Original Article
Formulation of Medicines

NEW FORMULATIONS FROM *ACACIA NILOTICA* L. AND *GLYCYRRHIZA GLABRA* L. FOR ORAL ULCER REMEDY

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SUMMARY: This study addresses the innovation of a stable new pharmaceutical formulation comprising natural botanical extracts of Acacia nilotica *L. and* Glycyrrhiza glabra *L., which are therapeutically effective in the treatment of oral ulcer and possess high patient acceptability.*

The pastes are prepared, characterized, and subjected to physical and chemical stability studies and then evaluated for therapeutic efficacy regarding ulcer size.

The novel adhesive paste is prepared and when applied to the oral mucosa, it remains on it for a considerable period of time. The stability tests of all the samples in the analysis showed satisfactory physical and chemical stability evaluated by the Normal Stability Test. The mucoadhesive paste incorporating the active; A. nilotica *L, when applied to oral ulcer, could promote the healing process, leading to a decrease in diameter of the inflammatory halo of the ulcer. However, the effect of liquorice extract on recurrent aphthous ulceration (RAU) was found even better than that of acacia extract. A combination of the two plants presented a synergism of both, leading to better healing with favorable reduction of the diameter of inflammatory halo of the ulcer together with a prolonged action.*

Therapeutically effective and stable oral pastes are dispensed with cost-effective benefits.

Key words: Acacia nilotica *L.,* Glycyrrhiza glabra *L., Adhesive paste, Stability, Oral ulcer*

INTRODUCTION

This study addresses the application of traditional folk medicine for developing new pharmaceutical preparations comprising natural biologically active compounds to treat oral diseases and reduce side effects of synthetic drugs.

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Acacia nilotica L., Willd Family Leguminosae "Fabaceae," is cultivated around the Nile banks of Egypt. The fruits of *A. nilotica* L. are rich in simple phenolics, tannins, flavonoids, quinines, saponins, coumarins, and polysaccharides (1,2). The condensed tannin content of *A. nilotica* L. was reported to be 15.7 mg/g dry matter (3). The condensed tannins are often referred to as proanthocyanidins (4).

The genus *Glycyrrhiza* (Leguminosae—Fabaceae) consists of about 30 species comprising *Glycyrrhiza glabra* (5). *G. glabra* L. is a perennial grass of Leguminosae Family, which has been used in folk medicine since ancient times. A list of biologically active compounds are isolated from various liquorice species including triterpenoids, individual phenolic compounds, several polysaccharides, amino, and lipids

(6,7). Liquorice root contains triterpenoid saponins ranging from 10-25% (8) according to one report, whereas 12-26% according to another report (9). Triterpenoid saponins consist mostly of glycyrrhizin, a mixture of potassium and calcium salts of glycyrrhizic acid (GA, also known as glycyrrhizinic acid and a glycoside of glycyrrhetinic acid), which is 50 times as sweet as sugar (10).

It is accepted that the successful treatment of disorders of the oral cavity using topical dosage forms is a difficult process (11,12). This difficulty arises from the wide range of stresses to which the dosage form is exposed and from the poor residence time of the dosage form/therapeutic agent at the required site (13). These factors limit both the concentration at, and the time of contact of the therapeutic agent with, the proposed site of action. One method proposed to correct this problem is the use of mucoadhesive formulations (14).

In this study new pharmaceutical mucoadhesive pastes have been designed for treatment of oral ulcer. These pastes comprise polymers as sodium carboxymethyl cellulose (Na CMC); pectin and gelatin; plasticizers as polyethylene glycol (PEG), glycerine and liquid paraffin together with an anionic surfactant; Tween 20; and therapeutic botanical extracts of *A. nilotica* and *G. glabra* L.

Several hydrophilic polymers have been reported to possess mucoadhesive/bioadhesive properties (15). Wong *et al.* (16) ranked the mucoadhesive properties of polymers as carbopols (polyacrylic acid) > gelatin > Na CMC > hydroxypropyl methyl cellulose > alginic acid, Eudragits, and chitosan. Peppas and Buri (17) demonstrated that the strong anionic charge on the polymer is one of the required characteristics for mucoadhesion. Classification of mucoadhesive polymers in buccal drug delivery shows pectin as a natural polymer, gelatin as a seminatural polymer, and Na CMC as a synthetic polymer. Also pectin and Na CMC possess anionic charge. Semisolid dosage forms have the advantage of easy dispersion throughout the oral mucosa (18).

