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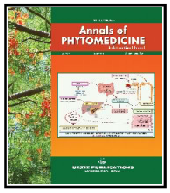

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Standardization and evaluation of the antiulcer potential of a traditional polyherbal formulation in experimental animals

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Abstract

Safoof Muqliyasa (SM) is a traditional polyherbal formulation reported in the classical Unani literature to treat the ulcerative condition of the gastrointestinal tract; however, its efficacy has to be established through scientific validation. The present study aims to standardise SM and evaluate its antiulcer activity in indomethacin and ethanol-induced peptic ulcers in rats. Standardisation of SM was carried through pharmacognostical evaluation, HPTLC analysis, and safety parameter evaluation. The preventive effect of SM in rat models of peptic ulcers induced by indomethacin and ethanol was tested at the dose of 250, 500, or 1000 mg/kg once daily for seven consecutive days, in comparison to ranitidine (50 mg/kg, p.o.) as standard drug. Outcome measures were ulcer score, per cent inhibition, and ulcer index. The obtained data was statistically analysed using GraphPad Prism 10 software. SM was standardised as per pharmacopoeial SOP's. In animal studies, SM-250 mg/kg and SM-1000 mg/kg showed significant protection against indomethacin-induced peptic ulceration in rats (51.8% and 52.1%, respectively) and SM-500 mg/kg (33.8%) while ranitidine showed 86% ulcer protection. In the ethanol-induced peptic ulcer model, ranitidine showed 75.72% protection against ulceration, however, none of the tested doses of SM provides any remarkable protection. Standardised SM may be beneficial for gastric ulcers and may specifically be co-administered with NSAIDs where long-term indication of NSAIDs is inevitable.

1. Introduction

Peptic ulcers are erosions on the mucosal wall of the gastrointestinal tract that reach the muscle layer (muscularis propria). It is reported that 5-10% of the global population is affected by peptic ulcers (Rothermel *et al.,* 2020). Aetiology peptic ulcer is multifactorial and a delicate balance is maintained between ulcer aggravating factors and protective factors under homeostasis. Peptic ulcers arise when the destructive effects of gastric acid and pepsin overwhelm the protective mechanisms of the gastrointestinal mucosa. The stomach and proximal duodenum are the typical locations (Karampour *et al.,* 2019). It could affect the distal duodenum, jejunum, or lower oesophagus (Malik *et al.,* 2024). The major forms of common peptic ulcer are duodenal ulcer and gastric ulcer, both of which are chronic diseases (Loscalzo *et al.,* 2022). Among the most prevalent illnesses in today's world are peptic ulcers; their main causes include NSAID use, excessive pepsin secretion, bile salts, gastric acid, alcohol consumption, *Helicobacter pylori* infection (Emin *et al.,* 2019) and stress (Shahzad *et al.,* 2022). In order to lower pain and fever in inflammatory diseases like osteoarthritis and rheumatoid arthritis, nonsteroidal anti-inflammatory drugs (NSAIDs) like indomethacin (IND) are widely used. NSAIDs damage the gastric mucosa, which leads to the development of gastric ulcers (Musumba *et al.,* 2009). Moreover, these medications disrupt the equilibrium between endogenous angiogenesis and the repair of the injured mucosa (Emin *et al.,* 2019), while the mortality rate from peptic ulcers is decreased by the use of antimicrobial agents, PPIs, prostaglandin analogues, and anti-histaminic drugs, research is still needed to find new medications that are less expensive and have fewer side effects (Karampour *et al.,* 2019).

Peptic ulcers are described as Quruh Hadmia in Unani medicine. It is described under the entities of Quruh al-Mari (oesophageal ulcer), Quruh al-Mi'da (gastric ulcer) and Quruh -Am'â' (intestinal ulcer) in Unani literature with their management and prevention (Hafeel *et al.,* 2018; Rahman, 2020). The eminent Unani Physician Ibne Sina (980-1037 A.D.) explained that gastric ulcers occur due to the damage of gastric mucosa which is caused by the irritant and corrosive humour (Khilt haad) which penetrates and breaches the continuity of gastric mucosa (Sina, YNM).

In Unani medicine, peptic ulcers are treated with demulcents, styptic drugs, haemostatic, and drugs having smoothening properties (Iqbal *et al.,* 2012). Safoof Muqliyasa consists of 10 ingredients, *viz.*, Tukhm-e-Aspaghol (*Plantago ovata* Forsk.), Tukhm-e-Bartang (*Plantago major* L.), Tukhm-e-Hummaz (*Rumex vesicarius* L.), Tukhm-e-Kanocha (*Phyllanthus maderaspatensis* L.), Tukhm-e-Khashkhash (*Papaver somniferum* L.), Tukhm-e-Khurfa (*Portulaca oleracea* L.), Tukhm-e-Raihan (*Ocimum sanctum* L.), Samagh Arabi (*Acacia arabica* L.), Nishashta-e-Gandum (*Triticum aestivum* L.), Gil-e-Armani (Armenian bole). These ingredients have primarily

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demulcent and soothing effects on gastrointestinal tract mucosa, which decreases pH and forms a protective layer on mucosa, as well as decrease the chance of ulceration and promote healing. *P. ovata*, *P. major*, *O. sanctum*, and *A. arabica* possess antiulcer activity in experimental animals. SM has not been scientifically evaluated as such. The study was conducted to develop standard operating procedures (SOPs) and standardization of SM and to evaluate the antiulcer potential in the treatment of gastrointestinal tract ulcerative conditions and to generate scientific data.

2. Materials and Methods

2.1 Chemicals

All the reagents and solvents were of analytical/HPLC grade.

2.2 Experimental animals

Adult male Wistar rats with body weight 220 ± 20 g were procured from the breeding facility of ICMR-National Institute of Nutrition, Hyderabad, India, and maintained at 22 ± 3, with 30-70% humidity with artificial 12:12 h illumination cycle. Animals were maintained on a standard diet (Krishna Valley Agrotech) and water *ad libitum*. Ethics guideline issued by the Committee for Control and Supervision of Experiments on Animals was adopted. Approval from the Institutional Animals Ethics Committee was taken *vide* protocol no. NRIUMSD/IAEC/17/2022/01/P06 held at NRIUMSD, Hyderabad.

