International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Antimicrobial Activity of Spray Dryer Licorice (*Glycyrrhiz glabra* L.) Extract Against Multi-Drugs Resistant Bacteria

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Abstract: ***The licorice has been used in pharmaceutical and confectionery industries because of the presence of glycyrrhizin. Stability study was done for spray dryer licorice extract (SDLE) according to the WHO guidelines on stability testing of active substances and pharmaceutical products. For long term stability study, SDLE was stored at 25 ºC, 65% R.H for 12 months and for accelerated stability, SDLE was stored at 40 ºC, 75% R.H for 6 months study physical examination and determination of glycyrrhizin content were done every 3 months. The extensive use of Antibiotics has led to growing resistance and spread of many bacterial pathogen, which now constituents a serious medical problem. Plant may be considered a new antibacterial activity. A study was conducted to determine the antibacterial activities of licorice root spray dried extract in methanol, water and ethanol against bacteria using the well diffusion method. The extracts showed significant antibacterial activities against five gram-positive (Bacillus subtilis and Staphylococcus aureus, Micrococcus lutes and Staphylococcus epidermes) and three gram-negative (Klebsiella pneumoniae, Escherichia coli and Pseudomonas aeruginosa) bacteria. The protein/peptides were isolated and separated on SDS-PAGE. The glycyrrhizin destroyed proteins of K. pneumoniae but in S. epidermes it resulted in appearance of even more protein bands when compared to the protein profile of the untreated bacteria. The extra protein bands were of both low and larger molecular weight than the native proteins present in untreated bacteria.***

Keywords: licorice, SDLE, Antibacterial activity and SDS-PAGE.

1. Introduction

Glycyrrhiza glabra L. belongs to the *Fabaceae* family. The extract obtained from the roots is called licorice, and this is the part of the plant that people use in foods and medicine. The main active constituent of licorice is Glycyrrhizin, which has a sweet taste [1]. For this reason, licorice is commonly used to mask bitter tastes in medicinal preparations. The plant has also been used as a mild laxative, antiarthritic, antinflammatory, antibiotic, antiviral, antiulcer, memory stimulant (being an MAO inhibitor), antitussive, aphrodisiac, antimycotic, estrogenic, antioxidant, anticaries agent, antineoplastic, anticholinergic, antidiuretic, and hypolipidemic agent [2]. Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. Spray drying technique of extraction is achieved by pumping a solution or a homogenized emulsion to an atomizer and sprays it as a fine mist of droplets into a drying chamber. Licorice contains as its major active principle the triterpene glycoside glycyrrhizin in concentrations ranging from 1 to 24%, depending on sources and methods of assay [3]. Determination of the antibacterial activities of licorice root extract in ether, chloroform, acetone on bacteria using the well diffusion method. The extracts showed significant antibacterial activities against two gram positive (*Bacillus subtili* and *Stapphylococcus aureus*) and two gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria[4].Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful tool to dissociate proteins into individual chains and separate them according to their molecular weight [5]and [6]. SDS-PAGE is an ideal technique to use for demonstrating antimicrobial affectivity and has previously been used to study resistance mechanisms in bacteria [7]. The main objective of this study was to determine the effect of licorice root spray dried extract on the bacterial cell proteins, using SDS-PAGE in order to elucidate the mechanism of antimicrobial action.

2. Material and Methods

2.1 Preparation of Spray dried licorice extract (SDLE)

The Egyptian *Glycyrrhiza glabra* were cultivated at the farm of Applied Research Center of Medicinal Plants (ARCMP) which collected from the Experimental Farm, Agriculture Faculty, Suez Canal University, Ismailia governorate in the season march 2004. This work was carried out in Applied Research Center of Medicinal Plants (Biotechnology, Microbiology and Pharmaceutics labs.), National Organization for Drug Control and Research (NODCAR), Giza, during the period of 2010 – 2013.

