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Research Article
Acute Toxicity and Gastroprotective Effect of 2-pentadecanone in Ethanol-induced Gastric Mucosal Ulceration in Rats

[1]Sareh Kamran, [2]Hamed Karimian, [3]Nurain Salehen, [2]Atin Khalajhedayati, [4]Bushroa Abdul Razak and [1]Nazia Abdul Majid

[1]Department of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
[2]Faculty of Health and Medical Sciences, School of Biosciences, Taylor's University Lakeside Campus, Subang Jaya, Malaysia
[3]Department of Biomedical Science, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
[4]Faculty of Engineering, Institute of Mechanical Engineering, University of Malaya, Kuala Lumpur, Malaysia

Abstract

Background and Objective: Gastric ulcer is a common gastrointestinal disease worldwide. Ketones have been reported to possess anti-inflammatory, antioxidant and antiulcer activities. This study aimed to evaluate acute toxicity and ulcer inhibitory effect of 2-pentadecanone as a ketone bioactive compound against ethanol-induced gastric ulcer in Sprague Dawley rats. **Material and Methods:** For acute toxicity test 300 mg kg^{-1} 2-pentadecanone was orally given to rats, followed by 2 weeks observation period to confirm the safety of the compound. To test gastroprotective effect of 2-pentadecanone, fasted rats were divided into 4 groups and orally received 1 mL of tween 20 as negative control, omeprazole as positive control, 10 and 20 mg kg^{-1} 2-pentadecanone. After 1 h, all groups received 1 mL of absolute ethanol orally. About 1 h later the animals were sacrificed and stomachs were collected. **Results:** Macroscopic and histological analyses of stomach tissues have revealed ulcer inhibitory effect of 2-pentadecanone. Enzymatic activity evaluation of superoxide dismutase and catalase and malondialdehyde production in tissue homogenate proved the antioxidant property of this compound. Immunohistochemistry results illustrated up regulation of HSP70 and down regulation of Bax protein in groups received 2-pentadecanone. **Conclusion:** In overall, results of this study have strongly suggested ulcer inhibitory effect of 2-pentadecanone in rats against ethanol-induced gastric injury.

Key words: Acute toxicity, gastric ulcer, 2-pentadecanone, antioxidant activity, HSP70/bax

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Corresponding Authors: Nazia Abdul Majid, Department of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia
Tel: +603-79675833 Fax: +603-79674173
Sareh Kamran, Department of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia Tel: +60-1161874898

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gastric ulcer is a common disease and it affects 4 million individuals worldwide yearly. It can become a life threatening illness with 10-40% mortality rate[1]. It is a condition of tissue destruction to the depth of gastric mucosa and it can appear as a result of factors such as stress, alcohol consumption and long-term anti-inflammatory medications. Also, disruption between the balanced level of endogenous offensive and defensive factors can lead into the formation of gastric ulcer[2]. Nowadays, several categories of drugs are being used against gastric ulcer disease, such as proton pump inhibitors, M1 receptor inhibitors and H2 receptor antagonists. However, there are side effects associated with these drugs; for example arrhythmia or hematopoietic changes[3]. In addition, recurrence and refractoriness to the treatment are being concerned and leads to the search for a new therapeutic agent[3,4].

Ethanol-induced gastric ulcer model has been widely used in testing gastroprotective activity of new therapeutic agents[3,5]. Ethanol results in the neutrophil infiltration into the site of injury, followed by the release of reactive oxygen spices (ROS) and oxidative damage, which contribute to lipid peroxidation and antioxidants depletion and has harmful effect on gastric mucosa[3].

2-pentadecanone was identified as a major compound in *Labisia pumila* as a result[6] of Gas Chromatography Mass Spectrometry in 2015. 2-pentadecanone ($C_{15}H_{30}O$), a ketone compound, is a flavouring ingredient. This compound was identified in *Humulus lupulus*, *Cocos nucifera* and other oils[7]. Plants such as *Peganum harmala* and *Eclipta alba*, which contain 2-pentadecanone analog (6, 10, 14-trimethyl-2-pentadecanone) were reported to support wound healing activity and also *Eclipta alba* has been reported to exert antioxidant effect[8]. Moreover, in 2019 a study has reported antibacterial, anti-inflammatory and skin wound healing effect of 2-pentadecanone[9]. However, potential effect of this compound on internal wounds such as gastric ulcer has not been studied. The current study aimed to evaluate acute toxicity and gastroprotective effect of 2-pentadecanone in ethanol-induced gastric ulcer in rats.