2.3 Procurement and authentication of the ingredient of study formulation

Formulation ingredients were procured from an herbal drug supplier in Hyderabad and their identification and authentication were performed by the Botanist. The authenticated crude drug samples were kept in the herbarium of the Institute for future reference. A specimen voucher no. for each ingredient was assigned, *viz*., *O. sanctum*: SMPU/CRI-Hyd15008, *A. arabica*: SMPU/CRI-Hyd15009, *P. maderaspatensis*: SMPU/CRI-Hyd15010, *R. vesicarius*: SMPU/CRI-Hyd15011, *P. ovata*: SMPU/CRI-Hyd15012, *P. oleracea*: SMPU/CRI-Hyd15013, *P. somniferum*: SMPU/CRI-Hyd15014, *P. major*: SMPU/CRI-Hyd15015, and *T. aestivum*: SMPU/CRI-Hyd15016.

2.4 Preparation of study formulation

The formulation SM was prepared in three different batches, in the GMP-certified pharmacy of the Institute. The composition and preparation method adopted was in confirmation to Qarabadin Najmul Ghani (Ghani, 2010), Sharah Asbab (Samarqandi, 2014), Tibb-i-Akbar (Arzani, YNM).

2.5 Standardization of SM

Organoleptic characteristics of SM such as appearance, colour, odour, and taste were recorded. SM was studied for its microscopic characteristics as reported earlier (Beg *et al.,* 2022; Anonymous, 1998). The physicochemical analysis of SM was carried out as per Pharmacopoeial parameters mentioned in Table 1 (Anonymous, 2009).

SM was also subjected to high-performance thin-layer chromatography (HPTLC) techniques. 10 g powder of SM was soaked in 50 ml of methanol for two hours at a benchtop orbital shaker filtered using the No.1 Whatman filter paper and evaporated to 10 ml. 10 µl methanolic extract sample was applied and different solvent systems were tried for effective separation of components (Zahid *et al.,* 2023, Naikodi *et al.,* 2011, Venkatesham *et al.,* 2021). The appropriate solvent system was identified as toluene: ethyl acetate: methanol (5:4:1, *v/v/v*) and developed in the Twin through TLC chamber. After the development, the TLC plate was dried, photographed under Camag TLC visualizer 2 and then scanned by Camag TLC scanner 4 under the UV visible chamber at 366nm, 254 nm. Then, the TLC plate derivatized with 1% Vanillin-sulfuric acid and heated on the TLC plate heater at 105^{0}C for 5 min then photographed under Camag TLC visualizer 2 and then scanned by Camag TLC scanner 4 under the UV visible chamber at 560 nm for detection of spots. CAMAG HPTLC system was used and 10 mm band of sample extract was applied and developed up to 80 mm in a pre-saturated TLC chamber for 20 min at 25 ± 2°C and scanned by CAMAG TLC SCANNER 4 at wavelengths 254 nm, 366 nm and 560 nm through Vision CATS 3.1 version software.

The assessment of microbial load for SM was performed as per the WHO /UPI (Anonymous, 2009). SM was determined for the presence of aflatoxins by TLC method. SM was analysed for pesticide residue at Bureau Veritas India Testing Services Private Limited, Hyderabad.

2.6 Antiulcer activity in rats

2.6.1 Indomethacin induced ulcer

Wistar rats were assigned into 05 groups (n=08), where test drug or vehicle (distilled water) were orally treated for seven consecutive days. Group 1 rats were orally given distilled water (10 ml/kg) and served as the negative control group. Group 2 received ranitidine (50 mg/kg/day, p.o.) and served as the positive control group. Groups 3, 4, and 5 received SM 250, 500 mg/kg/day, and 1000 mg/kg, orally, respectively. On the 7th day, after 18 h of fasting (water provided *ad libitum*), one hour after the last dosing, all animals were orally given a single dose of inducing agent indomethacin (30 mg/kg) and 4 hours after indomethacin administration animals were euthanised by CO_2 inhalation in euthanasia chamber (Figure 1). Stomach of each animal was removed and cut-opened, properly flushed with ice-cold saline and the glandular portion was analysed with a magnifier lens for ulcer scoring. Ulcer index was calculated as reported earlier (Jafari *et al.,* 2022).

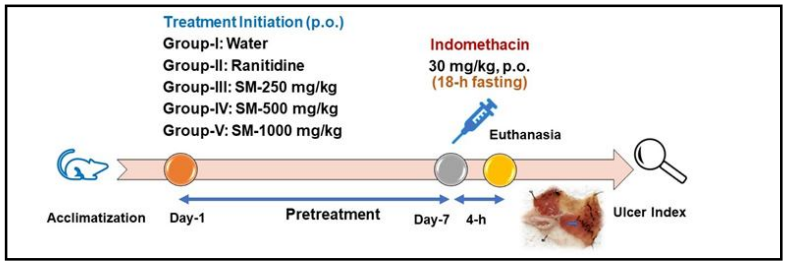

Figure 1: Experimental design of indomethacin-induced ulcer model in rats.

2.6.2 Ethanol-induced ulcer

Forty male Wistar rats were randomly assigned to five groups, each containing eight animals. Oral gavage was used to administer test agents and vehicle for 7 consecutive days. Group 1 received distilled water (10 ml/kg, p.o.; negative control group). Group 2 animals were orally administered with ranitidine (50 mg/kg/day; standard group) for seven days. Group 3, 4, and 5 were orally given 250, 500, 1000 mg/kg of SM for seven days. On 7th day, after 18 h of fasting (water was provided *ad libitum*) one hour after the respective treatment, all animals were subjected to a single dose of absolute ethanol (5 ml/kg, p.o.). Rats were euthanised by CO_2 inhalation 1 h after ethanol administration, and the stomach was removed, cut-opened along their greater curvature, and gently flushed with cold normal saline and spread on a sheet. Lesions in the glandular part of the stomach were visualised with the magnifier glass as reported earlier (Chaitra *et al.,* 2022; Shams *et al.,* 2022) and scored.

2.6.3 Assessment of gastric mucosal lesions

Ulcer index (U.I.) was calculated using a 0-3 scoring system (0: no lesions, 1: lesions < 1 mm length, 2: lesions 2-4 mm length, 3: lesions > 4 mm length). The lesion score was calculated as the number of lesions multiplied by their respective severity factor. The mean lesion score of all the rats in a group was calculated. The preventive index (P.I.) of SM was calculated as follows:

$$\text{P.I.} = \frac{\text{U.I. of the negative group} - \text{U.I. of the pretreatment group}}{\text{U.I. of the negative group}} \times 100$$

2.6.4 Histopathological examination

Stomach samples were preserved in 10% neutral buffer formalin immediately after scoring and subjected to histopathological examination. Tissues were subjected to dehydration in increasing concentrations of alcohol and then embedded in paraffin blocks. About 3 micrometre thin sections were prepared and treated with xylol and subjected to rehydration in a gradually reduced concentration of ethanol; stained with haematoxylin and eosin and fixed. Slides were observed under the Olympus trinocular microscope.

