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Lana Yousif Mutalib
M.Sc. pharmacognosy, Pharmacy College, Hawler medical university, Erbil city, Iraq.

Sheila Mufeed Nuraddin
M.Sc. microbiology, Pharmacy College, Hawler medical university, Erbil city, Iraq.

Safaa Toma Hanna Aka
Ph.D. medical microbiology, Pharmacy College, Hawler medical university, Erbil city, Iraq.

Correspondence:
Lana Yousif Mutalib
Ms.c. pharmacognosy, Pharmacy College, Hawler medical university, Erbil city, Iraq.

Phytochemical screening, antibacterial and antibiofilm evaluation of *Lagenaria siceraria* fruit growing in Kurdistan Region\Iraq

Lana Yousif Mutalib, Sheila Mufeed Nuraddin, Safaa Toma Hanna Aka

Abstract
Medicinal plants considered to be safe with low cost drugs to consumers and a source for developing new bioactive compounds. Dried fruit of *Lagenaria siceraria* was extracted, using ultrasonic method. Qualitatively evaluation was performed for the presence of natural products. *In vitro* antibacterial activity estimated, using agar well diffusion method, a 96-flat well microtiter plate was used to determination of minimal inhibitory concentrations (MIC), while plate cultures conducted for determination of minimal bactericidal concentrations (MBC).Biofilm formation was evaluated using crystal violet assay and measurement the optical density (OD_{630}) by ELISA reader. A phytochemical investigation shows the presence of flavonoid, hydrolysable tannin, sterol, quinine, and phenols. Antibacterial activity was recorded for the organic fraction against *Streptococcus pneumoniae* at (MIC 2.5 mg/ml and MBC 5 mg/ml). Highest antibiofilm activity was shown for an organic fraction against *Streptococcus pneumoniae* and *Staphylococcus aureus* for aqueous fraction. Generally, organic fraction of *Lagenaria siceraria* is more active than aqueous fraction according to antibacterial and antibiofilm activity view point.

Keywords: Ultra sonic, antibiofilm, *Streptococcus pneumoniae*, Quinon,

1. Introduction
Herbal remedies are considered to be more safe and less side effects to the human health and body was a most popular idea all over the world. Moreover, researchers and scientists are seeking for the biological and potential therapeutic values in plants. Local herbalists, practitioner and ethnomedicine emphasized the abundance of food and dietary items for treatment of diseases [1]. Relatively small percentage 1-10% of plant species are used as a food in comparison to the huge number (250,000 - 500,000) of plant species used in ethnomedicine [2]. At present, there is a variety of bacterial pathogens, which are resistant to most of the conventional antibiotics [3]. Therefore, to overcome this life-threatening problem, continuous research to discover new antimicrobial agents from plant source, since the usage of both plant extracts or their corresponding phytochemical constituent for their antimicrobial effect have great importance in therapeutic treatment [4, 5].
The antimicrobial property of most plants makes them to be valuable in the ethnomedicine [6]. An increase demand for drugs with less side effect, and hazard free for the environment and lower price, make the nature to be a rich source for drugs and bioactive compounds [7].
Bottle gourd botanically known as *Lagenaria siceraria* (Molina) standley (Family: Cucurbitaceae), is common widely distributed in Asia, Africa and America plant, specially the fruit part used as vegetable [8, 9].Various medicinal uses were documented for the plant immunosuppressant [10], cardio-tonic, cardio-protective [11], diuretic [12], nutritive agent [13], purgative, antidote for certain poisons [9], plant seed infusions were used for treatment of chills and headache, leave juice was used for jaundice and baldness [2, 14]. Different traditional uses were recorded for the plant, mainly in general tonic, diuretic, emetic, bronchodilator, antipyretic, alopecia, and aphrodisiac [15].
Variable chemical constituents were recognized for the *Lagenaria siceraria*, the fruit is consider as a good source for carbohydrates, vitamins (vitamin B complex and ascorbic acid), Beta carotene, minerals, dietary and fibers, in addition to the flavonoids, triterpenoids, sterols, and mucilage[16, 17,18, 19, 20, 21].
The study aimed to phytochemical screening and evaluation of the antibacterial and antibiofilm activity of the *Lagenaria siceraria* fruit growing in Kurdistan region against standard pathogenic bacte

2. Materials and methods

2.1. Plant material

The mid age fruit of *Legenaria siceraria* were collected during June 2014 in different places of Iraqi Kurdistan region, the plant was shade-dried, grinded, and stored in air tight container.