MATERIALS AND METHODS

Chemicals: 2-pentadecanone was purchased from Sigma-Aldrich Company (USA). About 10 and 20 mg kg^{-1} of this compound were prepared as low and high doses. Negative control and positive control used in this experiment are 5% tween 20 and 20 mg kg^{-1} omeprazole respectively[1]. Omeprazole, the standard anti ulcer drug, was purchased from the pharmacy of University of Malaya, Malaysia. This study was conducted in the faculty of Medicine, University of Malaya in October, 2016 and the whole experiment took about 8 months.

Animals: Animal experimental unit (AEU), Faculty of Medicine, University of Malaya (Malaysia), provided healthy male Sprague Dawley (SD) rats. Animals were housed under controlled condition at room temperature (25°C) with 12 h light/dark cycle. Access to water and normal pellet was available. This experiment was conducted after receiving the animal ethic approval from AEU committee (Ethic Number: 2016-190819/BMS/R/MAA).

Acute toxicity evaluation assay: According to OECD guideline 423, 300 mg kg^{-1} of 2-pentadecanone was given to treatment group (n = 6). Control group received 5% tween 20 (5 mL kg^{-1}) orally after overnight fasting with access to water. Any abnormal changes were recorded as signs of toxicity within 14 days of observation after dosing. Blood was collected at the last day of experiment to analyze biochemical parameters and liver and kidney were harvested for histology inspection. Animals were sacrificed by an overdose of ketamine (300 mg kg^{-1}) and xylazine (30 mg kg^{-1}).

Induction of gastric ulcer: For gastric ulcer induction, 24 rats were divided into 4 groups (n = 6). Pre-treatment was done orally. Negative control group was administered with 5% tween 20 (5 mL kg^{-1}). Positive control group received 20 mg kg^{-1} omeprazole in 5% tween 20 (5 mL kg^{-1}). Low dose and high dose groups were pre-treated with 10 and 20 mg kg^{-1} 2-pentadecanone respectively. All groups received 1 mL of absolute ethanol after 1 h of pre-treatment. Animals were sacrificed by an overdose of ketamine and xylazine, followed by euthanization by cervical decapitation. Stomachs were immediately harvested and preserved in 10% buffered formalin for further analysis[1,10].

Macroscopic evaluation of gastric lesions: To detect ulcer area (UA) in stomach tissue and Inhibition percentage (I%), length and width of the hemorrhagic bands were calculated by using a dissecting microscope (1.8x magnification). The following Eq. was used in this measurment[10]:

$$I\ (\%) = \frac{\text{UA control-UA treated}}{\text{UA control}} \times 100$$

Mucus content and acidity measurement: Mucus production was measured by gentle collection of gastric mucosa, followed by weighing it by an electronic balance[11]. Gastric juice acidity was measured by a pH meter[1].

Effect of 2-pentadecanone on antioxidant enzymatic expression and NO level: Superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were measured as antioxidant markers by using commercial kits (Cayman Chemical, USA). Nitric oxide (NO) expression level was measured in gastric tissue homogenate as a gastroprotective molecule by using commercial kit (Cayman Chemical, USA).

Histological examination of stomach tissues: Stomach tissues were fixed in 10% formalin prior to process and prepare sections for microscopic observation[1].

HSP70 and Bax proteins expression level: Immunohistochemistry (IHC) assay was performed, by using Polyvalent HRP/DAB detection kit (Abcam, ab64264) to detect protein expression level in stomach tissues. A light microscope (Nikon, Tokyo, Japan) was used to detect the appearance of brown color, which is an indicator of protein expression.

Statistical analysis: All data are reported as Mean±SEM. Experimental group were compared to negative control group by running one-way ANOVA analysis (SPSS, version 20). $p<0.05$ was considered as significant difference[12].

RESULTS

Safety determination of 2-pentadecanone: Dosed animals with 300 mg kg^{-1} 2-pentadecanone resulted in no mortality or signs of toxicity. One-way ANOVA analysis of liver and kidney parameters (Table 1 and 2) and histology assessment of liver and kidney (Fig. 1) have highlighted the safety of 2-pentadecanone by showing no significant difference between the 2 groups.