Recurrent aphthous ulceration (RAU) was reported as the most common inflammatory ulcerative condition of the oral mucosa (19,20). Aphthous ulcers are classified on the basis of ulcer size into major, minor, or herpetiform (21). Minor ulcers are small (<1 cm in diameter), well defined, shallow, and heal within two weeks without scars. Major ulcers are bigger, deeper, and require six weeks to heal leaving a scar behind. Herpetiform ulceration is characterized by small (3–6 mm), shallow ulcers that take weeks to heal (21).

The etiology of RAU is not understood (22); however, attacks may be precipitated by, or associated with, local trauma, stress, food hypersensitivity, hormonal changes, microorganisms, and vitamin and trace element deficiencies (23).

The efficacy and safety of previously prepared mouth washes from *G. glabra* L. and *A. nilotica* L. (6,24) encouraged the authors to proceed with clinical trials using new, stable pharmaceutical adhesive pastes containing the mentioned natural plant extracts for acquiring effective treatment of oral ulcer and possessing high patient acceptability.

MATERIALS AND METHODS

Plant Materials

Samples of dried roots and rhizomes of *G. glabra* L. Family Leguminosae "Fabaceae" were purchased from the local market; Harraz herbal store, Cairo, Egypt. Fruit samples of *A. nilotica* L. Willd were collected from trees growing along the bank of Egypt. The plant samples were authenticated by Dr Abdelhaleem Abdelmotagaly, Dept of Flora, the Agricultural Museum, Dokki, Giza, Egypt.

Materials

Glycerine, International; Tween 20, Loba Chemie, Mumbai, India; liquid paraffin and Na CMC, El-Goumhouria Co., Egypt. Pectin; pure, Sisco Research Laboratories, Mumbai, India; Acetone, ADWIC; pure reagent for analysis, El-Nasr Pharmaceutical Chemical Co., Egypt; hydrochloric acid, methanol, and n-butanol, BDH, AnalaR, Poole, England; PEG 400 and PEG 20000, Fluka Bio-Chemika; and gelatin from bovine skin, type B, Sigma Aldrich Chemie GmbH, Steinheim, Germany.

Methods

Preparation and Phytochemical Evaluation of Bioactive Fraction from *A. nilotica* L. and *G. glabra* L.

One kilogram of powdered dried roots and rhizomes of *A. nilotica* and *Glycyrrhiza glabra* were extracted separately and exhaustively in a contiuous extraction apparatus using solvents of increasing polarity in the following order: petroleum ether, ether, chloroform, methanol, and water. These extracts were evaporated to dryness under vacuum at 40°C, lyophilized, and saved for dispensing. The collected polar extracts were subjected to phytochemical investigation (2,6,24–26) using different chromatographic and spectral techniques (for the separation, identification, and confirmation of biologically active constituents) as; PC, CC, TLC, Preparative HPLC, LC/MS, High field NMR, ^{1}H-NMR, ^{13}C-NMR, HMBC, HMQC, H^{1}H1-COSY, and ESI-MS (6).

Assessment of Calibration Curves for Acacia and Liquorice Dry Botanical Extracts

Determination of condensed tannins (CT) in *A. nilotica* L. dry extract was conducted as reported by Al-Soqeer (3) with some modification; 75 mg CT was extracted thrice with a mixture of 5 ml acetone/water (7:3) in a water bath at 30°C. The tubes were vortexed for 3 min and filtered through filter paper No. 5; the filtrates

Tablo 1: Percentage Composition of Proposed Adhesive Pastes

	Single acacia	Single liquorice	Mix acacia and liquorice
Na CMC	5	5	5
Pectin	5	5	5
Liquid praffin	9.8	9.8	9.8
Gelatin	1.7	1.7	1.7
PEG 20000	2	2	2
Glycerine	18	18	18
PEG 400	16.7	16.7	16.7
Tween 20	2	2	2
Acaia extract	2		1
Liquorice extract		2	1
Distilled water	45	45	45

PEG: polyethlene glycol.
Na CMC: sodium carboxymethyl cellulose.

completed with distilled water to the 25 ml mark in volumetric flasks. Aliquot samples of 0.1, 0.2, 0.3, 0.5, and 1 ml were added each to 6 ml of n-butanol/HCl (95:5 v/v), vortexed, and heated at 95°C on a water bath for 1 h. The absorbance of the red anthocyanidin products (CT) was measured at 550 nm using UV-2401 PC, UV-Vis Recording Spectrophotometer, Schimadzu Corporation, Kyoto, Japan. The same method was adopted to assess a calibration curve for CT in *A. nilotica* L. in the presence of liquorice extract by adding an equal amount of dry liquorice extract to the acacia extract at the beginning of the procedure.

