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Research Article
Licorice (*Glycyrrhizza glabra*) Extract Prevents Production of Th2 Cytokines and Free Radicals Induced by Ova Albumin in Mice

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Abstract

Background and Objective: Previous studies demonstrated that licorice (*Glycyrrhiza glabra*) inhibits airway hyperresponsiveness, inflammation and remodeling in murine models of asthma. However, licorice use is faced with many side effects including, increased adrenal hydrocortisone secretion and hypertension which are dose-dependent. This study aimed to test the effect of three different doses of licorice extract on, lung pathology, bronchoalveolar lavage oxidative stress markers, plasma immunoglobulin E (IgE) and Th2 cell cytokine in a model of ovalbumin (OVA)-induced bronchial asthma. **Methodology:** Mice were sensitized with 10 mg OVA on days 0 and 14. On days 21, 22 and 23, mice were challenged with 1% OVA solution. Six groups of mice were used in this study: 1-normal saline (S), 2-OVA-sensitized and challenged (OVA), 3- OVA+montelukast (M), 4-OVA+10 mg kg^{-1} licorice (L10), 5-OVA+20 mg kg^{-1} licorice (L20) and 6-OVA+40 mg kg^{-1} licorice extract (L40). **Results:** The results revealed that the lowest dose of licorice (10 mg kg^{-1}) protected against OVA-induced lung inflammation and mucus secretion. It also, reduced interleukin (IL)-5, IL-13 and IgE. Moreover, it significantly reduced the raised malondialdehyde (MDA) and nitric oxide (NO) and restored superoxide dismutase (SOD) and catalase (CAT) activity. **Conclusion:** The results of this study offered clear evidence for the antioxidant and anti-inflammatory effects of licorice in a murine model of bronchial asthma. The lowest dose of licorice is the most effective and as the dose increase the antioxidant and anti-inflammatory action decrease. This provides an advantage as licorice has many side effects which appears with the high doses.

Key words: Licorice, Th2 cytokines, oxidative stress markers, ovalbumin, plasma immunoglobulin E (IgE)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Today, many people choose herbal remedies over the chemical drug therapy because they think herbs and the health beneficial nutrients are much safer choices[1].

Licorice, the root of *Glycyrrhiza glabra*, is a well-known plant of family Fabaceae that has been widely used in the folk medicine for centuries[2]. Previous experimental and clinical reports approved licorice multiple phytotherapeutic activities. It treats an assortment of ailments ranging from the common cold to liver illnesses. It especially used in the treatment of respiratory diseases, such as cough[3]. A previous study spotlights the potential capacity of the licorice for the treatment of bronchial asthma[4]. Licorice has anti-inflammatory and antioxidant properties that attributed to its major active constituent, glycyrrhizin[5,6].

Bronchial asthma (BA) is one of the widely prevalent chronic lung ailments among all ages and so far, it is an illness that presently unable neither to hinder nor to treat[3]. Reactive oxygen species (ROS) and inflammation have been shown to be directly associated with BA[7]. Allergen-specific $CD4^+$ T cells are confirmed to possess a pivotal role in the pathogenesis of BA[8]. Type 1 allergies are mediated by a distinctive immune reaction to allergens, mostly caused by T helper type 2 (Th2) cells. Th2 cells produce high concentrations of interleukin (IL)-4, IL-5 and IL-13, which resulted in increased formation of allergen-specific immunoglobulin (Ig) E and the liberation of mast cells mediators[9]. Potent anti-inflammatory corticosteroids are the most effective medications available for BA treatment[10]. However, these drugs are not completely satisfactory and there are bothers concerning familiar side effects of corticosteroids, like adrenal function disturbance and global immune suppression. The chronic nature of this ailment and the lack of definitive prophylactic and therapeutic medications lead up to 60% of patients to look alternative therapy[11].

Bronchial asthma is a common disease from childhood to adulthood and studies have reported more than 300 million infected people worldwide. Reactive oxygen species and inflammation play an important role in the pathogenesis of airway inflammation during asthma. Therefore, antioxidant and anti-inflammatory agents that could block the inflammatory signals and/or the transcription of inflammatory markers could be excellent for the treatment of airway inflammation. Licorice contain many bioactive compounds which exert an inhibitory effect on cyclooxygenase and lipoxygenase activities.

