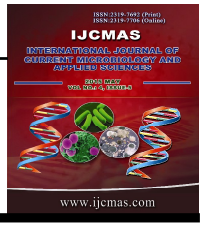
Int.J.Curr.Microbiol.App.Sci (2015) 4(5): 473-484

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 5 (2015) pp. 473-484**
http://www.ijcmas.com



Original Research Article

Study the Effect of Ethanolic Extract of *Glycyrrhiza glabra* on Pathogenic Bacteria

Alhan M.Alwan*, Zainab Nesrullah and Elaf Faraj

Department of Biology, College of Science, University of Diyala, Baquba, Iraq

**Corresponding author*

A B S T R A C T

The antimicrobial effect in the vitro of ethanolic extracts of the specified plant *Glycyrrhiza glabra* is implemented. It is against seven species of pathogenic bacteria (*Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Bacillus*). All isolates are sensitive to ethanolic extract of this plant specialized on 50% concentration. The extracted material from this plant has significant effects on pathogenic bacteria because of the containing many complexes such as Glabridin, Flabron, Licochalcone, and Glycerhetinic acid. So the mentioned complexes have fatal effect on pathogenic bacteria. The extracted materials have inhibition ability on both gram negative and gram positive bacteria. The highest inhibition zone is observed about 25mm with combination of extract on *Streptococcus pyogenes*.

Keywords

Ethanolic extract, *Glycyrrhiza glabra*, Pathogenic bacteria

Introduction

Licorice species are perennial herbs native in Iraq and Mediterranean region, central to southern Russia, Asia Minor to Iran, now widely cultivated throughout Europe, the Middle East and Asia (Blumenthal *et al.*, 2000). They have been used medically since at least 500 B.C and licorice has been described as 'the grandfather of herbs (Ody, 2000). The genus *Glycyrrhiza* consists of about 30 species including *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*. Spanish and Italian licorices are *G. glabra*var, the family of this species is Fabiaceae (Leguminosae) (Nomura *et al.*, 2002).

The roots and rhizomes of licorice (*Glycyrrhiza*) species have long been used worldwide as an herbal medicine and natural sweetener. Licorice root is a traditional medicine used mainly for the treatment of peptic ulcer, hepatitis C, and pulmonary and skin diseases, although clinical and experimental studies suggest that it has several other useful pharmacological properties such as anti-inflammatory, antiviral, antimicrobial, anti-oxidative, anti-cancer activities, immune modulator, hepatic

protective and cardio protective effects. A large number of components have been isolated from licorice, including triterpenesaponins, flavonoids, isoflavonoids and chalcones, with glycyrrhizic acid normally being considered to be the main biologically active component. This review summarizes the phytochemical, pharmacological and pharmacokinetics data, together with the clinical and adverse effects of licorice and its bioactive components (Anti-HIV, 2004).

Flavonoids: The other constituents include flavonoids and chalcones (which are responsible for the yellow color of licorice) such as liquiritin, liquiritigenin, rhamnoliquiritin, neoliquiritin, chalconesisoliquiritin, isoliquiritigenin, neoisoliquiritin, licuraside, glabrolide and licoflavonol (Williamson, 2003). Recently 5,8-dihydroxy-flavone-7- O-beta-D-glucuronide, glychionide A, and 5-hydroxy-8- methoxyl-flavone-7-O-beta-D-glucuronide, glychionide B were isolated from roots and rhizomes of *G. glabra* (Li *et al.,* 2005). The retrochalcones, licochalcone A, B, C, D and echinatin, were recently isolated from the roots of *G. inflate* (Haraguchi, 2001), and the minor flavonoids, isotrifoliol and glisoflavanone, from the underground part of *G. uralensis* (Hatano *et al.,* 2000; Jonsson *et al.,* 2015).

Isoflavones: this compounds derivatives present in licorice includeglabridin, galbrene, glabrone, shinpterocarpin, licoisoflavonesA and B, formononetin, glyzarin, kumatakenin (Williamson, 2003). More recently, hispaglabridin A, hispaglabridin B, 4′-O-methylglabridin and 3′-hydroxy-4′-O-methylglabridin (Haraguchi, 2001) and glabroisoflavanone A and Bglabroiso-flavanone B (Kinoshita *et al.,* 2005; Rowe, *et al.,* 2015) have been found.

