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Isolation and Purification *Glycyrrhizic acid* and Determination of the Biological Activate Against Microbial Pathogens

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Abstract

Background: The World Health Organization estimates that 80% of the world's population uses plant extracts or their active components in traditional therapies. Recently, there has been a lot of interest in the quest for new antioxidant and antibacterial plant-based medications. Additionally, *Glycyrrhiza glabra* is a popular food flavoring and sweetening ingredient.

Methods: This work was to isolate and purify licorice root's glycyrrhizin acid (GA) and investigate its antimicrobial properties against *S. aureus* and *K. pneumoniae* were isolated from urine, blood, wounds, ear water, sewage, soil, and water, totaling 12 isolates of each kind, Analytical method for the measurement of Glycyrrhizic acid from the root extract that has been suitably supported by a mass spectroscopic analysis.

Results: The result showed the effects of GA with different concentrations on *S. aureus* and *K. pneumoniae*. Obviously, the MIC range value was in concentration between 18.75 to 37.5 % for *S. aureus* isolates, and 4.68 to 75 for *K. pneumoniae* isolates. The result indicated that GA reduced the metabolic activity of cells in biofilm on *S. aureus* and *K. pneumoniae* with an inhibition percentage

Conclusion: GA may have an effect on cell membrane permeability, biofilm formation, and efflux activity, which inhibits bacterial growth.

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Introduction

The World Health Organization estimates that 80% of the world's population uses plant extracts or their active components in traditional therapies, since ancient times, plant sources of therapeutic chemicals have continued to play important role in preserving human health, Modern clinical medications with a greater than 50% natural product component [1]. The previous years, there has been a surge in interest in the study of plant-based natural medicines for the development of novel antioxidant and antimicrobial agents. In addition, the pharmaceutical and food industries are increasingly asking for plants that are currently used in conventional medicine, the licorice root, *Glycyrrhiza glabra* is a member of the *Fabaceae* family and is frequently used in food as a sweetener and flavor, it has also been suggested for use in a number of clinical procedures [2]. Although clinical and experimental studies indicate that licorice root has a number of other useful pharmacological properties, including anti-inflammatory, antiviral, antimicrobial, anti-oxidative, anti-cancer activities, immune modulator, hepatic protective, and cardio protective effects, it is primarily used as a traditional medicine to treat peptic ulcer, hepatitis, and pulmonary and skin diseases, There are numerous substances found in licorice that have been isolated, including triterpene saponins, flavonoids, isoflavonoids, and chalcones, while glycyrrhizic acid (GA) is typically thought to be the most physiologically active substance [3]. The therapeutic benefits of licorice are due to two of its constituents, glycone and aglycone, GA is a significant molecule that gives licorice its pharmacological and biological activities, GA is a triterpenoid disaccharide glycoside that has been shown to have anti-inflammatory, anti-diabetic, anti-allergic, and many other properties, GA is a yellow to orange powder with a molecular weight of 822.93 g/mol, the current investigation's goal was to separate GA from licorice roots, the physicochemical properties were used to validate the extracted GA (High-performance liquid chromatography) [4]. One molecule of the triterpenoid aglycone glycyrrhizic acid, which appears in 18- α or 18- β stereoisomeric forms, is conjugated to two molecules of glucuronic acid to generate the saponin complex known as GA, Commercial licorice products are frequently made from licorice root extracts GA, sometimes referred to as glycyrrhizin or glycyrrhizinate, is thought to be the main active component in licorice root extract and makes up 10% to 25% of the root extraction [5]. Licorice is used medicinally to treat respiratory conditions like dry cough and asthma, Licorice was found to induce apoptosis in melanoma and gastric cancer cells, and GA also have anti carcinogenic, antidote, and antioxidant