The objective of this study was to evaluate the effect of three different doses of licorice extract on, lung pathology, bronchoalveolar lavage (BAL) oxidative stress markers, IgE and Th2 cell cytokine production compared to montelukast in a model of ovalbumin (OVA)-induced bronchial asthma.

MATERIALS AND METHODS

Chemicals: Ovalbumin (OVA), albumin from chicken egg white grade V (Thermo Scientific, Rockford, IL, USA), montelukast (singulair tablets, 10 mg) (Merck Sharp and Dohme Limited, UK) and licorice (*Glycyrrhiza glabra*) powder (purity, 98.7%) (GNC, Jeddah, KSA).

Experimental animals: Female BALB/C mice (n = 36) (25-30 g) were obtained from the experimental animal unit of King Fahd Medical Research Center, KAU. The mice were housed at a temperature of 22±3°C, relative humidity of 50-55% and 12 h light/dark cycle for one week prior to the experiment. The mice were allowed free access to water and standard pellets chow *ad libitum*. The use of mice was carried in rigorous compliance with the basics and regulations settled by the Research Ethics Committee at KAU, Saudi Arabia. This protocol was also approved by King Abdul Aziz City for Science and Technology under the research number P-S-36-005.

Ovalbumin mouse model: Induction of asthma was done according to the method of Oh *et al.*[12]. Briefly, mice were sensitized intraperitoneally (i.p.) with 10 mg OVA and 20 mg aluminum hydroxide gel in 200 µL PBS (pH 7.4) on days 0 and 14. On days 21, 22 and 23 after initial sensitization, mice were challenged with 1% (w/v) OVA solution in PBS for 1 h using an ultrasonic nebulizer (IH-50; Beurer Co., Ulm, Germany).

Experimental protocol: Mice were randomly classified into six groups (n = 6): (1) mice sensitized and challenged with normal saline (S), (2) mice sensitized and challenged with OVA (OVA), (3) OVA-sensitized mice orally administered 30 mg kg^{-1} montelukast (M)[13], (4) OVA-sensitized mice orally administered 10 mg kg^{-1} licorice (L10), (5) OVA-sensitized mice orally administered 20 mg kg^{-1} licorice (L20), OVA-sensitized mice orally administered 40 mg kg^{-1} licorice (L40) (Fig. 1).

Collection of bronchoalveolar lavage fluid (BALF): On day 26th, mice were sacrificed and tracheostomy was performed. BALF was obtained via tracheal cannulation via three successive instillation and aspirations of ice-cold PBS[14]. BALF aliquots were centrifuged at 1500 rpm for 10 min at 4°C. The supernatants were stored at -80°C for biochemical analysis.