Saponins: Licorice root contains triterpenoidsaponins (4–20%), mostly glycyrrhizin, a mixture of potassium and calciumsalts of glycyrrhizic acid (also known as glycyrrhizinic acid, and a glycoside of glycyrrhetinic acid) which is 50 times as sweet as sugar (Blumenthal *et al.* 2000).

Coumarins: Coumarins present in *G. glabra* include liqcoumarin, glabrocoumarone A and B, herniarin, umbelliferone, glycyrin (Williamson, 2003; Tan, *et al.* 2015), glycocoumarin, licofuranocoumarin, licopyranocoumarin (Haraguchi, 2001) and glabrocoumarin (Kinoshita *et al.,* 2005; Park *et al.* 2015).

Stilbenoids: Four new dihydrostilbenes, dihydro-3,5-dihydroxy-4′-acetoxy-5′-isopentenylstilbene, dihydro-3,3′,4′-trihydroxy-5-O-isopentenyl-6-isopentenylstilbene, dihydro-3,5,3′-trihydroxy-4′-methoxystilbene and dihy. *glabra* grown in Sicily (Biondi *et al.,* 2005; Dreux *et al.* 2015).

Miscellaneous compounds: *G. glabra* extract also contains fatty acids (C2–C16) and phenols (phenol, guaiacol), together with common saturated linear γ-lactones (C6–C14). A series of new 4-methyl-γ-lactones and 4-ethyl-γ-lactones in trace amounts has also been found (Näf and Jaquier, 2006). Asparagines, glucose, sucrose, starch, polysaccharides (arabinogalactants), sterols (β-sitosterol, dihydrostigmasterol) are also present (Hayashi *et al.,* 1998; Blumenthal *et al.,* 2000, Brumbaugh, *et al.* 2015).

Glycyrrhizin, or glycyrrhizic acid,: It is the chief sweet-tasting constituent of *Glycyrrhiza glabra* (liquorice) root. It has also been given intravenously in Japan as a treatment for hepatitis C and is used as an

emusifier and gel-forming agent in foodstuff and cosmetics. Its aglycone is enoxolone.

This acid mechanism of action inhibits the enzyme 11beta-hydroxysteroid dehydrogenase, which likely contribute to its anti-inflammatory and mineral corticoid activity. It has broad-spectrum antiviral activity in vitro against.

Materials and Methods

Culture Media and solution prepared Laboratory

Blood agar: This medium was prepared according to the instruction of manufacturer company, sterilized by autoclaving at 121ºC for 15min. after cooled to 50ºC, 5ml of sterile defibrinated human blood was added for each 100ml of the medium, mixing well then poured in a sterile Petri-dish. This medium is suitable for the isolation and cultivation of bacteria and for the detection of haemolytic activity and the kind of haemolysis

Catalase reagent: This reagent was prepared as 3% of hydrogen peroxide (H_2O_2).

McFarland standard solution

McFarland's standard solution 0.5 it is the turbidity standard solution which is the most widely used method of inoculums preparation or standardization, this solution has specific optical density to provide a turbidity comparable to that of bacterial suspension containing 1.5×10^8 CFU/ml. This solution was prepared as the following:

Solution (A): 1g of barium chloride ($BaCl_2$) was dissolved in 100ml of distilled water.

Solution (B): 1ml of concentrated sulphuric acid (H_2SO_4) was added and the volume was completed to 100ml by distilled water, 0.5ml of solution (A) was added to 99.5ml of solution (B) and stored in dark bottle until used.