properties[6]. Alkaloids, glycosides, carbohydrates, starches, phenolic compounds, flavonoids, proteins, pectin, mucilage, saponins, lipids, tannins, sterols, and steroids were found in the *G. glabra* root during the phytochemical screening process, It shown pharmacological activities such as memory improvement, antidepressant, antibacterial, anticancer, antioxidant, and many more [7]. High-performance liquid chromatography, often known as HPLC or high-pressure liquid chromatography, is a particular type of column chromatography that is frequently used in biochemistry and analysis to separate chemicals [8]. The primary components of HPLC are a stationary phase (column holding packing material), a pump to drive the mobile phase(s) through the column, and a detector to show the molecules' retention times. Retention time is influenced by the interactions of the stationary phase, the molecules under investigation, and the utilized solvent(s) [9]. The analysis sample is added in a tiny amount to the mobile phase stream and is slowed down by particular chemical or physical interactions with the stationary phase, the kind of analyte and the makeup of the stationary and mobile phases both affect the amount of retardation; retention time refers to the time at which a particular analyte elutes (comes out of the end of the column); popular solvents employed include any miscible mixtures of water or organic liquids (methanol and acetonitrile are the most frequently used) , Gradient elution is a separation method applied to alter the composition of the mobile phase during the analysis [10]. Multiple techniques have shown that GA has strong inhibitory effects on both Gram-positive and Gram-negative bacterial activity; the majority of GA's pharmacological activities also involve antimicrobial activity, which prevents bacterial infection by lowering gene expression, impeding bacterial development, and lessening the synthesis of microbial toxins [11].

As a commensal bacterium in humans, *Staphylococcus aureus* can also be harmful, *s. aureus* is predominantly an opportunistic infectious agent and is responsible for a number of suppurative illnesses, food poisoning, pneumonia, and sepsis [12]. Is one of the typical flora on the skin surface, the mucosal surfaces of the respiratory tract, the upper part of the digestive system, and the urogenital tract, *Staphylococcus* is a Gram-positive bacteria genus that belongs to the Bacillus order's *Staphylococcaceae* family [13]. One of *Staphylococcus aureus* defense mechanisms is its ability to build biofilms. Bacteria submerged in biofilms are sometimes challenging to eliminate using conventional antibiotic regimens and are intrinsically resistant to host immune responses [14].

Additionally, *Klebsiella pneumoniae* contributes significantly to the transfer of environmental bacteria's

antimicrobial resistance genes to clinically significant bacteria [15]. A Gram-negative encapsulated bacterium called *K. pneumoniae* thrives in water, soil, and animal mucous membranes. *K. pneumoniae* typically colonizes the oropharynx and gastrointestinal tract in humans. From there, it can quickly spread to other tissues and the bloodstream to cause infections like bacteremia, septicemia, surgical site infections, urinary tract infections, ventilator-associated pneumonia, and pneumonias that are hospital acquired. Additionally, it helps explain why opportunistic infections are so common in those with immune system problems like diabetes or bladder neuropathy [16, 17]. Another characteristic of *K. pneumoniae* that is well known is its capacity to create biofilms, which are bacterial populations embedded in an extracellular matrix, proteins, exopolysaccharides, DNA, and lipopeptides make up this matrix [18]. The World Health Organization (WHO) identified antibiotic resistance as one of the three global health crises, citing a variety of mechanisms of antimicrobial resistance that may adversely affect therapeutic outcomes [19]. This study aimed to identify, and isolation of GA and investigation the effect of GA extract on *S. aureus* and *K. pneumoniae* and evaluate the biofilm effect.

Methods

Collection of Plant Samples

The roots of *G. glabra* were purchased from the local herbal merchandise, in Baghdad, Iraq and the plant has been diagnosed in the herbarium of the College of Science – Department of Biology. They were air-dried, ground to powder and stored overnight at 4°C.

Preparation of GA Extract

Extraction using a water bath apparatus at 50°C for 4 hours, 250 g of crushed *G. glabra* was weighed and added to 500 ml of 30% ethanol. After cooling with continuous slow mixing, the mixture was filtered in a rotary evaporator at 60°C until a thick solution was obtained; the solution was then dried in a vacuum evaporator [20]. After that, a powder was obtained and macerated in acetone and diluted nitric acid for two hours, after filtering the contents, 20 ml more of acetone was added to the marc and gently warmed, filtrate was produced when the contents were filtered; A suitable amount of diluted ammonia solution was added to this filtrate in order to finish the precipitation of ammonium glycyrrhizinate, the precipitate was gathered, rinsed with acetone for five milliliters, dried, and collected [4]. The resulting deposit was dissolved in DMSO to prepare the doses.