Table 1: Effect of 2-pentadecanone on liver function in SD rats

Groups	Albumin	ALP	ALT	GGT	T. protein	AST
Control	36.6±0.2	234.8±26.8	73.6±1.9	2±0.4	58.4±1.4	152.0±8
300 mg kg^{-1}	35.6±0.5	218.4±25.7	74.0±2.4	1±0.5	55.0±0.6	168.4±10.9

ALP: Alkaline phosphatase, ALT: Alanine transaminase, GGT: Gamma-glutamyl transferase, T. protein: Total protein, AST: Aspartate aminotransferase, $p<0.05$

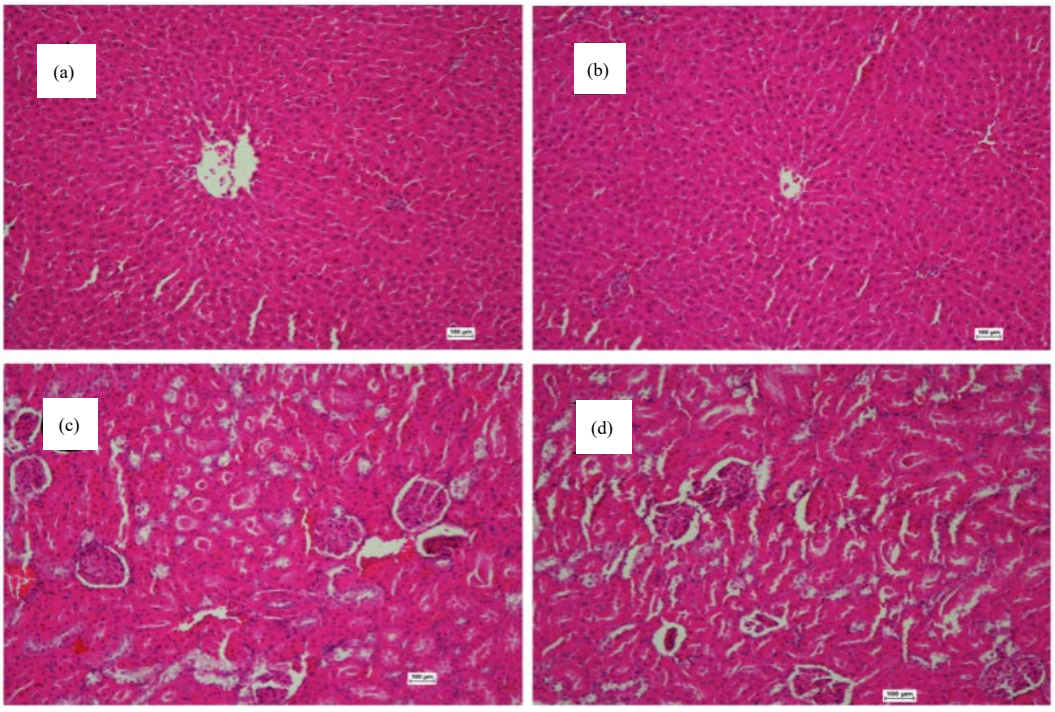

Fig. 1(a-d): Histological sections of (a) Control liver, (b) Treated liver, (c) Control kidney and (d) Treated kidney
Control group received 5% tween 20 and treated group received 300 mg kg^{-1} 2-pentadecanone, there was no histological changes in group received 300 mg kg^{-1} 2-pentadecanone, scale bar: 100 µm

Table 2: Effect of 2-pentadecanone on kidney function in SD rats

Groups	Sodium	Potassium	Chloride	Carbon dioxide	Urea	Creatinine
Control	142.0±0.4	4.58±0.1	101.0±0.3	35.2±0.8	7.82±0.2	33.2±0.6
300 mg kg^{-1}	141.2±0.6	4.32±0.05	100.6±0.8	34.6±0.8	6.96±0.2	31.6±0.4

p<0.05

Table 3: Effect of 2-pentadecanone on pH level and mucus secretion

Groups	pH	Mucus weight (g)
A	2.3±0.2	0.5±0.1
B	6.8±0.3*	2.7±0.1*
C	5.7±0.4*	2.0±0.1*
D	6.5±0.3*	2.3±0.2*

