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Phytochemical, anti-oxidant, anti-microbial, anti-inflammatory and anti-ulcer properties of *Helianthemum lippii*

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The medicinal plants have been considered a healthy source of life for all people and the therapeutical properties of the medical plants are very useful in curing range of diseases. The aims of the study are to evaluate the anti-oxidant, anti-microbial, anti-inflammatory and anti-ulcer properties of *Helianthemum lippii* (MHL) methanolic extracts. MHL samples were collected in the mountain region of Libya. The antioxidant properties of MHL were evaluated using free radical scavenging assay. Stomach ulcers were induced in rats by ethanol. Pretreatment with ranitidine and MHL samples were performed before the ulcer induction. Gastric mucosal histological changes in rat stomach tissue were evaluated. Antimicrobial efficacy of MHL was also studied against gram-positive bacteria *S. aureus*, gram-negative bacteria *E. coli* , and fungal strain *C. albicans*. The phytochemical screening of MHL showed presence of flavonoids, tannins, saponine and simple phenolic compounds. MHL exhibited a powerful anti-oxidant activity, where 31.17 ± 1.40 µg/ml of extract caused 50% inhibition on 2,2, Diphenyl-1-picryhydrazyl (DPPH) scavenging activity in comparison to standard ascorbic acid (15.35 ± **3**.2 µg/ml). MHL also showed substantial anti-microbial activity against a strain of gram-positive bacteria, *S. aureu*, with zone of inhibition (21 mm) and MIC (12.25 mg/ml) and a fungal strain, *C. albicans*, with zone of inhibition (20 mm) and MIC (**6**.25 mg/ml) compared to ciprofloxacin or amphotericin B. MHL at doses of 250 and 500 mg/kg produced statistical significant anti-inflammatory activity (23.6%, 50%) in comparison to aspirin (60%). It further showed significant anti-ulcer activity in doses of 250mg/kg and 500mg/kg with percentage of gastric lesions inhibitions of 48.78% and 76.82%, respectively, in comparison to standard anti-ulcer ranitidine (50 mg/kg), which showed 69.78%. MHL has clearly a protective effect against ethanol-induced gastric mucosal lesion, and this effect, at least in part, depends on the increase of anti-oxidant activity. In conclusion, the MHL extract showed anti-oxidant, anti-ulcer, anti-microbial activities against tested microbes, antioxidant and anti-inflammatory; mightily due to combined mechanisms of MHL's constituents.

Keyword: gastric ulcer, *Helianthemum lippii*, *Cistaceae*, anti-oxidant, anti-microbial, anti-inflammatory, anti-ulcer.

Abbreviations: DPPH: 2,2, Diphenyl-1-picryhydrazyl; MEHL: methanolic extract of *H. lippii*; MHL: *Helianthemum lippii*; MIC: Minimum inhibitory concentration

1. Introduction

Gastric ulcer is one of the most widespread diseases that is believed to be caused by an imbalance between aggressive (mainly gastric acid and pepsin) and protective factors (mucosal protections). This imbalance leads to potential

injuries in gastric mucosal cells[1,2]. There is a variety of commercial drugs available for the treatment of gastric ulcer, but most of these drugs are unsatisfactory because of their side effects[3,4]. Thus the lack of new anti-ulcer agents has become an important issue.

Herbal medicine is one of the oldest forms of healthcare known to man. About half of the drugs approved from 1998 to 2007 are based on natural products and thirteen natural product-related drugs were approved from 2005 to 2007[5]. Compared with synthetic anti-ulcer compounds many natural products exhibit significant activity against this disease without considerable adverse effects[6]. Several medicinal plants have been traditionally and extensively used to treat gastric ulcer[7,8]. *Helianthemum lippii* (H.L), is a plant which is used traditionally by people in Libya as anti-microbial treatment. It belongs to the plant family of *Cistaceae* which is used to treat cutaneous lesions and is known to exhibit gastro protective properties[9]. Because there are no scientific reports on the anti-ulcer activity of this plant we set out for the investigation of this issue.