2.6.5 Statistical analysis

Data is presented as the mean of a group (n=8) along with the Standard Error of Mean. Intergroup comparison was made by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test using GraphPad Prism (version 10) software. A value of $p<0.05$ was considered as statistically significant.

3. Results

3.1 Standardization of SM

SM is a powdered formulation, brick red due to the presence of Gil-e-Armani. It has characteristic odour and taste. On microscopic observation, the following salient features of the ingredients of the study formulation SM were observed.

Epidermal cells of the testa are filled with mucilage. Fragments showing endosperm cells with aleurone grains, pitted cells, xylem fibres, tracheids and calcium oxalate crystals are present. The epidermis is found to be filled with brown pigment. In surface view, palisade is like the epidermis of the testa. Epidermal cells of testa from a side view with pigment layer. Fragments of polygonal cells of endosperm filled with aleurone grains, rich in oil cells, are depicted in Figure 2.

The physicochemical evaluations of SM were conducted under the Unani Pharmacopoeia of India prescribed analytical procedures (Anonymous, 2009). The mean percentage of total ash value of SM was found in the range of 17.2806-17.3936%w/w in three different batches, whereas the mean percentage of acid insoluble ash value was observed in the range of 12.4415-12.6160%w/w. The mean values of alcohol-soluble, water-soluble and hexane-soluble extracts were found in the range of 11.4851-11.7583%w/w, 24.1185-24.5212%w/w and 2.1987-2.3468%w/w, respectively. Bulk density was found in the range of 0.5284-0.5316 g/ml in three different batches. The mean pH of 1% and 5% aqueous suspension was 4.73-4.85 and 4.72-4.74, respectively. Loss of weight on drying at 105°C was 4.4501-4.8683%w/w (Table 1). The presence of therapeutically active phytoconstituents was confirmed in qualitative phytochemical analysis (Table 2).

In HPTLC analysis, the TLC plate showed ten major spots under UV λ366 nm at R_f values 0.01, 0.05, 0.12, 0.23, 0.31, 0.48, 0.60, 0.72, 0.82, 0.98, the corresponding data mentioned in the Table 3 and shown in Figures 3 and 4. Microbial load were found within the permissible limit. No significant bacterial counts were found in SM. Aflatoxins such as B_1, B_2, G_1, and G_2 are found to be absent (Table 4).

Pesticide residue analysis for study formulation-SM was performed through validated test method BVTS/FOOD/INS/SOP-040 for 50 pesticide residues at Bureau Veritas India, Hyderabad. The pesticides in the SM were found below the detection limit and mostly nil.

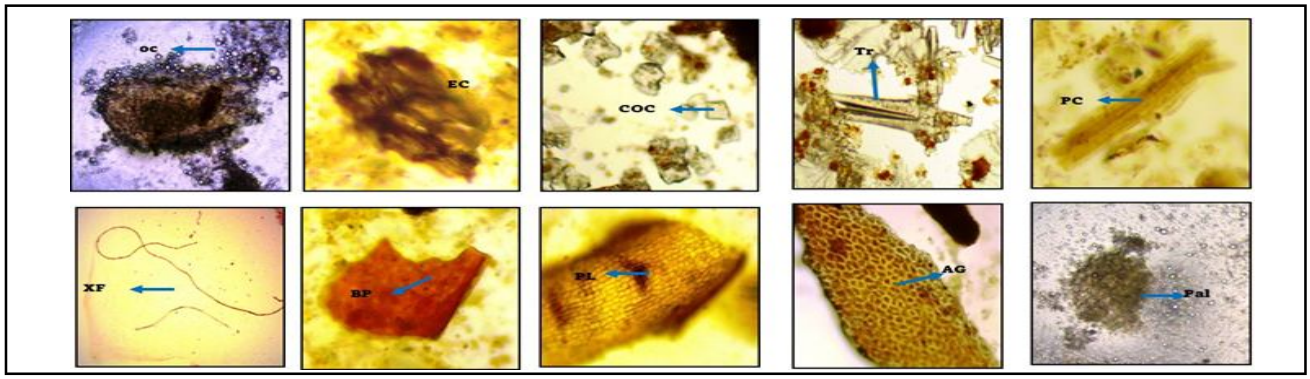

Figure 2: Powder microscopic observation of SM; EC: epidermal cells, COC: calcium oxalate crystals, Tr: tracheids, PC: pitted cells, BP: brown pigment, PL: pigment layer of underlined epidermal cells, OC: oil cells, Pal: palisade-like epidermis, AG: endosperm with aleurone grains, XF: xylem fibres.

Table 1: Physicochemical analysis of SM (Mean ± SEM)

S.No.	Physicochemical parameters	Batch I	Batch II	Batch III
1	Total Ash values (% *w/w*)	17.2806 ± 0.02	17.3783 ± 0.05	17.3936 ± 0.14
2	Acid Insoluble Ash (% *w/w*)	12.4415 ± 0.13	12.6160 ± 0.23	12.6017 ± 0.22
3	Alcohol soluble extractive values (% *w/w*)	11.7583 ± 0.34	11.4864 ± 0.14	11.4851 ± 0.09
4	Water soluble extractive values (% *w/w*)	24.5212 ± 0.11	24.1185 ± 0.37	24.1942 ± 0.15
5	Hexane soluble extract (% *w/w*)	2.1987 ± 0.08	2.3024 ± 0.02	2.3468 ± 0.14
6	pH of 1% aq. suspension	4.73 ± 0.01	4.84 ± 0.01	4.85 ± 0.02
7	pH of 5% aq. suspension	4.72 ± 0.02	4.73 ± 0.02	4.74 ± 0.04
8	Loss in weight on drying at 105°C	4.6788 ± 0.30	4.4501 ± 0.11	4.8683 ± 0.11
9	Bulk density	0.53 ± 0.003	0.53 ± 0.004	0.53 ± 0.004

Table 2: Qualitative phytochemicals analysis of SM

S. No.	Chemical constituents	Ethanolic extract	Aqueous extract	Chloroform extract
1	Alkaloids	+	-	-
2	Glycosides	-	+	+
3	Carbohydrate	-	-	-
4	Flavonoids	+	-	-
5	Saponins	+	-	-
6	Steroids	++	-	-
7	Tannin	+	-	-
8	Proteins	+++	-	-
9	Phenols	+	-	-
10	Fixed oil	-	-	-
11	Starch	-	-	++