HPLC Analysis of GA

A method for estimating the amount of GA in an extract once a mass spectrometric analysis has adequately supported it. However, numerous chromatographic estimation techniques have been published in which an isocratic elution is followed by a UV detector monitoring the eluent at 254 nm. A C18 column is employed with an aqueous phase containing either an acid modifier like acetic acid or phosphoric acid or a mobile phase made of either methanol or acetonitrile, an organic component [21].

Preparation of Different Concentrations of Glycyrrhizic Acid

Three grams of the dried extract were mixed with five milliliters of DMSO (dimethyl sulfoxide) to make stock solutions. Following that, various diluent concentrations (300, 150, 75, 37.5, 18.75, 9.37, and 4.68 mg mL⁻¹) were used.

Selection of the Isolates

Twelve isolates of *S. aureus* and *K. pneumoniae* from urine, blood, wounds, ear water, sewage, soil, and water were used in this study. By utilizing the Kirby- Bauer disc diffusion method, the isolates revealed enhanced resistance to widely used antibiotics. According to Bergey's Manual of Systematic Bacteriology, the bacteria were identified using common microbiological techniques (Gram staining, colony morphology, catalase test, cytochrome oxidase reaction, motility, and other biochemical tests)[22].

Antibiotic Susceptibility Test

4-5 colonies of bacterial isolates were taken from an overnight culture plate and suspended in 5ml of sterile normal saline until the turbidity was about similar to the McFarland No. 0.5 turbidity standard [23]. Ten minutes later, using sterile forceps, Mueller Hinton plates were covered with the antimicrobial disc Amoxicillin, Ampicillin, Cefotaxime, Methicillin, and Tetracycline. For 18 to 24 hours, the plates were incubated at 37°C. After incubation, the plates were checked to see if there was an area surrounding the antimicrobial discs that inhibited bacterial growth, using a metric ruler and the Clinical and Laboratory Standards Institute's standard inhibition zone as a reference, the diameter of the zone of inhibition was determined in millimeters [24].

Determination of minimum inhibitory concentration (MIC)

MIC of GA extracts was estimated by microdilution method in sterile 96-well microtiter plates according to the procedure described previously, different plant extracts concentrations (300, 150, 75, 37.5, 18.75, 9.37, 4.68 and 2.34 µg/ml) (W/V) were prepared to contain bacterial cells comparable to McFarland standard no.

0.5 in a final volume of 200 µl, after 24 h at 37°C, the MIC of each sample was determined. The MIC is regarded as the lowest antimicrobial concentration that, after a 24-hour incubation period, will prevent a bacterium from showing detectable growth [25].

Biofilm Formation Assays by using Tissue Culture Plate (TCP) Method

Hassan *et al.*, described this quantitative test. Regarded as the most effective technique for detecting biofilms [26]. In 10 ml of trypticase soy broth with 1% glucose w/v, isolated organisms from freshly prepared agar plates were inoculation; For 24 hours, broths were incubated at 37°C. Following a 1:100 dilution with fresh media, the culture was injected into each well of a sterile 96-well flat-bottom polystyrene tissue culture plate, for 24 hours, the plate was incubated at 37°C, following incubation; each well's contents were gently tapped out. Once, sterilized distilled water was used to clean the wells; This eliminated microorganisms that were floating around, (0.1%) w/v crystal violet was used to stain the bacterial biofilm adhered to the wells. Using distilled water, extra stain was removed, and the plates were kept to dry. Using a micro-ELISA auto reader at a wavelength of 630 nm, the optical density (OD) of stained adherent biofilm was measured. The experiment was performed in triplicate and repeated three times [27].