A: Negative control, B: Positive control, C: Low dose 2-pentadecanone, D: High dose 2-pentadecanone, data are reported as Mean±SEM/group, *p<0.05

Table 4: Effect of 2-pentadecanone on gastric mucosal SOD, CAT, MDA and NO

Groups	SOD (U mg^{-1})	CAT (nM min^{-1} mL^{-1})	MDA (μM g^{-1})	NO (μM g^{-1})
A	6.9±0.002	62.5±0.012	31.2±0.003	2.9±0.002
B	29.7±0.003*	140.0±0.020*	9.8±0.003*	11.2±0.002*
C	21.8±0.006*	120.0±0.033*	15.2±0.006*	8.9±0.006*
D	24.4±0.006*	130.9±0.005*	12.5±0.005*	10.1±0.003*

Data are reported as Mean±SEM/group, SOD: Superoxide dismutase, CAT: Catalase, NO: Nitric oxide, MDA: Malondialdehyde, A: Negative control, B: Positive control, C: Low dose 2-pentadecanone, D: High dose 2-pentadecanone, *p<0.05

Macroscopic analysis of stomach tissues: Macroscopic appearance of stomach tissues was illustrated in Fig. 2. Negative control group showed detectable hemorrhagic lesions in the gastric mucosa. Pre-treatment with positive control and 2-pentadecanone attenuated gastric lesions. Moreover, flattening of the folds was observed in pre-treated groups. Omeprazole, low dose and high dose of 2-pentadecanone inhibited ulcer formation by 90.18, 80.58 and 86.63% accordingly (Fig. 3). Statistical analysis has revealed significant (p = 0.00) ulcer inhibitory effect in pre-treated groups compare to negative control.

Mucus secretion and acidity level assessment: Ethanol caused a significant attenuation of mucus secretion and pH level in negative control group. 2-pentadecanone similar to omeprazole showed significant (p = 0.00) protective response against damaging effect of ethanol by inducing higher mucus production and reducing acidity level of gastric juice (Table 3).

Enzyme activities and NO measurement: The antioxidant activity of 2-pentadecanone in tissue homogenate was reported in Table 4. Ethanol has greatly reduced the antioxidant activity in negative control group. Pre-treatment with omeprazole and 2-pentadecanone at both doses revealed significant elevation of SOD (p = 0.008), CAT (p = 0.00) and NO (p = 0.004) and remarkable decrease in MDA production level (p = 0.00).

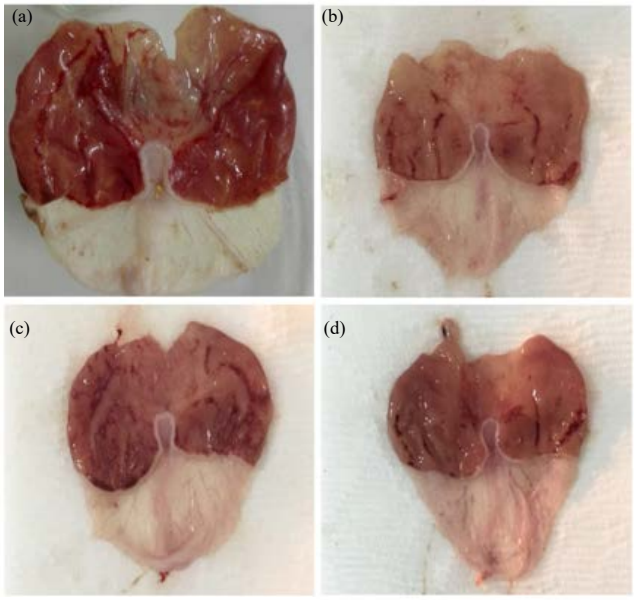

Fig. 2(a-d): Macroscopic analysis of stomach tissues, (a) Negative control, (b) Positive control, (c) Low dose 2-pentadecanone and (d) High dose 2-pentadecanone

Groups b-d showed less hemorrhagic bands as a result of pre-treatment with omeprazole and 2-pentadecanone

