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Phytochemical and clinical studies of the bioactive extract of *Glycyrrhiza glabra* L. Family Leguminosae

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Abstract

The aim of this study was the isolation, identification of the bioactive ingredients of *Glycyrrhiza glabra* L. Family Leguminosae, as well as the evaluation of their efficacy and ability to control plaque induced gingivitis in a preliminary clinical study. The dried powdered roots and rhizomes of *G. glabra* was subjected to successive extraction using organic solvents of increasing polarity. The total polar extract of the roots and rhizomes of *G. glabra* showed significant inhibition of carrageenan induced swelling of the hind rat paw (P>0.5).Seven phenolic compounds namely liquiriteginin, liquiritin apioside, neoliquiritin apioside, isoliquiritin, isoliquritin apioside, licuraside2-(5-P-coumaryl apiosyl) and isoliquiritin were isolated from the total polar extract utilizing different chromatographic techniques (PC, TLC, CC, HPLC and LC/MS) and identified using spectral analysis (^{1}H-NMR, ^{13}C-NMR, 2D-NMR and ESI-MS).The effectiveness of the isolated bioactive fractions of *G. glabra* mouth rinse in the reduction of plaque and gingivitis was studied clinically, after the approval of its safety, and the results were significant.

Keywords: *Glycyrrhiza glabra,* anti-inflammatory activity, bioactive constituents, clinical trial, gingivitis, plaque index.

Introduction

The world wide use of medicinal plants has become important in primary health care especially in developing countries. Many pharmacognostical and pharmacological investigations are carried out, to identify new drugs or to find new lead structures for development of novel therapeutic agents for the treatment of human diseases [1]. It is also important because the dangerous side effects of the treatment of inflammatory diseases may be more difficult to manage than the disease itself. The severe side effects of steroidal and non-steroidal anti-inflammatory drugs used in the treatment of several oral diseases encourage the search for new anti-inflammatory oral drugs from natural botanical sources which may have minimal drawbacks. The reviewed literature displays powerful anti-inflammatory activity of the plant under investigation. These activities come into accordance with the current study that aims to the development of preparations from natural origin that is useful in the treatment of some oral inflammatory diseases. Licorice is derived from Greek words Glykos "sweet" and Rhyza "root") or licorice. Licorice species are native to the Mediterranean region, central to Southern Russia and Asia minor to Iran, now widely cultivated throughout Europe, the Middle East and Asia [2]. Different species of Licorice have been used medically since at least 500 BC and licorice has been described as 'the grandfather of herbs [3]. The genus *Glycyrrhiza* which belong to Family Leguminosae consists of about 30 species, some of them are *G. glabra*, *G. uralensis*, *G. inflata*, *G. aspera*, *G. korshinskyi* and *G. eurycarpa*, *G. glabra* also includes three varieties: Persian and Turkish licorices are assigned to *G. glabra* var. *violacea*, Russian licorice is *G. glabra* var *gladulifera* and Spanish and Italian licorices are *G. glabra* var. *typical* [4]. It is also known as liquorice, kanzoh, gancao, sweet root and yasti-madhu [2,4]. Remarkable therapeutic properties of licorice as a medicinal plant are known from very ancient times. Licorice roots have been used as a raw plant material for medicinal purposes for several thousand years. Modern folk medicine employs licorice roots both independently and in dozens of multicomponent preparations (teas) possessing cholagogic, diaphoretic,expectorant, analgesic, wound-healing, antiseptic, antidotal, antiallergic and tonic properties. Liquorice is also used for the treatment of disorders in lungs, respiratory tracts, stomach, kidneys, and other organs [5]. With respect to the number of available and widely used preparations, licorice presently occupies the first line in the list of medicinal plants. Licorice has a long history as medicinal plant used in Europe and Asia. It is felt to be effective in the treatment of peptic ulcer disease, constipation, cough and other diseases. It seems that different parts of this herb may be useful to treat some diseases. During the past century, a number of components, including both complexes of biologically

active substances and hundreds of individual compounds belonging to various chemical classes and representing groups possessing different pharmacotherapeutic properties, were isolated from licorice. Among these components, the first to be mentioned is a water-soluble complex of biologically active substances which account for (40-50%) of the total dry raw material weight. In this complex, a considerable part is made up of triterpenes, saponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, microelements and some other substances [6].

