GASTROPROTECTIVE EFFECT OF AQUEOUS *TRIGONELLA FEONUM-GRACUM* AND *LINUM USSITATISSIMUM* SEED EXTRACTS IN MICE

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Abstract

The majority of Ethiopian population relies on traditional remedies and some of which may also have nutritional value. *Trigonella foenum-gracum* infusion and *Linum usitatissimum* water extract are used to manage peptic ulcer. This traditional practice supplements the modern medicine and fills the gap where the latter appears to be inadequate, ineffective or costly. However, the safety and efficacy of these remedies are not well known. The aim of this study is, therefore, to screen the aqueous extract of *T. foenum-gracum* and *L. usitatissimum* seeds for their anti-ulcer activity with the acute toxicity evaluation. The results indicated that both aqueous *T. foenum-gracum* and *L. usitatissimum* seed extracts reduced the ulcer index and ulcer number of ethanol induced lesions ($P<0.001$). The extracts showed dose dependent anti-ulcer activity. Similarly, *T. foenum-gracum* and *L. usitatissimum* extracts protected the indomethacin-induced gastric mucosal damage dose dependently. Per oral LD_{50} of both extracts was greater than 2000mg/kg whereas the intraperitoneal LD_{50} of the aqueous extract of *T. foenum-gracum* and *L. usitatissimum* seeds were found to be 4677.4 and 1698.2 mg/kg, respectively. From the present study it can be concluded that the aqueous extracts of *T. foenum-gracum* and *L. usitatissimum* have anti-ulcer effect supporting their claimed traditional use. They also appear safe at the anti-ulcer doses. However, further studies are required before the extracts are used as medicine.

Key words: *Trigonella foenum-gracum, Linum usitatissimum,* linseed, fenugreek, indomethacin, alcohol.

African ethnomedical systems employed numerous plant extracts for the treatment of diseases, including gastrointestinal disorders, in order to give a therapeutic choice to the population (1). *Linium usitatissimum* L. (Flux) (Linaceae) and *Trigonella foenum-gracum* L. (Fenugreek) (Leguminosae) are traditionally used in the management of peptic ulcer in Ethiopia.

Linium usitatissimum L. is 20-35 cm long annual herb. Its seed contains fixed oil, mucilage, protein and cyanogenic glycosides. *L. usitatissimum* seed has been used as a poultice for local inflammation (2). From experimental studies, *L. usitatissimum* seed inhibits breast tumor development (3) and the growth of prostate cancer (4). *L. usitatissimum* seed oil in men shows total cholesterol concentration reduction by 12.5 % (5).

Trigonella foenum-gracum L. is a herbaceous annual of 10-40 cm, aromatic and has compound leaves of 7 to 12 cm long. It has been used for labor induction, aiding digestion and lactation (6). The seed contains alkaloids, steroidal sapogenins, fixed oil and mucilage (2). Preliminary study on animal showed that *T. foenum-gracum* seed reduced total acid in ethanol-induced ulcer model (7**).** Other studies reveal the possible hypoglycemic and antihyperlipidemic properties of *T. foenum-gracum* seed (6).

The majority of Ethiopian population relies on traditional remedies such as *T. foenum-gracum* seed infusion and *L. ussitatissimum* seed water extract to manage peptic ulcer via oral route (8). This traditional practice may supplement the modern medicine and fills the gap where the latter appears to be inadequate, costly or ineffective. However, the safety and efficacy of these remedies are not well known. The aim of this study is, therefore, to screen the aqueous extracts of *T. foenum-gracum* and *L. usitatissimum* seed for their antiulcer activity (indomethacin and alcohol induced model) with acute toxicity evaluations.