The assessment of GA content in dry liquorice extract was done according to Raja *et al.* (27). Fifty milligrams of dry liquorice extract were sonicated in 20 ml methanol in a closed vial for 1 h, and then filtered through filter papers No. 4 and 5 in a weighed beaker. The filtrate was evaporated to dryness. The residue (GA) was weighed and dissolved in distilled water. Serial dilutions of this stock were prepared to give six concentrations in the range of 10–70 μg/ml. Absorbance was measured spectrophotometrically at λmax 256 nm. The same procedure was adopted for (GA) calibration curve assessment in *G. glabra* L. in the presence of an equal amount of dry acacia extract to obtain the slope appropriate for the calculation of (GA) concentration in the presence of acacia extract in the mix formula.

Preparation of Adhesive Paste Formulas

Adhesive pastes containing acacia and liquorice dry extracts either in single or mix formulas were prepared by mixing gelatin, PEG 20000, and the plant extract in glycerine, PEG 400, and distilled water at 70°C till complete dissolution of the components. Simultaneously, Na CMC, and pectin were dispersed in liquid paraffin and mixed well at 70°C. The aqueous portion was mixed with the oily component of the preparation with the addition of Tween 20. Ingredients were blended at 70°C till homogeneous, cooled to room temperature and stored in sealed containers. The composition of the pastes is presented in Table 1.

Determination of Condensed Tannins in Acacia and GA in Liquorice Extracts

Half a gram of single acacia and mix acacia and liquorice adhesive pastes, each, was weighed; 6 ml acetone/water (7:3) was added, followed by vortex mixing for 3 min. and filtration through filter paper No. 5. The filtrate volume was completed with distilled water to 10 ml. To 1 ml of this liquid extract, 6 ml n-butanol/HCl (95:5) was added, followed by vortexing for 1 min and then heating in a water bath at 95°C for 1 h. The absorbance was measured at λmax 550 nm versus blank n-butanol/HCl (95:5). Measurements were conducted six times.

One gram of single liquorice and 2 g of mix acacia and liquorice adhesive pastes, each, was weighed, 20 ml methanol was added, sonication was conducted for 1 h followed by filtration through filter papers No. 4 and 5 in a weighed beaker, the filtrate was evaporated to dryness, the residue dissolved in distilled water and filtered using millipore filter 0.45 μm, absorbance was measured at 256 nm. Six measurements were performed.

Stability Studies

Thermal stability was assessed according to ICH guidelines as described in section 2 (28). Physical and chemical stability evaluations for the prepared three formulations were conducted by storing samples at room temperature, 30 ± 2°C (long-term stability) and at 40 ± 2°C (accelerated stability) for 2 months (28). They were visually inspected for physical properties and evaluated for the residual amount of CT in single acacia and mix acacia and liquorice and for the amount of GA in single liquorice and mix acacia and liquorice samples, at zero time and after 2 months storage.

Assessment of Adhesive Pastes

A total of 28 patients (12 male and 16 female) having minor oral aphthae with a history of at least three times oral ulceration in 1 year were included. They were recruited from the patients attending at the Oral Medicine Clinic, National Research Centre (NRC), Cairo, Egypt. The patients were free of the exclusion criteria of RAU and assigned randomly to four treatment groups namely Group I (*Acacia*; A), Group II (*Liquorice*; L), Group III (Mix *Acacia* and *Liquorice*; A&L), and Group IV (control group receive no treatment). Patients of the tested groups received herbal preparations and applied the adhesive pastes three times daily. A detailed personal and ulcer assessment questionnaire was filled out, and the diameter of inflammatory halo was measured at 0, 48, and 72 h. Statistics was conducted using Tukey's Test. This study was approved by the Research Ethics Committee at NRC, Egypt.

RESULTS

This study addresses the innovation of stable new pharmaceutical formulations with natural botanical extracts of *A. nilotica* L. and *G. glabra* L., for the treatment of oral ulcer and possessing high patient acceptability. The pods of *A. nilotica* L. are rich in condensed tannins (2), gallic acid, and flavonoids (24).