Materials and methods

Materials

Plant Materials

Samples of the dried roots and rhizomes of *Glycyrrhiza glabra* L. Family Leguminosae were purchased from the Egyptian local market-Harraz herbal store, Cairo, Egypt and kindly authenticated by Dr. Abdelhaleem Abdelmotagaly, Flora Department, the Agricultural Museum, Dokki, Giza, Egypt. The samples were air-dried, powdered and reduced to mesh no. 36 and kept in tightly-closed containers. A voucher specimen was deposited in the National Research Center Herbarium ,Cairo ,Egypt.

Animals

Adult rats of both sexes weighing 150-200g and adult mice weighing 20-25g were used in the experiments. Animals were housed under standardized conditions of light and temperature and received standard rat chow and tap water *adlibitum.* Animals were randomly assigned to different experimental groups, each kept in separate cage. All animal procedures were performed after approval from the Ethics Committee of the National Research Centre, Cairo, Egypt and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication No. 85-23, revised 1985).

General Experimental Procedures

NMR Varian Inova –500, Varian Gemni unity plus 300 NMR and JEOL delta 400 spectrometer apparatus using DMSO-d_6, $CDCl_3$-d_1, CD_3OD-d_4 as solvent and TMS as internal standard. The data are expressed in δ-values in ppm and *J*-values in Hz. LC-MS were conducted using an Agilent LC-MSD ion trap mass spectrometer (Bruker Daltronics, Bremen, Germany) equipped with a 1100 series HPLC and a Cosmosil waters $5C_{18}$ (150 mm x 4.6 mm, 5 μm) column. The UV detection was set at 280 nm. Thin layer chromatography (TLC)was carried out on TLC Silica gel 60 F254 plates, Silica gel 60 F_{254} for CC(Merk). Sephadex LH-20 for CC (Pharmacia, Uppsala, Sweden).

Methods

Preparation of successive extracts with selective organic solvents

500 grams of the dried powdered roots and rhizomes of *Glycyrrhiza glabra* L. were extracted in a continuous extraction apparatus (Soxhlet) successively and exhaustively using solvents of increasing polarity in the following order: petroleum ether (60-80°C), ether, chloroform , methanol and 50% aqueous methanol. For each solvent the extraction was continued till exhaustion. The extracts were evaporated to dryness under vacuum at 40°C .

Plant extracts and fractions for biological tests

Both polar (methanol and 50% aqueous methanol) and non polar (petroleum ether (60-80°C), ether and chloroform) successives extracts previously prepared from *Glycyrrhiza glabra* L. were combined and evaporated under reduced pressure till dryness were administered orally to experimental animals in a dose equal to 500 mg/kg body weight separately.

Acute inflammation test (carrageenan- induced paw oedema assay) [7,8]

A. Twenty-four adult male albino rats, divided into four groups, each of six animals, were orally treated with the four mentioned dose of both polar and non-polar extracts, Indomethacin (2mg/100g.b.wt) (positive control) and saline (negative control).
B. One hour after oral administration, all animals were given a sub-plantar injection of 100 μl of 1 % carrageenan solution in saline 100 ml in the right hind paw. The contralateral hind paw received the same volume of saline and served as (negative control).
C. Hind foot-paw thickness was measured with a micrometer caliber before and at 1, 2, 3 and 4hrs. after carrageenan injection, as carrageenan caused visible redness and pronounced swelling that was well -developed after 4 hrs. and persisted for more than 48 hrs. [9].

Statistical Analysis

Results are expressed as mean ± S.E. Differences between vehicle control and treatment groups were tested using one-way ANOVA followed by the least significant difference (L.S.D). Methods of statistical analysis were done according to [10].