Table 3: Peak list of methanolic extract of SM under 366 nm wavelength

S.No.	Max Rf	Height	Height %	Area	Area %
1	0.013	0.2074	11.75	0.00287	4.73
2	0.051	0.4921	27.87	0.01492	24.58
3	0.123	0.2505	14.19	0.00656	10.80
4	0.233	0.3192	18.08	0.01495	24.63
5	0.313	0.0993	5.62	0.00555	9.14
6	0.489	0.0793	4.49	0.00223	3.67
7	0.606	0.0606	3.43	0.00356	5.86
8	0.720	0.1287	7.29	0.00519	8.54
9	0.823	0.0487	2.76	0.00253	4.17
10	0.984	0.0798	4.52	0.00235	3.87

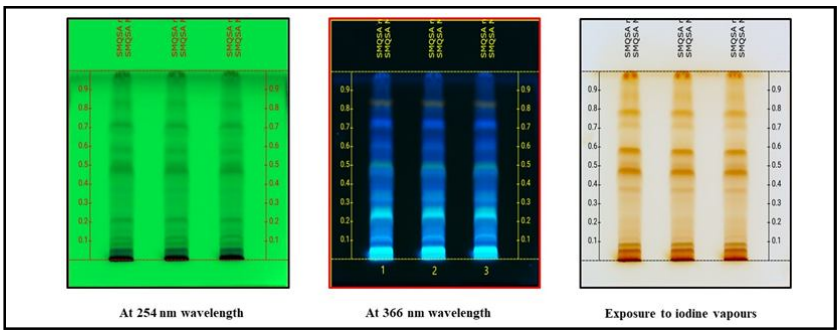

Figure 3: HPTLC analysis of methanol extract of formulation SM.

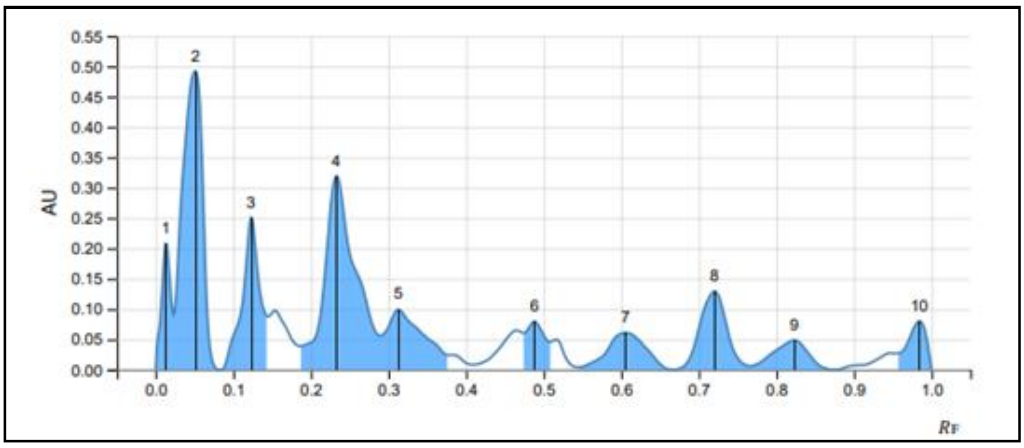

Figure 4: Chromatogram of TLC plate observed under 366 nm wavelength.

Table 4: Safety parameters of the formulation SM

		Microbial load determination results			
S.No.	**Parameter analysed**	**Batch I**	**Batch II**	**Batch III**	**WHO permissible limit**
1	Total microbial load	11×10^2	9×10^2	13×10^2	Not more than 10^5 /g
2	*Salmonella* spp.	Nil	Nil	Nil	Nil
3	*Escherichia coli*	Nil	Nil	Nil	Nil
4	Total fungal count	Nil	Nil	Nil	Not more than 10^2/g
Aflatoxin determination					
S.No.	**Parameter analysed**	**Batch I**	**Batch II**	**Batch III**	**WHO permissible limit (ppm)**
1	B 1	Nil	Nil	Nil	NMT 0.50
2	B 2	Nil	Nil	Nil	NMT 0.10
3	G 1	Nil	Nil	Nil	NMT 0.50
4	G 2	Nil	Nil	Nil	NMT 0.10

3.2 Antiulcer activity in rats

3.2.1 Indomethacin-induced ulcer

Data on indomethacin-induced ulcers in rats is presented in Table 5. Treatment with indomethacin resulted in significant ulceration in rats stomach as evident in Figure 5. Pre-treatment with ranitidine as well as SM inhibited ulcer formation to variable degrees as shown in the form of ulcer index in Figure 6.

Histopathological investigations, in negative control group animals (Figure 7A) showed moderate to severe inflammatory reaction/ ulcerative conditions including degenerative changes in mucosal epithelial cells, sub-mucosal glands degeneration indicating development of gastric ulcer in this group. In ranitidine group animals (Figure 7B) showed no inflammatory reaction / ulcerative conditions. 5/8 rats indicated healing of ulcerated mucosal and sub-mucosal layers; sub-mucosal ulcers were replaced with fibrous tissue. 1/8 rats showed severe degenerative changes. In, SM-250 group (Figure 7C), 1/8 of the animals showed no inflammatory/ulcerative pathology. 4/8 rats indicated healing/ regeneration condition of ulcerated mucosal and sub-mucosal layers. 2/8 rats showed moderate degenerative changes in mucosal and submucosal layers while 1/8 rats showed severe inflammatory reaction and submucosal degeneration. In, SM-500 group (Figure 7D), 2/8 animals showed mild inflammatory/ ulcerative pathology. 6/8 rats showed moderate degenerative changes in mucosal and submucosal layers. In, SM-1000 group (Figure 7E), 2/ 8 animals showed no inflammatory/ulcerative pathology. 2/8 rats indicated healing/ regeneration condition of ulcerated mucosal and sub-mucosal layers; sub-mucosal ulcers were replaced with fibrous tissue. 3/8 rats showed moderate degenerative changes in mucosal and submucosal layers while 1/8 rats showed severe inflammatory reaction and submucosal degeneration. Based on the above observation, ranitidine showed best protection in indomethacin model. SM-250 and SM-1000 showed good protection against ulceration as indicated by absence of inflammatory condition and presence of sign of regeneration with fibrous tissue in these groups. Only a low level of protection was observed in SM-500 mg/kg.

