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Antibacterial Activity of Selected Palestinian Wild Plant Extracts against Multidrug-Resistant Clinical Isolate of *Streptococcus pneumoniae*

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ABSTRACT

Streptococcus pneumoniae is one of the most important bacterial pathogens that affect respiratory system. The occurrence of multidrug-resistant strains of *S. pneumoniae* necessitates the discovery of new classes of anti-pneumonia drugs. As some of the medicinal plants and their extracts have antibacterial activity, we aimed to investigate the antibacterial potential of five Palestinian wild medicinal plants including *Echinops adenocaulos, Parietaria judaica, Urtica urens, Verbascum fruticulosum* and *Vitex agnus-castus* against multidrug-resistant strain of *S. pneumoniae*, which was screened for its antibiotic resistance profile showing 67% resistance of the tested antibiotics. In this research micro-broth dilution method was used to study the inhibitory activity of ethanol, distilled water and Zamzam water extracts of all plants under investigation. The obtained results showed that all Zamzam extracts of the five studied plant species inhibited the growth of *S. pneumoniae*. It was clearly noticed that there was a difference in the antibacterial activity between distilled water and Zamzam water extracts recording higher activity in the Zamzam water extract of some of the studied plant species. *Verbascum fruticulosum* distilled water extract was the most effective one with MIC equal to 0.195 mg/ml. In general, both water extracts were more bioactive than the ethanol extracts for all the examined plants. These results confirmed the possibility of using these plant species in medicine and pharmaceutical industry of new drugs for the treatment of multidrug-resistant clinical isolates of *S. pneumoniae.*

KEYWORDS : Wild Plant Extracts, Antibacterial Activity, *Streptococcus pneumoniae*, Palestine.

INTRODUCTION

The bioactive materials isolation concept from natural wild plants was based on the consumption of many medicinal plants in traditional medicine. Therefore, the herbal medicine market has spread and became prosperous in pharmacies against many types of bacteria [1]. The development of new antimicrobial agents for the treatment of many infections is an urgent demand in recent years due to the cumulative bacterial resistance against common antibiotics [2]. This challenge posed by the resistant strains of microorganisms can be countered by the increase of the chemotherapy efficacy depending on the continuous search for new drugs. Recently, the range of available antibacterial agents against Gram-positive bacteria is significantly lower than the range effective against Gram-negative pathogens [3]. Thus researchers are faced with major obstacles to develop drugs effective against problematic Gram-positive organisms. Hence the antimicrobial assay investigation of indigenous plants may yield the discovery of new potential bioactive plant components, which aid significantly in the therapeutic applications against human pathogens bacteria, fungi or viruses [4].

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One of the most common Gram-positive pathogens that cause pulmonary inflammations is *Streptococcus pneumoniae*, which is known as pneumococcal in medical microbiology. It was recognized as a major cause of pneumonia diseases in the late 19th century causing many types of pneumococcal infections in children and the elderly other than pneumonia in many communities acquired respiratory infections [5, 6].

Palestine is considered as one of the most countries containing a wide variety of vegetation in the world. The utilization of complementary and alternative medicine in Palestine is very common [7]. It is interesting to determine whether their traditional uses are supported by actual pharmacological effects or merely based on folklore [8]. Several studies have been published concerning many plant extracts biological active properties such as antibacterial, antitumor, antifungal and antioxidant of wild plants in Palestine [8-26]. Nevertheless, Documentation of scientific information about the efficacy and safety of wild plants in West Bank, Palestine is one of our research goals based on their accurate taxonomical identification. Systematic screening of folk medicine plants may result in the discovery of novel effective compounds exploring unknown medicinal wild plants.

Echinops adenocaulos, is one of our target plants in this study. The

members of the genus *Echinops* in the family Asteraceae are widely used in herbal medicine for the treatment of various diseases [27]. Hydroalcoholic extracts of different plant parts of several *Echinops* species were investigated against several Gram-positive and Gram-negative bacteria in different countries. Results of those studies showed variation among different *Echinops* species in respect to the extract type, the used plant part and the bacterial species [27, 28, 29]. Hence, we thought that antibacterial activity investigation of *E. adenocaulos* in Palestine might reveal the potentiality of this plant species as a medicinal plant adding to its impervious antibacterial activity assay.

The second studied plant species is *Parietaria judaica*, which is known as Pellitory of the wall belonging to the family Urticaceae which, has been valued in herbal medicine [30]. However, different extract types of several plant parts of *P. officinalis* were studied in respect to their bioactivity showing that they have medicinal as well as antibacterial effect on both Gram-positive and Gram-negative bacteria [31, 32]. This out finding was the clue for the examination of *P. judaica* antibacterial effect against *S. pneumoniae*, which has not been examined before in Palestine.