Isolation and identification of active constituents from the biologically active polar extract

Both the successives methanol and 50% aqueous methanol extracts previously prepared were combined and evaporated under reduced pressure till dryness to give (60 gm) of a dark brown residue .The residue was partitioned between water and organic solvents (chloroform , ethyl acetate and finally *n*-butanol). The ethyl acetate soluble fraction (4 gm) was dried and chromatographed over VLC column (10 cm x 4 cm) packed with silica gel 60 F_{254} for CC. and eluted with chloroform followed with increasing

percentages of methanol (up to 100%) under vacuum (adjusted to flow rate 3 ml/min). A total of 25 fractions were collected (100 ml each). These were combined according to TLC examination using silica gel 60 F_{245} (Merk) plates and developed using (methanol:chloroform) as eluents (80:20)(v/v). Components were detected under UV and by spraying with $AlCl_3$ to give six major fractions (1-6).On standing fraction no.2 yielded colorless needle crystals which were further dissolved in methanol and purified by passing on Sephadex LH-20 column using methanol as mobile phase to yield (15 mg) of (compound I) as colorless needle crystals. Fraction no.4 was then applied to Sephadex LH-20 column (40cm×3 cm) using (methanol: water) (1:1) (v/v) as mobile phase to yield eight major fractions (from A to H).Fractions B, C and D were fractionated separately using preparative HPLC on C_{18} column utilizing [methanol/ 0.1%TFA in water] as mobile phase and UV detector (λ=280) to detect the eluted compounds. Compound II (3mg) and Compound III (2mg) were isolated from fraction B as yellow amorphous powder. While gradient elution of fraction C was carried out starting with 15% methanol in(0.1%TFA in water) for 20 minutes and gradually increased to(35% ,50% and 70%) , for (45 min, 60 min and 75 min) respectively, to yield (compound IV) (8mg), (compound V) (6 mg) and (compound VI) (10 mg) which were isolated as pale yellow powder. Gradient elution for fraction D was carried out starting 10% methanol in(0.1%TFA in water) for 20 minutes and gradually increased to(30% ,50% and 70%) , for (50 min,70 min and 80 min) respectively, to yield (compound VII) (4mg), which was isolated as yellow powder. The structures of all the isolated compounds were then investigated by spectral analysis, using ^{1}H-NMR , ^{13}C-NMR HMQC, HMBC and ESI-MS spectroscopy , as well as comparison with reported data.

Clinical trials

Subjects

Twenty volunteers aged 20-45 years from outpatients` clinic of Misr University of Science and Technology (MUST) Egypt were enrolled in this study.

Inclusion criteria

-Subjects willing to participate
-Subjects having at least 20 natural teeth
-The gingival condition was evaluated for all subjects using the gingival index of Loe and Silness [11], they were selected having moderate gingivitis.
-All were willing to refrain from any form of mechanical or chemical means of plaque control during the study period.
-Subjects were free from any systemic diseases according to dental modification of Cornnell medical index.

Preparation of the mouth rinse formulations

Thirty percent of the biologically active polar extract of *G. glabra* was formulated in the form of mouth rinse according to the method described in [12]. The negative control mouth rinse (placebo) consisted of 0.5% sodium carbonate in 5% alcohol/sterile water.

Exclusion criteria

The selected subjects must be free of antimicrobial therapy during the past three months, smokers, pregnant females, as well as subjects undergoing orthodontic treatment or using intra-oral artificial prosthesis, or large fillings were excluded.