2. Material and Methods

2.1 Chemicals and Standards

Graded solvents, 2,2, Diphenyl-1-picryhydrazyl (DPPH), carboxymethyl cellulose, carrageenan, standard ascorbic acid, standard ranitidine, aspirin, ciprofloxacin, and amphotericin B were purchased from Sigma/Aldrich, Sternheim, Germany.

2.2 Plant Collection

The whole aerial part of *Helianthemum lippii* (L.) Dum.Cours. (*H.lippii*) family *Cistaceae* was collected from Al-gabel Al- gharbi (Gharian, Libya) during the spring season. It was identified and authenticated by the Department of Botany Faculty of Sciences Tripoli University, Tripoli, Libya.

2.3 Preparation of Extract

The plant material was dried in the shade and grind to coarse powder in a mechanical grinder (Peruzzo, Italy). The powdered plant material was successively extracted with different organic solvents using a maceration method for extraction. 1000 g of plant powder were extracted using petroleum ether, chloroform or methanol (72 h for each solvent), respectively. The three crude extracts were then dried by using a rotary evaporator and stored at -20°C.

2.4 Preliminary Phytochemical Screening

The phytochemical screening the methanolic extract of *H. lippii* (MEHL) was performed according to standard literature methods in which the extracts were exposed to different reagents to identify the primary metabolites, like carbohydrates (Molisch reagent test), Proteins (Biuret test) and the secondary metabolites such as alkaloids (Mayer's test), flavonoids, terpenoids (Salkowski test), tannins (Ferric chloride test), saponins (Frothing test), cardiac glycosides (Keller-Killiani test) and anthraquinones(Borntrager's test)[10].

2.5 Anti-oxidant activity

The free radical scavenging activity of the methanol extract was evaluated by using 2,2, Diphenyl-1-picryhydrazyl (DPPH)[11], form where a stock solution of methanolic extract (1mg/ml) was prepared. 400 µl of 0.1 µM of DPPH solution was added to a 1 ml cuvette. Then MEHL solution of different doses (1 – 50 µg) was added. 600 µl of ethanol 99% was added and the mixture was shaken vigorously and allowed to stand in a dark place at room temperature for 5 min. Then the absorbance was measured at 517 nm using a UV-Visible NIR spectrophotometer (Varian Cary 5000, USA). Low absorbance of the reaction mixture indicates substantial free radical scavenging activity. The radical scavenging activities of the test samples expressed as percentage of inhibition were calculated according to the following equation[12]:

$$\text{Percent of DPPH inhibition} = [(A_A - A_B)/AB] \times 100$$

where A_A and A_B are the absorbance values of the test and the blank sample, respectively. The percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

2.6 Anti-microbial activity

2.6.1 Microorganism

The microorganisms employed in the current study were procured from the American type of cell culture collection (ATCC). Strains of gram-positive bacteria *S. aureus* (ATCC 29213), gram-negative bacteria E. coli (ATCC.25922), and fungal strain *C. albicans* (ATCC 10231) were employed.

2.6.2 Media

Nutrient broth, Mueller Hinton agar and Sabouraud dextrose agar, all products of Liofelshem company (Italy) were used.

2.6.3 Agar well Diffusion Bioassay

The anti-microbial activity of the extracts was determined by using the agar well diffusion technique[13,14]. Mueller Hinton agar plates were seeded each with 0.1 ml of bacterial suspension (equivalent to $10^7 - 10^8$ CFU/ml), while the Sabouraud dextrose agar plates were similarly seeded with fungal strain. The 12 hrs broth culture of each bacterium and fungus culture were used to seed on sterile Mueller Hinton agar and Sabouraud dextrose agar at 37°C. The agar was allowed to set and cool and wells drilled by a sterile standard cork borer. An aliquot of 50 µl of 20 mg/ml solution of the extract were added into each well. Then bacterial plates and fungal plates were incubated at 37°C for 24 hrs and the diameter of inhibition zones measured. Ciprofloxacin and Amphotericin B were used as standards.

