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Chemical Composition, Antioxidant, Antimicrobial and Anticancer Activities of Licorice (*Glycyrrhiza glabra* L.) Root and Its Application in Functional Yoghurt

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Abstract Recently, consumers are paying much more attention to natural products from plants, mainly because of the general feeling that natural components are safe. The present research was undertaken in order to evaluate the potential application of licorice roots extract as a nutritional plant used in food industry. Chemical compositions, antimicrobial potency, and the potential antioxidant capacities of licorice root extract were determined. Furthermore, phytochemical, identification of bioactive compounds and antitumor potentials on breast cancer cell MDA-MB-361 was also carried out. The results revealed that the most licorice root contents were sugars and fiber (40.47 and 38.41 g/100g, respectively), and calcium was the main element (112.2 mg/100g). The licorice aqueous and ethanol extracts showed broad spectrum against tested microorganisms Gram positive and negative bacteria, yeast and fungi. Licorice aqueous extract showed strong antioxidant potential with IC_{50} (29.92±2.43 mg/g) and exhibited considerable proapoptotic property on breast cancer cell, due to its high concentration of total phenolic compounds in the extract. Gallic acid was observed to be the most abundant phenolic component in licorice root aqueous extract. The sensory evaluation revealed that the fortified licorice yoghurt was preferred. Obtained results indicate that the tested licorice extract could have pharmaceutical potential in prevention of breast cancer and can be recommended as natural antioxidant and antimicrobial agent to be used in food industrial.

Keywords: *licorice, phytochemical, antioxidant, antimicrobial, breast cancer, phenolic compound*

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1. Introduction

In the last years, there has been a great interest in the research for natural medicines from plants for the discovery of new antioxidant and antimicrobial agents. Moreover, there is a growing request by industry for plants used in conventional medicine that could be combined in foods or pharmaceuticals. The root of licorice (*Glycyrrhiza glabra* L.) belongs to the Fabaceae family, is widely used as sweetening and flavoring agent in food and tobacco industries but has also been proposed for various clinical applications [1]. Traditions from ancient Egyptian, Assyrian, Chinese and Indian cultures have documented its extensive medicinal use as demulcent, expectorant, ulcer healing, treating kidney and urinary system diseases [2]. It has gained a highly common interest, especially due to its main medicinal bioactive component, glycyrrhizin, a triterpenoid saponin glycoside. Its bioactive compounds showed to have anti-inflammatory, gastroprotective, antitumoral, antiviral [3], antihepatotoxic, antifungal, antimicrobial [4], anticancer effects, relieving ulcers, rheumatoid arthritis and hot flashes of menopause, and decreasing low density lipoprotein [5]. Licorice contain natural antioxidants such as, phenols, saponins, flavanones, isoflavonoids, isoglycyrrhizin, 18β-glycyrrhetinic acid, liquiritigenin, licochalcone A, licochalcone E and glabridin [4], that might help to prevent oxidative damage [6,7] and could also offer protection against cancer, pathogens and oxidative stress-induced physiological malfunctions [8]. Antioxidants have long been concerned

as crucial for helping to overcome oxidative stresses and disorders caused by free radicals [9]. The antioxidants of licorice not only inhibit oxidative stress, but also increase the nutritional value of the end product, especially with their mineral, vitamin, polyphenol and anthocyanin contents. Furthermore, antibacterial activities against anti-helicobacter pylori of flavonoids such as glabridin and glabrene from the licorice extract have already been reported [10].

Given Egypt's rapidly increasing human population and growing demand for natural health products, accompanied with consumption of medicinal plants. Licorice root, as a strategic and highly valuable plant in Egypt, is known for its nutritive value, natural sweetness, favorable flavor and soothing properties attributed to glycyrrhizic acid (5-9%) and anethole (up to 3%) contents [11]. Egyptian licorice root, due to its sweet taste, is used in the manufacture of confectionery, as an additive to different beverages (brandy, liqueur, etc.) and widely used as a cold beverage [12].

In order to deepen the knowledge on licorice root plant, the main objective of this study was to evaluate the potential application of Egyptian licorice root extract as a new source of valuable bioactive compounds in functional foods with health benefits. To achieve that aim, investigations of antimicrobial and antioxidant activities, chemical and mineral composition, and breast anticancer property were conducted. In addition to application in functional fortified yoghurt with different concentrations and evaluate its impact on organoleptic properties which are the main pillar of consumers' acceptability, were examined.

2. Material and Methods

2.1. Plant Material and Extracts Preparation

Egyptian licorice roots were purchased from local market, Alexandria, Egypt. The roots were dried at 60°C/3h, grinded, then subjected for extraction as follows. Extraction in boiled Milli-Q water with ratio (1:10 w/v) follows, stirring for 3 h at temperature (60°C) and centrifugation (using Pro-Centrifuge, Centurion Science Limited, UK) at 4000 rpm for 30 min, and then the filtered aqueous extract is stored at 4°C until use. Dried grinded licorice roots were also extracted in 80% ethanol (100g/ L) by maceration for 72 hours. The extract was filtered using Whatman No1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator [13].