Materials and Methods

Plant materials and extract preparations:

The seeds used in this study were purchased from a supermarket in Addis Ababa. The plants were identified and authenticated by Mr. Melaku Wondafrash of the National Herbarium, Department of Biology, Faculty of Science, Addis Ababa University. A voucher specimen of the plant was deposited in the National Herbarium. Unpowdered whole seeds of *L. usitatissimum* were macerated in distilled water in a ratio of 1 gm: 6 ml for 48 hrs and the aqueous viscous material was separated from the mixture by straining using muslin. Powdered *T. foenum-gracum* seeds were soaked in distilled water in a ratio of 1 gm: 100 ml for 12 hrs. And the supernatant (aqueous layer) was separated from the mixture by decantation. The aqueous extracts of both seeds were then lyophilized using a freeze-drier. Portions of the same extracts that were subjected for the pharmacological investigation were used for the identification of the major secondary metabolites (9, 10). The extracts and standard drugs used for the pharmacological investigations were dissolved in distilled water freshly.

Animal preparation:
Swiss Albino mice (25-30 gm) of either sex were used for the study. These were purchased from Ethiopian Health and Nutrition Research Institute and the animals were acclimatized for a week at the animal house of Faculty of Medicine. Animals were handled in this study as per the International Guidelines for handling experimental animals. Animals were fed on pellet and water. They were deprived from food before the experiment but were allowed free access to water. To prevent coprophagy, animals were kept in cages with grating floors (11). The animals were sacrificed by cervical dislocation for histological examination. The total animals used were 228 mice. Six to eight animals per group for pharmacological test and ten animals per group for LD_{50} determination were used during the investigation. The number of groups arranged was 22 for pharmacological test and 8 for LD_{50} determination.

Chemicals and drugs:
The following drugs and chemicals were used: absolute alcohol (Changshu, China); Cimetidine (Kwang Myung Pharm Co.Ltd, Korea); Formaline (Alpha Laboratory Reagent, India); Indomethacin (Indukern Chemie AG, Switzerland; granted from East African Pharmaceuticals P.L.C.); Omeprazole (Ambalal Sarabha Enterprises, India); Sodium bicarbonate (BDH Chemicals Ltd Poole, England).

Extract and vehicle administration:
The animals were given a volume of 0.8ml per 30g body weight of the dissolved extract which is equivalent to each dose or vehicle *per os*. A volume of 2 ml per 30g that is equivalent to each dose (g/kg) calculated on basis of the weight of each mice was administered intraperitoneal (i.p.) for acute toxicity assessment.

Pharmacological investigations:

i. Gastroprotective effect of the extracts in ethanol induced gastric ulceration

Fifty minute before absolute alcohol (99.9 %, 0.2ml *per os*), twenty-four hours fasted mice were treated with extracts (500, 1000 and 1500 mg/kg), omeprazole (50 mg/kg) or distilled water *per os*. One hour after administration of absolute ethanol, each animal was killed by cervical dislocation. The stomach was excised and injected with 3 ml of 5 % formalin solution. After 15 minutes, the stomachs were opened along the greater curvature, rinsed with tap water, and then inspected visually for destructive mucosal lesions (12). The extent of damage was expressed by the sum length of (mm) all lesions, lesion index, in the glandular area of stomach and by the mean total number of lesions (13).

$$\% \text{ Inhibition} = \frac{\text{Lesion index in control} - \text{Lesion index in test}}{\text{Lesion index in Control}} \times 100$$

The percent inhibition of lesion number was also calculated similarly.

ii. Gastroprotective effect of extracts in indomethacin induced gastric ulceration

Twenty-four hours fasted mice were intragastrically treated with extracts (500, 1000, and 1500 mg/kg) or vehicle. One hour later, the ulcerogenic agent (indomethacin) prepared in 2 % sodium bicarbonate solution was given to mice at an oral dose of 30 mg/kg according to the method described by Sartori, *et al* (14). The animals were sacrificed after 6 hrs; the stomachs were removed and injected with 3 ml of 5 % formalin solution. After 15 minutes, the stomachs were opened along the greater curvature, rinsed with tap water, and examined for ulcers. The ulcers were counted with the aid of a hand lens (5 times magnification power) and each was given a severity rating as follows: less than 1mm = 1; 1 - 2mm = 2; and greater than 2 mm = 3. The summation of the score was divided by a factor of 10, to derive ulcer index for each animal as described by Makonnen (8). The percent inhibition of ulcer was determined in the same way as that for ethanol-induced lesions.

