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.

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,
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 ml ethanol (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], anthaquinone 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 liebemann-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 °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 °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 °C 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- well microtiter broth dilution method [12]. 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 °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 °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 (OD₅₉₀) 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 shown in Table 1.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Plantescape siceraria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>-</td>
</tr>
<tr>
<td>Glycoside</td>
<td>+</td>
</tr>
<tr>
<td>Cardioactive</td>
<td>-</td>
</tr>
<tr>
<td>Glycoside</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
</tr>
<tr>
<td>Sterol</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>+</td>
</tr>
<tr>
<td>Quinone</td>
<td>+</td>
</tr>
<tr>
<td>Phlobatannin</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td>+</td>
</tr>
</tbody>
</table>

*(−) 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>
<thead>
<tr>
<th>Plantescape siceraria Fraction</th>
<th>Extractive values</th>
<th>Percentage (%) (w/w)</th>
<th>Color</th>
<th>Consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic Fraction (OF)</td>
<td>1</td>
<td>Green</td>
<td>Non sticky</td>
<td></td>
</tr>
<tr>
<td>Aqueous Fraction (AF)</td>
<td>8</td>
<td>Brown</td>
<td>Non sticky</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Antibacterial activity
*In vitro* antibacterial activity of the two fractions (organic and aqueous) of *Legenaria 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 Figure 1.

<table>
<thead>
<tr>
<th>PlantExtract Fraction</th>
<th>Mean±SD Inhibition zone in (mm) of bacterial Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa (ATCC27853)</td>
</tr>
<tr>
<td>OF(10mg/ml)</td>
<td>R</td>
</tr>
<tr>
<td>AF(10mg/ml)</td>
<td>R</td>
</tr>
<tr>
<td>AF(100mg/ml)</td>
<td>R</td>
</tr>
<tr>
<td>AF(200mg/ml)</td>
<td>R</td>
</tr>
<tr>
<td>AF(300mg/ml)</td>
<td>R</td>
</tr>
<tr>
<td>Gentamycin(30µg/ml)</td>
<td>20±0.013</td>
</tr>
</tbody>
</table>

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

3.5. Determination antibiofilm activity of *Legenaria 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 1: Blood agar plate shows antibacterial activity of the organic fraction (OF) of *Legenaria siceraria* fruit against *S. pneumoniae* ATCC 6303.](image)

![Fig 2: Biofilm formation of different standard ATCC bacterial strains at different concentrations of (OF) of *Legenaria siceraria*](image)
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- value 5 ±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.

6. References

Pharmacognosy and Phytochemistry