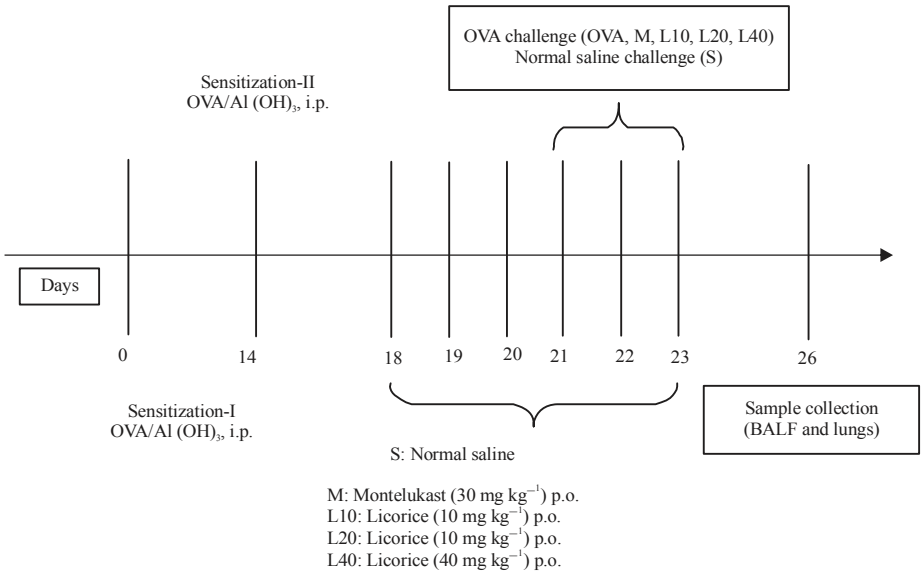

Fig. 1: Experimental protocol

Histopathological study: Lungs were collected and fixed in 10% (v/v) neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 4 µm thickness and stained either with hematoxylin and eosin (H and E) for assessment of inflammation or with periodic acid-Schiff (PAS) for assessment of mucus-secreting goblet cells and mucus production. The slides were examined microscopically by a blind pathologist.

Biochemical analysis: Plasma IL-5, IL-13 and IgE concentrations were measured using mice specific ELISA assay kits (Cusabio Biotech Co., LTD. China)[15]. BALF concentrations of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) and catalase (CAT) were measured using commercially available kits (Biodiagnostic, Egypt) based on the manufacturer's instructions.

Statistical analysis: Statistical analyses were carried out using Minitab Inc. software (13.1). Data were presented as the Mean±SE. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. Statistical significance occurred at $p \leq 0.05$.

RESULTS

Histopathological results of H and E stained sections: Microscopically, the lung of mice from S group showed normal bronchiole and alveoli (Fig. 2a). Lung of mice from OVA group showed inflammatory cells infiltration around the pulmonary blood vessel (Fig. 2b and c) and focal interstitial pneumonia (Fig. 2d). Lung of mice from M group showed normal bronchiole and normal alveoli (Fig. 2e). Lung of mice from L10 group showed normal bronchiole and normal alveoli (Fig. 2f). Lung of mice from L20 group showed focal emphysema (Fig. 2g). Lung of mice from L40 group showed inflammatory cells infiltration around the bronchiole and focal atelectasis (Fig. 2h).

Histopathological results of PAS stained sections: Representative PAS stained lung sections from S, OVA, M, L10, L20 and L40 treated groups. In S group, lung showed no histochemical reaction (Fig. 3a). In OVA group, lung showed hyperplasia and hyperactivity of mucus-secreting goblet cells with accumulation of excessive mucus in the bronchial lumen (Fig. 3b and c). In M group, lung showed no histochemical reaction (Fig. 3d). In L10 group, lung showed no histochemical reaction (Fig. 3e). In L20 group, lung showed slight activation of mucus-secreting goblet cells (Fig. 3f). In L40 group, lung showed hyperplasia and hyperactivity of mucus-secreting goblet cells (Fig. 3g and h).

BALF malondialdehyde (MDA): OVA group showed a significant increase in MDA concentration compared to the S group. Treatment with M, L10 and L20 significantly decreased MDA compared to the OVA group. There is no significant difference in MDA between L10 and L20 group. Treatment with L10 and L20 significantly decreased MDA compared to the M group. Treatment with L40 showed a non-significant change in MDA compared to the OVA group. Furthermore, in L40 group the MDA level is still significantly high compared to the S, M, L10 and L20 groups (Table 1).

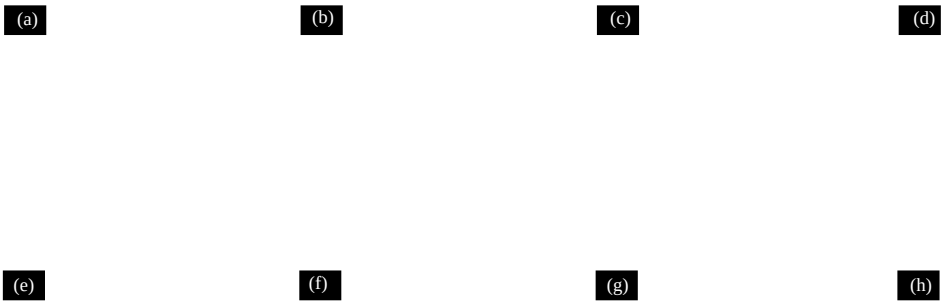

Fig. 2(a-h): (a) Representative H and E stained lung sections from S, OVA, M, L10, L20 and L40 treated groups. In the S group, (A) Lung showed normal alveoli, (B) Normal bronchiole, (b-c) In the OVA group, lung showed inflammatory cells infiltration around pulmonary blood vessel (IC), (d) Focal interstitial pneumonia (IP), (e-f) In the M and L10 groups, lung showed apparently normal alveoli (A) and normal bronchiole (B), (g) In the L20 group, lung showed focal emphysema and (h) In the L40 group, lung showed focal atelectasis (AT). (H and E×400)