Table 5: Data of indomethacin-induced model in rats

S.No.	Group	Ulcer index (Mean ± SEM)	Preventive index	95% CI of diff
1.	Vehicle + IND (Negative control)	11.15 ± 1.76	-	-
2.	RAN + IND (Positive control)	01.56 ± 0.56***	86%	4.406 - 14.77
3.	SM-250 mg/kg + IND	05.37 ± 1.63*	51.8%	0.5960 - 10.96
4.	SM-500 mg/kg + IND	07.38 ± 0.71	33.8%	–1.414 - 8.954
5.	SM-1000 mg/kg + IND	05.33 ± 1.24*	52.1%	0.6360 - 11.0

RAN (Ranitidine), IND (Indomethacin), SM (Safoof Muqliyasa), CI (Confidence interval); One-way ANOVA followed by post hoc Tukey's test (n=8); * $p<0.05$; *** $p<0.001$ compared with the indomethacin control group.

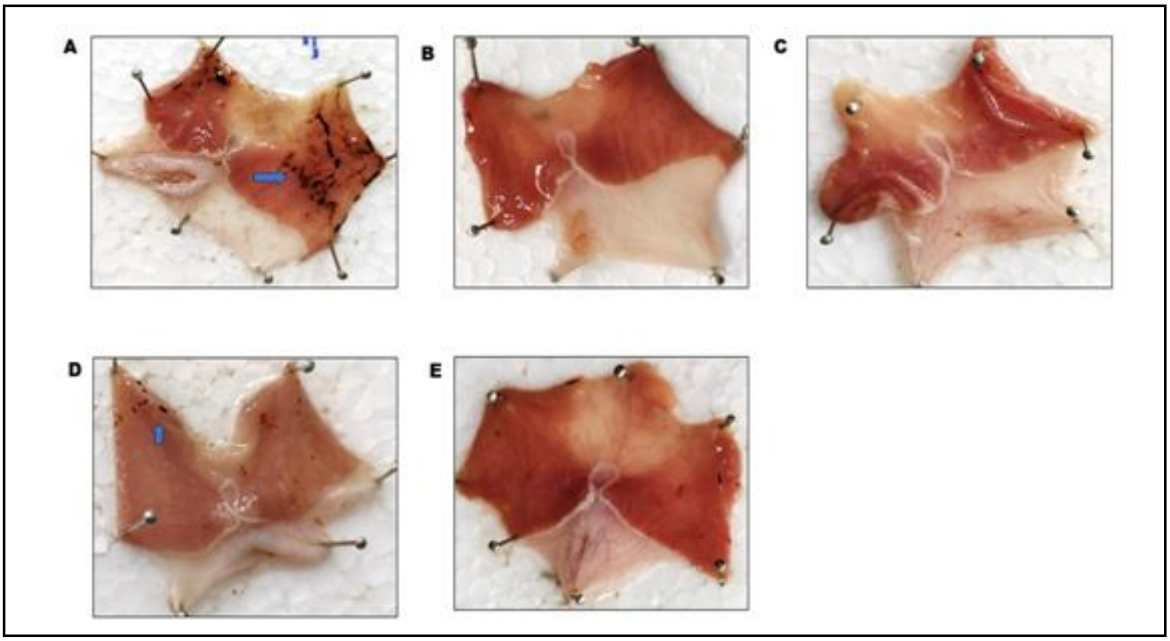

Figure 5: Indomethacin-induced peptic ulcers in rats A: negative control (vehicle + IND); B: positive control (RAN + IND); C: SM-250 mg/kg + IND; D: SM-500 mg/kg + IND; E: SM-1000 mg/kg + IND.

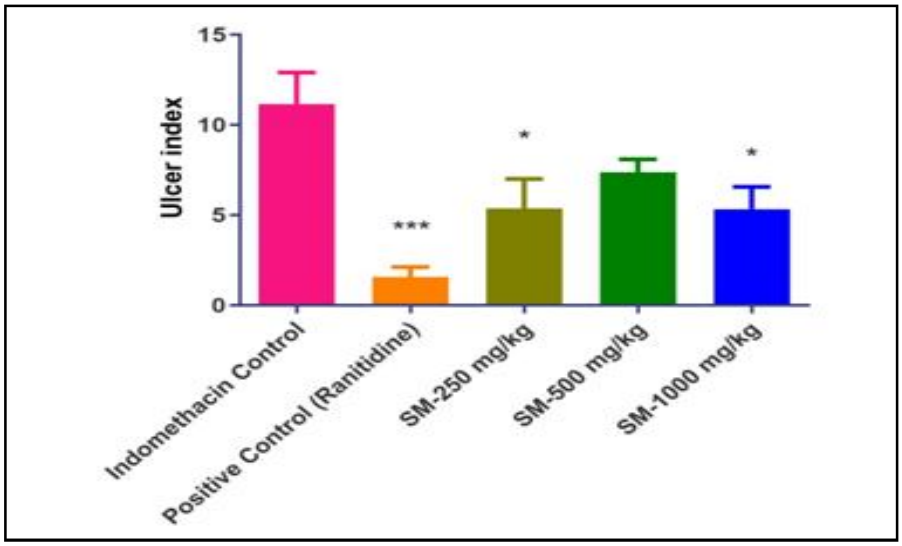

Figure 6: Ulcer index in different treatment groups in indomethacin-induced ulcer model. *$p<0.05$; *** $p<0.001$ *vs.* indomethacin control.