2.2. Proximate Analysis of Licorice Root Extract

The protein, fat and ash contents of licorice root aqueous extract were determined according to Fahey et al [14]. Moisture, fiber and total sugars contents were measured according to AOAC [15]. Different minerals were determined according to AOAC [16] using atomic absorption spectrophotometer. The energy value was obtained by multiplying the mean value of the protein, fat, and carbohydrate by Atwater factors of 4, 9, and 4, respectively.

2.3. Licorice Extracts as Antimicrobial Agents

The antimicrobial potency of both licorice aqueous and ethanol extracts were tested to identify the more efficient extract to control the growth of pathogens via agar-based disc diffusion assay. Sterile filter paper discs were kept as stored at 6°C in sealed containers desiccant and protected from light until amended with 25 µL of the licorice aqueous and ethanol extracts just before applied to inoculated plates. After overnight culture activation of three Gram-positive bacteria strains; *Bacillus cereus* EMCC1006, Staphylococcus aureus EMCC1351 and *Staphylococcus epidermidis* EMCC 1353, five Gram-negative bacteria strains; Salmonella *spp.*, Escherichia coli ATCC25922, *Proteus mirabilis* EMCC 1312, Klebsiella pneumonia ATCC12296 *and Pseudomonas flourescens* EMCC1221 *and a yeast strain Candida albicans* EMCC105, they were diluted to 0.5 McFarland standard and were then swapped over LB plates, extract-impregnated disks were applied, then the plates were incubated at 37°C for 24 h. The antifungal potentials of both aqueous and ethanol extracts were evaluated against two fungi strains; *Aspergillus niger* EMCC 72 and *Aspergillus flavus* EMCC 274. The organisms were sub-cultured on potato dextrose agar (PDA) at 30°C for 4 to 7 days. Following growth, conidia were harvested in sterile saline; the conidial suspension was adjusted to 0.5 McFarland standards. Potato dextrose agar (PDA) plates were streaked evenly with a swab dipped into the standardized inoculum suspension. Lids were left ajar for 3 min in a laminar flow cabinet to allow for any excess surface moisture to be absorbed into the agar before the extract-impregnated disks were applied to the surfaces of inoculated plates. Plates were inverted and incubated at 30°C for 4 to 7 days to allow for fungal growth. *A set of 5 concentrations of reconstituted licorice water and ethanol extracts (100, 75, 50, 25 and 12.5 mg/ mL), were examined to determine the minimum inhibitory concentration (MIC) of each against a specific pathogenic strain.* The plates were eventually checked for the Inhibition zone diameters (IZD) which were measured in millimeters. All strains were obtained from Microbiological Resources Centres (MERCIN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The strains were maintained by; the Department of Food Technology, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, Egypt in 60% glycerol/ LB culture at -80°C [17].

2.4. Antioxidant Potentials and Total Phenolic Content of Licorice Root Extract

The total phenolic content in licorice aqueous extract was determined according to Hamad et al [17] using gallic acid as a standard. The ability of licorice extract to scavenge DPPH˙ free radical was evaluated as IC_{50} according to Mohamed et al [18]. The process involved mixing DPPH· solution with licorice aqueous extract. After 30 min, the remaining DPPH free radicals were measured at 517 nm using a spectrophotometer.

2.5. Phytochemical Qualitative Analysis in Licorice Root Extract

The presence of phytochemical compounds: tannins, phenols, flavonoids, alkaloids, reducing sugars, glycosides, amino acids and proteins, saponins and terpenoids were determined in aqueous licorice extract according to Hamad et al [19].

2.6. HPLC analysis of Licorice Root Extract

HPLC (Agilent 1000) analysis of ten phenolic and flavonoid compounds; Gallic acid, itaconic acid, protocathechuic acid, catechin, esculetin, catechol, tannic acid, ferulic acid, pyrogallol and cinnimic acid, were performed on a reverse phase Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5µm), using a gradient program with two solvent systems; A: 0.5 % acetic acid in 50:50 acetonitrile: water (1:1); B: 2 % acetic acid in water at a constant solvent flow rate of 1.2 ml/min with injection volume of 20µL. The signals were detected at 280 nm by UV–VIS detection [17]. The phenolic compounds' separation from licorice aqueous extract was accomplished with 1.0 mL/min and fractions were collected using 100-ml injections of 100 mg/ml phenolic resin solutions with the same columns as in the analytical separation according to Li et al [20].

2.7. Cell Toxicity Assay by Neutral Red

Neutral red cytotoxicity assay was used to determine the IC_{50} of licorice root extract on MDA-MB-231 cell line. Neutral red solution (19% acetic acid and 50% ethanol) was freshly prepared from a 0.4% aqueous stock solution that had been shielded from light. A 1:80 dilution of the stock solution was prepared in DMEM (Dulbecco's Modified Eagle's medium), allowed to precipitate for 24 h at room temperature, and then centrifuged for 10 min at 1500 xg. The clear red solution was used for the assay. The cells were washed with PBS (150 µL/well) and the plates were tapped then washing solution was aspirated, neutral red medium (100 µL) were then added to each well and the plate was incubated for 2 h at 37°C. The neutral red medium was aspirated after 2 h then cells were washed with 150 µL PBS. The plate was tapped gently then washing solution was aspirated. Neutral red destain solution (19% acetic acid and 50% ethanol) were added as 150 µL/well and the plate was shaked rapidly on a microtiter plate shaker (Shaker PSU 2T plus, BOECO, Germany) for at least 10 minutes.