iii. Influence of indomethacin pretreatment on the extracts gastroprotection

In an experiment designed to determine whether mucosal protection by the extract is dependent on the synthesis of prostaglandins, mice were given indomethacin (20 mg/kg, Intraperitoneal (I.P)) 60 minutes before administration of extracts or distilled water. In three other groups, mice were given 2 % sodium bicarbonate (vehicle for indomethacin) before the extracts or distilled water (15). All the animals received 0.2ml absolute ethanol 50 minutes after the extract administration and were killed 1 hr later. The remaining procedure was in the same way as that for ethanol-induced lesions (12).

iv. Acute toxicity

Acute toxicity of both plant extracts was assessed using 12 hrs fasted mice. The safety evaluation of the extracts was performed by both oral and i.p. routes. The administration of the extracts orally did not show any toxic manifestation up to a dose of 2000 mg/kg. For a dose ranges exceeding 2000 mg/kg the material become more viscous and makes it difficult to administer *per os* through intra gastric tube. Hence, i.p. routes were used in order to determine the LD_{50} of the extracts. Increasing doses of extracts were administered i.p. to groups of 10 mice of either sex at a dose range of 2000 – 8000 mg/kg (*T. foenum-gracum* seed extract) and 750- 2500 mg/kg (*L. usitatissimum* extract). Animals that received the vehicle (distilled water) served as control. The groups were examined for observable toxic effect. The number of survivors within 24 hrs was recorded; and the acute toxicological effect was inferred on the basis of mortality expressed as i.p. LD_{50} by probit method (16).

Statistical analysis

The data, expressed as mean $\pm$ standard error of the mean, were analyzed using one-way ANOVA. *Post hoc* comparisons between the two groups were made with Dunnett's test using SPSS 10 statistical software package. When appropriate, independent student t-test was used. P value of less than 0.05 was considered statistically significant.

Results

Phytochemical screening:
Screening of the powdered lyophilized aqueous *T. foenum-gracum* seed extract for its chemical constituents showed the presence of polyphenols, alkaloids, saponins, polysaccharides and chromophoric compounds. Similar screening of the whole seeds aqueous extract of *L. ussitatissimum* indicated the presence of peptides and polysaccharides.

Gastro protective effects of the extracts on ethanol induced lesion:
The effects of *T. feonum-gracum* and *L. usitatissimum* on ethanol induced gastric lesions are shown in Table 1. Vehicle treated control mice showed extensive gastric mucosal lesions in the glandular segments. Dose dependent gastro protective effect against ethanol-induced lesion was observed with both extracts. *T. foenum-gracum* extract was found to be more potent than that of *L. usitatissimum* in protecting the animals from ethanol induced lesion. Both extracts, however, were less potent than omeprazole, the standard drug, considering the much smaller dose of the standard employed here.

Table 1 Effect of *L. usitatissimum and T. feonum-gracum* extracts on ethanol induced gastric lesion in mice

Treatment	Dose(mg/kg)	n	Lesion Index	% ILI	Lesion No.	% ILN
Control	--	8	29.3 ± 2.7	---	14.6 ± 1.5	--
L. usitatissimum	500	6	15 ±1.3*	48.8	8.2 ± 0.5*	43.8
	1000	6	11.1 ±3.1**	62.1	5 ± 1.4**	65.8
	1500	6	7.33 ±2.3**	74.9	4.5 ± 2.6**	69.2
T. foenum-gracum	500	6	8.3 ±3.2**	71.7	5.2 ±2.2**	64.4
	1000	6	6.8 ± 3.3**	76.8	4.7 ±1.3**	67.8
	1500	6	4.5 ± 1.8**	84.6	3.3 ± 1.3**	77.4
Omeprazole	50	6	8.8 ± 2.7**	70	5.7 ±1.8**	61.1

*P<0.01 and **P<0.001, statistically significant relative to control (Dunnet's t-test)
% ILI = % Inhibition of lesion index; % ILN = % Inhibition of lesion No,
n = number of animals.