Powdered licorice root, 750 grams were mixed with 2.25 liters hot water at 500C, containing 6 grams of sodium benzoate as a preservative and macerated in covered percolator for 24 hours, 0.75 liter hot water was gradually added to the percolator, the received percolate was filtered,

2.25 liters of licorice aqueous extract were received as a filtrate. Aqueous extract was applied to Fluidized Spray Dryer-FSD, Size 6.3, (NIRO, Switzerland) connected to Compressor and Dryer (Fiac, Italy). Several trials were done till reaching best conditions (includes inlet and outlet temperature, feed rate and nozzle pressure) that give the higher yield of dry extract, lower moisture content and higher amount of active constituent. The SDLE extract was examined for its color, odor, taste, solubility and pH for 1% aqueous solution at 25 ºC.

2.2 Determination of glycyrrhizin content in SDLE

Glycyrrhizin was determined applying HPLC method [8]. 200 mg of SDLE and 100 ml of 0.8% w/v ammonia solution were added and treated in ultrasonic bath for 30 min. as a test solution. Part of supernatant layer was centrifuged and diluted 1 to 5 ml with 0.8% w/v ammonia solution.130 mg of monoammonium glycyrrhizinate was dissolved in 0.8 % w/v ammonia solution to 100 ml as stock solution. Reference solutions RS (a, b, c, d, e) were prepared by diluting (1, 3, 5, 10, 15) ml of stock solution to 100 ml with 0.8% w/v ammonia solution respectively. Column 0.1 m long and 4 mm in internal diameter packed with octadecylsilyl silica. Mobile phase at flow rate 1.5 ml/min. a mixture of 6 volumes of acetic acid R, 30 volumes of acetonitrile R and 64 volumes of water R. Spectrophotometric detector set at 254 nm. The reference solutions (e), (d), (c), (b) and (a) were injected and the peak areas were determined. Calibration curve was established with concentration of the reference solutions (g/100 ml) as the abscissa and the corresponding areas as the ordinate. Test solution was injected and the peak due to glycyrrhizin was located and integrated in the chromatogram obtained with the test solution. Percentage content of glycyrrhizin (%w/w) was calculated according to equation: (A) X (5/m) X (B) X (822/840), A= concentration of monoammonium glycyrrhizate in the test solution determined from the calibration curve, in g/100 ml, B = declared percentage content of monoammonium glycyrrhizinate RS (75%), m = mass of drug in grams (0.2 gram), 822= molecular weight of glycyrrhizin, 840= molecular weight of monoammonium glycyrrhizinate (without any water of crystallization).

2.3 Stability study for SDLE

Stability study was done for SDLE according to the [9].On stability testing of active substances and pharmaceutical products. For long term stability study the SDLE was stored in well closed amber colored glass bottles at 25 0C, 65% R.H for 12 months, physical examination and determination of glycyrrhizin content were done at the following time intervals: 0, 3, 6, 9 and 12 months and for accelerated stability study the SDLE was stored in well closed amber colored glass bottles at 40 0C, 75% R.H. for 6 months, physical examination and determination of glycyrrhizin content were done at the following time intervals: 0, 3 and 6 months.

2.4 Antibacterial preparation:

Clinical *Klebcilla pneumonia*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermis* and *Pseudomonas aeruginosa* isolates were collected from the period of January 2013 to July 2013, were identified by Microscopically examination and Biochemical Tests according to[10].American Type Culture Collection strains such as *Bacillus subtlus* ATCC 6633; *Staphylococcus aureus* ATCC-29213; *Micrococcus luteus* ATCC 10240 and *Staphylococcus epidermis* ATCC-12228; as sensitive bacteria. The bacterial strains were tested for their susceptibility to antibiotics using disk diffusion method. The diameters of inhibition zones were measured. Strains were classified as susceptible (S), intermediate (I) or resistant (R) in accordance to [11].

Preparation of crude extracts: Extracts were prepared in polar solvents (Ethanol & Methanol), at room temperature by simple extraction method [12].