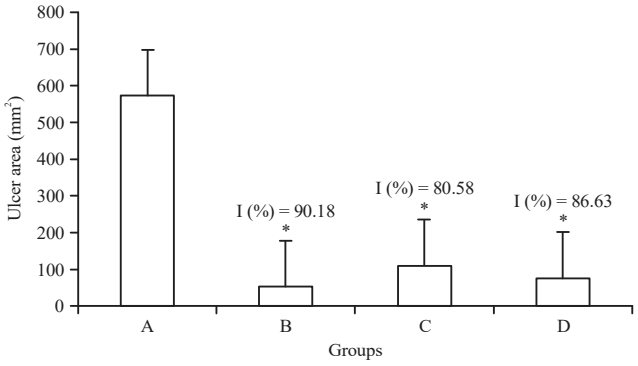

Fig. 3: Effect of 2-pentadecanone on ulcer area an inhibition percentage (I %), A: Negative control, B: Positive control, C: Low dose 2-pentadecanone and D: High dose 2-pentadecanone

Omeprazole and 2-pentadecanone significantly reduced ulcer area and revealed notable ulcer inhibitory percentage (b, c, d), *p<0.05

Histology examination of stomach tissues: Microscopic characterizations of stomach tissues were illustrated in

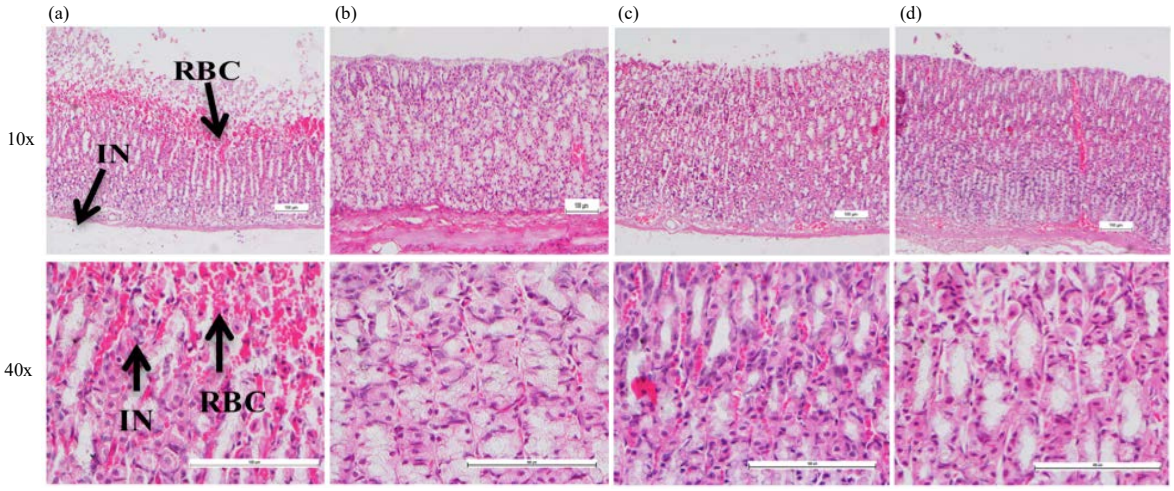

Fig. 4(a-d): Histology (H and E staining) of the gastric tissues, (a) Negative control, (b) Positive control, (c) Low dose 2-pentadecanone and (d) High dose 2-pentadecanone

IN: Inflammatory cells, RBC: Red blood cells, pre-treated groups with omeprazole and 2-pentadecanone (b, c, d) showed mild disruption of epithelium surface with reduction in submucosal edema and less inflammatory infiltration in comparison to negative control group, scale bar: 100 µm

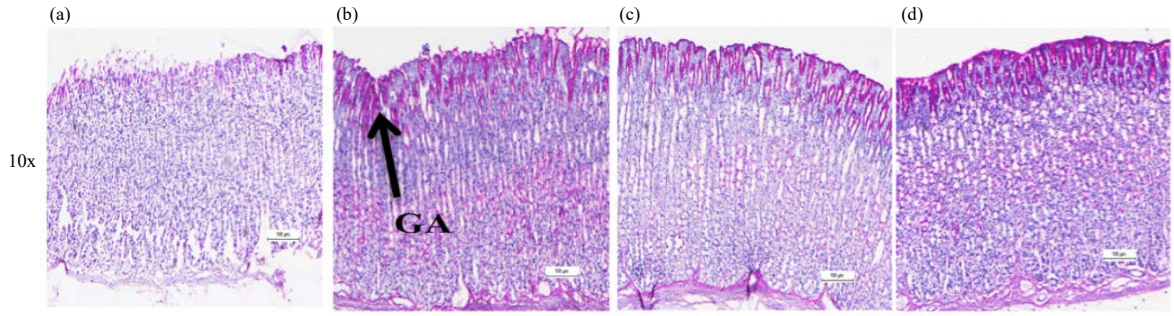

Fig. 5(a-d): Effect of 2-pentadecanone on glycogen accumulation in stomach tissue as a result of PAS staining, (a) Negative control, (b) Positive control, (c) Low dose 2-pentadecanone and (d) High dose 2-pentadecanone