Gastroprotective effects of the extracts on indomethacin induced ulcer:
Intragastric administration of indomethacin (30 mg/kg) resulted in production of gastric lesions on glandular segment of the stomach. Dose dependent gastroprotective effect against indomethacin induced ulcer with both *L. usitatissimum* and *T. foenum-gracum* extracts, with insignificant difference in potency at all dose level, were observed as shown in Table 2. The potency of both extracts, however, was less than that of cimetidine, the standard drug, considering the smaller dose of the standard used in the experiment.

Influence of indomethacin pretreatment on the extracts gastro protection:
Indomethacin (20 mg/kg, i.p.) pretreatment significantly aggravated the ethanol-induced lesions ($P < 0.001$) (Table 3). In mice groups that did not receive indomethacin, the *L. ussitatissimum* extract (1000 mg/kg) significantly decreased the lesion index by 56.9 % ($P < 0.001$), while *T. foenum-gracum* extract reduced the lesion index by 52 %. In the indomethacin-pretreated groups the lesion index due to ethanol was 52.6 ± 1.9 for distilled water group. The lesion index in *L. ussitatissimum* extract treated group was 22.8 ± 1.6 ($P < 0.001$) and for the group treated with *T. feonum-gracum* it was 21.8 ± 4.2 ($P < 0.001$).

Table 2 Effect of *L. usitatissimum and T. feonum-gracum* on indomethacin induced gastric ulcer in mice

Treatment	Dose (Mg/kg)	n	Ulcer Index	% IUI	Ulcer No.	% IUN
Control	--	7	3.07 ± 0.42	--	22.00 ±2.98	--
L. ussitatissimum	500	7	2.04 ± 0.24	33.6	13.43 ±2.56*	39.9
	1000	7	1.24 ± 0.11**	59.9	10.86 ± 0.80**	50.6
	1500	6	1.06± 0.18**	65.5	7.5±0.8**	65.9
T. foenum-gracum	500	6	1.78 ± 0.29*	42	14.83 ±2.24	32.6
	1000	6	1.27 ± 0.17**	58.6	11.00 ±1.06**	50
	1500	7	0.99 ±0.15**	67.8	8.43 ± 1.27**	61.7
Cimetidine	100	8	1.4 ±0.16**	54.4	11.89 ±1.11**	46

* $P < 0.01$ and ** $P < 0.001$, statistically significant relative to control. (Dunnet's t-test)
% IUI = % Inhibition of Ulcer index; % IUN = % Inhibition of Ulcer No, n=number of animals.

Table 3 Influence of indomethacin pretreatment on the gastroprotective effect of *L. ussitatissimum* and *T. foenum-gracum* extract against ethanol induced gastric lesions

Treatment	Dose (mg/kg)	Vehicle pretreatment		Indomethacin pretreatment	
		Lesion Index	(%) ILI	Lesion Index	(%) ILI
Control (vehicle)	--	36.9 ± 1.6	----	52.6 ± 1.9[a]	----
L. usitatissimum	1000	15.9 ± 2.8[b]	56.9	22.8 ± 1.6[b]	56.7
T. foenum-gracum	1000	17.7 ± 2.5[b]	52	21.8 ± 4.2[b]	58.6

n =6, a=P<0.001, statistically significant compared with the control in vehicle pretreated group (student's t -test); b=P<0.001, statistically significant compared with the respective control group (student's t- test); % ILI = inhibition of lesion index

Acute toxicity:
Intragastric administration of *T. foenum-gracum* and *L. ussitatissimum* seed extracts did not show any toxic manifestation up to 2000 mg/kg. The i.p. LD_{50} in mice for *T. foenum-gracum* and whole *L. ussitatissimum* seed aqueous extracts were 4677.4 mg/kg and 1698.2 mg/kg, respectively (Figure 1 and 2). Toxic manifestations such as diarrhea, forced respiration and altered motor activity were observed prior to death by administering lethal dose of aqueous whole *L. ussitatissimum* extract while *T. foenum-gracum* seed aqueous extract caused excessive urination, diarrhea, vocalization and altered motor activity prior to death.