Previously published results (6,26) as well as available data reveal that the biologically active fractions isolated from the roots and rhizomes of *G. glabra* L. are rich in triterpenoid saponins, mostly glycyrrhizin (10) (reaching up to 26% (9)), flavonoids (29), and flavan type glycosides.

Calibration curves for the quantitative estimation of CT were found to be linear regarding single and mix acacia and liquorice extracts. Absorbance was measured at 550 nm; the values of r^2 were 0.9963 and 0.9752 for single and mix extracts, respectively. Straight line equations were $y = 32 x + 0.0220$ for single acacia and $y = 38 x + 0.0794$ for mix acacia and liquorice extracts, where y is the absorbance and x is the concentration in mg/ml. As for the single liquorice and mix acacia and liquorice extracts, the absorbance corresponding to the GA content was measured at 256 nm; r^2 was 0.9920 in both single and mix extracts. The UV-spectrophotometric method adopted provided a linear relationship of absorbances measured versus concentrations ranging from 0.01–0.07 mg/ml regarding single liquorice extract and 0.003–0.020 mg/ml for mix extract. The regression equations are $y = 0.007 x + 0.045$ and $y = 0.015 x + 0.051$ referring to single and mix extracts, respectively.

The novel adhesive paste was applied to the oral mucosa and was retained on it for a considerable period of time.

Physical and chemical stability tests describe approaches to predicting how well pharmaceutical products will resist common stress such as temperature extremes. High-temperature testing is commonly used as a predictor of long-term stability. The accelerated aging assay is also known as Normal Stability Test (30). The Normal Stability test in the present article was conducted through 60 days in two storage conditions of temperature and luminosity. The packaging material was neutral glass and the stability assays were realized in replicates of six. In physical stability assay, several subjective tests were used to evaluate the appearance of the products as shown in Table 2: storage at ambient temperature and 40°C, result in a smoother texture, and ease of application. The chemical stability of the adhesive pastes under investigation was obtained in function of CT and GA determination, concerning acacia and mix acacia and liquorice, and liquorice and mix acacia and liquorice samples, employing spectrophotometric methods at 550 nm and 256 nm, respectively. The content of active ingredients content dropped no more than 9% on storage at ambient and high temperatures. Results are illustrated in Table 3.

Concerning the results of the clinical trials (Table 4 and Figures 1 and 2) it was found that, before treatment, there was no statistically significant difference between the four groups: Group I (A), Group II (L), Group III (A&L), and Group IV (Control).

After 48 and 72 h, there was no statistically significant difference between group A and Control; both showed the statistically significantly highest mean diameter. There was no significant difference between A&L and L groups; both showed significantly lowest mean diameter, $P \leq 0.05$. As regards the % decrease in diameter after 48 h, there was no significant difference between A&L and L groups; both showed the significantly highest mean % decrease. This was followed by Group (A). Control group showed the significantly lowest mean % decrease. As for the % decrease in diameter after 72 h, A&L group showed the significantly highest mean % decrease. This was followed by Group L then Group A. Control group showed the statistically significantly lowest mean % decrease, $P \leq 0.05$.

DISCUSSION

Semisolid dosage forms have the advantage of easy dispersion throughout the oral mucosa. These dosage forms provide an extended retention time, adequate drug penetration, as well as high efficacy and patient acceptability (18). In general, the more concentrated polymer would result in a longer penetrating chain length and better adhesion (18). Movements within the oral cavity continue even during sleep,

Tablo 2: Physical Properties of Adhesive Pastes 24 h after Preparation (t 0) and at the End of the Normal Stability Test (60th Day of analyses)

Parameter	Single acacia			Single liquorice			Mix acacia and liquorice		
	t 0	RT	HT	t 0	RT	HT	t 0	RT	HT
Color	dark brown	S	S	dark brown	S	S	dark brown	S	S
Odor	characteristic	S	S	characteristic	S	S	characteristic	S	S
Opacity	opaque	S	S	opaque	S	S	opaque	S	S
Elegance in appearance	elegant	S	S	elegant	S	S	elegant	S	S
Integrity	integral	S	S	integral	S	S	integral	S	S
Texture	smooth	M	M	smooth	M	M	smooth	M	M
Homogeneity	homogeneous	S	S	homogeneous	S	S	homogeneous	S	S
Grittiness	non-gritty	S	S	non-gritty	S	S	non-gritty	S	S
Fungal growth	absent	S	S	absent	S	S	absent	S	S
Dehydration	absent	S	S	absent	S	S	absent	S	S
Application	easy	M	M	easy	M	M	easy	M	M
Aqueous W	feasible	S	S	feasible	S	S	feasible	S	S
Visual appearance (thick or thin)	thick	M	M	thick	M	M	thick	M	M

t 0 : 24 h after preparation, RT: room temperature, HT: high temperature, S: stable (unaffected parameter), M: modified (slightly modified parameter).