2.6.4 Determination of MIC

Minimum inhibitory concentration (MIC) of any compound is defined as the lowest concentration which completely inhibits visible growth (turbidity on liquid media). MIC values were determined using the microdilution method[15]. Solutions of the tested extract of MHL were prepared in phosphate buffer at a concentration of 50 mg/ml. From this stock solution serial dilutions of the extracts (50, 25, 12.25, 6.25 and 3.12 mg/ml) were prepared to determine the MIC. All measurements were done in triplicate. The standard antibiotics (ciprofloxacin and ketokenazol) were used as positive controls. At the end of the incubation period MIC values were determined.

2.6.5 Animals

Male, wistar albino rats and Swiss albino mice were used in this study. They were obtained from the animal house of our National Medical Research Center (NMRC), Zawia, Libya. The animals were housed in cages and maintained at 22°C ± 1°C under 12h light/dark cycle, they had free access to water and were fed with standard laboratory chow. The study was conducted in accordance with the nationally accepted guidelines for laboratory animal use and care (NMRC35/2009).

2.6.6 Acute Toxicity

Forty two male Swiss albino mice (25 - 40 g) were fasted for 24 hour and divided into 6 groups, each group containing 6 animals. All the groups were orally fed with methanolic extract of *H. lippii* (MEHL) suspended in 1% carboxy methyl cellulose in increasing doses of 100, 500, 1000, 3000,6000,10000 and 15000 mg/kg[16]. The animals were observed for 2 h for any behavioral changes, neurological and autonomic profiles or cases of death after 24 h and 72 h[17].

2.7 Anti-inflammatory Activities

The carrageenan induced mice paw edema model was used in this study [18,19]. Twenty four fasted male albino mice (25 - 40 g) were used and divided into four groups (6 in each). All groups were injected intraperitoneal. First group was subjected to 0.5 ml normal saline (negative control), the second group was treated with standard aspirin 100 mg/kg (positive control), whereas the third and fourth groups were treated with MEHL (250, 500 mg/kg). The acute inflammation was produced by the sub-plantar administration of 0.1 ml of 1% carrageenan into the right Paw. Paw volume was measured at 0 and 3 h after the administration of the carrageenan using a plethysmometer (Ugo Basile, Italy). The anti-inflammatory effect of MEHL was calculated by the following equation[20]:

Anti-inflammatory activity (%) = (1-D/C) × 100, where D represents the percentage difference in paw volume after drugs administration and C represents the percentage difference of volume in the control groups.

2.8 Anti-ulcer Activity

Twenty four fasted male wistar albino rats (150 - 250 g) were used. The anti-ulcer effect of methanolic extracts of *Helianthemum lippii* (MHL) was investigated using the ethanol-induced ulcer model[21]. The animals were divided into four groups, six rats each. All the groups were treated by oral route. The first group was treated with 1 ml normal saline (negative control). The second group was treated with 50 mg/kg standard ranitidine[22]. The third and fourth groups were treated with MHL 250 mg/kg and 500 mg/kg, respectively. The different treatments were administered one hour before ulcer induction which was caused by oral administration of absolute ethanol (99%) (1ml/animal). One hour after the administration of ethanol, animals were sacrificed by suffocation with chloroform, and stomachs were incised along the greater curvature. Stomach contents were collected to measure gastric pH (HANA pH meter). Ulcers found in the gastric mucosa, appeared as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. Each specimen of gastric mucosa was examined for damage. The length and width (mm) of the ulcer on the gastric mucosa were measured by a micrometer and ulceration was scored. Mean ulcer score for each animal was expressed as ulcer index (U.I). The ulcer index was determined using the following formula[23]:

$$\textbf{Ulcer index} = 10/X,$$

where X = total mucosal area / total ulcerated area. The ulcers were given scores based on their intensity as follows: **0** = normal stomach, **0.5** = red coloration, **1** = spot ulcer, **1.5** = hemorrhagic streak, **2** = ulcers, **3** = perforation.

