volatile polyphenols have been demonstrated to possess many health guarding pharmacological effects.[15,29] Though many preclinical study reports are available on the volatile oil of clove and its major component eugenol, systematic information on the bioactivity of clove polyphenols is limited. Since plant derived polyphenols have already been shown to be able to provide protection against the development of many oxidative stress mediated diseases, including cardiovascular, diabetes, neurodegenerative, and gastrointestinal disorders and even cancer,[30–32] the present contribution attempted the preparation of a standardized polyphenol-rich extract of clove (Clovinol), and an examination of its pharmacological effects. The method of extraction and formulation and its physicochemical/microbial properties were optimized and developed as a taste and odour minimized water soluble powder suitable for functional applications. It was prepared by hydro-ethanolic extraction followed by purification and encapsulation under controlled conditions of temperature and pressure. Further UPLC-ESI-TOF-MS analysis revealed its major polyphenols as flavonoids, phenolic acids, hydrolysable tannins and their glycoside derivatives in addition to eugenol and eugenyl acetate. Most of these phenolic compounds found in Clovinol have already been shown to possess potent antioxidant and anti-inflammatory activities.[29] The Oxygen Radical Absorbance Capacity (ORAC) of Clovinol, a measure of antioxidant capacity, was found to be more than 10 000 μM TE g^{-1}, indicating its high antioxidant potential.

Clovinol showed significant *in vivo* anti-inflammatory activity when tested using a carrageenan-induced paw oedema model in mice, a most widely used reliable and convenient primary test model to screen new anti-inflammatory agents.[33] Carrageenan-induced inflammation was believed to be a biphasic process, with an early phase (1 to 2 h) mediated by histamine, serotonin and increased synthesis of prostaglandins, and a late phase sustained by prostaglandins and mediated by bradykinin and leukotrienes, produced by tissue macrophages.[33,34] Oral supplementation with Clovinol at 100 mg per kg b.w. could produce an anti-inflammatory response (47.3% inhibition) similar to 10 mg kg^{-1} of intraperitoneal administration of diclofenac (52.7% inhibition), a non-steroidal anti-inflammatory drug very often considered as the first choice in the treatment of acute and chronic painful inflammatory conditions.[35] Thus, Clovinol could be a simple and safe antioxidative anti-inflammatory food component useful against various inflammatory responses usually originating from diet, exercise, alcoholism, smoking and various disease states such as obesity, Alzheimer's, diabetes, allergies, gastrointestinal disorders *etc.*[36–38] Though our body tends to over-produce pro-inflammatory chemicals as a result of oxidative stress mediated by any of the above states of disease or diet/life style, most of the time our body does not get enough nutrients that can naturally reduce inflammation.

Traditional use of clove buds against gastrointestinal disorders, earlier reports on the gastroprotective activity of clove buds[39] and the high antioxidant and anti-inflammatory activities of Clovinol motivated us to further evaluate its potential as a safe natural anti-ulcerogenic agent. Moreover, botanical extracts derived from food components having a history of safe human consumption are always of great significance as a functional ingredient against many human disorders.[8,9] Considering the ever increasing global interest in alcoholism and its gastrointestinal complications, the present study was attempted on an ethanol-induced gastric ulcer rat model to demonstrate the effectiveness of Clovinol as an aid to reduce

the complications and discomforts due to chronic alcohol consumption. Intragastric administration of absolute ethanol to fasted rats was found to produce linear hemorrhagic lesions, extensive sub-mucosal edema, mucosal friability, inflammatory cells, and epithelial cell loss similar to chronic alcohol injury in humans. This is due to the direct action of ethanol upon the gastric epithelium and perturbation of mast cells leading to the release of a vasoactive mediator such as histamine.[40] Ethanol also causes constriction of sub-mucosal venules with subsequent stasis of blood flow in mucosal microcirculation and arteriolar dilation leading to plasma leakage from the vascular bed, which ultimately produces band like blisters in the gastric mucosa.[41] Such alcoholic injury is already evident from Fig. 3. Since prostaglandins play a vital, protective role in the stomach by stimulating the secretion of bicarbonate and mucous, maintaining mucosal blood flow, and regulating mucosal cell turnover and repair, the alcohol induced inhibition of prostaglandin synthesis may escalate the mucosal injury and gastro-duodenal ulceration.[42,43] The gastric mucous coat is thus hypothesized to be important in both preventing the damage and in facilitating the repair of the gastric epithelium.[44] It was observed that Clovinol efficiently enhanced the gastric mucosal content and significantly reduced the ulcer index in the ethanol-induced ulcer model. When pre-treated with 50 mg per kg b.w. of Clovinol, the ethanol induced ulcer formation was inhibited by about 41%, which was nearly equivalent to the effect of the standard gastro-protective drug ranitidine, a histamine H_2-receptor antagonist that is commonly engaged in the treatment of peptic ulcer disease (PUD) and gastro-esophageal reflux disease (GERD).[45] At 100 mg per kg dose of Clovinol, the ulcer inhibition was nearly 80%, indicating the ethanol induced ulcer preventive potential of Clovinol. Simultaneous treatment with Clovinol at 50 and 100 mg per kg b.w., on the other hand, was found to completely inhibit the ethanol induced ulceration, indicating its potent anti-ulcerogenic efficacy.