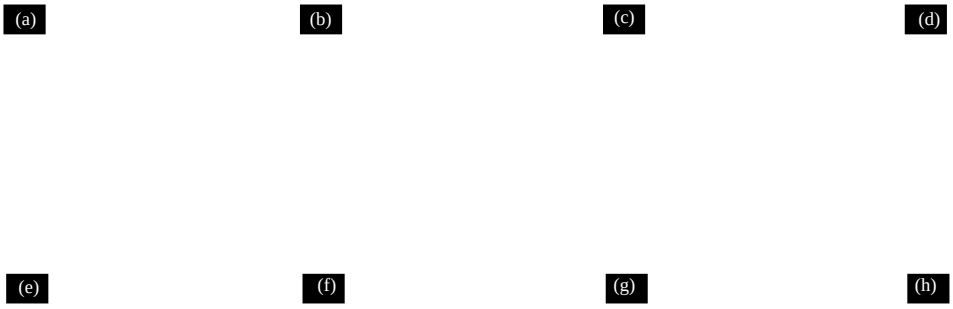

Fig. 3(a-h): (a) Representative PAS stained lung sections from S, OVA, M, L10, L20 and L40 treated groups. In the S group, lung showed no histochemical reaction, (b-c) In OVA group lung showed hyperplasia and hyperactivity of mucus-secreting goblet cells with accumulation of excessive mucus in the bronchial lumen, (d-e) In the M and L10 groups, lung showed no histochemical reaction, (f) In the L20 group, lung showed slight activation of mucus-secreting goblet cells and (g-h) In the L40 group, lung showed hyperplasia and hyperactivity of mucus-secreting goblet cells (PAS×400)

BALF nitric oxide (NO): OVA group showed a significant increase in NO concentration compared to the S group. Treatment with M, L10, L20 and L40 significantly decreased NO compared to the OVA group. There was no significant difference in NO between M and L10 and L10 and L20. Furthermore, in L40 group the NO level is still significantly high compared to the S, M, L10 and L20 groups (Table 1).

BALF superoxide dismutase (SOD): OVA group showed a significant decrease in SOD concentration compared to the S

Table 1: Effect of licorice extract on BALF malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) and catalase (CAT) concentration measured in OVA-sensitized/challenged mice

Groups	MDA (nmol mL^{-1})	NO (μM)	SOD (U mL^{-1})	CAT (U mL^{-1})
S	5.4±0.24	3.2±0.57	1.82±0.12	0.39±0.02
OVA	6.2±0.15^{a*}	11.1±0.60^{a***}	0.75±0.02^{a***}	0.08±0.03^{a***}
M	5.6±0.17^{b*}	3.3±0.67^{b***}	1.84±0.18^{b***}	0.40±0.03^{b***}
L10	4.8±0.1^{b***}	4.9±1.11^{b***}	1.99±0.09^{b***}	0.42±0.04^{b***}
L20	4.9±0.09^{b***}	5.2±0.99^{b***}	1.55±0.13^{b***}	0.51±0.03^{b***}
L40	6.4±0.34	7.9±0.20^{b***}	0.82±0.05	0.18±0.03

Data are Mean±SE n = 6. ^{a}Significant from S, ^{b}Significant from OVA. *p<0.05 and ***p<0.001

Table 2: Effect of licorice extract on plasma immunoglobulin E (IgE), interleukin 5 (IL-5) and IL-13 concentration measured in OVA-sensitized/challenged mice