The percentage of ulcer protection was determined as follows[24]:

$$\% \text{ protection} = \frac{\text{control mean ulcer index} - \text{test mean ulcer index}}{\text{control mean ulcer index}} \times 100$$

Gastric juice pH was determined by taken aliquot of 1ml gastric juice and diluted with 1ml of distilled water and the pH of the solution was measured using a pH meter**.**

2.9 Histopathological Evaluation

Specimens of the gastric walls from each rat were kept in 10% buffered formalin for 24 h and processed in a paraffin tissue processing device. Sections of the stomach were made at a thickness of 5 µm using a rotary microtome and stained with hematoxylin and eosin for histological evaluation[25].

2.10 Statistical Analysis

Values for anti-oxidant, anti-microbial, anti-inflammatory and anti-ulcer activities were expressed as mean ± standard error of the mean (S.E.M.). The significance of difference between means was determined by one-way analysis of variance (ANOVA) and values of $p < 0.05$ and $p < 0.01$were considered as significant and highly significant, respectively.

3. Results

3.1 Preliminary Phytochemical Screening

Preliminary phytochemical tests revealed that the main active constituents of MHL are polyphenols including flavonoids, tannins, glycosides, simple phenolics, free reducing sugers and saponines, while free anthraquinones, steroids, terpenoids and alkaloids were absent.

3.2 Anti-oxidant Activity

MHL exhibited good anti-oxidant activity, where 31.17 ± 1.40 µg/ml of extract showed 50% inhibition on DPPH scavenging activity if compared to ascorbic acid (20.73 ± **3**.2 µg/ml) as anti-oxidant standard[26].

3.3 Anti-Microbial Activity

MHL also showed a good anti-microbial activity (Table 1) against gram-positive bacteria *S. aureus* with zone of inhibition (21 mm) and MIC (12.25 mg/ml) and the fungal strain *C. albicans* with

zone of inhibition (20 mm) and MIC (**6**.25 mg/ml) in comparison to ciprofloxacin and amphotericin B, but did not show any activity against gram-negative bacteria *E. coli*.

Table1: anti-microbial activity and MICs of MHL

	Zone of inhibition (mm) (MIC in mg/ml)		
	S. aurious (ATCC 29213)	*E. coli* (ATCC.25922)	*C. albicans* (ATCC 10231)
MHL	21 (12.25 mg/ml)	n.a.	20 (**6**.25 mg/ml)
*Ciprofloxacin	24 0.25	32 10	///////
*Amphotericin B	///////	///////	22 0.5

The results summarized are mean values of n = 3; n.a.: no activity; * zone of inhibition and MICs of standard antibiotics (µg/ml).

3.4 Anti-Inflammatory Activity

The MHL at doses 250 and 500 mg/kg produced a statistical significant ($P < 0.05$) anti-inflammatory activity (23.6%, 50%) in comparison to controls. The result at a dose of 500 mg/kg was comparable to that of aspirin (60%) as a standard anti-inflammatory drug with no significant difference (table 2).

Table 2: Anti-inflammatory activity of different doses of MHL and aspirin on carrageenan-induced paw edema.

Treatment	Edema volume (µl)	% of anti-inflammatory activity
normal saline (negative control)	94.0 ± 3.54	-------
aspirin 100 mg/kg (positive control)	37.3 ± 2.27	60.00
MHL500 mg/kg	47.5 ± 2.09	50.00
MHL 250 mg/kg	71.8 ± 5.05	23.60

Results are means ± S.E.M. and data are evaluated by using one-way analysis of variance (LSD and Duncan tests) *p <0.05, n= 6.

3.5 Anti-Ulcer Activity

Oral administration of ethanol (99%) produced multiple mucosal lesions in the glandular portion of the rat stomach, which appeared as elongated bands of thick, black and dark red lesions (Fig. 1a). Pre-treatment with MHL or ranitidine were found to inhibit the ethanol-induced gastric mucosal injury in rats. Preventive effects of 250 and 500 mg/kg MHL occurred in a dose dependent manner. However, animals treated with methanol extract of MHL at 250 and 500 mg/kg doses showed significant (P<0.05) reduction in the number of ulcer and ulcer index (Table 3). The results showed 48.78% and 76.82%, (Table 3 and Fig. 1c, 1d) ulceration inhibition at a dose of 250 and 500 mg/kg, respectively, whereas ranitidine showed 69.78% inhibition of ulceration (Fig. 1b). The Anti-ulcer effect of MHL in ethanol induced ulcers was comparable to that of ranitidine (50 mg/kg).