2.2. Phytochemical analysis

2.2.1. Extract preparation

Fifty grams of powdered plant material introduced for extraction by 100 mlethanol (70%), using ultrasonic assisted extractor [22].

2.2.2. Phytochemical screening

Preliminary qualitative tests (phytochemical screening) were carried out on the extract to identify the chemical nature and the functional groups of compounds [23]. The extracts obtained by different methods where introduced for identification of alkaloids, using dragendorff test [24, 25, 26], flavonoids by alkaline test [27, 28], anthraquinone glycoside with borntrager test, cardioactive glycoside by keller killiani test [27], saponin glycoside by means of foam test [23], tannin with braymers test [23, 28, 29], sterol using liebermann-burchard test terpenoid steroids by salkowkis test, quinone using quinone test, phlobatannin with precipitate test, and phenols by ferric chloride test [28].

2.3. Antibacterial activity evaluation

2.3.1. Plant extraction

One hundred grams of powdered plant material introduced for extraction using chloroform (organic solvent) yielding organic fraction, the residue dried and re-extracted by 75% ethanol (hydroalcoholic solvent) yielding aqueous fraction using ultrasonic assisted extraction method [22]. The two extract fraction were concentrated and dried under vacuum, fractions were kept at 4 ^{0}C until used for biological activity. Extraction values were estimated.

2.3.2. Tested microorganisms

Five standard pathogenic bacteria were selected for the antibacterial evaluation: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615), *and Streptococcus pneumoniae* (ATCC 6303). All strains were maintained on Muller Hinton and blood agar slant, and then stored at 4 ^{0}C until used. McFarland standards 0.5 are used as a reference to adjust the turbidity of bacterial inoculums [30].

2.3.3. Evaluated methods

Two different methods were evaluated as follows: agar diffusion wells for antibacterial activity evaluation and microdilution method used for the determination of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) test.

2.3.4. Tested natural products

Different concentrations (100, 200 and 300) mg/ml of ethanolic extract was prepared, while 10mg /ml of organic fraction was arranged. Dimethyl sulfoxide (DMSO) (10% (v/v) and tween 20 (20% (v/v) used as diluents for extract fractions respectively. Gentamycin (30 µg/ml) was used as positive control.

2.3.5. Agar well diffusion method

Agar well diffusion method used for evaluation of antibacterial activity of plant extract as described by Sandhya *et al.* [31] with slight modification. The bacterial inoculum was uniformly spread using sterile cotton swab on a sterile nutrient agar plate. About 0.1 ml of natural products was added to each of the wells (6 mm) diameter holes cut in the agar. The plates were incubated for 24 h at 37 ºC under aerobic conditions. Following incubation, the inhibition of the bacterial growth around the wells was measured in mm. Tests were performed in triplicate, the average of the results were taken.

2.3.6. Determination of MIC & MBC

The MIC for the biologically active fraction was estimated using 96- flat well microtiter broth dilution method [32]. The test was performed in sterile 96 well. The two folds serial dilution of products was added to the wells, starting from 10 mg/ml as higher active concentration. Around 50 µl bacterial suspensions adjusted to 0.5 McFarland turbidity were added. Bacterial suspension were used as negative control, while broth contains standard drug were used as positive control. The plates were covered and incubated for 24 hr at 37 ^{0}C. The MIC-value was estimated as lowest concentration of extract that shown no turbidity after incubation, while MBC of extract were determined by sub-culturing the wells that showed non-turbid only. Following incubation for 24 h at 37 ^{0}C the concentration of extract that does not showing bacterial growth after sub-culturing considered as MBC [33].