The Effect of GA on Biofilm Formation

Biofilm formation assays were performed using 96_ well microtiter plate, based on the protocol with some modification; TSB + 1% w/v glucose was added to the cultured *S. aureus* and *K. pneumoniae* after it had been cultivated in trypticase soy broth for an overnight period, each microtiter plate well was filled with 100 µl of medium and 100 µl of glycyrrhizic acid, each concentration for every Glycyrrhizic acid tested was assayed triplicate, the plate then incubated at 37°C for 24 hrs.; Shaking the dish over a garbage tray loaded with sterile distilled water eliminated the planktonic bacteria, then, each well received 0.1% w/v crystal violet solution, and the plate was allowed to stain for 10 minutes at room temperature, the plate was then placed in a water pan to drain the crystal violet solution; The plate was then turned upside down, placed on paper towels, and allowed to dry by air, the dye was then solubilized by treating the stained wells with 95% v/v ethanol for 10 min at room temperature [28]. Each well's bacterial solution was well mixed before having its optical density measured at 630 nm in a microplate reader [29, 8]. The following formula was used to determine how much biofilm development was inhibited-mediatedly reduced [30].

$$\% \text{ of biofilm inhibition} = \frac{\text{O.D. of control} - \text{O.D. of treatment}}{\text{O.D. of control}} \times 100$$

Statistical Analysis

One-way analysis (ANOVA) tests were used for data analysis in SPSS version 25, the significant difference between means was compared using the least significant difference (LSD), and differences were deemed significant when $P \leq 0.05$ [31].

Results

Identification of GA by HPLC

For the identification of the main bioactive of licorice like GA, an HPLC method was achieved by comparison of retention time of *GA extract* with standard. The result in figure 1 (A) showed the peak of standard and retention time (8.00), area (100%), and in figure 1 (B) showed peak of GA extract. The result showed the appearance GA compound in licorice extract (90%) of the total area and concentration (101 mg/gm). The concentration of GA extract calculated by the following formula:

$$C_{\text{sample}} = C_{\text{standard}} * \frac{A_{\text{sample}}}{A_{\text{standard}}} * D.F / wt_{\text{gm}}$$

C: concentration, A: area, D.F : dilution factor, wt: weight

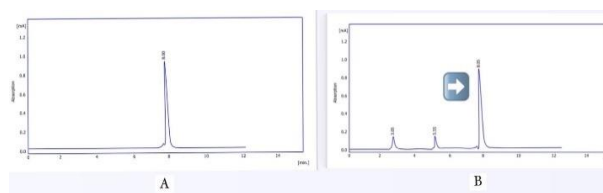


Figure 1: (A) Representative HPLC of GA standard. (B) representative HPLC of GA extract. Absorption of GA is represented on the Y-axis and X-axis is represented retention time.

Staphylococcus aureus and *K. pneumoniae* isolates' antibiotic susceptibility

Bacteria are more resilient to certain antimicrobial treatments when they are in biofilm form. In accordance with CLSI [35] recommendations, results of susceptibility tests for isolates of *S. aureus* and *K. pneumoniae* to five different antibiotics are summarized in Tables 1 and 2. Due to their method of action, which prevents the production of cell walls and results in the release of bacterial cell DNA into the environment, these antibiotics were used in this investigation [36]. Table 1 and Table 2 indicate that all isolates were resistant to Ciprofloxacin and Ceftriaxone, all isolates of *K. pneumoniae*, and 5 isolates of *S. aureus* (S2, S7, S8, S10, S12) resistant to Cefoxitin and other isolate sensitive. All isolate sensitive to Ampicillin-sulbactam and Azithromycin.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) value is essential for assessing an antibacterial agent's

effectiveness. A low MIC value could mean that a bioactive substance is highly effective or that microorganisms are unable to become resistant to it. Figure 2 represents the effects of GA concentrations with varying MIC values on *S. aureus* and *K. pneumoniae*. Obviously, For *S. aureus* isolates, the MIC concentration ranged from 18.75 to 37.5%, with the S1, S9 isolate having the most effect and other isolates having the lowest effect. And 4.68 to 75 for *K. pneumoniae* isolate, the highest effect was observed on *K. pneumoniae* in S7, and the lowest effect was observed in S12.