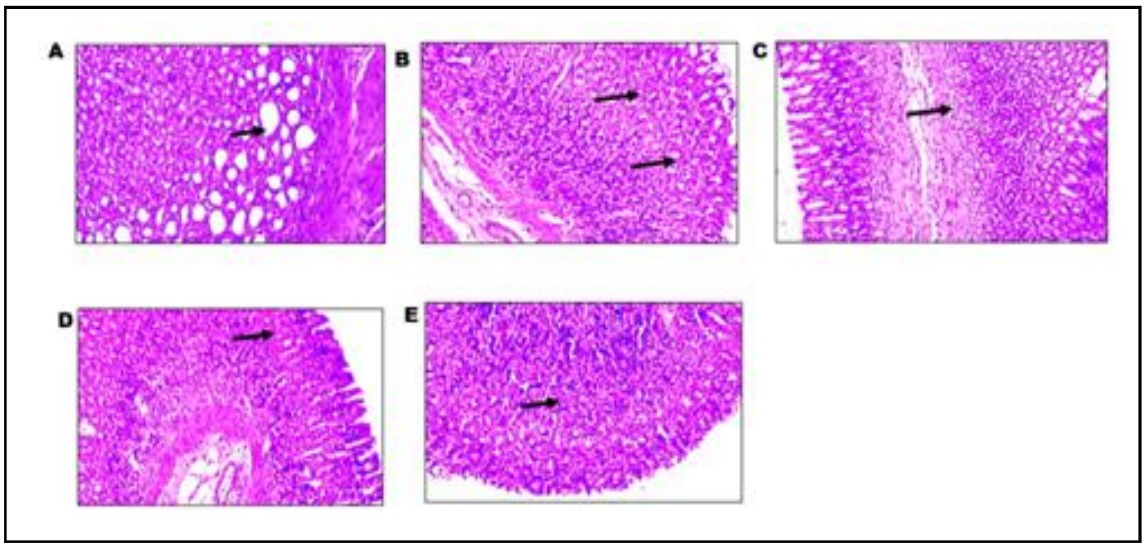

Figure 7: Photomicrograph of the stomach in indomethacin-induced ulcer in rats (100X) A: negative control showing multi-focal mucosal epithelial cells degeneration noticed in glandular stomach; B: positive control showing normal morphology of mucosal epithelial cells layer; C: SM-250 mg/kg showing normal morphology of mucosal epithelial cells layer; D: SM-500 mg/kg showing normal morphology of mucosal epithelial cells layer; E: SM-1000 mg/kg showing normal morphology of mucosal epithelial cells layer.

3.2.2 Ethanol-induced ulcer

Data of the effect of SM on ethanol-induced ulcers in rats is presented in Table 6. Ethanol administration caused significant ulceration in rat stomach as evident in Figure 8. Pre-treatment with ranitidine significantly inhibited (p<0.01) ulcer formation in stomach as depicted in Figure 9 though SM pre-treatment did not protect the ulcer formation.

Based on individual observation of pathology report, inflammatory reaction in the negative control group (Figure 10A) four out of eight animals showed inflammatory/ulcerative conditions and two out of eight animals showed degenerative changes in the stomach. This observation indicated that 75% and above animals showed the incidence of ulcerative/inflammatory and degenerative conditions in the negative control group. In the positive control group (Figure 10B), two out of eight animals showed degenerative changes and the remaining animals showed normal morphology conditions and these animals recovered back to normal conditions which indicated a protective response to the standard drug ranitidine. In the SM-250 group (Figure 10C), 5/8 animals showed inflammatory reactions including infiltration of inflammatory cells, mucosal ulceration, and sub-mucosal gland degeneration. 3/8 rats showed healing conditions of ulcerated mucosal and sub-mucosal layers. In the SM-500 group (Figure 10D), 6/8 animals showed inflammatory reactions including infiltration of inflammatory cells, mucosal ulceration, and sub-mucosal gland degeneration. 2/8 rats showed signs of recovery. In the SM-1000 group (Figure 10E), 6/8 animals showed inflammatory reactions including infiltration of inflammatory cells, mucosal ulceration, and sub-mucosal gland degeneration. 2/8 rats showed the healing condition of ulcerated mucosal and sub-mucosal layers. Based on the above histopathological observation, only ranitidine (positive control) appeared to be effective in protecting against mucosal damage/ulceration. However, none of the SM-treated groups exerted any noteworthy protection against ethanol-induced assault in the stomach.

Table 6: Data of ethanol-induced ulcer in rats

S.No.	Group	Ulcer index (Mean ± SEM)	Preventive index	95% CI of diff.
1	Vehicle + Ethanol (Negative control)	11.33 ± 1.13	-	-
2	RAN + Ethanol (Positive control)	02.75 ± 0.68**	75.72%	1.838 - 15.32
3	SM-250 mg/kg + Ethanol	09.06 ± 2.09	20.03%	–4.472 - 9.012
4	SM-500 mg/kg + Ethanol	10.46 ± 2.40	7.67%	–5.872 - 7.612
5	SM-1000 mg/kg + Ethanol	10.37 ± 1.36	8.47%	–5.782 - 7.702

RAN (Ranitidine), SM (Safoof Muqliyasa); One-way ANOVA followed by post-hoc Tukey's test; **p<0.01; *vs.* ethanol control.

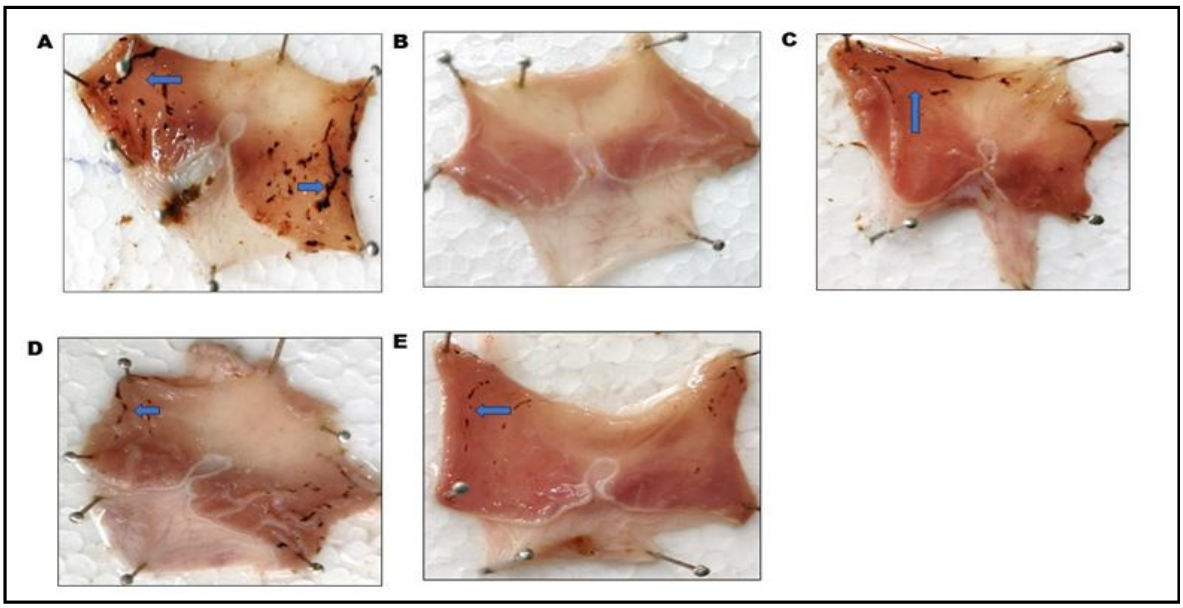

Figure 8: Ethanol-induced peptic ulcers in rats A: negative control (placebo + Eth); B: positive control (RAN + IND); C: SM-250 mg/kg + IND; D: SM-500 mg/kg + IND; E: SM-1000 mg/kg + IND.