The optical density (OD) of the neutral red extract was measured at 540 nm in a microtiter plate reader spectrophotometer (Spectrostar^{Nano}, BMG Labtech), using blanks which contain no cells as a reference. Grubb's test for outliers, also called the extreme studentized deviate (ESD) method, to determine whether one of the values in the list was a significant outlier from the rest [21]. The percentage of cytotoxicity (inhibition) was calculated according to the following formula:

$$\% \ inhibition = \frac{O.D \ Control - O.D \ Treatment}{O.D \ Control}$$

2.8. Organoleptic Properties of Licorice Fortified Yoghurt

2.8.1. Licorice Fortified Yoghurt Preparation

Mixed cow/buffalo milk (1:1) (8.5% SNF) was standardized using (skimmed milk powder (SMP) to reach (13% SNF, 3% fat). The milk then was homogenized at 200 bar and pasteurized at 85°C for 15 min. Hot milk was divided into three equal portions, C; Control plain yoghurt, T_1; yoghurt fortified with licorice aqueous extract 1: 100 (w/v) of milk, T_2; yoghurt fortified with licorice aqueous extract 2: 100 (w/v) of milk. The mix was cooled to 42±1°C then inoculated with 0.03 g/kg of yoghurt culture YC-X11, poured into 100 mL plastic cups and incubated at 42±1°C until set coagulation at pH ~4.6 (About 5hrs), then cooled and stored at 4°C [22].

2.8.2. Sensory Evaluation

Ten panelists, (6 men and 4 women, aged between 27 to 51 years), conducted sensory evaluation on fresh licorice fortified yoghurt samples (the second day of the yogurt manufacturer) at Food Technology Department, Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt. The criteria for selection depended on their experience and background related to yoghurt products. The samples were allowed to rest at room temperature (25°C), 10 min before evaluation. Panelists were instructed to evaluate the yoghurt with respect to their degree of acceptance as follows; flavor (10 points), body & texture (10 points) appearance and color (10 points) and an overall acceptance grade out of (10), the scale from 1 to 10, where 10 is the best as described by Darwish et al [23] with some modifications. The average of sensory evaluation data with standard deviations was determined.

2.9. Statistical Analysis

All data were expressed as mean values ± SD. Statistical analysis were performed via Statistical analysis system (SAS) software program (SAS Institute 2004) using two-way analysis of variance (ANOVA) test followed by t Tests (LSD). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Proximate and Mineral Composition of Licorice Aqueous Root Extract

Results of analysis of the chemical composition of licorice aqueous extract (Table 1) revealed that sugars (40.47 g/100g) and fiber (38.41 g/100g) are the most abundant constituents that show potential health benefits. While ash and protein contents were represented with moderate amount 7.61 and 7.19 g/100g, respectively. Low moisture content (4.11 g/100g) was found in licorice root extract so remains an asset in storage attributes and preservation of the nutrients. Licorice root extract had a

really low amount of fat (2.21 g/100g) so it might be helpful for weight loss. It has revealed that licorice aqueous extract has reducing sugars as a major component (40.47 g/100g) that corresponds to its high energy (210.53 kcal/100g). These data are in agreement with the results obtained by Rosa et al [9] and Giulia et al [24].

Table 1. Chemical composition and minerals contents of licorice aqueous extract

Chemical composition (g/100g)	
Protein	7.19±1.55
Crude fat	2.21±0.61
Sugars*	40.47
Crude fiber	38.41±0.54
Moisture	4.11±0.19
Ash	7.61±0.14
Food energy value (Kcal/100g)	210.53
Minerals contents (g/100g)	
Calcium, Ca	112.2
Iron, Fe	1.70
Zinc, Zn	0.5
Copper, Cu	0.14

Data represented are the mean ± standard deviation, n=3. * Sugars estimated in this fashion includes fiber.

Data of mineral/ash content analysis (Table 1) revealed that calcium is the main element in the licorice root extract (112.2 g/100g). Relatively low amount of iron (1.70 g/100g) was found in licorice extract which is required for the production of red blood cell and enzymes. While the rest of assessed elements; zinc and copper were presented in small quantities 0.5 and 0.14 mg/100g, respectively. Obtained results agreed with Soni et al [25], while, Al-Bachir et al [26] reported higher content of calcium and Ansari et al [27] reported higher content of iron in licorice water extract. The presence of important components like proteins, sugars and fiber, mineral salts (such as calcium, phosphorus, iron, potassium, manganese, zinc, and copper) have been reported in licorice [4].