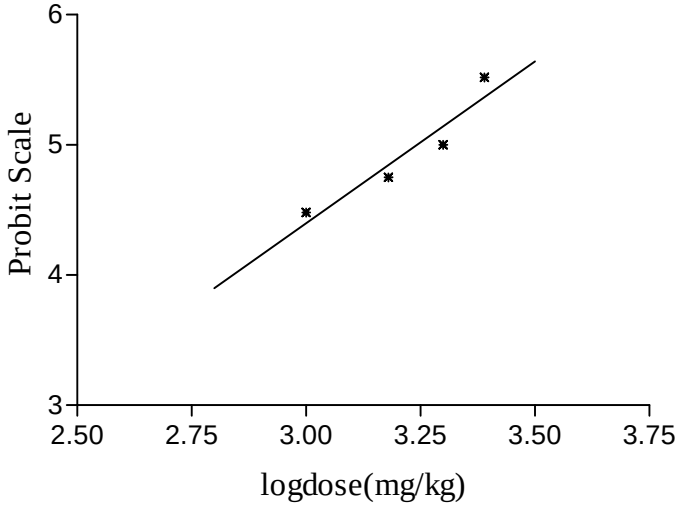

Figure 1 Probit scale transformed versus log dose (mg/kg) of aqueous extract of *L. ussitatissimum* seed; LD_{50}= 1698.2mg/kg
Equation: $Y= 2.488X - 3.06$, $r^2= 0.898$.

Discussion

The presence of poly-phenols in the *T. foenum-gracum* aqueous extract, and peptides and polysaccharides in the extract of *L. ussitatissimum*, might have attributed to their gastro protective effect, which could be useful for managing the traditional claim, peptic ulcer as supported by the pharmacological tests.

Cytoprotective action of drugs has been investigated using animal models of acute gastric injury induced by necrotizing agents such as ethanol, hydrochloric acid and hydrochloric acid – ethanol (12). Ethanol serves as the most common ulcerogenic agent and produces severe gastric hemorrhagic erosions when given intragastrically (17). Oxygen free radicals, which lead to an increased lipid per oxidation and damage to the cell and cell membranes, are implicated in the pathogenesis of ethanol induced gastric mucosal injury (18).

In the present study, ethanol was observed to increase the lesion index of control animals. This effect however, was counteracted by pretreatment with both extracts.

One of the previous studies showed that *T. feonum-gracum* reduced peroxidation index with increased antioxidant enzyme activity and gastric lesion reduction in hydrochloric acid - ethanol treated rats **(**7**).** From *in vitro* study, aqueous and alcoholic extract of *T. foenum-gracum* leaves were shown to have antioxidant property. *T. foenum-gracum* leave extracts were classified in high antioxidant activity from plant source antioxidants (19). The present phyto constituent investigation of the aqueous extract of *T. foenum-gracum* seed revealed positive result for the polyphenol test. *T. feonum-gracum* leaves were found to have high phenolic contents (19). The same study also showed the correlation between phenolic content and antioxidant effect *in vitro*. It is possible to say that the gastroprotective effect of *T. feonum-gracum* may be attributed to antioxidant properties of poly-phenols.

The free radical scavenging activity of *L. usitatissimum* polysaccharide might have contributed for the reduction of the gastric lesion (20), as free radicals mediate gastric injury induced by ethanol. Ethanol, in addition to its direct damage of gastric mucosal cells by the development of free radicals, causes solubilization of mucus constituents and depresses tissue levels of proteins, leading to flow stasis in gastric blood (21). The mucilage, which is composed of neutral and acidic polysaccharide, may prevent penetration of necrotizing agent in to the gastric mucosa. Perhaps, it forms a protective layer and averts the deep necrotic lesions and extensive exfoliation of surface epithelium induced by ethanol.

Indomethacin produces gastric lesions by inhibiting the synthesis of cytoprotective prostaglandins, leading to disruption of gastric mucosal barrier. Agents, which have cytoprotective and/or anti-acid secretory effect, prevent gastric lesions induced by indomethacin (22). The present study demonstrated that pre-treatment with both *T. foenum-gracum* and *L. usitatissimum* extract produced a dose-dependent protection against indomethacin-induced gastric lesions. Involvement of oxygen derived free radicals such as the super oxide anion, hydrogen peroxide, and hydroxyl radical are well established in the pathogenesis of non-steroidal anti-inflammatory drug induced ulcer (23). *L. usitatissimum* gum extract is known to have film-forming properties (24). This film forming property may be important in the gastroprotective effect. Therefore it is conceivable that the observed gastro protection against indomethacin-induced gastric damage may be due to antioxidant and mucosal protective constituents present in both plant extracts.