Table 3: Effects of different doses of MHL and ranitidine on ethanol induced gastric mucosal injury.

groups	ulcer index	PH of gastric juice	% of protection

control	8.24 ±0.28	2.16±0.19	--------
ranitidine 50 mg/kg	2.49±0 .29	6.27±0.28	69.78
MHL 250 mg/kg	4.22±0.51	4.26±0.30	48.78
MHL 500 mg/kg	1.91±0.22	5.89±0.40	76.82

Results are means ± S.E.M. and data are evaluated by using one-way analysis of variance (LSD and Duncan tests)*p <0.05, n = 6.

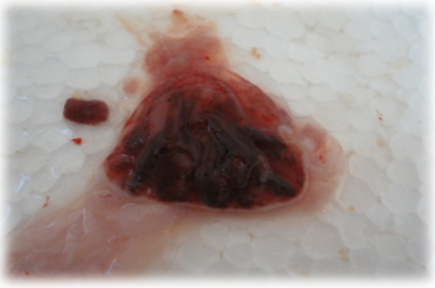

Figure 1a: Gross appearance of hemorrhagic gastric mucosal lesions after ethanol treatment to induce ulcer. Application of 1 ml of normal saline still shows severe injuries of the gastric mucosa (negative control). Untreated ethanol induced ulcers did not differ from normal saline treated ulcers.

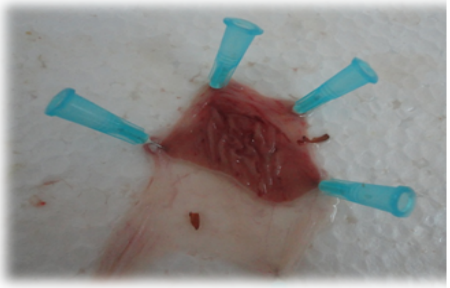

Figure 1b: Gross appearance of the gastric mucosa group pre-treated with standard ranitidine (50 mg/kg, positive control) after ethanol treatment. Mild injuries are seen in the gastric mucosa compared to the injuries seen in the negative control group (1a).

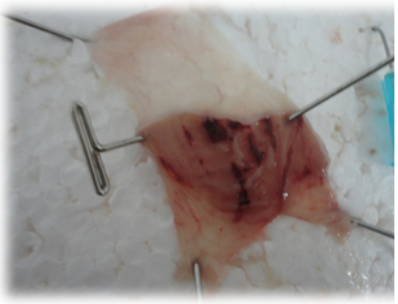

Figure 1c: Gross appearance of the gastric mucosa in group pre-treated with MHL (250mg/kg) after exposure to ethanol. The figure shows milder injuries to the gastric mucosa compared to negative control group (1a)

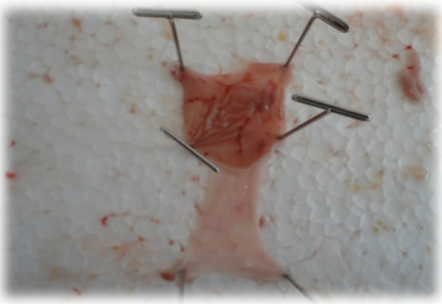

Figure 1d: Gross appearance of the gastric mucosa in group pre-treated with MHL (500mg/kg) after ethanol application. No injuries to the gastric mucosa are seen and gastric mucosa appears flat compared to the negative control group. The 500 mg/kg dose of MHL shows an enhanced correction of the architectural distortions created by ethanol.

3.6 Histopathological Evaluation

The histological observation of ethanol induced gastric lesions of the ulcer control group untreated or pre-treated with normal saline, showed extensive damage and discontinued villi in the gastric mucosa and inflammation of the

submucosal layer as seen in Fig. 2a. Animals pretreated with MHL at doses of 250 or 500 mg/kg yielded considerably improved protection of the gastric mucosa as seen in Fig. 2c, 2d, indicated by reduced or the absence of damage, continuation of villi in the gastric mucosa, and reduced or absence of submucosal inflammation compared to control groups. The group pre-treated with MHL 500 mg/kg showed a higher protection in comparison with the positive control group pre-treated with standard anti-ulcer ranitidine (50 mg/kg). The cytoprotective effect of MHL therefore shows dose-dependency.