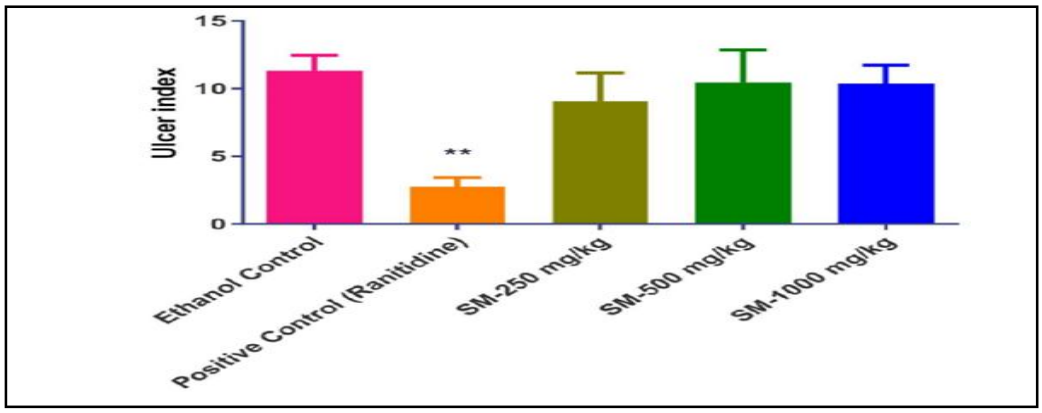

Figure 9: Ulcer index in different treatment groups in ethanol-induced ulcer model; $^{**}p<0.01$ *vs.* negative control (ethanol) group.

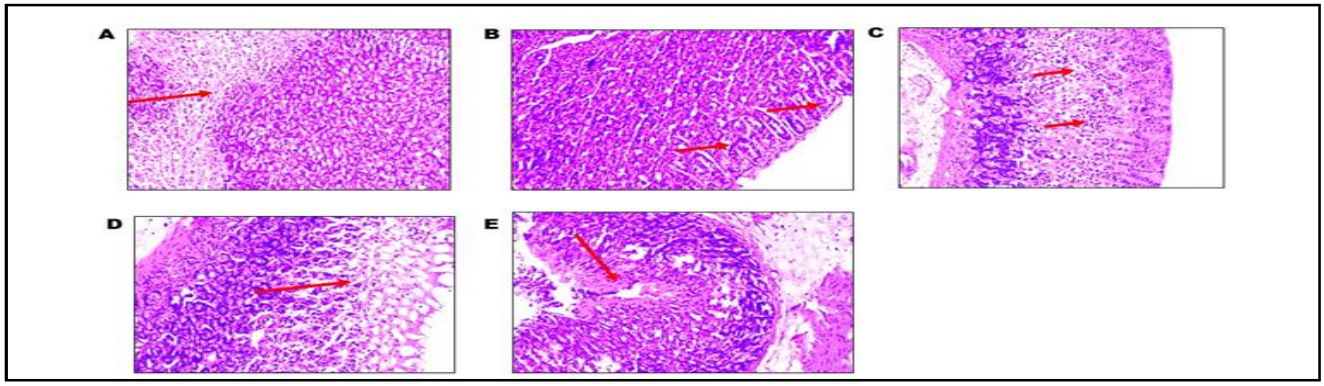

Figure 10: Photomicrograph of the stomach in ethanol-induced ulcer in rats (100X). A: negative control showing multi-focal mucosal ulceration, necrosis and inflammation with fibrosis and infiltration of plasma cells was observed in the glandular stomach; B: positive control showing normal morphology of mucosal epithelial cells layer; C: SM-250 mg/kg showing multi-focal mucosal ulceration, necrosis and inflammation with fibrosis and infiltration of plasma cells were observed in the glandular stomach; D: SM-500 mg/kg showing multifocal mucosal necrosis and inflammation with fibrosis in the mucosal layer of the glandular stomach; E: SM-1000 mg/kg showing multiple large foci of mucosal ulceration with infiltration of inflammatory cells.

4. Discussion

Standardization in Unani formulations ensures the establishment of standards for raw material quality and purity, quality control during the drug manufacturing process, production of a high-quality finished product, storage, and distribution to ensure the final product's quality, efficacy, safety, and reproducibility. The organoleptic and physicochemical properties of drugs are crucial for identifying both crude and compound formulations. The mean % ash value and acid insoluble ash was observed as 17.2806-17.3936 %w/w and 12.4415-12.6160 %w/w in three different batches, respectively. The formulation included a significant amount of Gil-e-Armani, a drug of mineral origin, which may have contributed to the high total and acid-insoluble ash values. The pH of SM is acidic in nature which indicates easy absorption ability in a low pH medium such as in the stomach. The study formulation was assessed for solvent extracts such as hexane for non-polar compounds, water, and ethanol for polar and medium-polar compounds. The range of 24.1185-24.5212%w/w was found to have the highest mean percentage of extractive values in water, followed by alcohol and hexane (11.4851-21.7583%w/w and 2.1987-2.3468%w/w, respectively), which indicate the formulation's purity index. A drug's extractive value in a particular solvent is a measure of its purity and is crucial in identifying adulteration. Among three separate batches, the mean % of weight loss on drying at 105°C for the formulation SM was found to be between 4.4501 and 4.8683%w/w. Low moisture content is sign of stable, high-quality, and suitable plant material. It is reasonable to assume that a formulation with less moisture will remain safe over time. Microbial content, aflatoxins and pesticide content in SM were found nil/or within the permissible limits of WHO. Phytochemical analysis revealed the presence of alkaloids, glycosides, starch, phenols, proteins, steroids, saponins, tannins, and flavonoids, whereas carbohydrate, and fixed oils were not detected in SM.

From the above discussion, it can be concluded that quality control parameters evaluated for study formulation is in the line assigned by WHO or other regulatory bodies. These quality control metrics can be used as a reference to certify the authenticity and quality of drugs to maintain the efficacy and safety of drugs.

Two animal models were used to evaluate the antiulcer activity of SM *viz.* indomethacin-induced ulcer and ethanol-induced ulcer in Wistar rats. Ranitidine was used as a standard drug. NSAIDs exert their ulcerogenic effect by locally acting on the gastric mucosa and damaging the layer of membrane phospholipids which causes reperfusion of the H^+ ions and tissue injury (Boeing *et al.,* 2016). Systemically, NSAIDs inhibit COX-1 and COX-2 isoforms (rate-limiting enzymes in the synthesis of prostaglandins), which reduces the protective prostaglandins synthesis and secretion of mucus, and bicarbonate. Adequate blood flow was disrupted leading to peptic ulcers (Serafim *et al.,* 2020). The administration of NSAIDs especially indomethacin has been shown to induce oxidative stress in stomach tissue and mucus cells, suppress antioxidant defence, and start lipid peroxidation, all of which can lead to gastric damage. Indomethacin is reported to decrease endogenous antioxidants glutathione and nitric oxide (Zaghlool *et al.,* 2015). On the other hand, alcohol consumption resulted in ulcer formation due to physiological and psychological stress.