Method of Plant Extraction and Antimicrobial activity

Plant Extraction: The following step are conducted based on Harbome (1973) for detecting the phenolic compounds for the studied species:

1- The leaves and roots of the flowering plants have been taken from *Glycyrrhiza glabra* and have been grinned by using electric grinned.
2- 50 gm from the specimen are weight, and 500 ml of ethyl alcohol (70%) is added, left in the room temperature for 24–48 hour.
3- Filtration has been done by using ederol filter paper (medium pores filtering).
4- The extract has been concentrated to a suitable volume to discard the ethyl alcohol by using air dryer in a moderate temperature.
5- As many as the extract volume, petroleum ether (with boiling point 40-60°C) has been added to the extract, the mixture was shaken well, then has been put in separating funnel, and has been left till separate in to two obvious layer, in this point discarding take place from a large part of the chlorophyll that dissolved in the petroleum ether and floated above because it has less density than the aqueous extract of the phenolic compound and then withdrawn from funnel bottom.
6- The extract of the phenolic compounds were concentrated to half of its volume by leaving it in dry air current

Antimicrobial activity

Sample collection: The samples was collection from patients reside in Baqubah hospital during period time from 15/11/2013

to 30/11/2013 the sample include different swabs from Throat and Vaginal and burns and wounds.

The isolation: The collected specimens were inoculated on the blood agar which prepared as mentioned in (2-1-3-2-2), incubated at 37ºC for 24 hours. The isolates were examined for theirshape, size, colour, pigments, and haemolytic activity. Then transferred and streaked on mannitol salt agar (2-1-3-1) which considered as selective and differential medium for the isolation, purification and identification of isolates, and for detecting the ability of each isolate to ferment mannitol. All plates were incubated at 37ºC for 24 hours then a single pure isolated colony was transferred to Nutrient agar medium (2-1-3-1) for the preservation and to carry out other biochemical tests that confirmed the identification of isolates.

Identification

Gram stain: All the bacterial isolates were examined under light microscope after stained by Gram stain (2-1-4-1), to detect their response to the stain, sizes, shapes, and arrangement of the cells.

Catalase test Single isolated colony of bacteria was removed from culture plate by wooden applicator stick and placed on a glass slide then 1-2 drops of 3% H_2O_2were mixed with the cells on the slide. The appearance of gas bubbles indicates a positive test.

Procedure of antimicrobial activity: The antibacterial activity of *Glycyrrhiza glabra* was determined by agar well-diffusion method 0.1 ml of 12-16 hrs incubated cultures of bacterial species were mixed in molten Mueller Hinton Agar medium and poured in pre-sterilized petri plates. A cork borer (6 mm diameter) used to punch wells in solidified medium and filled with extracts of 100 µl and used the water as Negative control The efficacy of extracts against bacteria was compared with the broad spectrum antibiotic Gentamicin and Keflex (positive control) distill water (negative control). The plates were incubated at 37°C for 24 hrs in BOD incubator and the diameter of the zone of inhibition was measured in mille meter. Each sample was assayed in triplicate and the mean values were observed. The antibacterial activity was interpreted from the size of the diameter of zone of inhibition measured to the nearest millimetre (mm) as observed from the clear zones surrounding the wells

Results and Discussion

Isolation

Collected samples from Baquba hospital which included swabs from burns and wounds and throat and urine. The specimens were cultured on Blood agar at 37 c for 24 hrs the transported to Mannitol Salt Agar as the G+ve isolate were selective differential *Staphylococcus* species, selective because it contains 7.5% NaCl Differential because it contains Mannitol Sugar and phenol red as PH indicator which differentiated between mannitol fermented *Staphylcocci* e.g. *S. aureus* and mannitol non-fermented e.g. *S. epidermdis. Streptococcus pneumoniae* grow on blood agar for type haemolysis

Bacilli when grown on blood agar plates, the organisms produce non hemolytic gray to white round colonies with a rough texture and have a "cut glass" appearance in transmitted light.

The G-ve bacteria isolate transported to MacConkry agar selective and differential media selective because contain the bile salts and crystal violet that inhibition the growth of gram-positive and some

Fastidious gram-negative bacteria Differential because contain lactose as source of carbon and pH indicator neutral red that differential between lactose fermenter and lactose non-fermenter (Benson, 2001; Peres, *et al.*, 2015).

Strepto.neumonia growth on blood agar because the growth of *Streptococci* tend to be poor on solid media or in broth unless enriched with blood or nutritive tissue fluid (Benson, 2001).