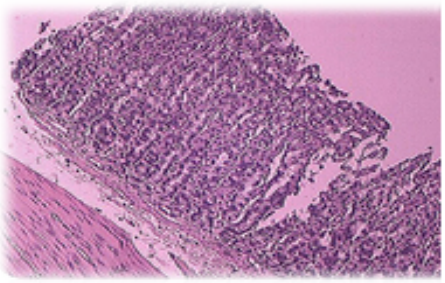

Figure 2a: Photomicrographs section of gastric mucosa in negative control group (after ethanol induced ulceration and saline pre-treated) shows severe disruption to the surface epithelium and inflammation (H&E stain, 10x).

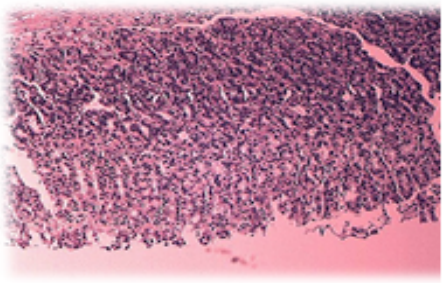

Figure 2b: Photomicrographs section of gastric mucosa (after ethanol induced ulceration and 50 ml/kg ranitidine pre-treated), shows a mild disruption to the surface epithelium with mild inflammation (H and E stain 10x).

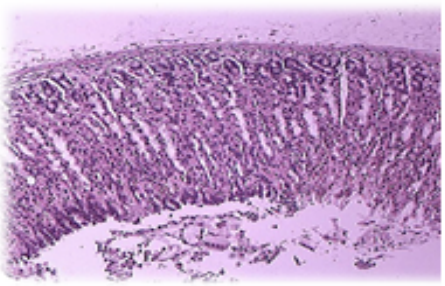

Figure 2d: Photomicrographs section of gastric mucosa (after ethanol induced ulceration and 250 mg/kg MHL pre-treated) shows disruption to the surface epithelium with mild inflammation (H and E stain 10x).

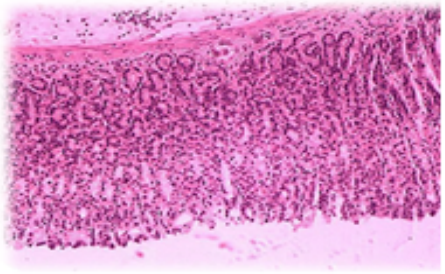

Figure 2c: Photomicrographs section of gastric mucosa (after ethanol induced ulceration and 500 mg/kg MHL pre-treated) shows no disruption to the surface epithelium and no inflammation. (H and E stain10x).

3.7 Acute Oral Toxicity Study

Acute oral toxicity was carried out by as mentioned in the material and methods. It was found that MHL was safe at a limit dose of 15000 mg/kg with no mortality of studied subjects.

4. Discussion

4.1 Anti-Oxidant Activity

MHL extract, which contains high amounts of flavonoid and phenolic compounds, showed consistent and significant anti-oxidant activity. The high scavenging property of MHL extract may be due to hydroxyl groups existing in the phenolic compounds' chemical structure that can provide the necessary component as a radical scavenger. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases is well established and has been reported previously[27,28]. The potent scavenging of free radicals may therefore serve as a possible preventative intervention for the diseases. The anti-oxidant activity of MHL extract could be related to the high amount of flavonoid and phenolic compounds in this plant extract.