Experimental design

The eligible subjects were informed regarding the purpose of this study. Before entering the study, each of them provided a signed (witnessed) consent to participate in this study. The protocol was approved by Misr University of Science and Technology (MUST) University Ethical Committee, Egypt.
Each subject then received a full mouth scaling and professional cleansing of the teeth, by rubber cups and polishing paste, until almost zero plaque index scores were reached.
On day zero of the experiment (baseline), the following clinical parameters were assessed plaque index (P1) and gingival index (GI).The previous parameters were recorded again after two weeks. The twenty volunteers were allotted into two groups:
Group I Instructed to rinse with mouth rinse containing 30% of the bioactive polar extract of *G. glabra* twice daily for 2 weeks
Group II Instructed to rinse with a placebo mouth rinse twice daily for 2 weeks that serve as a negative control.
All volunteers were asked not to undertake any other oral hygiene measures and to refrain from brushing their teeth the whole experimental period. The condition of the mucous membrane was also examined during the study. Any ulceration was recorded as well as any other clinical manifestation.

Statistical Analysis

Data analysis was performed with Wilcoxon signed rank test between groups at both investigation periods. Statistical analysis was performed using Graphpad Prism-4 statistics software for Windows. P values ≤ 0.05 are considered to be statistically significant in all tests.

Results

Acute inflammation test (carrageenan- induced rat paw oedema assay)

The anti-inflammatory effect of both polar and non–polar extracts of the roots and rhizomes of *G.glabra* were evaluated adopting the carrageenan- induced rat paw oedema assay.
A-The subplanter injection of 100 µl of 1% sterile carrageenan into the rat hind paw elicited an inflammation (swelling and erythema) and a time-dependent increase in paw oedema that was maximal

at 4hrs post carrageenan. In control group, the paw thickness increased by (59.83±2.04%), (102.18±8.07%), (101.41±6.09%) and (100.58±4.22%). after 1hr., 2hrs., 3hrs and 4hrs.respectively of carrageenan injection as compared with pre-carrageenan control values.

B-The polar extract (50 mg/100g.b.wt) of *G. glabra* roots and rhizomes showed significant inhibition of the oedema formation by 45.81%, 28.35%, 27.10% and 21.13% at 1, 2, 3 and 4 hrs. respectively after (carrageenan injection), as compared with saline-treated control group, with a potency of 58.36%, 37.88%, 40.44% and 34.06% at 1, 2, 3 and hrs. after (carrageenan injection) respectively as compared with Indomethacin-treated control group which being most potent at 1 hr. after carrageenan injection.

C- The non-polar extract (50 mg/100g.b.wt) of *G. glabra* roots and rhizomes showed no significant inhibition of the oedema formation by 8.52%, 13.31%, 12.03% and 11.00% at 1, 2, 3 and 4 hrs. after carrageenan injection, respectively as compared with saline-treated control group with a potency of 10.90%, 17.79%, 17.95% and 17.73% at 1, 2, 3 and 4 hrs. after carrageenan injection respectively as compared with Indomethacin-treated control group .

D-Comparison of the activity of the extracts of *G. glabra* roots and rhizomes to the activity of Indomethacin is shown in [Table 1].

Identification of isolated compounds from the bioactive extract

Compound I was isolated as a colorless needle crystal (15 mg). melting point 206-208 °C. ESI–MS (*m/z*) (negative): 255 [M−1], further spectral investigation was carried out for confirmation of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) and ^{13}C-NMR. The spectrum of the compound displays signals characteristic of the flavanone nucleus .The structure of (compound I) was established as liquiritigenin comparing with reported literature[13].

Compound II was isolated as a amorphous pale yellow powder(3mg). Further spectral investigation was carried out for confirmation. of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) ,^{13}C-NMR and 2D-NMR. The structure of (compound II) was established as liquiritin apioside comparing with reported literature

Compound III was isolated as a amorphous pale yellow powder(2 mg). Further spectral investigation was carried out for confirmation. of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) ,^{13}C-NMR and 2D-NMR. The structure of (compound III) was established as neoliquiritin apioside comparing with reported literature [14].

Compound IV was isolated as a amorphous yellow powder(8mg).Further spectral investigation was carried out for confirmation. of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) and ^{13}C-NMR, The structure of (compound IV) was established as isoliquiritin comparing with reported literature [13].

Compound V was isolated as a yellow amorphous powder(6mg). Further spectral investigation was carried out for confirmation. of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) ,^{13}C-NMR and 2D-NMR.The structure of (compound V) was established as isoliquritin apioside comparing with reported literature[14].