Identification

Only isolate Staph appear has ability to grow on the Mannitol salt agar which considered selective and differential media for genus *Staphylococcus*. The colonies appeared round, smooth, raised, mucoid and glistening. Consequently, the isolates belong to the genus *Staphylococcus*. Some isolates had the ability to ferment mannitol and form large golden colonies surrounded by wide yellow zones and turned the color of the medium from pink to yellow, others were non mannitol-fermenter and appeared as small white colonies and no color change was observed on the medium. Microscopic examination was applied to the all 4 isolates after staining by Gram stain and the staph appeared as Gram-positive cocci arranged in grape-like irregular clusters. For further identification, the catalase test was performed which Staph bacteria isolates gave positive results (Fig. 2).

Streptococcus pneumoniae grow on blood agar because the growth of streptococci tend to be poor on solid media or in broth unless enriched with blood or nutritive tissue fluid after 24 hrs incubation the colonies are small 1mm in diameter semitransparent and surrounded by alpha-hemolysis the colonies first are dome shaped and later become draughtsman colonies. Microscope examination applied after staining by Gram stain appear Gram positive diplococcic often lancet shaped or arranged in chains possessing capsule the catalase was performed *Strepto. pneumoniae* gave negative results (Fig. 3).

Pseudomonas aeruginosa grow on MacConkey media because selective and differential media for Enterobacteriaceae family *Pseudomonas aeruginosa* forms smooth round colonies with pale colonies non lactose fermenter. When stained with Gram stain gave negative result appeared under microscope examination rod shaped and has polar flagella also catalase test has performed and gave positive results (Fig. 4).

Klebsiella aerogense grow on MacConkey media because selective and differential media the colonies appeared on this media are large and mucoid and pink when stained with Gram stain gave negative results and appeared bacilli shaped also catalase test has preformed and gave negative results.

Proteus grow on Macconkey media because selective and differential media the colonies appeared swarming on this medi, and grow on blood agar to test swarming and haemolysis (Fig. 6).

E.coli: Eosin methylene blue agar (EMB) (Selective and Differential media). Selective because contains: the aniline dyes (eosin and methylene blue) that inhibited the growth of Gram-positive bacteria and some fastidious Gram-negative bacteria. Differential because contains: the aniline dye also combines to form a precipitate at acid pH (appearing as a metallic green sheen) thus serving as indicator of acid production from lactose (Fig. 7).

Bacilli When grown on blood agar plates, the organisms produce non hemolytic gray to white round colonies with a rough texture

and have a "cut glass" appearance in transmitted light (Fig. 8).

The results of seven bacteria species have been checked. The obtained results present high sensitivity of those seven bacteria species to the extract (root and leave of *Glycyrrhiza glabra* extract). The obtained results are presented as in the following tables 1 and 2.

When we compare the above results with standards of antibiotics, Gyntamycin and Keflex, (Table 3 and Table 4), all isolations of bacteria is inhibited. Diyala University, the highest inhibition zone was only 16mm compared with our results of highest inhibition zone 25mm for same Streptococcus. In this study, the extract effects on both gram negative and gram positive bacteria because of containing more chemical compound that have bactericidal effect (Lynskey *et al.*, 2015).

It can be concluded that all objectives of the study have been achieved. This study presented new extract of *Glycyrrhiza glabra.* The obtained results were very encouraged, where they are compared with the standards.

Recommendations for Other study

This study provides a sufficient approach for extract of *Glycyrrhiza glabra* but a few issues addressed require further research. For further development related to this research, future studies are suggested as follow:

1. Compare the effect of this extract with other antibiotics.
2. Study the effect other plant extract on this pathogenic bacteria for example:
Nigeria sativa, Oleaeuropaea, Mentha putegium.
3. Study the effect of ethanolic extract of this plant with other isolate bacteria for example: *Mycobacterium tuberculosis, Salmonella, Shigella, Vibro cholera, Aeromonas hydrophila.*
4. By water extraction plant.