Dried powder of plant parts (10mg) was mixed with 100 ml solvent (10%w/v) in 250 ml conical flask. All conical flasks were kept on shaker for 48 h then it was allowed to stand for five hours to settle the plant materials. Thereafter, it was filtered through wattman filter No. 1.The supernatant was collected and the solvent was evaporated at 45°C in vacuum evaporator to make the final volume 1/5th of the original volume. It was dissolved in Dimethyl sulfoxid and stored at 4ºC for further studies. The antibacterial activity of plant extracts on resistant and sensitive, were screened using Agar well diffusion assay [13].Wells of 6mm diameter were cut into solidified agar media using a sterilized cup-borer. 100µl of each extract was poured in the respective well and the plates were incubated at 37°C overnight. The antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by each extract at the end of incubation period. Dimethyl sulfoxid used in dissolving of extracts was also used as negative controls during the study.

22.5 Sample preparation for SDS-PAGE

Effect of Licorice root spray dried extract on protein samples by SDS- PAGE. The protein(s) was determined using 12 % SDS - PAGE as described by [14].To investigate a possible mechanism of action of antibacterial activity from licorice were performed on *Staphylococcus epidermis* and *klebsiella pneumoniae* cells exposed to Licorice root spray dried extract for 24 hs to determine if this extract induced any alteration to protein profile of the tested bacteria.

0.05 mg/ml of methanol extract were added to S. epidermis and K .pneumonia cells and grown on Nutrient broth for 24 h at 37ºC .The bacterial suspensions were transferred into Falcon 50 ml (PPCO-AOR, Sigma, USA) and centrifuged for 10 min at 10.000 rpm using Sigma Laboratory Centrifuge 3K30,USA.The supernatant was discarded, and the pellet was washed four times with 20 ml phosphate buffer of pH 6.8.The pellet was resuspended in 1 ml of phosphate buffer and then transferred to a preweighed Eppendorf tube. Cells were centrifuged for 11 min at 10.000 rpm and the supernatants were discarded. The mass of the pellet was determined by weighing the tubes. 100 µl of 20% SDS were mixed with 900 µl of STB (sample treatment buffer) for use with proteins. 100 µl of SDS-STB solution was added to the pellet and heated for 3 min at 96ºC. Cells

were lysed by sonication for about 5 s using homogenizer IKA LABORTECHNIK Janke & Kunkel GmbH &Co KG, D-79219 Stafen, Germany. Another 100 µl of SDS-STB mixture was added and cells were centrifuged for 11 min at 10.000 rpm. The supernatant was transferred to the sterile Eppendorf tube and stored according to [15].

The proteins were boiled at 95°C for 5 min after mixing with 5X sample buffer at ratio of 1:1 (V:V). The resolving and staking were prepared as described in (Table.1) 20µl of sample were loaded separately into slots, vertical gel electrophoresis (Multigel - Long cat. ≠ 010-400, Biometra®, Germany), which used first at 80 Volt until the bromophenol blue dye reached the resolving gel and continued at 150 Volt for 5 hr. The protein bands were observed after staining the gel with Commassie Brilliant blue (R-250) about 12 hr and incubated in destining buffer at RT until the background was colorless then photographed. BlueRanger™ Protein Molecular weight Marker Mixes cat No#26681 PIERCE, USA was used to detect the molecular weight of the different bands.

3. Results & Discussion

3.1 Pharmaceutical study

The best conditions of spray dryer which produce the highest yield of powder and the higher content of glycyrrhizin in the dry powder are: Inlet temperature: 145-155 0C, Outlet temperature: 95-105 °C, Feed rate: 6 ml/minute, Nozzle Pressure: 30 bars. Liquorice dry extract yield was approximately 120 grams dry extract from 750 grams plant (i.e.) 16% w/w of plant was received. The SDLE was brown in color and had a characteristic odour, sweet taste. It's solubility in water was 1 gram in 10 ml water, the pH was 6.09 for 1% aqueous solution at 25 °C. Table (1) shows the concentrations of reference solutions (a), (b), (c), (d) and (e) in g/100 ml and corresponding peak areas in mAU.min. Figure (1) shows the calibration curve with concentration of the reference solutions (g/100 ml) as the abscissa and the corresponding areas in mAU.min. As the ordinate, the slope calculated and was found to be 10384. Figure (2) shows the chromatograms of standard solution (Reference solution e) and the test solution (conc. 0.2 g/100 ml) indicating that the glycyrrhizin peak was located in standard and test solutions at retention time (about 4.3 min.). Applying the equation, the content (% w/w) of glycyrrhizin in SDLE was about 13.314 (% w/w).Tables (2, 3) show the results of physical examination, the peak area of glycyrrhizin in test solution and glycyrrhizin content (% w/w) in SDLE after 0, 3, 6, 9 and 12 months storage and at 0, 3 and 6 months storage respectively indicating that there was no change in glycyrrhizin content and its physical properties. The chromatograms of test solution (conc. 0.2 g/100 ml) and standard (Reference solution e) at 0, 3, 6, 9 and 12 months storage and at 0, 3 and 6 months storage are shown in figures (3, 4) respectively, indicating that the glycyrrhizin peak was located in both standard and test solutions at retention time (about 4.3 min.).