2.3.7. Total activity (TA)

Total activity is the measurement of potency that defined as the volume at which test extract can be diluted without losing the ability to kill microorganisms. It is estimated by dividing the amount of extract from 1 g plant material introduced to extraction by the MIC of the same extract and is expressed in ml/g [34].

2.4. Antibiofilm activity evaluation

A modified crystal violet assay was employed to test the effect of plant extract on biofilm formation as described by O'toole and Kolter [35]. Twofold serial dilutions of plant extract were made in sterile 96 flat wells microtiter plates containing 150 µl of Mueller–Hinton broth per well. The tested concentration ranged from (0.0195 up to 5) mg/ml for organic fraction (OF) and (0.586 -150) mg/ml for aqueous fraction (AF). A 50 µl of fresh bacterial suspension adjusted with (0.5 McFarland) was added to each well. Positive control (bacterial suspension in broth) and negative control (extract in broth), were included. Following incubation at 37 °C for 24 h, the content of each well was gently removed by tapping the plates. The wells were washed with 200 µl of sterile distilled water to remove free-floating bacteria. Biofilms formed by adherent cells in plate were stained with 0.1% crystal violet and incubated at the room temperature for 30 minutes. Excess stain was rinsed off thorough washing with distilled water and plates were fixed with 200 µl of ethanol 70%. Optical densities (OD_{630}) of stained adherent bacteria were measured using an ELISA microplate reader.

2.5. Statistical analysis

All procedures were repeated at least three times and the mean value ± standard deviations were estimated using Microsoft Excel 2007 and data were presented using Graphpad Prism 6 program.

3. Results

3.1. Phytochemical screening

According to the phytochemical screening for the ethanolic fruit extract of *Legenaria siceraria*, only 6 out of the 11 tested phytochemicals were detected as shows in Table 1.

Table 1: Phytochemical screening results of *Legenaria siceraria* Fruit:

Plant	Phytochemicals											
	Alkaloid	Flavonoid	Anthraquinone glycoside	Cardioactive glycoside	Saponin	Tannin		Sterol	Terpenoid	Quinone	Phlobatannin	PhenPhenol
						HT	CT					
Legenaria siceraria	-	+	-	-	-	+	-	+	+	+	-	+

*(-) stands for the absence of the phytochemical, (+) stands for the presence of the phytochemical

3.2. Extractive values:

The extractive value was expressed as percentage (w/w), color and consistency is shows in Table 2.

Table 2: Extractive values of *Lagenaria siceraria* fruit:

***Lagenaria siceraria* Fraction**	**Extractive values**		
	Percentage % (w/w)	**Color**	**Consistency**
Organic Fraction (OF)	1	Green	Non sticky
Aqueous Fraction (AF)	8	Brown	Non sticky

3.3. Antibacterial activity

In vitro antibacterial activity of the two fractions (organic and aqueous) of *Lagenaria siceraria* fruit were evaluated against five standard bacterial strains: *Pseudomonas aeruginosa* (ATCC 27853) *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615), and *Streptococcus pneumoniae* (ATCC 6303). All bacterial strains shown resistance to the aqueous fraction (AF) of tested extraction, while *S. pneumoniae* was the only strain showed sensitivity against organic fraction (OF) as shown in Table 3 and Figure1.

Table 3: Antibacterial activity of *Lagenaria siceraria* fruit against five standard ATCC bacterial strains

***Lagenaria siceraria* Fraction**	**Mean±SD Inhibition zone in (mm) of bacterial Strains**				
	***P. aeruginosa* (ATCC27853)**	***E. coli* (ATCC 35218)**	***S. aureus* (ATCC 25923)**	***S. pyogenes* (ATCC 19615)**	***S. pneumoniae* (ATCC6303)**
OF(10mg\ml)	R	R	R	R	19± 0.021
AF(10mg\ml)	R	R	R	R	R
AF(100mg\ml)	R	R	R	R	R
AF(200mg\ml)	R	R	R	R	R
AF(300mg\ml)	R	R	R	R	R
Gentamycin(30µg\ml)	20±0.013	23± 0.021	21±0.014	35±0.041	24±0.022

* OF stands for organic fraction, AF stands for aqueous fraction, R stands for resistant to the bacterial species.