Table.1 Leaves extract

Leave Extract			
Bacterial genus	**12.5%**	**25%**	**50%**
Pseudomonas	10	13.5	16
Proteus	13	14	16
Bacillus	14	16	16
Staphylococcus	10	10.5	15
Klebsiella	10	11.5	18
Streptococcus	14	15	18
E. coli	8	10	11

Table.2 Root extract

Roots Extraction			
Bacterial genus	12.5%	25%	50%
Pseudomonas	11	14	18
Proteus	12	13.5	18
Bacillus	11	18	19
Staphylococcus	14	15	19
Klebsiella	13	14	23
Streptococcus	-----	23	25
E. coli	10	13	20

Table.3 Antibiotic (Gyntamycin)

Gyntamycin			
Bacterial genus	**12.5%**	**25%**	**50%**
Pseudomonas	20	20	23
Proteus	15	17	23
Bacillus	22	25	30
Staphylococcus	18	20	35
Klebsiella	_	_	_
Streptococcus	_	_	_
E. coli	21	21	29

Table.4 Antibiotic (Keflex)

KEFLEX			
Bacterial genus	**12.5%**	**25%**	**50%**
Pseudomonas	_	_	_
Proteus	23	24	26
Bacillus	38	41	45
Staphylococcus	25	35	35
Klebsiella	18	21	24
Streptococcus	22	25	32
E. coli	17	22	25

Fig.1 The adult plant shows flowers, fruits and the most important is rhizomes, which consists the bioactive components

Fig.2 *Staph. aureus* bacteria, isolates gave positive results

Fig.3 *Strepto. pneumoniae* gave negative results

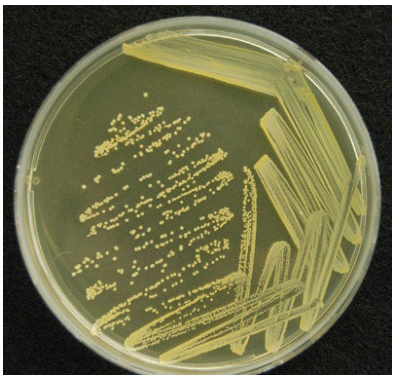
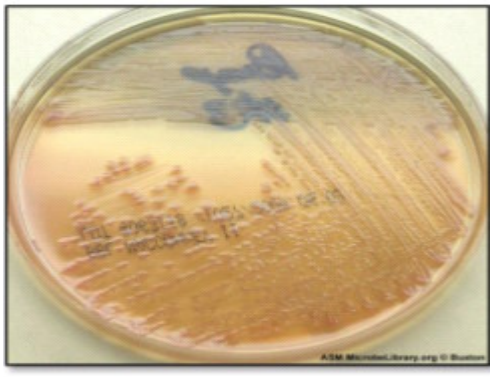


Fig.4 Growth of *Pseudomonas aeruginosa*, gave positive results



P. aeruginosa on MacConkey agar

Fig.5 growth of *Klebieslla aerogense*, gave negative results

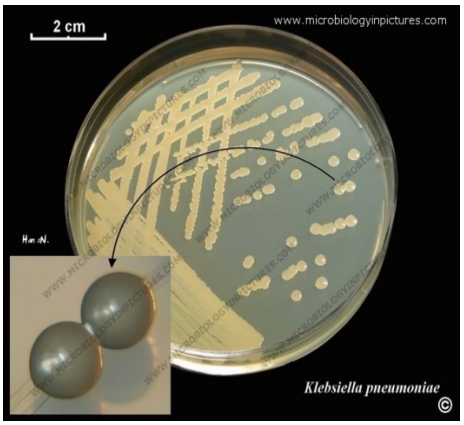
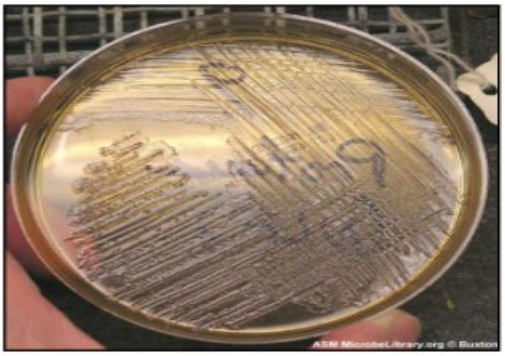