Table 1: The concentrations of reference solutions (a), (b), (c), (d), (e) and their corresponding peak areas

Reference solutions	*Concentration (g/100 ml)*	*peak area (mAU.min)*
Reference solution (a)	0.0013	12.66
Reference solution (b)	0.0039	38.85
Reference solution (c)	0.0065	69.15
Reference solution (d)	0.013	137.56
Reference solution (e)	0.195	200.65

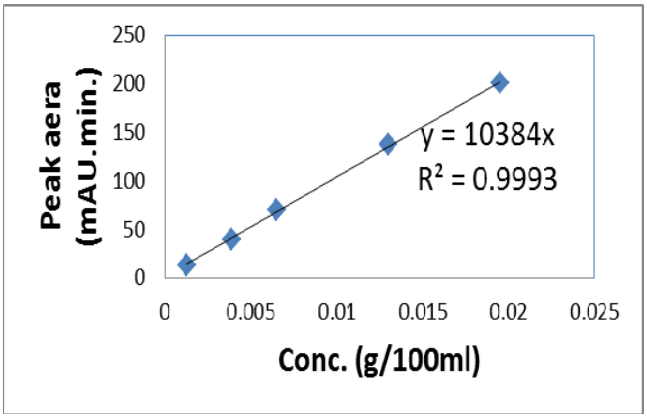
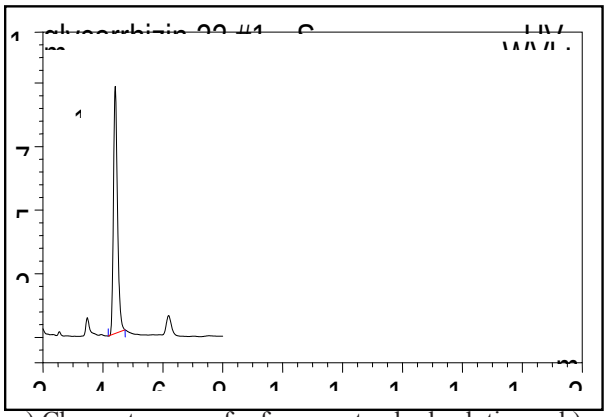


Figure 1: Calibration curve of monoammonium glycyrrhizinate standard



a) Chromatogram of reference standard solution e. b) Chromatogram of test (conc. 0.2 g/100 ml).

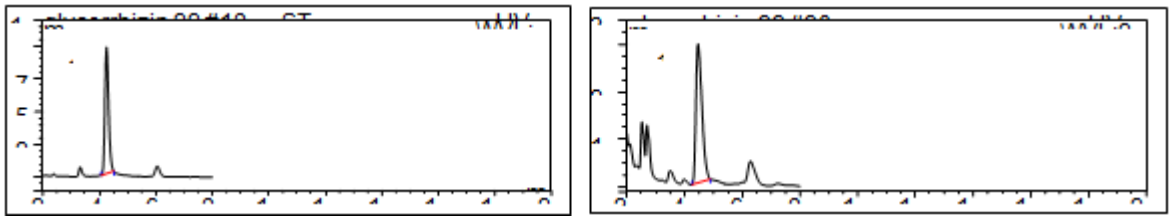
Figure 2: Chromatograms of SDLE

Table 2: Physical examination and glycyrrhizin content (% w/w) in SDLE during long term stability study (25 ^{0}C and 65% R.H)