| <i>S. aureus</i> isolates | Ampicillin-sulbactam | Azithromycin | Cefoxitin | Ceftriaxone | Ciprofloxacin |
|---------------------------|----------------------|--------------|-----------|-------------|---------------|
| 1 | S | S | S | R | R |
| 2 | S | S | R | R | R |
| 3 | S | S | S | R | R |
| 4 | S | S | S | R | R |
| 5 | S | S | S | R | R |
| 6 | S | S | S | R | R |
| 7 | S | S | R | R | R |
| 8 | S | S | R | R | R |
| 9 | S | S | S | R | R |
| 10 | S | S | R | R | R |
| 11 | S | S | S | R | R |
| 12 | S | S | R | R | R |

R=Resistant, S=Sensitive

Table 1: Antibiotic susceptibility of *S. aureus* isolates according to CLSI.

| <i>K. pneumoniae</i> isolates | Ampicillin-sulbactam | Azithromycin | Cefoxitin | Ceftriaxone | Ciprofloxacin |
|-------------------------------|----------------------|--------------|-----------|-------------|---------------|
| 1 | S | S | R | R | R |
| 2 | S | S | R | R | R |
| 3 | S | S | R | R | R |
| 4 | S | S | R | R | R |
| 5 | S | S | R | R | R |
| 6 | S | S | R | R | R |
| 7 | S | S | R | R | R |
| 8 | S | S | R | R | R |
| 9 | S | S | R | R | R |
| 10 | S | S | R | R | R |
| 11 | S | S | R | R | R |
| 12 | S | S | R | R | R |

R=Resistant, S=Sensitive

Table 2: Antibiotic susceptibility of *K. pneumoniae* isolates according to CLSI.

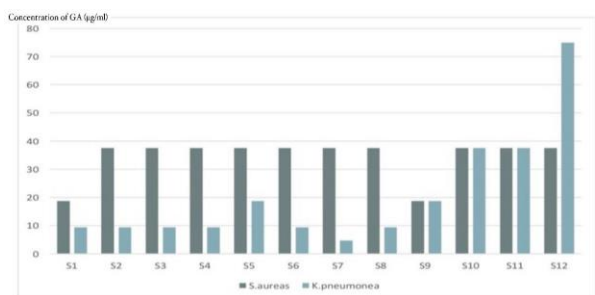


Figure 2: *S. aureus* and *K. pneumoniae*'s minimum inhibitory concentration (MIC) values (µg/ml). The Y-axis represented concentration of GA (Glycyrrhizic acid) and the challenge with bacterial isolate on the X-axis. Concentration of GA from 300 to 4.68 mg mL⁻¹, respectively.

Effect of GA extract on biofilm formation

Along with MIC studies, the effects of GA on existing bacterial biofilms were also examined. These included

S. aureus and *K. pneumoniae*. Due to the fact that biofilms naturally resist antibacterial therapy, based on the MIC data, the treatment concentrations were chosen. The result indicated that of GA extract reduced metabolic activity of cell in biofilm on *S. aureus* and *K. pneumoniae* showing an inhibition percentage, in comparison to the *S. aureus* and *K. pneumoniae* biofilm control groups that weren't treated with GA for 24 hours, there was a substantial decrease in absorbance A630. Figure 3 below shown that their signification differences between bacterial isolates and GA extract inhibit the formation of biofilm, the highest effect of GA in S2 isolate could inhibit 66% and lowest effect in S4 isolate could inhibit 34% of *S. aureus* biofilm. Moreover, the highest effect of GA in S6 isolate could inhibit 80% and lowest effect in S12 isolate could inhibit 54% of *K. pneumoniae* biofilm.