GA: Glycogen accumulation, groups that were pre-treated with omeprazole and 2-pentadecanone showed significant accumulation of magenta color in mucosal cell layer in comparison to negative control group, this is an indication of significant accumulation of glycoprotein in pre-treated groups (b, c, d), scale bar: 100 µm

Fig. 4 and 5. Sever damage of gastric mucosa with submucosal edema was observed in negative control group (Fig. 4a). Pre-treatment resulted in reduction of inflammation (Fig. 4b-d). This observation was in consonance with the macroscopic analysis.

Glycogen deposition in gastric epithelium was detected by PAS staining. Magenta color represented positive result. Negative control group (Fig. 5a) showed negligible glycogen production in stomach tissue. Pre-treated groups showed similar glycogen production level (Fig. 5b-d). These observations were analyzed by Image J software (Fiji version) (Fig. 6). Statistical analysis has confirmed the significant positive effect of 2-pentadecanone on glycogen production ($p = 0.00$).

HSP70 and Bax proteins expression level in stomach tissue:

Protein expression was analyzed among experimental groups. Negative control group (Fig. 7a) showed extremely faint HSP70 and significant Bax protein expression, while up regulation of HSP70 protein and down regulation of Bax protein in gastric mucosa of pre-treated groups (Fig. 7b-d) was detected. These results were analyzed by Image J software

(Fiji version), (Fig. 8). Statistical analysis of these results showed significant positive effect of 2-pentadecanone on HSP70 and Bax proteins expression ($p = 0.00$).

DISCUSSION

As it was reported in 2019, stomach ulcer is listed among the most common health problems all over the world[13]. Several studies were conducted to prevent formation of gastric ulcer. A high number of these studies tested the potential effect of whole plant extracts, which include a combination of different compounds with unknown concentrations[14-16]. In the current study, potential gastroprotective effect of 2-pentadecanone was tested to introduce a new synthetic pure compound, since pharmaceutical industries usually focus on synthetic compounds as drug discovery sources[17]. However, some studies tested the gastroprotective effect of pure compounds and detected promising results[11,18,19]. But, the effective doses of these compounds were reported far higher (100 and 200 mg kg^{-1}) than the effective doses of the available drug in market (20 mg kg^{-1} omeprazole) and 2-pentadecanone (10 and 20 mg kg^{-1}). The current study did not exceed the dose of omeprazole.

Although proton pump inhibitors (PPIs) are the most common prescribed drugs against stomach ulcer, there are side effects such as iron and vitamin B12 deficiency and bone fracture associated with the consumption of these drugs[20]. Therefore search for a safer and more effective drug is necessary. This study has provided promising preliminary results to build a base for nano-encapsulation of 2-pentadecanone in future. This is to improve overall drug delivery and its effectiveness.

Some studies reported promising gastroprotective effect of various compounds without testing the acute toxicity of the compound[18,19]. In the current study, safety of 2-pentadecanone was tested prior to evaluate its protective effect against gastric ulcer. The biochemical function and

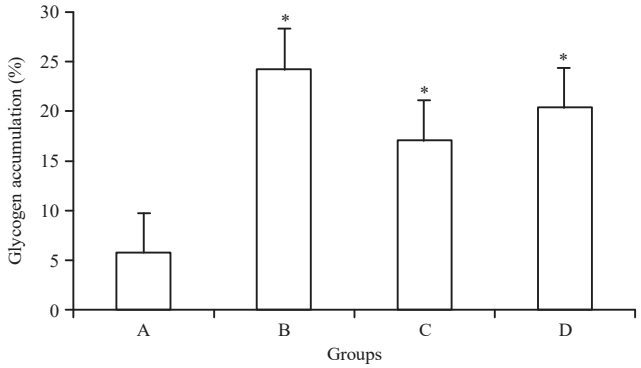

Fig. 6: Glycogen accumulation (%) in stomach tissues, A: Negative control, B: Positive control, C: Low dose 2-pentadecanone and D: High dose 2-pentadecanone (20 mg kg^{-1})