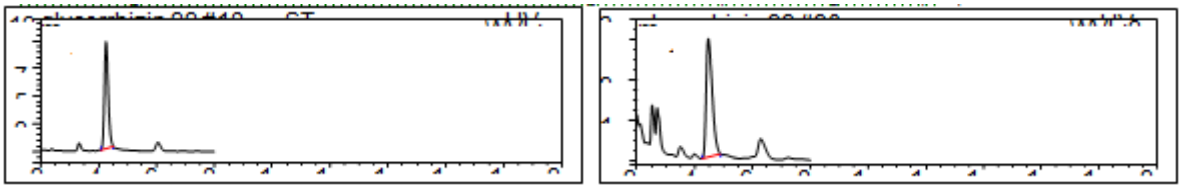
Storage period (months)	*Physical Examination*				*Glycyrrhizin content (% w/w)*	
	Colour	*Odour*	*Taste*	*pH (1% aqueous solution)*	*Glycyrrhizin peak area (mAU.min)in test solution (conc.0.2g/100 ml)*	*Content of glycyrrhizin (% w/w)*
zero time	Brown	Characteristic	Sweet	6.09	78.617	13.314
3	No change	No change	No change	6.08	78.569	13.310
6	No change	No change	No change	6.08	78.591	13.309
9	No change	No change	No change	6.07	78.572	13.306
12	No change	No change	No change	6.08	78.562	13.305

Table 3: Physical examination and glycyrrhizin content (% w/w) in SDLE during accelerated stability study (40 ^{0}C and 75% R.H)

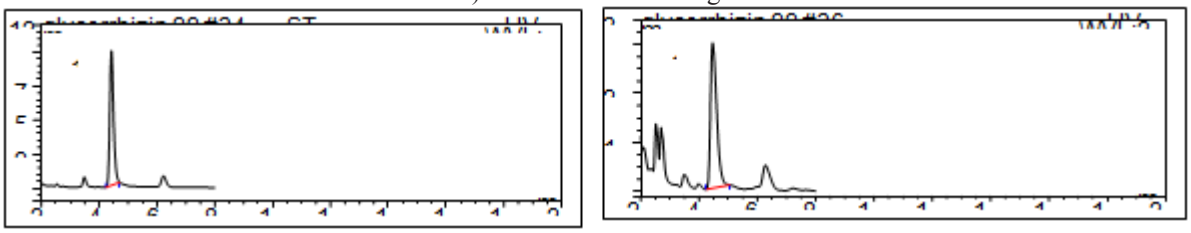
Storage period (months)	*Physical Examination*				*Glycyrrhizin content(% w/w)*	
	Colour	Odour	Taste	pH (1% aqueous solution)	Glycyrrhizin peak area in test solution (conc.0.2g/100 ml) (mAU.min)	Content of glycyrrhizin (% w/w)
zero time	Brown	Characteristic	Sweet	6.09	78.617	13.314
3	No change	No change	No change	6.07	78.482	13.291
6	No change	No change	No change	6.07	78.469	13.289



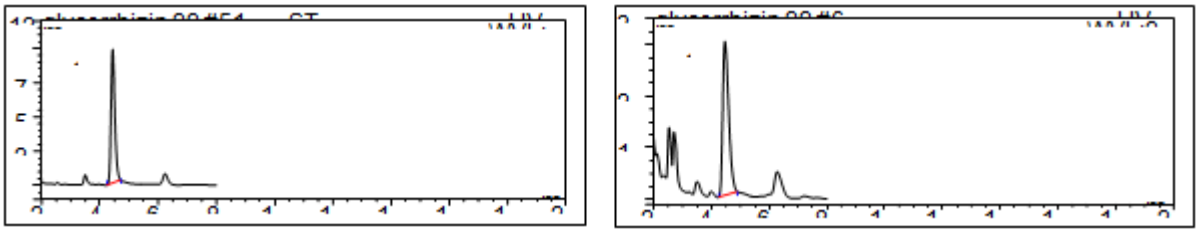
Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml).
a) At zero time.