4.2 Anti-microbial and anti-inflammatory activities

The methanolic extract isolated from MHL also showed anti-bacterial (*S. aurious*) and anti-fungal (*C. albicans*) activities. Polyphenols such as tannins and flavonoids in the methanol extract were detected in this study. Tannins are known for their astringent property and anti-microbial activity[29,30]. It is well known that tannins and flavonoids are also responsible for strong free radical scavenging activity and anti-inflammatory properties[31]. Free radical scavengers can inhibit the process of an inflammatory response[32]. In a recent review article, cellular mechanisms for anti-inflammatory activity of flavonoids have been discussed in detail[33]. Flavonoids possess anti-oxidative, radical scavenging activities and regulate cellular activities of inflammation-related cells: mast cells, macrophages, lymphocytes, and neutrophils. For instance, some flavonoids inhibit histamine release from mast cells and others inhibit T-cell proliferation. Additionally, some flavonoids alter metabolizing enzymes such as phospholipase A2,cyclooxygenase, lipoxygenase and the nitric oxide (NO) producing enzyme, nitric oxide synthase. An inhibition of these enzymes by flavonoids reduces the production of prostaglandins, leukotrienes, and NO, crucial mediators of inflammation. Consequently, the inhibition of these enzymes by flavonoids may be considered as one of the important cellular mechanisms of anti-inflammation[34,35].

4.3 Anti-ulcer activity

Ethanol-induced gastric ulceration is a suitable model for investigating the gastroprotective effects of herbal medicines. The causes of peptic ulcer are still unknown in most cases. Nevertheless, it has been proposed that it results from an imbalance between destructive factors and the maintenance of mucosal integrity through the endogenous defense mechanisms[2,36]. To restore this balance, different therapeutic agents like H2-blockers, proton pump inhibitor and plant extracts are used[37,38]. MHL extract is one such herbal drug was used in the present study to further evaluate the anti-ulcerogenic in ethanol induced ulcers in rats. Ethanol has been reported to cause turbulences in gastric secretion, destruction of the mucosa, alters in the permeability of the gastric membrane, gastric mucus depletion and free radical production. This has been attributed to the release of superoxide anion and free hydroperoxy radicals produced during metabolism of ethanol. Oxygen derived free radicals have been found in the mechanism of acute as well as chronic ulceration in the gastric mucosa[39]. Ethanol induced gastric ulcer might be due to stasis in gastric blood flow which contributes to the development of hemorrhage and necrotic features of tissue injury. Alcohol quickly penetrates the gastric mucosa and causes cell and plasma membrane damages leading to increased intracellular membrane permeability to sodium and water. Furthermore, an enormous intracellular increase of calcium corresponds to a major step in the pathogenesis of gastric mucosal injury[37]. These changes lead to cell death and exfoliation in the surface epithelium[37]. Our results show that the MHL extract significantly reduced ethanol-induced ulcer. This could be due to a cytoprotective effect of MHL extract through anti-oxidant effects. The extract shows protection against distinctive lesions produced by ethanol administration. The anti-ulcer effect of MHL may be due to two factors, reductions in gastric acid

secretion and gastric cytoprotection. A preliminary phytochemical analysis of MHL extract showed the presence of polyphenols including flavonoids, tannins, glycosides, simple phenolics, free reducing sugars and saponines. The significant increase in the anti-ulcer activity of MHL might be attributed to the presence of flavonoids (quercetin), tannins, saponin glycosides and phenolic compounds. Flavonoids are among the cytoprotective materials for which anti-ulcerogenic efficacy has been reported[40-42]. It is suggested that, these active compounds would be able to stimulate mucus, bicarbonate and the prostaglandin secretion and counteract with the distorting effects of reactive oxidants in gastrointestinal lumen[43-46]. Hence, the anti-ulcer activity of MHL may be attributed to its flavonoids content. Our study suggests that the methanol extract of MHL may be useful in the treatment of gastric lesions. Possibly not only a single molecular species contained in the plant extract is responsible for its gastroprotective effect but it may be the combination of various compounds. Further studies to identify the active moieties and elucidation of the mechanism of action are needed.

5. Conclusion

Research in medicinal plants has recently attracted considerable attention, particularly in order to avoid side effects that often lead to serious complications of classical drugs. Plant based drugs are being investigated with the intent to circumvent some of these problems. Our study using the plant *Helianthemum lippii* indicates that its methanolic extract offers a new gastroprotective treatment with less side effects, it is less costly, affordable and more effective in the treatment of different types of gastric diseases.

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