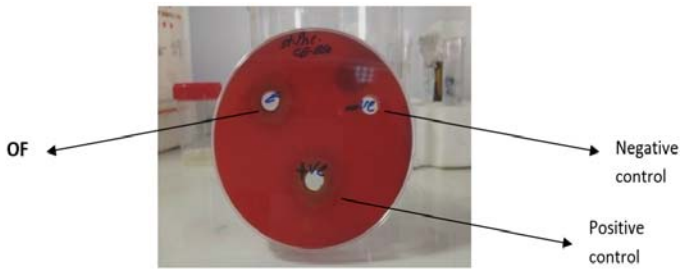

Fig 1: Blood agar plate shows antibacterial activity of the organic fraction (OF) of *Lagenaria siceraria* fruit against *S. pneumoniae* ATCC 6303.Negative control stands for the extract diluent tween 80 (20%), positive control stands for the gentamycin antibiotic:

3.4. Determination of MIC, MBC and TA

These tests were performed on the *S. pneumoniae* as the only sensitive strain. Table 4 shows the biological active fraction, which evaluated for the MIC, MBC, and TA expressed in (mg/ml) and (ml/g) respectively.

Table 4: Determination of MIC, MBC and TA for the organic fraction of *Lagenaria siceraria* fruit against *S. pneumoniae*

Plant Extract Fraction	**MIC (mg/ml)**	**MBC (mg/ml)**	**TA (ml/g)**
Concentration	2.5 mg/ml	5 mg/ml	0.002 ml/g
Standard deviation	± 0.002	±0.0012	±0.003

3.5. Determination antibiofilm activity of *Lagenaria siceraria* fruit

Anti-biofilm activity of both extraction forms (OF) and (AF) shows biofilm inhibition against the five tested strains. Same inhibition levels of biofilm have seen in all strains except *P. aeruginosa*, since high concentrations were required to the same inhibition rate (Figure 2) and (Figure 3).

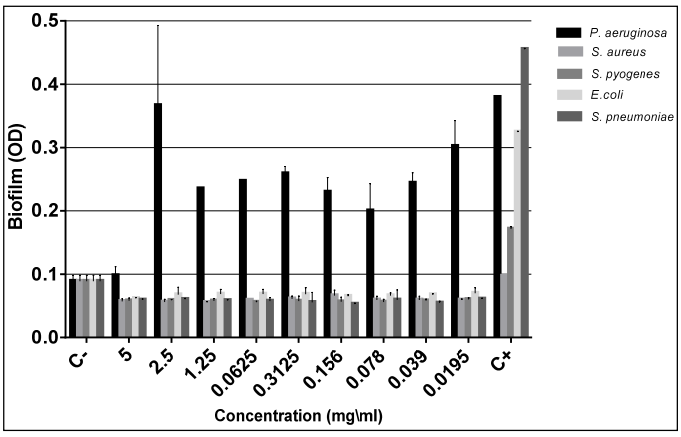

Fig 2: Biofilm formation of different standard ATCC bacterial strains at different concentrations of (OF) of *Lagenaria siceraria* [C-: stands for control negative, C+: stands for positive control]

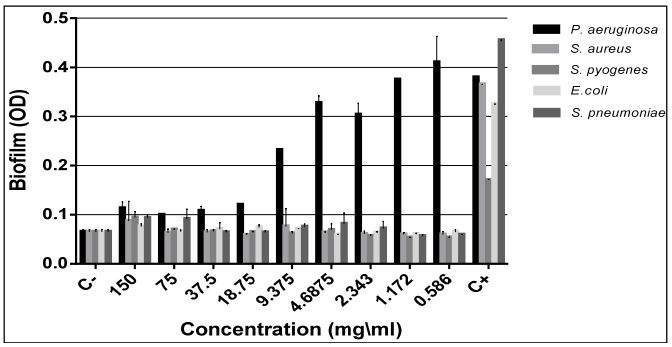

Fig 3: Biofilm formation of different standard ATCC bacterial strains at different concentration of (AF) of *Lagenaria siceraria* [C- : stands for control negative, C+: stands for positive control]