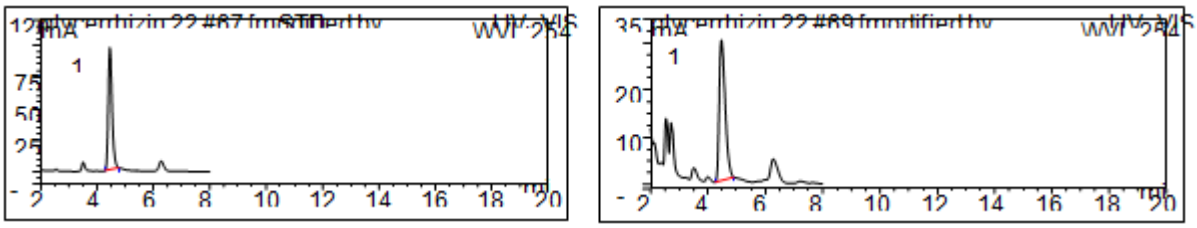
Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml).
b) After 3 months storage.



Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml). c) After 6 months storage.

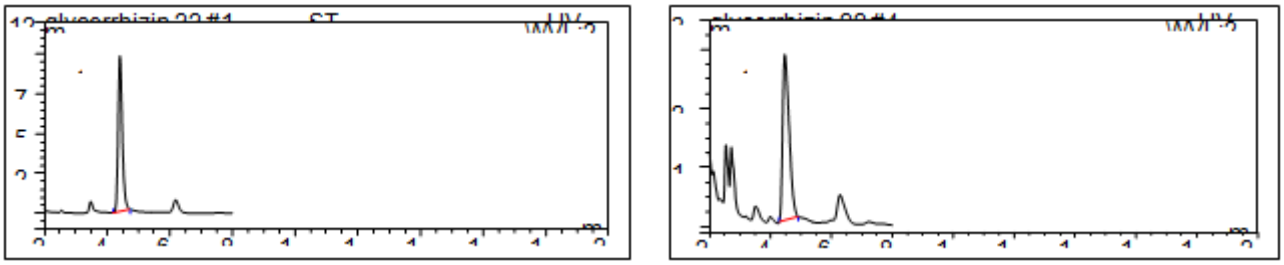


Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml). d) After 9 months storage.

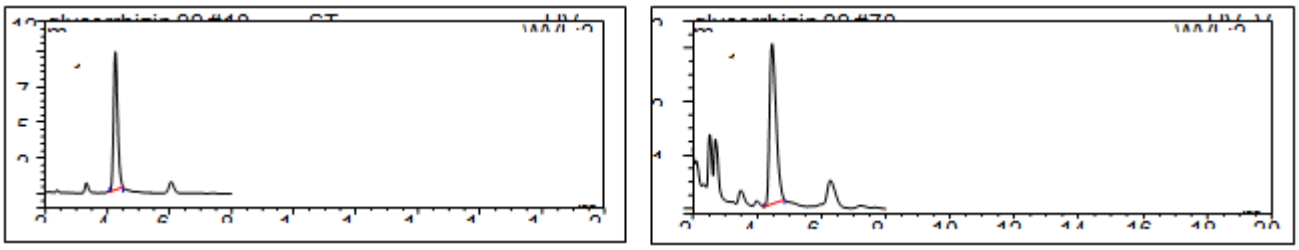


Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml).
e) After 12 months storage.

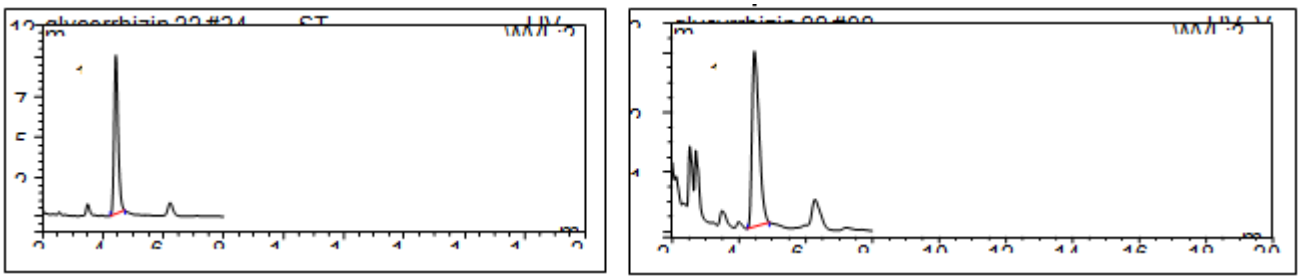
Figure 3: Chromatograms of SDLE during long term stability study (25 ^{0}C and 65% R.H)



Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml).
a) At zero time



Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml).
b) After 3 months storage



Chromatogram of ref. standard solution e. Chromatogram of test (conc. 0.2 g/100 ml).
c) After 6 months storage.

Figure 4: Chromatograms of SDLE during accelerated stability study (40 ^{0}C and 75% R.H).

3.2 Antibacterial activity of SDLE

The methanol extracts of the SDLE has shown magnificent antibacterial effect. Although methanol extract has shown good effect on all except E. Coli strain. The water extracts of the SDLR has shown non significant antibacterial effect. The ethanol extract of SDLR has shown excellent effect than some antibiotics. Overall the extract has shown significant antibacterial effect on reference organisms as well as clinical isolates. From the previous data we can deduce that the plant methanol extract have potentioal activity on resistant clinical isoltes .Plants are important source of potentially useful structures for the development of new chemotherapeutic agents.

Table 4: Detection of multi-drug resistant *clinical strain (RCS)* by disc diffusion method

Antibiotics	*Staphylococcus epidermis*	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa* s	*Klebcilla pneumonia*
Vancomycin 30µg	I	R	R	R	R
Amikacin 30µg	R	S	R	R	I
Cefaclor 30µg	S	R	R	R	R
Amoxicillin/ Clavulinic 30µg	I	R	R	R	R
Meropenem 30µg	S	S	R	S	S
Levofloxacin 30µg	S	I	S	S	I
Cefotrocin 30µg	R	R	R	R	R
Erythromycin 30µg	I	R	R	R	R
Gentamycin 10µg	R	R	I	R	R
Cefepime 30µg	S	R	R	I	I
Ciprofloxacin 5µg	R	R	R	S	I

Table (4) showed that highest resistance percentages were achieved by Cefotrocin. Meropenem showed the lowest resistance. Three strains were sensitive to Levoloxacin and intermediate to *E.coli* and *Klebcilla pneumonia*. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants [16]. Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings.

Table 5: Antibacterial activity of the licorice *Glycyrrhiza glabra* L. *against clinical strain (RCS)*

Extracts	***Staphylococcus epidermis***	***Micrococcus lutes***	***Staphylococcus aureus*(CRS)**	***Klebcilla pneumonia*(CRS)**	***Escherichia coli*(CRS)**	***Pseudomonas aeruginosas* (CRS)**	***Bacillus subtlus,***	***Staphylococcus epidermis*(CRS)**	***Staphylococcus aureu***
Methanol	35	20	23	28	-	26	23	22	25
Water	-	-	-	-	-	-	-	28	-
Ethanol	-	16	20	-	-	15	-	20	25

[17] Stated that *G. glabra* root extract showed antibacterial activity against S. aureus. Also [18] stated that the extracts of *Glycyrrhiza glabra* L. showed activity against S. aureus and can be used as raw materials for phytotherapy. Table (5) showed the different level of activity with different type of solvents, methanol, water and ethanol; however methanol one was the best accordance with [19] who confirmed that plants differ significantly in their activity against tested organisms using different solvents.

In addition most likely the water extract of tested plant failed to inhibit the growth of investigated microorganisms. Consistently, [20] demonstrated that ethanolic extracts were found to be more effective than aqueous extracts. Also in present study, the extract showed response on both G+ve and G-ve bacteria. The demonstration of activities against both G+ve and G-ve bacteria is an indication that plant can be a source of bioactive substances that could be broad spectrum of activity [21].Since plants produce a variety of compounds with antimicrobial properties, it is expected that plant compounds showing target sites other than those currently used by antibiotics and will be active against drug –resistant microbial pathogens [22].