Compound VI was isolated as a yellow needle crystals (10 mg) melting point 188-190°C. .Further spectral investigation was carried out for confirmation. of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) ,^{13}C-NMR and 2D-NMR. The structure of (compound VI) was established as licuraside which was previously reported in literature [13].

Compound VII was isolated as a yellow amorphous powder(4mg). Further spectral investigation was carried out for confirmation. of the structure by ^{1}H-NMR spectrum recorded in methanol (CD_3OD-d4) and ^{13}C-NMR. The structure of (compound VII) was established as isoliquiritin[14], which was previously reported in literature.All structures of the isolated compounds are compiled in [Table 2].

Preliminary clinical study of the bioactive polar extract of *G. glabra*

Gingival index

The results revealed that the difference between gingival index mean values for experimental group before (base line) and after treatment (2 weeks) was statistically significant (Wilcoxon=210, P=0.0001 < 0.05).Mean while the difference between gingival index mean values for control group before (base line) and after treatment (2 weeks) was statistically significant (Wilcoxon=121, P=0.0087 <0.05).Also it was found that the difference between gingival index mean values for experimental and control groups before treatment (base line) was statistically non- significant (Wilcoxon =8, P=0.8462>0.05). Meanwhile the difference between gingival index mean values for experimental and control groups after treatment (2 weeks) was statistically significant (Wilcoxon=210, P=0.0001 < 0.05) as shown in[Table 3] and [Figures1 and 2].

Plaque index

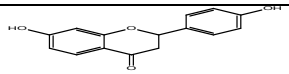
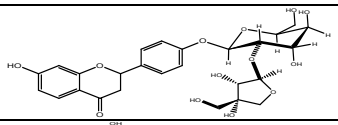
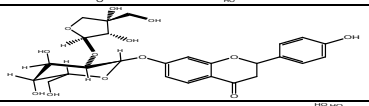
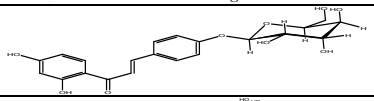
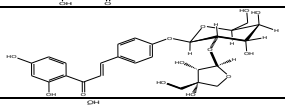
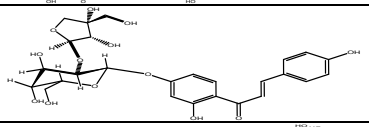
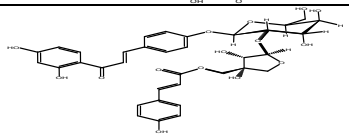
It was found that the difference between plaque index mean values for experimental group before (base line) and after treatment (2 weeks) was statistically significant (Wilcoxon=109, P=0.0290< 0.05). Meanwhile the difference between plaque index mean

Table 1 Results for mean thickness inflammation (cm) of the hind paw of control and tested rats at different time intervals of carrageenan injection in case of *G. glabra* extracts

Group	1 hour			2 hour			3 hour			4 hour		
	Edema rate (%)	Inhib. rate (%)	Potency (%)	Edema rate (%)	Inhib. rate (%)	Potency (%)	Edema rate (%)	Inhib. rate (%)	Potency (%)	Edema rate (%)	Inhib. rate (%)	Potency (%)
Control	59.83±2.04	0	-----	102.18±8.07	0	-----	101.41±6.09	0	-----	100.58±4.22	0	-----
Non-polar	54.73±5.08	-8.52	10.90	88.58±5.60	-13.31	17.79	89.21±5.05	-12.03	17.95	89.52±5.11	-11.00	17.73
Polar	32.42±2.94 [a]	-45.81	58.36	73.22±4.64 [a]	-28.35	37.88	73.93±5.81 [a]	-27.10	40.44	79.33±6.63 [a]	-21.13	34.06
Indomethacin	13.07±1.34 [a]	-78.15	100	25.71±2.97 [a]	-74.84	100	33.45±1.87 [a]	-67.02	100	38.19±2.47 [a]	-62.03	100