3.2. Antimicrobial Activity of Licorice Aqueous Extract

The antimicrobial activity of licorice aqueous and ethanol extracts were assessed against some pathogenic microorganisms such as Gram positive bacteria, Gram negative bacteria, yeast and fungi to identify the more efficient extract to inhibit their growth. Data presented showed that, both aqueous and ethanol extracts exhibited significant antimicrobial activity against all the examined strains (Table 2 and Table 3). All the examined bacterial and fungal pathogenic strains showed sensitive and no growth against the aqueous extract except for *Salmonella* spp. and *Staphylococcus epidermidis* EMCC1353 with MIC values (12.5 and 25 mg/mL), respectively. The licorice aqueous extract was found to possess higher antibacterial activity against Salmonella *spp*. The results from the disc diffusion assay followed by measurement of minimum inhibitory concentration (MIC) (Table 3), exhibited that ethanol extract showed good activity against Salmonella *spp*, *Staphylococcus epidermidis* EMCC1353 and *Escherichia coli* ATCC25922, showing the highest inhibition zones (28, 27 and 19 mm, respectively) with lowest MIC value of 12.5 mg/mL.

Table 2. Inhibition zone diameters and MICs of licorice aqueous extract against pathogenic strains

Pathogenic strain	Inhibition zone diameter (mm)*					
	100	75	50	25	12.5	MIC
Gram-positive bacteria						
Bacillus *cereus*	nd	nd	nd	nd	nd	nd
Staphylococcus aureus	nd	nd	nd	nd	nd	nd
Staphylococcus epidermidis	22	18	15	12	nd	25
Gram-negative bacteria						
Salmonella *spp*.	25	20	16	14	7	12.5
Escherichia coli	nd	nd	nd	nd	nd	nd
Proteus mirabilis	nd	nd	nd	nd	nd	nd
Klebseilla pneumonia	nd	nd	nd	nd	nd	nd
Pseudomonas flourescens	nd	nd	nd	nd	nd	nd
Yeast						
Candida albicans	nd	nd	nd	nd	nd	nd
Fungi						
Aspergillus niger	nd	nd	nd	nd	nd	nd
Aspergillus flavus	nd	nd	nd	nd	nd	nd

Data represented are average of triplicates. MIC; Minimum Inhibition Concentration. * Diameter included 5 mm well diameter; Concentrations of extract and MIC are in mg/mL. nd; Not detected.

On contrary, *Klebseilla* pneumonia *ATCC12296*, *Pseudomonas flourescens* EMCC1221, *Candida albicans* EMCC105 and *Aspergillus flavus* EMCC 274 showed sensitive against the licorice ethanol extract. These results are in lines with those of Jalal and Zahra [28] who reported that the extracts of licorice (*Glycyrrhiza glabra*) exhibited various antimicrobial activities (7-11 mm inhibition zone) against both Gram-positive and Gram-negative bacteria by the agar diffusion method.

The results of antibacterial activity assay (Table 3) also showed that Bacillus *cereus* EMCC1006 was the most resistant species at concentration of 100 mg/mL with 16 mm inhibition zone. This finding is in agreement with Karami et al [29] who showed that B. *cereus* (with MICs 1.0 ± 0.0 mg/mL) was the most resistant species against G. *glabra* ethanol extract. Asad et al [30] studied the antibacterial activity of the G. *glabra* and reported that 80% methanolic extract had high activity against B. *subtilis* and E. *coli* strains with inhibition zones (30 and 28.5 mm) with lowest MIC values (12.2 and 20.1 mg/mL), respectively, while S. *aureus* strain was the least activity with inhibition zone (19 mm) and highest MIC value (110 mg/mL).

The majority of antimicrobial effects from licorice is due to isoflavonoid components particularly hispaglabridin and ß,4'-O-methylglabridin, glabridin, glabriol and 3-hydroxyglabrol [30]. Rosa et al [9] examined the relationship between *flavonoid* structure and *antibacterial activity* in licorice-root and found that its antibacterial activity is caused by reducing the fluidity in hydrophilic and hydrophobic regions of the cell membrane. All tested fungal strains showed sensitive to licorice aqueous and ethanol extracts except *Aspergillus niger* EMCC 72 in ethanol extract, showing the MIC of 25 mg/mL. Obtained results are in agreement with Karami et al [29] and Ahmed

et al [31]. Due to the inhibitory effects of a licorice root extract on cultures of *Candida albicans*, which had been obtained from mouth lesions of infants. Therefore, licorice aqueous extract is of considerable use to formulate cosmetic products with antiseptic and purifying activities as the use of licorice based mouthwash to treat candida-induced lesions in HIV (human immunodeficiency virus) patients.