3.3 Protein analysis of bacterial cells after and before treatment with glycyrrhizin

Two kinds of bacteria were chosen for example of G-ve bacteria (*Klebcilla pneumonia*) and example of G+ve (*Staphylococcus epidermis*) to determination the effect of SDLE on both bacterial protein profiles.

A. Klebcilla pneumonia.

The protein profiles of untreated bacteria (control) differed from those of treated bacteria with glycyrrhizin for 24 h at 37ºC. Several protein bands were observed for untreated *K.penumonia* {Fig. 5, lane 1(control) and lane 2(treated)} .There was a single band for about 25 KDa on control cells and disappeared in treated cells.

The protein bands for treated bacteria in band 70KDa was very sharp and more density than it found in treated cells. In general it is clearly that glycyrrhizin decreased the numbers of protein bands after the bacterial strains were treated with glycyrrhizin. This suggests that glycyrrhizin may have caused the death of the bacteria through the destruction of cellular proteins. These results are in agreement with the findings of [23] who observed the disappearance of protein bands after exposing E. coli to an anolyte solution with an ORP of 1000 mV. Oxidizing compounds present in anolyte probably because destruction of proteins by breaking down the covalent bonds in proteins. The 10-2 anolyte dilution destroyed proteins of *P. aeruginosa* but in E. coli it resulted in appearance of even more protein bands when compared to the protein profile of the untreated bacteria.

B. Staphylococcus epidermis.

The results obtained for *S.epidermis* differed from those of *K.penumonia*. Similar to the observations made for *S.epidermis*, there were fewer protein bands from *S.epidermis* cells treated with glycyrrhizin than those of untreated cells {Fig. 5, lane 3(control) and lane 4(treated)} However, reduced the numbers of viable cells and also resulted in more protein bands.

The extra protein bands were of both low and larger molecular weight than the native proteins present in untreated bacteria. Low molecular weight protein bands probably resulted from fragmentation the proteins into smaller peptides [24] and [25]. Presence of dilute anolyte caused an unfavorable environmental condition which induced a stress response in bacteria. The level of stress response varies with the type of organism and the type of environment [26]. During exposure to stressful conditions, bacteria synthesize and replace damaged proteins from mainly recycled amino acids, and this may give rise to the altered profiles from the native proteins [27]. Also, stress initiates the activation of a variety of defense genes which encode for the scavengers of reactive radical and other stress related proteins [28] and [27].This condition may account for the presence of the additional, large molecular weight protein bands as evidenced.

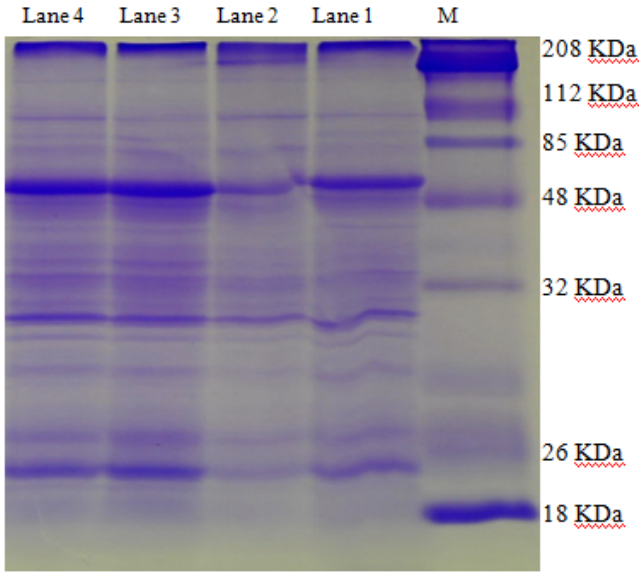


Figure 5: SDS-PAGE whole protein profiles from bacteria treated and untreated with glycyrrhizin. Lane 2 and lane 4 treated cells of *K. pneumonia* and *S. epidermis*, respectively, Lane 1 and Lane 3 untreated cells of *K. pneumonia* and *S. epidermis*, respectively, M: represent the molecular weight marker.

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