When tested for the mucous antioxidant status, a significant reduction in antioxidant enzyme levels was noticed among animals with ethanol-induced ulcers, indicating that the inflammatory conditions of ulcers result in the development of free radicals and hyperoxidation of lipids.[40,41,46] Administration of Clovinol subsequently enhanced the antioxidant enzyme levels in mucosa and offered a significant reduction in lipid peroxidation, indicating the usefulness of dietary antioxidants capable of acting at the cellular level as anti-ulcerogenic agents. This was further evident from the ulcer preventive potential of Clovinol when it was simultaneously administered along with ethanol, which completely prevented the ulcer formation at 50 to 100 mg per kg b.w. and restored the antioxidant enzyme levels and lipid peroxidation to the normal range.

Albeit the encouraging anti-ulcerogenic activity of Clovinol, the basic property that has to be evaluated before the next levels of detailed investigations such as its molecular mechanism of action and randomized placebo controlled human trials is the evaluation of the probable toxicity effects that may arise upon its continuous consumption.[47] In the acute toxicity evaluation, Clovinol administered animals showed neither any toxicity nor any mortality up to the dose of 5 g per kg b.w. and survived beyond the observational period, indicating the LD_{50} to be higher than 5 g kg^{-1} for both male and female rats. Upon sub-acute toxicity studies, Clovinol administration even at 2.5 g kg^{-1} for 28 days did not produce any indications of toxicity. Hematological and biochemical parameters along with the histopathology data indicated the safety of Clovinol when translated from animal data,[48] and substantiated its suitability for further human trials and development as a functional ingredient of gastro-protective functions.

5. Conclusion

The present contribution has demonstrated the safety and efficacy of the clove bud-derived polyphenol-rich standardized extract (Clovinol) as an alcohol-induced stomach ulcer protective/curative agent. The potent anti-ulcerogenic activity of Clovinol was found to be mainly due to its high antioxidant and anti-inflammatory effects in addition to its mucous producing effect. Lethal dose oral administration (5 g per kg b.w.) and sub-acute 28-days of oral administration (2.5 g per kg b.w.) studies indicated no toxicity or adverse effect for Clovinol. In a view of its possible development as a functional ingredient of GRAS-listed kitchen spices, a commercially feasible preparation and formulation of Clovinol with minimized taste and aroma was also achieved in the present study.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

The authors are grateful to M/s Akay Flavours& Aromatics Ltd, Cochin, India for financial support; for the research project "*Spiceuticals: functional ingredients from spices'* (AK/R&D/02/10-11). We are also grateful to M/s Agilent Application Laboratory, Bangalore, India for mass spectrometric studies.

References

1 Y. Gulia and M. Choudhary, *Pharmacologyonline*, 2011, **3**, 48–70.
2 J. Y. Lau, J. Sung, C. Hill, C. Henderson, C. W. Howden and D. C. Metz, *Digestion*, 2011, **84**, 102–113.
3 E. J. Kuipers, *Dig. Dis.*, 2014, **32**, 202–205.
4 K. Takeuchi, *World J. Gastroenterol.*, 2012, **18**, 2147.
5 A. Bhattacharyya, R. Chattopadhyay, S. Mitra and S. E. Crowe, *Phys. Rev.*, 2014, **94**, 329–354.
6 J. K. Desai, R. K. Goyal and N. S. Parmar, *Indian J. Physiol. Pharmacol.*, 1997, **41**, 3–15.