Table 3. Inhibition zone diameters and MICs of licorice ethanol extract against pathogenic strains

Pathogenic strain	Inhibition zone diameter (mm)*					
	100	75	50	25	12.5	MIC
Gram-positive bacteria						
Bacillus *cereus*	16	13	10	7	nd	25
Staphylococcus aureus	22	18	15	9	nd	25
Staphylococcus epidermidis	27	24	20	15	7	12.5
Gram-negative bacteria						
Salmonella *spp.*	28	25	21	19	9	12.5
Escherichia coli	19	16	14	12	6	12.5
Proteus mirabilis	20	16	13	11	nd	25
Klebseilla pneumonia	nd	nd	nd	nd	nd	nd
Pseudomonas flourescens	nd	nd	nd	nd	nd	nd
Yeast						
Candida albicans	nd	nd	nd	nd	nd	nd
Fungi strains						
Aspergillus niger	21	18	15	12	nd	25
Aspergillus flavus	nd	nd	nd	nd	nd	nd

Data represented are average of triplicates. MIC; Minimum Inhibition Concentration. * Diameter included 5 mm well diameter ; Concentrations of extract and MIC are in mg/mL. nd; Not detected.

In general, both extracts exhibited antimicrobial activity against both Gram-positive and negative bacteria, yeast and fungi strains. Moreover, the licorice aqueous extract was found to possess higher antimicrobial activity than licorice ethanol extract. This antimicrobial activity can be due to glycosides, alkaloids, terpenoids, proteins and amino acids found in licorice aqueous extract. These phytochemical groups are known to posses' antimicrobial compounds.

3.3. Antioxidant Potential of Licorice Aqueous Extract

Recently, the antioxidant activity of plants and foods can be used as one of the indices for estimating food functionality. In our present work, DPPH radical scavenging ability ~~method~~ was determined to test the antioxidant efficiency of licorice extract with different concentrations and the results are given in Table 4 and Figure 1. The antioxidant capacity (IC_{50} value) as determined by DPPH assay was found to be good (29.92±2.43 mg/g GAE) in the licorice extract (Table 4) and this value was higher than reported by Sanja et al [32] who reported that the IC_{50} of *G. glabra* from Serbia was 11.50 mg/g GAE. Somaris et al [33] obtained licorice extracts with IC_{50} values in the range of 21.9–248.1µg/g GAE using SC-CO_2 extraction at different pressures.

Table 4. Antioxidant activity and total phenolic content of licorice aqueous extract

Parameters	Licorice extract
DPPH scavenging activity** (IC_{50})	29.92 ± 2.43
Total phenolic content* (TPC)	7.88 ± 1.05

Data represented in means of duplicates ± standard deviation. *Total phenolic was expressed as gallic acid equivalents (GAE) mg/ g sample ** IC_{50} (mg/g): Inhibitory concentration at which 50% of DPPH radical is scavenged.

According to the findings shown in Figure 1, the scavenging activity of licorice extract against DPPH$^{\bullet}$ is directly proportional with concentration in dose-dependent manner up to 30 µg/mL, then it gave adverse pattern in higher concentrations, 40, 50, 60 and 70 µg/mL, and showed no antioxidant activity of extracts at concentration above 80 µg/mL. Similar observation was reported by De Marchi et al [34], monitoring antioxidant capacity of quercetin, which relied to the higher rate of superoxide radical ($O_2^{\cdot-}$) generation. Conformingly, the namely flavonoids; quercetin and fisetin at low concentrations (10-25 µg/mL), were informed to protect the rat H4IIE (*Rattus norregicus* liver hepatoma) cells against H_2O_2-induced cytotoxicity, DNA strand breaks and apoptosis, whereas the higher concentrations (50-250 µg/mL) caused cytotoxicity [35]. This phenomenon was also observed by Yu-Jin et al [8] who reported that the excessive consumption of licorice (more than 2 mg/kg/day of pure glycyrrhizinic acid, a liquorice component) may result in reverse effects, such as muscle weakness and hypokalemia.

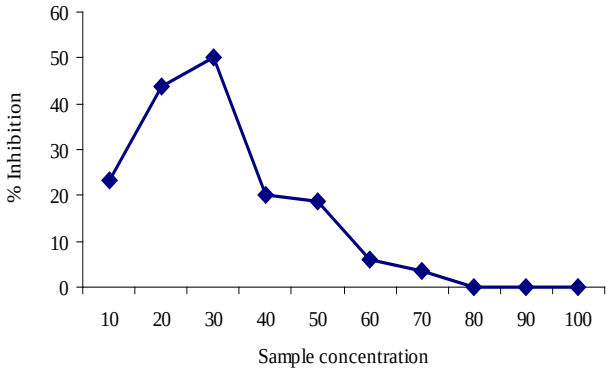

Figure 1. Dose dependent radical scavenging activity (DPPH) of licorice aqueous extract

In contrast, adverse results were reported that the licorice root extract showed antioxidant capacity that increased along with increasing the sample concentrations due to the DPPH hydrogen donating ability in addition to the correlation with increasing of extracted plant phenolic compounds [9]. In this study we assume that the best concentration of the licorice which has high antioxidant activity was 30 µg/mL and this results confirmed with a previous report that the root of G. *glabra* from different localities possesses antioxidative activity, due to the high concentration of phenolic compounds in the extract [32]. Phenolic compounds such as flavonoids, alkaloids, tannins and saponins contained in liquorice extract have good antioxidant activity [10]. It can be concluded that the licorice aqueous extract may be used as a great source of natural antioxidants in reducing oxidative damage in the human body.