Tablo 3: Chemical Stability Data of Adhesive Pastes by Active Ingredient Determination

Formula	CT (mg/g DM) content ± SD		
	t 0	RT	HT
Single acacia adhesive paste	16.515 ± 1.37	15.941 ± 1.69	16.50 ± 1.10
Mix acacia and liquorice adhesive pastes	15.90 ± 1.55	15.84 ± 1.84	15.40 ± 1.37
	GA (mg%) content ± SD		
Single liquorice adhesive paste	25.184 ± 0.928	25.600 ± 2.060	23.615 ± 0.389
Mix acacia and liquorice adhesive pastes	26.810 ± 0.106	26.560 ± 3.401	24.620 ± 2.700

Results are means ± standard deviation, n= 3–4, CT: condensed tannins, DM: dry matter, GA: glycyrrhizic acid, t 0: 24h after preparation, RT: room temperature, HT: high temperature.

and can potentially lead to detachment of the dosage form. Therefore an optimum time span for administringthe dosage form is necessary to avoid many interfering factors (31).

The novel pharmaceutical paste dispensed comprises a water soluble or swellable polymer, gelatin, which can adhere to a wet mucous surface; the paste also comprises pectin and Na CMC, which are anionic and thus promote mucoadhesion. The plasticizers involved comprise paraffin oil (mineral oil), polyhydric alcohol; glycerine, and glycols (PEG 400 and PEG 20000). Permeation of the buccal mucosa can be increased by various penetration enhancers (32). In the present study, penetration enhancers are compiled as nonionic surfactant Tween 20 (generally regarded as safe GRAS according to WHO), polyhydric alcohol, glycer-

Tablo 4: Inflammatory Halo Diameter (mm)

		A	A&L	L	Control	P-value
	Before treatment	9.2 ± 1.8	9 ± 1.5	8.9 ± 1.7	8.3 ± 1.6	0.481
	48 h	6.7 ± 1.4^{a}	5 ± 1.1^{b}	5.4 ± 1.4^{b}	7.4 ± 1.3^{a}	0.001*
Inflammatory halo	72 h	6.1 ± 1.2^{a}	2.2 ± 0.4^{b}	3.4 ± 0.9^{b}	7.1 ± 1.3^{a}	<0.001*
(mm)	% reduction (48 h)	27.2 ± 8.4^{b}	44.4 ± 8.4^{a}	39.3 ± 8.9^{a}	10.8 ± 2.7^{c}	0.001*
	% reduction (72 h)	33.7 ± 7.8^{b}	75.6 ± 11^{a}	61.8 ± 10.3^{b}	14.5 ± 4.1^{d}	<0.001*

Values are mean ± SD, *: Significant at P≤0.05, Relations between a and b, a and c, a and d, b and c, b and d, c and d, indicate significant difference.

ine, and PEG. PEG is a neutral, water soluble, and nontoxic polymer, used by the FDA for internal consumption and injection and introduced into several biomaterials and drugs because of good immunogenicity (33).

According to the data presented in Table 3, all the samples in analysis show satisfactory chemical stability evaluated by the Normal Stability Test.

It is worth mentioning that the % decrease in halo diameter is higher after 72 h compared with 48 h indicating prolonged action of products.

The bioactive extract of *A. nilotica* L. showed previously, a remarkable inhibitory effect against Gram positive and Gram negative bacteria as well as *Candida albicans* (25,34). It has been reported that CT reduce the rate of proteolysis and inhibit growth of proteolytic rumen microorganisms. CT not bound to protein can inhibit fermentation to structure carbohydrates in the rumen by forming indigestible complex with cell wall carbohydrates, rendering them undegradable. It can also form complex with microbial enzymes, rendering them inactive (3). In the present study, the antimicrobial effect of *A. nilotica* L. paste led to the reduction in bacterial growth, whichpromote the healing process leading to decrease in diameter of inflammatory halo of the ulcer. However, acacia paste did not present good results as a single preparation, and the diameter of inflammatory halo of the ulcer was still higher than the liquorice paste alone or mix acacia and liquorice paste.