7 H. Ahmad, A. Wadud, N. Jahan, M. Khazir and G. Sofi, *Int. Res. J. Med. Sci.*, 2013, **1**, 7–12.
8 H. L. Madsen and G. Bertelsen, *Trends Food Sci. Technol.*, 1995, **6**, 271–277.
9 S. Dragland, H. Senoo, K. Wake, K. Holte and R. Blomhoff, *J. Nutr.*, 2003, **133**, 1286–1290.
10 K. Pramod, S. H. Ansari and J. Ali, *Nat. Prod. Commun.*, 2010, **5**, 1999–2006.
11 R. Shukri, S. Mohamed and N. M. Mustapha, *Food Chem.*, 2010, **122**, 1116–1121.
12 D. F. Cortés-Rojas, C. R. F. de Souza and W. P. Oliveira, *Asian Pac. J. Trop. Biomed.*, 2014, **4**, 90–96.
13 S. P. Dibazar, S. Fateh and S. Daneshmandi, *J. Immunotoxicol.*, 2014, 1–8.
14 M. I. Nassar, *Carbohydr. Res.*, 2006, **341**, 160–163.
15 M. I. Nassar, A. H. Gaara, A. H. El-Ghorab, A. Farrag, H. Shen, E. Huq and T. J. Mabry, *Rev. Latinoam. de Quím.*, 2007, **35**, 47–57.
16 S. A. Adefegha and G. Oboh, *Asian Pac. J. Trop. Biomed.*, 2012, **2**, 774–781.
17 S. E. Atawodi, J. C. Atawodi, B. Pfundstein, B. Spiegelhalder, H. Bartsch and R. Owen, *Electron. J. Environ., Agric. Food Chem.*, 2011, **10**, 1970–1978.
18 V. Dwivedi, R. Shrivastava, S. Hussain, C. Ganguly and M. Bharadwaj, *Asian Pac. J. Cancer Prev.*, 2011, **12**, 1989–1993.
19 V. L. Singleton and J. A. Rossi, *Am. J. Enol. Vitic.*, 1965, **16**, 144–158.
20 D. Lorke, *Arch. Toxicol.*, 1983, **54**, 275–287.
21 S.-H. Lee, J.-M. Mo, M.-N. Park and Y.-S. Lee, *FASEB J.*, 2008, **22**, 869–867.
22 J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 1969, **244**, 6049–6055.
23 H. Aebi, in *Methods of enzymatic analysis*, ed. H. U. Bergmeyer, Academic Press, New York, 1974, pp. 673–677.
24 M. S. Moron, J. W. Depierre and B. Mannervik, *Biochim. Biophys. Acta*, 1979, **582**, 67–78.
25 D. G. Hafeman, R. A. Sunde and W. G. Hoekstra, *J. Nutr.*, 1974, **104**, 580–587.
26 H. Ohkawa, N. Ohishi and K. Yagi, *Anal. Biochem.*, 1979, **95**, 351–358.
27 S. J. Corne, S. M. Morrissey and R. J. Woods, *J. Physiol.*, 1974, **242**, 116P–117P.
28 W. Mullen, T. Yokota, M. E. J. Lean and A. Crozier, *Phytochemistry*, 2003, **64**, 617–624.
29 K. Chaieb, H. Hajlaoui, T. Zmantar, A. B. Kahla-Nakbi, M. Rouabhia, K. Mahdouani and A. Bakhrouf, *Phytother. Res.*, 2007, **21**, 501–506.
30 A. Scalbert, C. Manach, C. Morand, C. Rémésy and L. Jiménez, *Crit. Rev. Food Sci. Nutr.*, 2005, **45**, 287–306.
31 W. Guo, E. Kong and M. Meydani, *Nutr. Cancer*, 2009, **61**, 807–810.
32 A. Borriello, V. Cucciolla, F. Della Ragione and P. Galletti, *Nutr., Metab. Cardiovasc. Dis.*, 2010, **20**, 618–625.
33 E. O. Agbaje and M. S. Fageyinbo, *Int. J. Appl. Res. Nat. Prod.*, 2012, **4**, 7–14.
34 C. J. Morris, in *Inflammation Protocols: Methods in Molecular Biology*, ed. P. Winyard and D. Willoughby, Springer, Humana Press, 2003, pp. 115–121.
35 P. A. Todd and E. M. Sorkin, *Drugs*, 1988, **35**, 244–285.
36 C. N. Lumeng and A. R. Saltiel, *J. Clin. Invest.*, 2011, **121**, 2111–2117.
37 H. Y. Chung, M. Cesari, S. Anton, E. Marzetti, S. Giovannini, A. Y. Seo, C. Carter, B. P. Yu and C. Leeuwenburgh, *Ageing Res. Rev.*, 2009, **8**, 18–30.
38 E. O. Iwalewa, L. J. McGaw, V. Naidoo and J. N. Eloff, *Afr. J. Biotechnol.*, 2007, **6**, 2868–2885.
39 J. R. Santin, M. Lemos, L. C. K. Júnior, I. D. Machado, P. Costa, A. P. de Oliveira, C. Tilia, J. P. de Souza, J. P. B. de Sousa, J. K. Bastos and S. F. de Andrade, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 2011, **383**, 149–158.
40 S. Park, K.-B. Hahm, T.-Y. Oh, J.-H. Jin and R. Choue, *Dig. Dis. Sci.*, 2004, **49**, 384–394.
41 P. J. Oates and J. P. Hakkinen, *Gastroenterology*, 1988, **94**, 10–21.
42 J. L. Wallace, *Phys. Rev.*, 2008, **88**, 1547–1565.
43 A. Tarnawski, D. Hollander, J. Stachura, W. J. Krause and H. Gergely, *Gastroenterology*, 1985, **88**, 334–352.
44 J. L. Wallace and B. J. R. Whittle, *Gastroenterology*, 1986, **91**, 603–611.
45 R. N. Brogden, A. A. Carmine, R. C. Heel, T. M. Speight and G. S. Avery, *Drugs*, 1982, **24**, 267–303.
46 G. Pihan, C. Regillo and S. Szabo, *Dig. Dis. Sci.*, 1987, **32**, 1395–1401.
47 C. D. Klaassen and D. L. Eaton, in *Toxicology: The Basic Science of Poisons*, ed. M. O. Amdur, J. D. Doull and C. D. Klaassen, Pergamon Press, New York, 1991, pp. 11–32.
48 H. Olson, G. Betton, D. Robinson, K. Thomas, A. Monro, G. Kolaja, P. Lilly, J. Sanders, G. Sipes and W. Bracken, *Regul. Toxicol. Pharmacol.*, 2000, **32**, 56–67.