3.4. Total Phenolic Content of Licorice Aqueous Extract

Polyphenols, secondary metabolites, are very important plant constituents because of their purported health-promoting properties. They have the ability to steady the unpaired electron and prevent harmful oxidation through their scavenging ability on free radicals because of their hydroxyl groups. Thus, total phenols content of fruits and herbs may contribute directly to their antimicrobial and antioxidant activities. Therefore, in the present research, total phenolic content (TPC) of licorice aqueous extract was investigated by Folin-Ciocalteau method. Results in Table 4 revealed that the licorice aqueous extract contained 7.88 mg gallic acid/ g extract. Mircea et al [7] reported that the antioxidant activity of any plant extract mainly depends on its content of phenolic compounds in quality and quantity, and that the licorice extract contained eleven different phenolic compounds. The TPC content in licorice extract in the present study was found to be lower than extracts of G. *glabra* from Fruska Gora, Serbia (13 mg GAE/ g extract) [32]. It is known that total phenolic content (TPC) in plant varies greatly with genotype, harvest time, growing conditions and processing.

3.5. Phytochemicals Analysis

Phytochemicals qualitative assessment of licorice aqueous extract was determined to check the presence of secondary metabolites. Results indicated the absence of tannins and reducing sugars, in contrast, glycosides, alkaloids, flavonoids, saponins, terpenoids, proteins and amino acids were present in the licorice aqueous extract (Table 5). These results are in accordance with that reported by Asif et al [6] and Karami et al [29]. In another study by Asad et al [30], it has been reported that the mthanolic extract of licorice from Faisalabad, Pakistan, was found to be positive for the presence of flavonoids, alkaloids, saponins and tannins while steroids and anthraquinones were absent. It was reported that alkaloids were isolated and identified from licorice [36]. High amount of terpenoids was found to be present in licorice extract. Phenolics are bioactive compounds formed inside the plant cells; which play a vital role as antioxidant materials.

Table 5. Phytochemical analysis of licorice aqueous extract

Phytochemical constituents	Licorice extract
Tannins	-
Reducing sugars	-
Glycosides	+
Alkaloids	+
Flavonoids	+
Terpenoids	++
Protein + amino acids	+
Saponins	+

Key: + =low concentration, ++ =high concentration and (-) means absent.

It was reported that phytochemicals such proteins and amino acids and/or terpenoids and alkaloids in the licorice extract showed anti-cancer and anti-microbial activities [7]. Moreover, alkaloids are phytochemical compounds present in medicinal plants and it could exert an antibacterial role [37]. Abd El Azim et al [38] reported that licorice extract contains 17 different phenolic compounds; protocatechuic acid, vanillic acid, benzoic acid, quercetin-3-O-α-rhamnosyl (1→6)-β-glucoside (rutin), quercetin 3-*O*-a-rhamnoside (quercetrin), naringenin 7-*O*-rhamnoglucoside (naringin), (2*S*)-4',7 dihydroxyflavanone 4'-O-β-D-glucopyranoside (liquiritin), ferulic acid, *p*-coumaric acid, cinnamic acid, myricetin, quercitin, kaempferol, apigenin, 5,7,4'-trihydroxyflavanone (naringenin), liquiritigenin and flavone. These results are in agreement with what previously reported by Yu et al [39]. In another study by Hassan et al [12], who found that the major active component of licorice root is the triterpenoid saponin glycyrrhizin (also known as glycyrrhizic acid or glycyrrhizinic acid).

3.6. HPLC Analysis of Licorice Aqueous Extract

High-performance liquid chromatography (HPLC) method was used for the identification of bioactive compound in the extracts of licorice. HPLC standard chromatogram and phenolic compounds analysis of licorice aqueous extract are represented in Figure 2. The HPLC analysis results revealed that, comparing to HPLC standard chromatogram contained six phenolic and four flavonoids, only 6 peaks at the specific retention time of standards' peaks were identified in licorice extract which represent their phenolic content profile; gallic acid, itaconic acid, protochatechuic acid, catechin, esculetin, catechol, tannic acid, ferulic acid, pyrogallol, and cinnimic acid with different concentration. These results are similar to what previously reported by Rosa et al [9] and Abd El Azim et al [38]. Previous reports of Laura et al [40], reported that in licorice fifteen peaks were identified dihydrostilbenes (nine compounds) and flavonoids (six compounds). Gallic acid was detected to be the major phenolic component in licorice aqueous extract. Itaconic acid, catechol and pyrogallol were also predominant in the extract of licorice. Liquiritigenin and isoliquiritigenin were identified in earlier researches of G. *glabra* originating from different regions, but the amount of these compounds was very low [41]. Esculetin and protochatechuic acid was observed in the extracts of licorice, which was not found in previous studies. In another study by Giulia et al [24], who reported that the triterpene saponins are the major characteristic constituents of licorice, being responsible for the sweet taste. It could be explained that, the variations of these bioactive compounds content maybe due to the geographical location, harvesting, and processing, affecting the medicinal effects of licorice. These results were in accordance with previous report of Giulia et al [24]. The identified compounds which have varying capacities could contribute the biological activities and treatment of numerous pathophysiological conditions of the licorice extract.