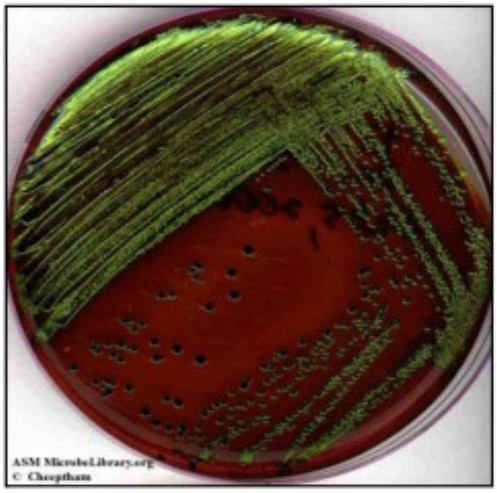
Fig.6 *Proteus spp*, grow on lactose and on Blood agars



Proteus spp. on MacConkey agar (Lactose non-fermenter)

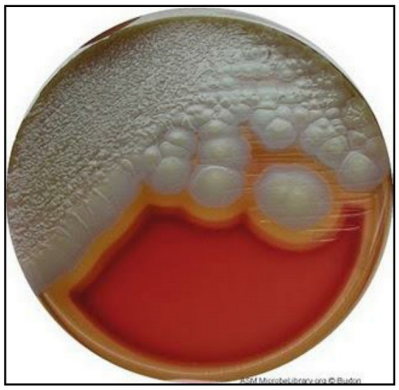
Proteus spp. on Blood agar (swarming)

Fig.7 *E. coli* grown on blue agar



***E. coli* on Eosin methylene blue agar (EMB) (Metallic green sheen)**

Fig.8 *B. cereus* grown on blood agar



Colonies of on blood agar *B. cereus*

References

Allison, C., Coleman, N., Jones, P.L., Hughes, C. 1992. Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect. Immun.*, 60: 4740–4746**.**

Anti-HIV, 2004. Aromatic compounds from higher plants. In: Tringali, C. (Ed.), Bioactive compounds from natural sources: isolation, characterization and biological properties. Taylor and Francis, New York. 325 Pp.

Ash, M. 1997. *Staphylococcus aureus* and Staphylococcal Enterotoxins. In: Hocking, A.D., Arnold, G., Jenson, I., Newton, K., Sutherland, P. (Eds), Food borne microorganisms of public health importance, 5th edn, Pp. 313–332. AIFST (NSW Branch), Sydney, Australia.

Ayashi, H., Hiraoka, N., Ikeshiro, Y., Yamamoto, H., Yoshikawa, T. 1998. Seasonal variation of glycyrrhizin and isoliquiritigenin glycosides in the root of *Glycyrrhiza glabra* L. *Biol. Pharm. Bull.,* 21: 987–989.

Becker, H., Schaller, G., von Wiese, W., Terplan, G. 1994. Bacillus cereus in infant foods and dried milk products. *Int. J. Food Microbiol.*, 23: 1–15.

Biondi, D.M., Rocco, C., Ruberto, G. 2005. Dihydrostilbene derivatives from *Glycyrrhizin glabra* leaves. *J. Nat Prod.,* 68: 1099–1102.

Blumenthal, M., Goldberg, A., Brinckmann, J. 2000. Herbal medicine, expanded commission E Monographs. *Am. Bot. Council, Newton*, Pp. 233–236.

Brumbaugh, A.R., Smith, S.N., Himple, S.D 2015. Blocking yersiniabactin import attenuates extra intestinal pathogenesis *Escherichia coli* in cystitis and Pyelonephritis and represents a novel target to prevent urinary tract infection. *Am. Soc. Microbiol. Infect. Immun.,* 83: 1443–1450.

Chase-Topping, M., Gally, D., Low, C., Matthews, L., Woolhouse, M. 2008. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat. Rev. Microbiol*., 6(12): 904–12.