Values represent the mean ± S.E. of five animals for each group.
[a] P<0.05: Statistically significant from control. (Dunnett's test)

Table 2 Chemical structures of compounds isolated from the bioactive extract

Compound No.	Name	Structure
I	Lquiriteginin	
II	Liquirtin apioside	
III	Neoliquritin apioside	
IV	Isoliquiritin	
V	Isoliquiritin apioside	
VI	Neoisoliquiritin apioside(licuroside)	
VII	2-(5-P-coumaryl apiosyl), isoliquiritin	

Preliminary clinical study of the bioactive polar extract of *G. glabra* Gingival index

Table 3 The gingival index score (Mean ± SD) for the experimental and control groups before (baseline) and after treatment (two weeks)

Group	Before Mean ± SD		After Mean ± SD		Mean difference	Wilcoxon (W) test	
						W value	P value
Experimental	1.915 ± 0.08952		1.210 ± 0.09288		0.7050	210	0.0001*
Control	1.955 ± 0.1545		2.320 ± 0.1315		0.3650	121.0	0.0087*
Wilcoxon (W) test	W	28	W	210			
	P value	0.5148ns	P value	0.0001*			

*; significant (p<0.05) ,ns; non significant P>0.05).

Table 4 The plaque index score (Mean ± SD) for the experimental and control groups before (baseline) and after treatment (two weeks)

Group	Before Mean ± SD		After Mean ± SD		Mean difference	Wilcoxon (W) test	
						W value	P value
Experimental	2.330 ± 0.1248		2.120 ± 0.1178		0.21	118	0.0284*
Control	2.240 ± 0.1130		2.410 ± 0.09865		0.3650	61	0.1874
Wilcoxon (W) test	W	28	W	109			
	P value	0.5148ns	P value	0.2900			

*; significant (p<0.05) ,ns; non significant P>0.05).

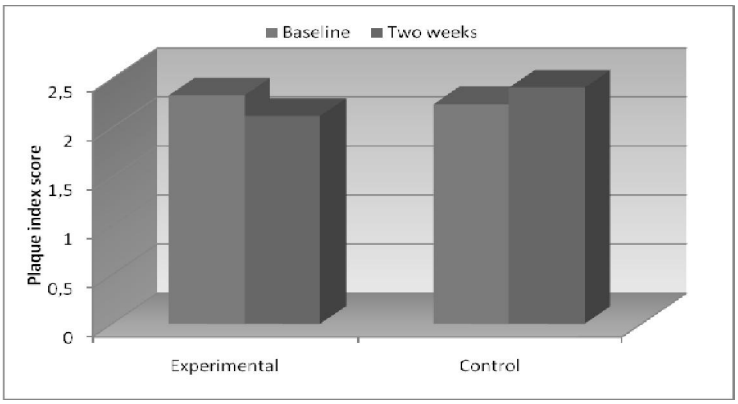


Figure 1 A column chart of plaque index score mean values determined in both groups at different investigation time

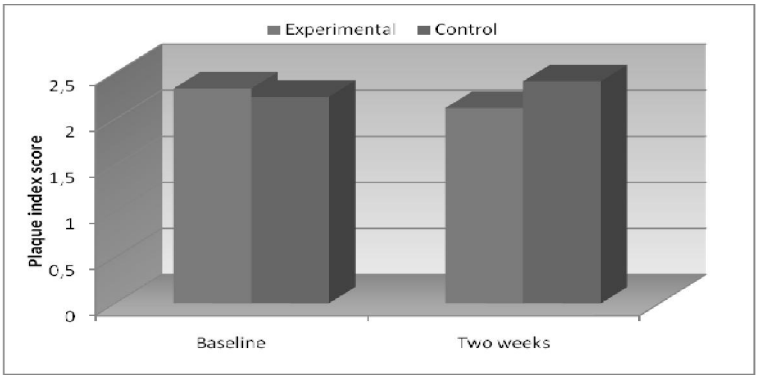


Figure 2 A column chart of plaque index score mean values determined at different investigation time in both groups

values for control group before (base line) and after treatment (2 weeks) was statistically non-significant (Wilcoxon=61, P=0.1874> 0.05). Also it was found that the difference between plaque index mean values for experimental and control groups before treatment (base line)was statistically non- significant (Wilcoxon =28,P=0.5148>0.05). Meanwhile the difference between plaque index mean values for experimental and control groups after treatment (2 weeks) was statistically significant (Wilcoxon=109, P=0.0284 < 0.05),as shown in[Table4] and [Figures3 and 4].