4. Discussion

Medicinal plants contain many natural products, which are abundant source of bioactive compounds. Plants are source for development of new antimicrobial agents [37]. However a small number of plants have been investigated for their antimicrobial activity. *Lagenaria siceraria* is a fruit, vegetable cultivates in Kurdistan used for culinary purposes, with numerous therapeutic properties and physiological activity. Preliminary phytochemical screening of the ethanolic extract of *Lagenaria siceraria* fruit revealed the presence of flavonoid, terpenoid, sterol, hydrolysable tannin, and phenol. These finding were similar the results of previous works done in the same field [38, 39, 40] while absence of alkaloid, glycosides and saponin agreed with findings of Sharma *et al.* [41]. New natural product quinone was detected in the ethanolic extract of *Lagenaria siceraria* fruit.

The extractive values regarding the consistency and color of yield extract fraction were similar to the yields of Suresh *et al.* [40]. Screening for the antibacterial activity of extracts of *Lagenaria siceraria* fruit against five standard ATCC bacterial strains shows a strong antibacterial activity were recorded for the organic fraction against standard *S. pneumoniae*(inhibition zone 19± 0.021) with MIC- value 2.5 ± 0.002 mg/ml and MBC- value5 ±0.0012 mg/ml. These results were also supported by the finding of Goji *et al.* [42], who documented the activity of the fruit against clinically isolates of *S. aureus, S. pyogenes,* and *E. coli* to the fruit extract. In contrast to our finding Parasa *et al.* [43] described the activity of the methanolic extract of the fruit on different standard bacterial strains have been recorded against *S. aureus* (ATCC6538), *E. coli* (ATCC8739) and *P. aeruginosa* (ATCC9027). Aqueous fractions of *Lagenaria siceraria* fruit dose not exhibit any activity against tested bacterial strains. The inhibition zone recorded for the organic fraction was close to the value recorded for the positive control (gentamycin).

Biofilm is a community of bacterial cell attached to a surface consider as cause of 60% of infectious diseases in human [44, 45, 46]. The inhibition rate of biofilm were estimated for both extract fraction of the plant in comparison the optical density of the plant extracts at different concentrations with those of the positive control. The results revealed a strong antibiofilm activity for both fractions against tested bacterial species (Figure. 2. & Figure. 3.), since highest antibiofilm was documented for the organic fraction against *S. pneumoniae* (ATCC6303) at concentration (5 mg/ml), while (150 mg/ml)of aqueous fraction was against *S. aureus* (ATCC25923). Lower antibiofilm activity for both organic and aqueous fractions at high concentrations 5 mg/ml & 150 mg/ml respectively were recorded against *S. pyogenes* (ATCC19615). Interestingly the OD-values described low concentrations of the aqueous fraction could promote the biofilm formation instead of inhibition against *P. aeruginosa* (ATCC27853). The two fractions extract show less antibiofilm activity against *P. aeruginosa* (ATCC27853) in comparison with other species. Our results were in correspondence to the results of study on phenolic compound extracted by ethanol [47]. Antibiofilm activity of plants on *P. aeruginosa* PAO1, which revealed that phenolic compounds at a concentration that did not or weakly inhibit bacterial growth, increased biofilm formation, in which they suggested that have been a relationship between biofilm formation enhancement and las quorum-sensing system of this bacterium [48]. Generally the organic fraction was stronger than aqueous fraction in inhibition the biofilm formation of the tested bacterial species, the concentration and dose of bioactive compound in plant extract is a crucial point in the plant extract fractions which effect on their biological activities [49].

5. Conclusion

In an attempt to finding new antimicrobial medicinal plant from our wealthy culture of plants we found that *Lagenaria siceraria* is a fruit vegetable cultivated in Kurdistan region used for culinary purposes containing many important phytochemicals with antibacterial activity and inhibition of biofilm formation, which consider the first step in series infections.

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