Previous work focused on the bioactive fraction of *G. glabra* mouth rinse demonstrated significant remarkable anti-inflammatory and analgesic effects (6). In the present study, it was found that the effect of liquorice paste on RAU was even better than that of acacia paste. This effect may be attributed to anti-inflammatory effect that is reported for liquorice, resulting in less inflammatory tissue damage and accelerating healing and decreasing diameter of inflammatory halo of the ulcer. However this effect of liquorice paste was still less than that of the mix paste of *A. nilotica* L. and *G. glabra* L. GA in liquorice extract is reported to be a promising neutraceutical for remedying inflammation (35). In another report, results indicated that GA might serve as a potential agent for the treatment of inflammatory mediated diseases, where GA would provide an anti-inflammatory effect by atten-

Group I = Acacia (A), Group II = Liquorice (L), Group III= Acacia and Liquorice (A&L), Group IV = Control.

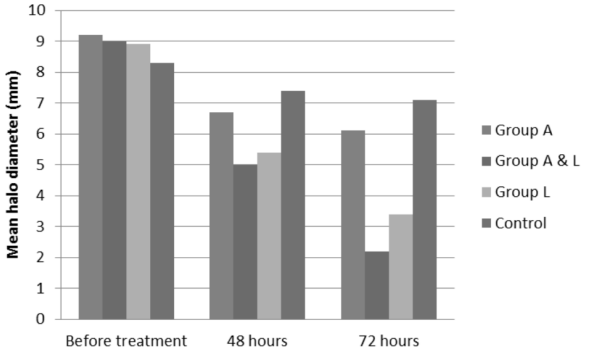

Figure 1: Bar chart representing mean halo diameter before and after the treatment in the four groups

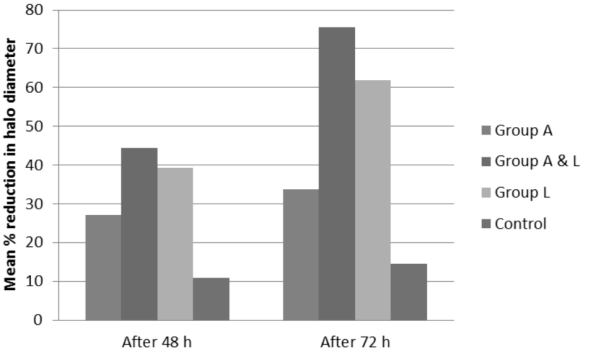

Figure 2: Bar chart representing mean halo diameter before and after the treatment in the four groups

uating the generation of excessive nitric oxide (NO), prostaglandin E (2) (PGE (2)), and reactive oxygen species (ROS) and by suppressing the expression of pro-inflammatory genes. In addition, Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR) analyses revealed that GA significantly reduced the protein and mRNA levels of COX-2 in lipopolysaccharide (LPS)-induced microphages (36). Also, GA is reported to possess antibacterial (37) and antiviral (38) effects. It is reported that, today, root of *G. glabra* is known to have anti-oxidative properties, and anti-inflammatory and anti-viral effects (39). A significant decrease in the rate of ROS and an increase in secretion of insulin by dose of 1 ppm GA were reported. GA has been previously found to reduce ROS in other cells like neuronal cells by elevating the intracellular antioxidant system (40). Also, GA is the main active component of *G. glabra*'s root that is responsible for most of positive biological effects (41). Glycyrrhizin, extracted from the roots of liquorice and its aglycone, and glycyrrhetinic acid have been shown to exert antihepatotoxic activity. Furthermore, glycyrrhizin has been used in the treatment of chronic hepatitis (42,43).

The combination of the biologically active extracts of the two plants in a single paste formulation presented a synergism of both anti-inflammatory and antimicrobial effects of liquorice and acacia, respectively, leading to better healing with favorable reduction of the diameter of inflammatory halo of the ulcer.

CONCLUSION

The results obtained suggest that the herbal, acacia and liquorice, single and mix adhesive pastes are simple to dispense and cost-effective formulations. They are physically and chemically stable on storage at room temperature and at accelerated condition; 40°C. They are effective, on topical application, for the treatment of minor aphthae, especially the mix paste formulation that is the most therapeutically effective.

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