Discussion

This study was focused on the roots and rhizomes of *G. glabra* L, which can be useful as a source of medicine for some oral diseases and as an important component of the health care system. Since the main topic is to find a medicine from natural source that can be useful in the treatment of some oral diseases, therefore some biological activities must be taken into consideration mainly the analgesic , anti-inflammatory and antimicrobial activities of the medicinally active principles.The roots extract of licorice possesses powerful anti-inflammatory activity as *G. radix* increased corticosterone levels in rats. Also, glycyrrhizin and glycyrrhetinic acid are known to inhibit phospholipase A2[15]. Not only active as analgesic, anti-inflammatory and antimicrobial, licorice is also very active as antiulcerogenic agent [16,17] which increases its potential for the treatment of oral ulcers like aphthus ulcer and lichen planus. In this study the investigation of the biological activity of licorice successive extracts as analgesic and anti-inflammatory agent using in vitro models namely writhing technique and hind rat paw oedema, together with separation of the major phytchemical constituents which may be responsible for these activities, in addition to the preparation of pharmaceutical dosage forms of the active extracts and performing clinical trials on patients suffering from oral diseases were carried out. The results revealed a remarkable and significant anti-inflammatory activity of the polar extract (P>0.05) by using the carrageenan induced hind rat paw oedema and this result represents 58% potency comparing with Indomethacin at the first hour of carrageenan injection.The bioactive polar extract of *G. glabra* was then subjected to further chemical investigation to isolate the phytochemical constituents which may be responsible for the anti-inflammatory activity.The phytochemical study resulted in the isolation of seven compounds by using a combination of classical and modern chromatographic techniques (PC, CC, TLC, preparative HPLC , LC/MS) , these compounds were further identified using spectral analysis (^{1}H-NMR, 13C-NMR, HMBC,HMQC and ESI-MS) three of these isolated compounds are belonging to the flavan type glycosides which are liquiritigenin, liquirin apioside and neoliquritin apioside and four of them belong to the chemical class of chalcones namely isoliquiritin apioside, isoliquiritin and licuroside, 2-(5-P-coumaryl apiosyl) isoliquiritin. The anti-inflammatory effect of both polar and non-polar extracts of the roots and rhizomes of *G. glabra* were evaluated adopting the carrageenan-induced rat hind paw oedema . Both flavans and chalcones are reported for their anti-inflammatory activity therefore the isolated compounds may be responsible for the anti-inflammatory activity of the biologically active extract [18,19].The efficacy and safety of the bioactive polar extract of *G. glabra* on experimental animals enhanced us to start the preliminary clinical trials on patients suffering from chronic gingivitis, after the approval of the protocol by Misr University of Science and Technology Ethical Committee, Egypt. Since little research has been conducted to determine the efficacy of herbal mouth wash and its ability to control plaque-induced gingivitis, the present work was focused on studying the effectiveness of the bioactive fraction of *G. glabra* mouth rinse in the reduction of plaque and gingivitis. The results of our study demonstrated that the *G. glabra* mouth rinse formulation was effective and useful in controlling plaque and gingivitis. The formulation demonstrated a statistically significant effect on the reduction of mean gingival index and plaque index scores when compared to base line, with significant reduction that is accompanied by a remarkable anti-inflammatory and analgesic effects. Therefore it can be concluded that the mouth wash containing biologically active fraction of licorice may be used as adjunct in treating gingivitis for long periods without side effects.

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