Groups that were pre-treated with omeprazole and 2-pentadecanone (B, C, D) showed higher percentage of glycogen accumulation compare to negative control group, *p<0.05

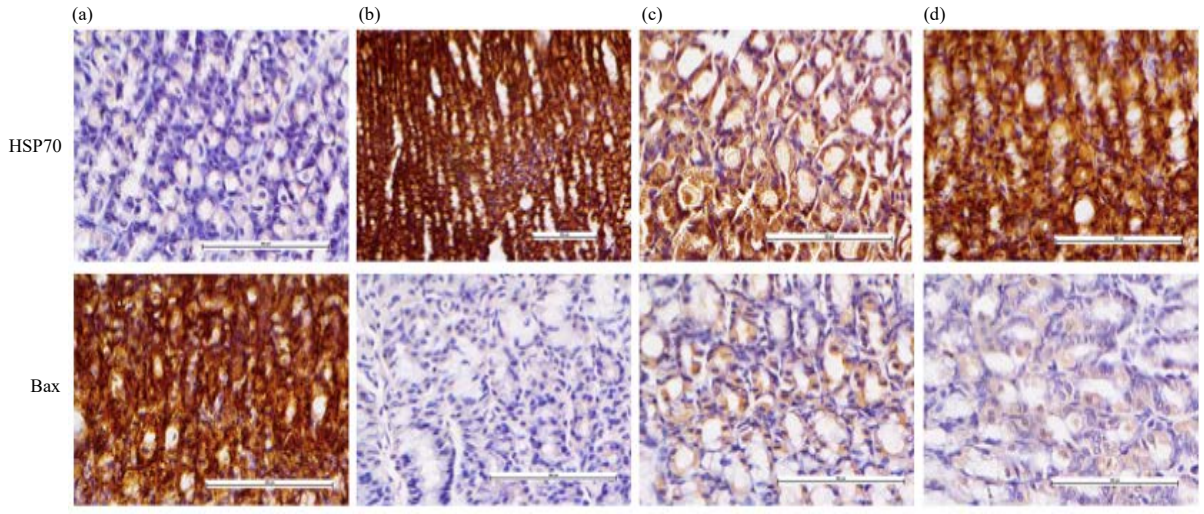

Fig. 7(a-d): Immunohistochemical analysis of HSP70 and Bax expression in stomach tissue, (a) Negative control, (b) Positive control, (c) Low dose 2-pentadecanone and (d) High dose 2-pentadecanone

Immunohistochemistry staining indicated up-regulation of HSP70 protein and down-regulation of Bax protein in group b-d (greater covered area with brown color), scale bar: 100 μm

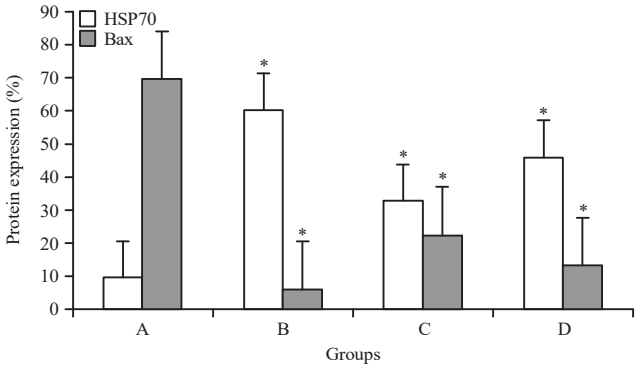

Fig. 8: Effect of 2-pentadecanone on HSP70 and Bax expression in gastric tissue, A: Negative control, B: Positive control, C: Low dose 2-pentadecanone and D: High dose 2-pentadecanone

Higher HSP70 expression percentage and lower Bax expression percentage was detected in pre-treated groups with omeprazole and 2-pentadecanone (B, C, D), *p<0.05

histology of liver and kidneys were assessed to detect safety of 2-pentadecanone prior testing the gastroprotective activity of this compound, as drug metabolism occur in liver and its metabolite is excreted by kidneys[21].