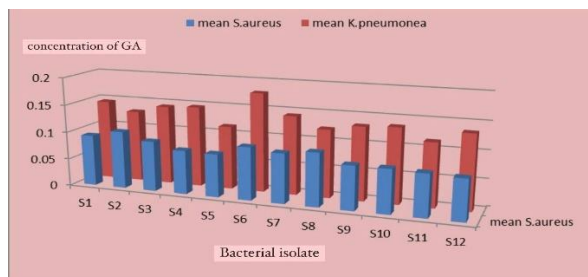


Figure 3: Effect of GA (µg/mL) on biofilm of *S. aureus* (blue) and *K. pneumoniae* (red). Concentration of GA is represented on the Y-axis (mean) and the challenge with bacterial isolate on the X-axis. GA: Glycyrrhizic acid.

Discussion

The results are in line with Rohinishree and Negi (2016), whereby they used 80% of GA as an external standard to detect GA in licorice extract using HPLC and a C18 column [32]. Liquiritin, liquiritigenin, and isoliquiritigenin are three flavonoids, and glycyrrhizin, 18-glycyrrhetic acid, 18-glycyrrhetic acid, and 18-glycyrrhetic acid methyl ester are four triterpenoids, all from licorice [33]. For the purpose of identifying the active ingredients in licorice extract, such as glycyrrhizin or glycyrrhizic acid, an upgraded high-performance liquid chromatographic technology with a photodiode-array detection system is used [34].

These findings concur with Gamal M. Hamad et al., findings. According to MIC results, ethanol extract of licorice root was effective against *K. pneumoniae*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella* spp., [37]. In this investigation, we showed that GA had antibacterial properties against *K. pneumoniae* and *S. aureus* isolates. These compounds exhibited strong antibacterial activities. Similar observation was reported by Kentaro Oyama *et al.*, GA displayed potent antibacterial action against a number of *S. aureus* strains, and this activity may have been influenced by

the blockage of a number of metabolic pathways for carbohydrates and amino acids [38]. Investigations were conducted on the antibacterial activity of (GA) against *S. aureus* and its impact on the production of *S. aureus* alpha-haemolysin (Hla) [39]. De Leon et al.'s subsequent research with *Bacillus subtilis*, another Gram-positive bacterium, demonstrated that triterpenoids disrupt cellular membranes as part of their mode of action [40]. Triterpenic acids show many pharmacological effects, among them an anti-inflammatory or an antitumor activity [41]. Glycyrrhizic acid, one of the proposed chemo preventive drugs, was used to inhibit arylamine *N*-acetyltransferase (NAT) activity in *Klebsiella pneumoniae* [42]. *B. pneumoniae*, *Pseudomonas fluorescens ATCC12296* and *EMCC1221* shown sensitivity to licorice ethanol extract [43]. They observed that using the agar diffusion method, licorice extracts (*Glycyrrhiza glabra*) showed a variety of antibacterial properties against both Gram-positive and Gram-negative bacteria. A microdilution technique and enzyme-linked immunosorbent test (ELISA) were used to determine the antibacterial activity of *Glycyrrhiza glabra* root extract against gram-positive and gram-negative bacteria, including *Salmonella enteritidis*, *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus* [44]. The treatment of MRSA biofilms with GA for 24 h caused a significant reduction in absorbance in comparison to the untreated MRSA biofilm control groups, according to Alan J. Wevaer et al., GA itself had no significant effect on biofilm viability, but it seems to be an effective biofilm disruptor, In addition, CFUs were considerably lower after GRA treatment, and these findings suggested that GRA therapy might improve biofilm dispersal [45]. Gram-positive bacteria are the only ones that triterpenes' antibacterial action has been shown to affect [40,46,47] including biofilm inhibition and reduction [48,49]. It inhibits the growth and generation of bacterial biofilms as well as the coaggregation of *P. gingivalis* and plaque bacteria, GA is one of a good candidate for a natural agent that reduces the onset and progression of periodontal disease. Additionally, the same effect was confirmed with supragingival plaque bacteria [50]. Additionally, *Pseudomonas aeruginosa* bacteria are prevented from growing by glycyrrhizic acid due to its effects on cell membrane permeability, biofilm formation, and efflux activity [51].

Author Contributions

All authors contributed equally to the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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