Groups	IgE (ng mL^{-1})	IL-5 (pg mL^{-1})	IL-13 (pg mL^{-1})
S	1.15±0.17	124.0±3.52	368.0±16.33
OVA	7.93±0.20^{a***}	378.0±55.1^{a**}	1488.0±68.16^{a***}
M	0.82±0.24^{b***}	89.0±21.63^{b**}	824.0±131.43^{b**}
L10	1.56±0.36^{b***}	195.0±1.65^{b**}	94.0±60^{b***}
L20	5.95±0.21^{b***}	133.0±6.53^{b**}	108.0±86.53^{b**}
L40	7.00±1.14	359.0±29.8	1230.0±154.29

M: Montelukast, L10: Licorice 10 mg kg^{-1}, L20: Licorice 20 mg kg^{-1}, L40: Licorice 40 mg kg^{-1}. Data are Mean±SE (n = 6). ^{a}Significant from S, ^{b}Significant from OVA, **p<0.01 and ***p<0.001

value. Treatment with M, L10 and L20 significantly increased the SOD compared to the OVA group. There is no significant difference in SOD between M and L10. There is a significant difference in SOD between L10 and L20. Treatment with L40 showed a non-significant change in SOD compared to the OVA group. Furthermore, in L40 group the SOD level is still significantly low compared to the S, M, L10 and L20 groups (Table 1).

BALF catalase (CAT): OVA group showed a significant decrease in CAT concentration compared to the S group. Treatment with M, L10 and L20 significantly increased the CAT compared to the OVA group. There is no significant difference in CAT between M and L10 values. There is a significant difference in CAT between M and L20 values and between L10 and L20 values. Treatment with L40 showed a non-significant change in CAT compared to the OVA group. Furthermore, in L40 group the CAT level is still significantly low compared to the S, M, L10 and L20 (Table 1).

Plasma Th2 cytokines: OVA group showed a significant increase in plasma IgE, IL-5 and IL-13 concentration compared to the S value. Treatment with M, L10 and L20 significantly decreased the plasma IgE, IL-5 and IL-13 compared to the OVA group. Treatment with L40 showed a non-significant change in IgE, IL-5 and IL-13 compared to the OVA group. There is no significant difference in plasma IgE between M and L10. There is a significant difference in plasma IL-5 and IL-13 between M and L10. There is a significant difference in plasma IgE between L20 and S, L20 and M and L20 and L10. There was a significant difference in plasma IgE between L40 and S, L40 and M and L40 and L10 (Table 2).

DISCUSSION

The results of this study revealed that, compared to montelukast the most effective dose of licorice extract was the 10 mg kg^{-1} which clearly inhibited OVA-induced recruitment of inflammatory cells in the lung, goblet cell hyperplasia in the airway, plasma IgE and Th2 cytokines. Allergic asthma is a chronic disease of the airways characterized by airflow obstruction and bronchial hyperresponsiveness. Inflammation plays a pivotal role in the pathophysiology of asthma. Eosinophils, mast cells, macrophages and neutrophils, are implicated in the pathogenesis of asthma by releasing ROS that contract the bronchi and trigger histamine release[16].

Th2 cell, a sub-group of lymphocytes, plays an important role in the initiation and progression of asthma by releasing of IL-5, IL13 cytokines and IgE[17]. IL-5 is pivotal for growth, differentiation, recruitment and survival of eosinophils that plays a key role in the pathogenesis of asthma[18,19]. IL-13 plays an important role in isotype switching of β cells to immunoglobulin IgE production[20]. The level of systemic IgE is positively correlated with the severity of clinical asthma[21]. Inactivating IgE by antibodies shows a potent therapeutic effect against asthma in both animal and clinical studies[22]. Glycyrrhizic, the main active constituent of licorice composed of two molecules of glucuronic acid and one molecule of 18 β-glycyrrhetinic acid[23]. Glycyrrhizic acid significantly decreases IL-4, IL-5 and IL-13 cytokine levels and significantly increases IFN-γ cytokine levels that is responsible to suppress Th2 immune responses[2]. The 18 β-glycyrrhetinic acid (18β-GA) exhibits potential anticancer, anti-inflammatory and antimicrobial activities[24]. It has been demonstrated that glycyrrhizic acid and its hydrolysis product 18 β-glycyrrhetinic acid prevent the passive skin contact inflammation in mice[25]. Glycyrrhetinic acid has a triterpenoid structure that is almost identical to the adrenal cortex hormones[26]. Many researches have reported that glycyrrhetinic acid inhibits 11-beta-

hydroxy steroid dehydrogenase enzyme, which converts cortisol into inactive metabolites. Thus, inhibition of the enzyme by glycyrrhetinic acid significantly elevates cortisol concentration and stimulates glucocorticoid receptors. This augments the action of the adrenal hydrocortisone. Thus, hydrocortisone is accompanied with and explain glycyrrhizin and glycyrrhetinic acid anti-inflammatory and antiallergic actions[27].