Present findings revealed that indomethacin induces ulcers in the glandular area which is confirmed by histological findings. Pre-treatment with Unani formulation SM-250 mg/kg, 1000-mg/kg and positive control RAN significantly attenuated the ulcers induced by indomethacin, which was reflected in a significant decrease in ulcer index and improved preventive index in the above-said groups compared to the negative control group. Though in the SM-500 mg/kg treated group, there was a decrease in ulcer index, but it was non-significance when compared to the negative control group.

Ethanol is physiologically a gastric mucosal destructive factor with its hydrolytic and proteolytic activity. Ethanol causes infiltration of neutrophils in gastric mucosa, which are key for the generation of reactive oxygen species, that cause increased production of pro-oxidative substances and inflammatory cytokines such as TNF-α, IL-1β, and IL-6, resulting in mucosal injuries and damage leading to gastric ulcers (Serafim *et al.,* 2020). Alcohol dehydrogenase metabolizes ethyl alcohol to acetaldehyde and to acetic acid which causes cytotoxicity to gastric cells (Liju *et al.,* 2015). Ethanol decreases the levels of nitric oxide, which is required for physiological processes like maintenance of haemodynamics in gastric mucosa. Lack of nitric oxide leads to haemorrhagic lesions and subsequent depletion of gastric mucus (Ribeiro *et al.,* 2016). On the other hand, ethanol-induced gastric mucosal injury increases the permeability of sodium and water, leading to increased intracellular calcium level which exacerbates gastric damage that leads to cell death and exfoliation of epithelial cells (Ragheb *et al.,* 2021).

The present study evaluated the gastroprotective effect of SM against ethanol-induced peptic ulcers in rats. Oral administration of ethanol resulted in several macroscopic and microscopic changes, confirming the ethanol-related damage. The result shows that ranitidine-treated group (positive control) has significantly less ulcerative index and improved preventive index compared to the negative control group but none of the SM-treated group showed a statistically significant difference in the ulcerative index compared to the negative control group. Hence, we can draw the conclusion that, study formulation SM does not have any significant ulcer protective effect in ulcers produced by this mode of action.

The observed antiulcer activity in the indomethacin model may be attributed to the reported anti-ulcer potential of the ingredients of SM. Aqueous extract of *P. ovata* seeds is reported to have antiulcer potential in indomethacin-induced ulcers in rats at the dose of 100 mg/kg (Bagheri *et al.,* 2018). Ethanolic extract of *P. ovata* seeds at 400 mg/kg bw protected gastric ulcers in rats induced by ethyl alcohol (Khedher *et al.,* 2022). Aqueous and methanolic extracts of *P. major* are reported to have significant antiulcer potential against indomethacin in rats and its effect was attributed to the antioxidant potential of this plant due to the rich content of flavonoids and phenols (Ragheb *et al.,* 2021). Seed extract of *P. major* at 400 and 700 mg/kg showed a protective effect against acetic acid-induced ulcerative colitis in rats. Fixed oil of *O. sanctum* seed showed protection in various chemical-induced as well as stress-induced ulceration in animals (Singh *et al.,* 1999). *O. sanctum* aqueous extract (400 mg/kg) showed significant ulcer protection against aspirin-induced ulcers in rats (Singh *et al.,* 1999). Ethanolic extract of *P. oleracea* seeds at the doses 50, 100 and 150 mg/kg showed significant protection and healing effects in acute ulcers induced by heterogeneous inducing agents and pyloric ligation model as well as the curative effect in chronic acetic acid-induced ulcers (Kumar *et al.,* 2010). 50% Ethanolic extract of *P. oleracea* showed gastric ulcer healing properties

in aspirin-induced gastric ulcers in diabetic rats (Garg *et al.,* 2014; Sahoo *et al.,* 2016). Acacia gum dose-dependently prevented ulcer induction by ethanol in rats when combined with a standard diet containing 2.5, 5, or 10% powder (Khedr, 2017). Gum Arabic contains fibres, total sugar, polysaccharide, phenolic compounds and flavonoids. Study has reported that Arabic gum contains arabinogalactan, which may be responsible for antiulcer activity (Goodrum *et al.,* 2000). According to Cipriani *et al.* (2006), mechanisms anticipated for the antiulcer activity of polysaccharides may be attributed to its mucosal surface biding ability which forms a physical protective coating against acid or scavenging radicals (Cipriani *et al.,* 2006). Gum acacia is reported to have an ulcer protective effect by suppressing gastric inflammation by reducing the level of cytokines like TNF-α and IL-6 levels while increasing the level of IL-10 in ethanol-induced rats (Jameel *et al.,* 2022).

The ulcer preventive effect of SM may be due to flavonoids present in *P. major*, *R. vesicarius*, *P. oleracea,* as studies have shown that flavonoids have antiulcer properties such as antacid activity, reducing pepsin level and activity, and increased gastric mucus and bicarbonate secretion. Flavonoids improve mucosal defence, have antioxidant activity, anti-inflammatory, and antibacterial defences against gastric ulcers. Hence, we can conclude that SM is showing the antiulcer effect due the presence of above ingredients and particularly gum is having a leading role in the observed activity in indomethacin-induced ulcer models (Zhang *et al.,* 2020).

5. Conclusion

In the present study, Safoof Muqliyasa (SM) was subjected to develop pharmacopeial standards with modern analytical techniques and evaluate antiulcer activity in rats. The developed SOPs of SM may serve as standards for confirmation of identity, purity, safety and quality assurance which will be helpful for future evaluation. In preclinical studies, our results indicated that SM might be beneficial in NSAID-induced ulceration even at smaller doses and no proportionate benefit was observed by increasing the SM dose. The observed effect may be attributed to the reported anti-ulcer effect of individual ingredients of SM like *P. ovata, P. major, O. sanctum, R. vesicarius, P. oleracea* and gum acacia. Further, no beneficial effect of SM was established in ethanol-induced mucosal damage/ulceration in our study. The result of the study supports the traditional use of SM in the treatment and prevention of peptic ulcers, especially induced by modern-day NSAIDs. Further studies are warranted to illustrate the mechanism of action of SM.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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