Gastric ulcer appears as a result of imbalance status among aggressive and protective factors of the gastric mucosa. Gastric acid secretion was reported as the main aggressive factor in gastric mucosal injury. High gastric acid secretion cause digestion of gastric mucosa and break down of its barrier. Hence, any compound that has the potential to suppress or reduce gastric acid secretion is known to have a mucosal defensive effect[22].

In the current study, great mucosal surface area is evidenced by flattening of the mucosal folds, which was detected in pre-treated groups (Fig. 2b-d). The flattening is due to muscle relaxation, which result in higher area exposure to the activity of compound[23]. More interesting, 2-pentadecanone resulted in greater mucosal area and muscle relaxation compare to zingerone compound that has been reported recently for its gastroprotective effect[18].

In 2014, another study was conducted by Rouhollahi *et al.*[14] which reported promising results against gastric ulcer. However, in a comparative manner to this study, they tested plant extract (*Curcuma purpurascens*) with a combination of compounds and the ulcer inhibitory percentage of the extract at the dosage of 200 and 400 mg kg^{-1} was lower than the effect of 2-pentadecanone at dosage of 10 and 20 mg kg^{-1} of omeprazole. Hence, 2-pentadecanone can be introduced as a pure and more potent protective compound against stomach ulcer in comparison to zingerone and the plant extract.

Excessive release of ROS such as superoxide anion and hydrogen peroxide due to neutrophil infiltration leads to tissue detruction[24]. Antioxidants provide protection against ROS by maintaining them at their physiological level, thus prevent tissue damage[25]. SOD and CAT are antioxidant enzymes that defense cells against damaging effect of ROS[26]. ROS affect cells by reacting with lipids and producing MDA product under lipid peroxidation process, therefore MDA is known as a marker of oxidative damage[27]. Measurement of MDA level is commonly suggested as it is a biomarker to evaluate lipid peroxidation level in tissue[28]. Pre-treatment with 2-pentadecanone exhibited potent antioxidant response by increasing SOD and CAT and reducing the level of MDA in gastric tissue homogenate, suggested its potential defensive effect in ethanol-induced ulcer in rats.

NO was reported as a protective molecule against gastric ulcer. This molecule exerted its defensive effect by supporting gastric mucosal blood flow, mucus secretion and diminishing inflammation[18]. Following this fact, in this manuscript 2-pentadecanone was suggested as a potential agent against gastric ulcer formation due to its potential to increase the level of NO in stomach tissue of rats.

The latest gastroprotective study that was conducted on the potential effect of zingerone did not include protein expression measurement in tissue or cellular level. The current study focused on the analysis of protein expression in stomach tissue to detect gastroprotective effect of 2-pentadecanone from different point of view.

Investigation of diseases at the cellular level and the related molecular pathways have provided a new insight in drug development studies[29]. The defensive mechanism of HSPs at the intracellular level was reported previously. HSP70, a chaperone molecule, was reported to be associated with diminishing gastric ulcer and cellular protection from apoptosis[30] and also play a role in cellular recovery[31]. In addition, Bax protein supports the initiation of apoptosis in cells undergoing stress. Clearly, application of an suppressive effect on Bax protein expression attenuate cellular damage and prevent necrosis of gastric tissue[1]. In this study, 2-pentadecanone elevated HSP70 protein expression and reduced Bax protein level in stomach tissue (Fig. 7). This can be associated with the gastroprotective mechanism of the tested compound.

CONCLUSION

The results of this study showed a significant gastroprotective effect of 2-pentadecanone against ethanol-induced stomach ulcer formation. The protective

activity of this compound is expected as a result of its antioxidant, anti-inflammatory activity and its protein modulating effect under stressed condition. Nano-encapsulation of 2-pentadecanone can be considered in future studies to improve overall drug delivery.

SIGNIFICANCE STATEMENTS

The current study discovered the safety and gastroprotective effect of 2-pentadecanone at doses not more than the standard anti-ulcer drug (omeprazole). The discovery can be beneficial to consider 2-pentadecanone as a new potential gastroprotective agent. This study by providing preliminary results offered a base for further research on the possible side effects of 2-pentadecanone and their comparison to the side effects of omeprazole and also Nano-encapsulation of 2-pentadecanone for a higher drug delivery level.

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