3.7. Antitumor Potentials of Licorice Extract on Breast Cancer

In spite of early detection and apparently complete surgical resection, breast cancer remains to cause rising

cancer death rates among women worldwide. Searching of natural plant capable of blocking tumor process could be a good approach to treat human breast cancer cells. So, the cytotoxic potential of licorice was evaluated for its potential medical and anti-cancer applications, serial concentrations of licorice were plotted against their corresponding % inhibition curve for MDA-MB-231 cell line.

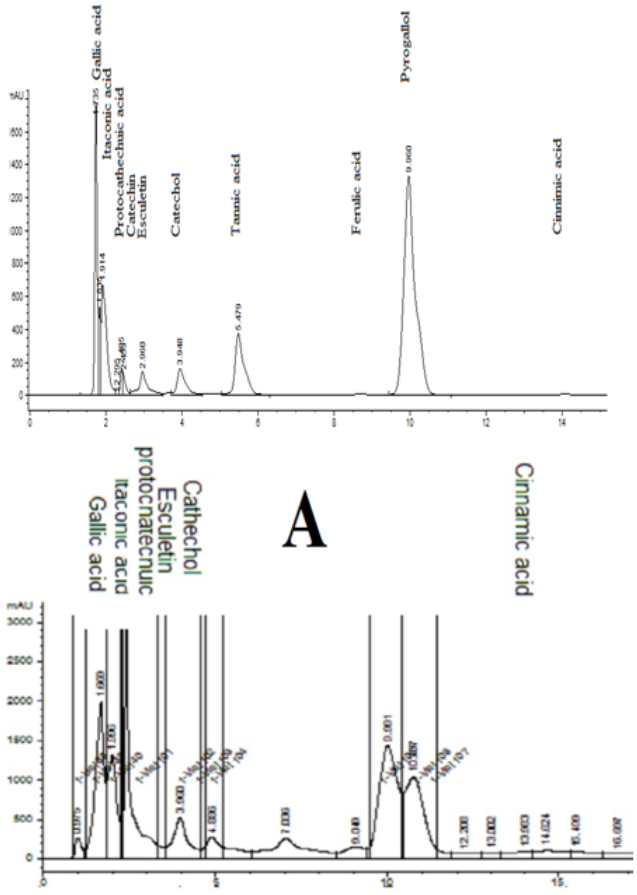

Figure 2. HPLC chromatograms of licorice aqueous extracts, S, standard chromatogram; A: licorice aqueous extract

The results of the neutral red assay are presented in Figure 3 where cytotoxicity was expressed as % inhibition upon 48 h contact time. The licorice extract dose-dependently inhibited the growth of MDA-MB-231 cells within the concentration range used. At different concentrations of licorice extract, reaching a maximum effect at about 100 µg/mL. A similar observation was reported by Chunyan et al [42] that the ehthanolic extract of licorice inhibited cell growth above 100 µg/mL.

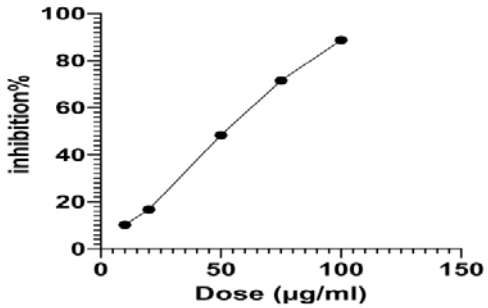

Figure 3. Dose-dependent effects of liquorice on cell proliferation inhibition in MDA-MB-231

Morphological changes of MDA-MB-231 cells upon treatments with different concentrations of licorice were studied as an indicator of cytoxicological changes. After 48 hrs cells started to detach from the monolayer and become rounded in shape, aggregated and started to fluff off from the attached bottom of the plate. The untreated negative control cells showed original spindle shape even after 48 h of incubation (Figure 4).