A prominent role of oxidative stress and decreased antioxidant defense in asthma pathophysiology is evident from several studies[27]. Activated macrophages, neutrophils and lymphocytes, release ROS[28]. Increased oxidative stress exacerbates inflammation by inducing proinflammatory mediators and contributes to the development of asthma[29]. Many investigators have reported that increased levels of ROS in the asthmatic inflammatory process aggravate airway inflammation and damages molecules, such as proteins, DNA and lipids[29]. Therefore, it was suggested that a combination of antioxidants may have positive impact on the treatment of asthma[29]. MDA, the end product of lipid peroxidation has been estimated as an indicator of damage to membrane lipids and its measurement provides an estimate of free radical activity[30]. NO plays a key role in allergic asthma-induced airway inflammation. It was reported that iNOS worsens allergic asthma-induced airway inflammation. Inhibition of iNOS reduces asthma associated airway constriction, inflammation and remodeling processes, by reducing both collagen and elastic fibers[31]. Asthma is closely related with increased ROS production in the airway[32]. SOD is a key enzyme of the antioxidant defense system of body, which plays an important role in scavenging superoxide radicals during oxidative stress. CAT catalyzes the decomposition of H_2O_2 produced by the action of superoxide dismutase, to water, thus protecting the living system from deleterious effects of H_2O_2. SOD and CAT are the other two crucial antioxidant enzymes, which work in co-operation[33]. The results of the present study showed that, licorice extract at both 10 and 20mg kg^{-1} doses significantly inhibited OVA-induced oxidative stress measures, MDA and NO. At the same dose levels, licorice extract significantly increased the activity of SOD and CAT. We concluded that the protective effect of licorice extract might be attributed to its reduction of oxidative stress. Flavonoids exert many physiological effects including antioxidant action, ROS scavenging activity, metal chelation, inhibition of ROS formation via enzyme inhibition and modulation of signal transduction pathways which regulates antioxidase expression[33,34]. Chopra *et al.*[34] demonstrated that glabridin isoflavan extracted from *Glycyrrhizza glabra*, inhibited copper ion and 2,2-azobis dihydrochloride-induced low density lipoprotein oxidation. Siracusa *et al.*[35] also, reported antioxidant power of *Glycyrrhizza glabra* isolated flavonoid. Currently, licorice flavonoid has been used in health and skin care preparations due to its antioxidant capacity. Li *et al.*[23] demonstrated that glycyrrhizin increases the antioxidant status, decreases lipid peroxidation and enhances the immunity in a mouse model of allergic rhinitis.

The results from this study showed that licorice extract at 40 mg kg^{-1} dose exacerbated lung pathology and function. At the same higher dose, licorice extract exerted neither antioxidant activity nor anti-inflammatory activity. Findings from previous research have indicated the possible prooxidative or autoxidation effect of carotenoids and guava leaf extracts, respectively, at a high concentration *in vitro*[36]. In addition, glycyrrhetinic acid, an ingredient of licorice root extract induced HL60 cell line apoptosis followed by increase in the intracellular reactive oxygen species[37].

CONCLUSION

The lowest dose of licorice is the most effective and as the dose increase the antioxidant and anti-inflammatory action decrease. This provides an advantage as licorice has many side effects which appears with the high doses.

SIGNIFICANCE STATEMENT

This study provided an evidence of the antioxidant and anti-inflammatory action of licorice in experimentally-induced asthma. Consequently, licorice may be recommended as an adjuvant therapy for asthmatic patients. Furthermore, the results showed that low licorice dose exerted antioxidant action while the high dose exerted a prooxidant action. This finding opens the field for researchers to try high doses of licorice in the treatment of cancers and the induction of apoptosis.

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