The cytoprotective actions of some anti-ulcer drugs are mediated by the action of endogeneous prostaglandins, which are known to play an important role in maintaining mucosal integrity (22). In the present study, pretreatment with indomethacin alone increased the injury after ethanol administration. The gastroprotective effect of the extracts, however, was not abolished when prostaglandin biosynthesis had been inhibited by indomethacin. This suggests that the presence of endogenous prostaglandins might not be essential to the expression of mucosal protective activity of the extracts.

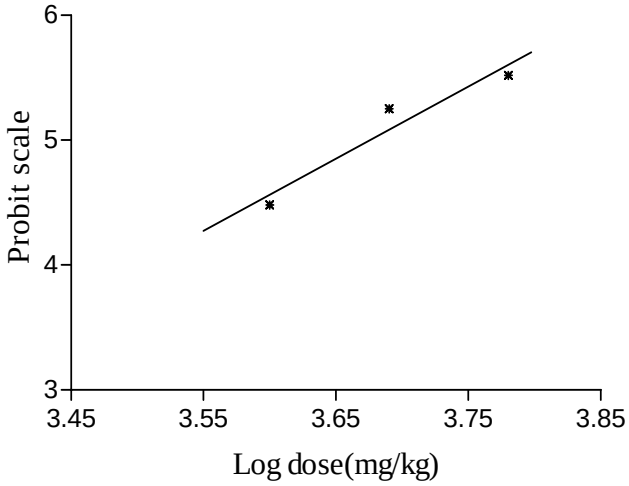

Figure 2 Probit scale transformed versus logdose(mg/kg) of aqueous extract of *T. foenum-gracum* seed; LD_{50}= 4677.4mg/kg
Equation: Y= 5.78X ⁻16.2, r^2= 0.928.

The increased human use of medicinal plants entails the generation of toxicity data in experimental animals. In the present study, intragastric administration of *T. foenum-gracum* and *L. ussitatissimum* seed extracts did not show any toxic manifestation up to 2000 mg/kg suggesting that the oral LD_{50} of both extracts is > 2000mg/kg. It was not possible to administer higher doses of extracts orally because it was too viscous to administer through intragastric tube. Therefore i.p. route was employed. The present study showed that the i.p. LD_{50} of aqueous *T. foenum-gracum* and *L. ussitatissimum* seed extract was 4677.4 and 1698.2 mg/kg, respectively. Toxicological effect on the basis of mortality revealed that *T. foenum-gracum* had been safer than *L. ussitatissimum* extracts by the same route of administration.

Though i.p. LD_{50} and the highest effective dose seem to be close, this does not imply the extracts are not safe as i.p. doses should be much smaller than oral dose. i.p. and oral LD_{50} of the aqueous extract of *T. foenum-graecum* leaf in mice were reported to be 1900mg/kg and 10,000 mg/kg, respectively (25). It will not be difficult to deduce from this that the oral LD_{50} of the present extracts to be much larger.

In conclusion, the results of the study suggest that both *T. foenum-gracum* and *L. ussitatissimum* extracts have gastroprotective action and wide safety margin. And these findings may support the traditional uses of *T. foenum-gracum* and *L. ussitatissimum* extracts to manage peptic ulcer in Ethiopia. Nevertheless, further studies on efficacy, mechanism, kinetics and safety (sub-acute and chronic toxicity studies involving the evaluation of the biochemical parameter) need to be carried out.

Acknowledgements

The authors would like to acknowledge Addis Ababa University for sponsoring this study, the Ethiopian Health and Nutrition Research Institute for allowing access to their laboratory facilities and East Africa Pharmaceuticals P.L.C for donating active indomethacin.

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