Dreux, N., Cendra, M.M., Darfeuille-Michaud, A., Barnich, N. 2015. Ribonucleotidereducasenrdr as a noval regulator for mortality and chemotaxis during adherent-invasive *Escherichia coli* infection. *Am. Soc. Microbiol. Infect. Immun.,* 83: 1305–1317.

Haraguchi, H. 2001. Antioxidative plant constituents. In: Tringali, C. (Ed.), Bioactive compounds from natural sources: isolation, characterization and biological properties. Taylor and Francis, New York. Pp. 348–352.

Harkness, J.E., Wagner, J.E. 1983. The biology and medicine of rabbits and rodents, 2nd edn. Lea and Febiger, Philadelphia.

Hatano, T., Aga, Y., Shintani, Y., Ito, H., Okuda, T., Yoshida, T. 2000. Minor flavonoids from licorice. *Phytochemistry*, 55: 959–963.

Jonsson, R., Struve, C., Boisen, N., Mateiu, R.V. 2015. Novel aggregative adherence fimbria variant of enter aggregative *Escherichia coli. Am. Soc. Microbiol. Infect. Immun.,* 83: 1396–1405.

Kinoshita, T., Tamura, Y., Mizutani, K. 2005. The isolation and structure elucidation of minor is flavonoids from licorice of *Glycyrrhiza glabra* origin. *Chem. Pharm. Bull.,* 53: 847–849.

Li, J.R., Wang, Y.Q., Deng, Z.Z. 2005. Two new compounds from Glycyrrhizaglabra. *J. Asian Nat. Prod. Res.,* 7: 677–680.

Lynskey, N.N., Turnur, C.E., Heng, L.S. 2015. A. Truncation in the regulator RocA underlies heightened capsule expression in serotype M3 group A *Streptococci*. *Am. Soc. Microbiol. Infect. Immun.,* 83: 1732–1733.

Nomura, T., Fukai, T., Akiyama, T. 2002. Chemistry of phenolic compounds of licorice (*Glycyrrhiza*species) and their estrogenic and cytotoxic activities. *Pure Appl. Chem.,* 74: 1199–1206.

Ody, P. 2000. The complete guide medicinal herbal. Dorling Kindersley, London. 75 Pp.

Park, J.U., Kim, J.W., Moon, B.Y. 2015. Characterization of a novel two-component regulatory system, hptRS, the regulator for the hexose phosphate transport system in

Staphylococcus aurous. Am. Soc. Microbiol. Infect. Immun., 83: 1620–1628.

Peres, A.G., Stegen, C., Li, J., Xu, A.Q. 2015. Uncoupling of pro- and anti-inflammatory properties *Staphylococcus aurous. Am. Soc. Microbiol. Infect. Immun.,* 83: 1587–1597.

Poirel, L., Héritier, C., Tolun, V., Nordmann, P. 2004. Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. *Antimicrob. Agents Chemosphere,* 48: 15–22. doi: 10.1128/AAC.48.1.15-22.2004.

Rowe, H.M., Hanson, B.R., Runft, D.L., Lin, Q. 2015. Modification of the CpsA protein reveals a role in alteration of the *Streptococcus agalactiae* cell envelope. *Am. Soc. Microbiol. Infect. Immun.,* 83: 1497–1506.

Tan, A., Petty, N.K., Hocking, D., Bennett-Wood, V. 2015. Evolutionary adaptation of an AraC-like regulatory protein in *Citrbacter rodentium* and *Escherichia* species. *Am. Soc. Microbiol. Infect. Immun.,* 83: 1384–1395.

Villegas, M.V., Lolans, K., Del Rosario Olivera, M., Suarez, C.J., Correa, A., Queenan, A.M., Quinn, J.P. 2006. First detection of metallo-b-lactamase VIM-2 in Pseudomonas aeruginosa isolates from Colombia. *Antimicrob. Agents Chemosphere,* 50: 226–229.

Williamson, E.M. 2003. Liquorice. In: C.W. Daniels (Ed.), Potter's cyclopedia of herbal medicines. Saffron Walden, UK. Pp. 269–271.