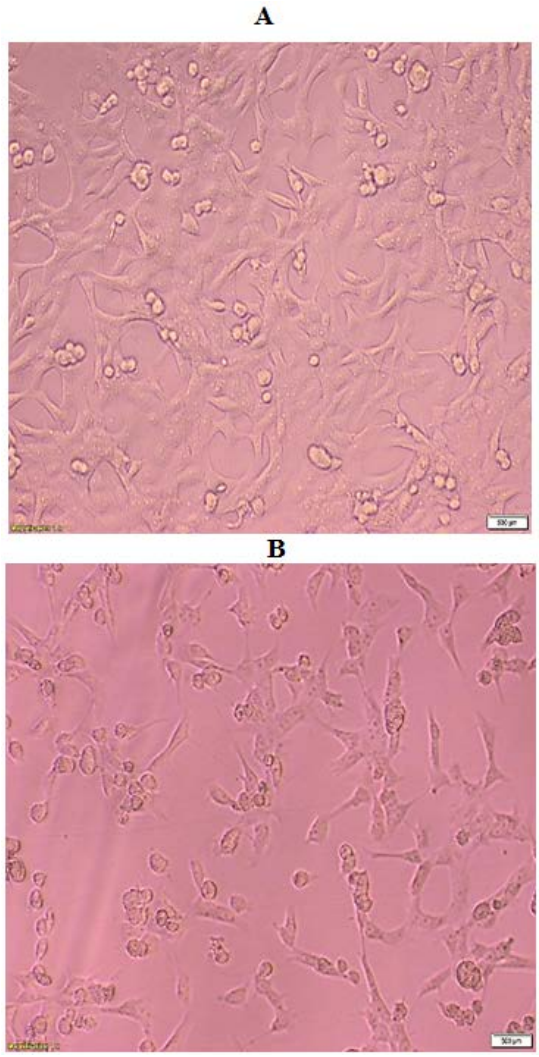

Figure 4. Anti-cancer activity of licorice on breast cancer cell line MDA-MB-231 by neutral red assay, (A) control, (B) licorice treated cells 50µg/ml

Different studies suggest that the extract of G. *glabra* may be a potential supplemental source for different cancer treatments [24]. According to Wang et al [43], licorice extract has antitumour activities in breast and ovarian cancer, gastric tumours, and leukaemia have also presented promising cytotoxicity on human breast cancer cells. These observations indicate that the licorice aqueous extract contains a variety of phytoestrogen compounds that might be considered as powerful chemo-preventive agents. Therefore, the amount of the extract of licorice in normal and/or supplemented food should be taking into considerable account in the treatment of postmenopausal women affected by hormone sensitive breast cancer. Zhou and Ho [44] reported that the anti-neoplastic activity of licorice had been considered as an effective utilization in the treatment of cancer.

3.8. Organoleptic Properties of Licorice Fortified Yoghurt

The addition of natural food additives to food products to improve nutritional value, shelf life, texture, taste and appearance of the product is a popular practice that has become very important [45]. Therefore, in the present work we evaluated the effect of licorice supplementation in organoleptic properties of yoghurt. Data presented in Table 6 exhibits organoleptic properties of licorice aqueous extract fortified yoghurt products; control, T1 (yoghurt fortified with licorice extract 1: 100) and T2 (yoghurt fortified with licorice extract 2: 100). Data revealed that the insignificant effect of fortifying with licorice extract on organoleptic properties; taste, odor, color, texture, appearance and overall acceptance scores. According to panelists' opinion, both of licorice fortified yoghurt products T1 and T2 showed an overall acceptance of 6.7 and were higher than control (6.4). In spite of the panelist did not detect any difference among treatments for the sensory analysis, color, texture and appearance attributes were bound tightly to the control treatment. On the other hand, the fortified yoghurt products were significantly favorable than control in taste with $p<5$ (0.38). These results are in lines with those of Gabriel et al [46]. Based on these results, it can be concluded that the licorice aqueous extract fortified yoghurt products were sensorial accepted which encourage its fortification in functional dairy products to be used as a vehicle delivering for its functional properties as antimicrobial, antioxidant and anticancer potentials to consumers.

Table 6. Organoleptic properties of licorice fortified yoghurt

Products	Control	T1	T2	Probability
Odor	6.4±1.8	7.0 ±1.7	7.0±1.6	0.55382
Taste	5.4±2.3	6.9 ±1.8	6.4±1.8	0.37956
Color	6.8±1.1	6.1 ±1.5	6.2±1.8	0.50352
Texture	6.5 ±1.7	6.7 ±1.3	6.5 ±1.4	0.94873
Appearance	7.0 ±1.9	6.5 ±1.6	6.6 ±1.6	0.63031
Overall acceptance	6.4 ±1.8	6.7 ±1.6	6.7 ±1.3	0.96492

Data represented in means of ten panelists' ± standard deviation.T_1; yoghurt fortified with licorice aqueous extract 1: 100 (w/v) of milk. T_2; yoghurt fortified with licorice aqueous extract 2: 100 (w/v) of milk.

4. Conclusion

Licorice is one of the considerable economically valuable plants ~~in the~~ worldwide. The present study was carried out to evaluate the antimicrobial activity of ethanol and aqueous extracts of *Glycyrrhiza glabra* root in parallel with their antioxidant and anticancer potential. Obtained results revealed the *In-vitro* pharmacological potentials of Egyptian licorice, especially as a valuable source of natural antioxidants which might be used as an antioxidant food additive. Moreover it can used as an alternative novel secondary anticancer pro-oxidant which might synergistically kill tumor cells disabling free radicals which could be recommended for long term use in prevention and/or therapy. Biological and pharmacological potential of the tested licorice aqueous extract is based on its phytochemical characteristics. On the other hand, fortified functional food applications using Egyptian licorice root extract could be recommended as natural food preservative due to its antimicrobial potency controlling food pathogens.

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