While, *Urtica urens*, which is commonly known as Annual Nettle or Dwarf Nettle [33,] was another studied plant in this work. The antibacterial effect of three plant extracts of *U. urens* against Gram-negative bacteria was studied showing their resistance to all extracts [34]. Moreover, *U. urens* was considered as medicinal plant for the treatment of pulmonary diseases [35]. Accordingly, it was explored for its antibacterial activity against *S. pneumoniae* in Palestine, in spite of its use locally as anticancer medicinal plant [1].

The flowers and leaves of some *Verbascum* species have been used in traditional medicine in Turkey [36]. Moreover, the susceptibility of *Klebsiella pneumoniae* and *Staphylococcus aureus* to *Verbascum* extracts revealed the usage of *Verbascum* in folk medicine to treat respiratory diseases [37]. In addition to that, other *Verbascum* species were tested for their antimicrobial activity against several bacterial species confirming their inhibitory effect against Gram-positive bacteria with no similar activity against the tested Gram-negative bacteria [38, 39, 40]. This medicinal history of the *Verbascum* species encouraged us to examine the antibacterial efficacy of *V. fruticulosum* in Palestine as most of the previous studies indicated the potential antibacterial efficacy of *Verbascum* species against Gram-positive bacteria rather than Gram-negative ones.

In addition to that, *Vitex agnus-castus* was another point of focus in this study, which belongs to the family Verbenaceae. The antibacterial activity of the aqueous extract of *V. agnus-castus* was examined against six different Gram-positive and Gram-negative bacteria. The studied extracts showed inhibitory effect on the bacterial growth of *Escherichia coli* [41]. However, *V. agnus-castus* was also reported to have antimicrobial effect against different Gram-positive and Gram-negative bacteria [42]. Therefore, a motivation for studying its antibacterial activity against *S. pneumonia* in this research has risen.

A new research approach and first of its kind has been studied in this work relaying on the unique properties of what is called Zamzam water. According to Arab histories, Zamzam well water has been used for around 4000 years. The properties of Zamzam water were investigated in European laboratories as samples were examined revealing the special unique physique properties which advantageous potable water. This fact has been confirmed by a group of Pakistani researchers. They proved that the quantity of calcium and magnesium salts is higher in Zamzam water than potable water. However, the significant difference was recorded in the fluorides, as they are higher in Zamzam water, in addition to its higher alkalinity. Nevertheless, it is still characterized by the European as fit for drinking. Moreover, the sterility nature with no germs in Zamzam water as no biological growth was ever recorded in it [43].

The high percentage of calcium, magnesium, sodium, potassium and fluorides in addition to other minerals and salts led us to the idea of the present study to explore the Zamzam water plant extract antibacterial effect comparing it with the standard distilled water extract type. Thus the present work was designed to investigate the antibacterial potential of selected five wild plants in Palestine against a multidrug-resistant clinical isolate of *S. pneumoniae.*

MATERIALS AND METHODS

Antibiotic screening assay

The *in-vitro* antibacterial activities of the plant extracts were evaluated against a multidrug resistant clinical isolate of *S. pneumoniae*, which was obtained from Rafidia hospital and identified by catalase, a-hemolytic, optochin and Gram stain tests [44]. The susceptibility of this clinical isolate was tested on blood agar plates by using twelve antibiotics which are: Vancomycin, Chloramphenicol, Erythromycin, Clindamycin, Tetracycline, Bacitracin, Penicillin G, Amoxicillin, Oxacillin, Methicillin, Streptomysin and Gentamicin [45].

Plant materials

Five plant species (*E. adenocaulos*, *P. judaica*, *U. arens*, *V. fruticulosum* and *V. agnus-castus*) were collected from different localities in West Bank area of Palestine. The plants were identified by Ghadeer Omar, department of biology & biotechnology, An-Najah National University, Palestine. Representative specimens of the studied plant species were collected, pressed till drying, mounted on herbarium sheets, provided with voucher numbers and deposited at An-Najah National University herbarium. For the antibacterial activity, plant materials were washed, air-dried, ground into powder and stored at room temperature until use.

Aqueous extraction

Five grams of each plant powder were soaked in 100 ml distilled water that previously warmed to 70°C and then incubated at 37°C with interval shaking for one week. The mixtures were centrifuged for 5 minutes at 5000 rpm and the supernatants were frozen at -20°C and evaporated by freeze-drying. The extracted powder of each plant species was dissolved in sterile distilled water to obtain 200 mg/ml

final concentration and the extracts were stored at -20°C. The same procedure was carried out using Zamzam water as extract solvent instead of distilled water.

Ethanol extraction

Five grams of each plant powder were soaked in 100 ml of 70% Ethanol for one week with interval shaking at 37°C. The mixtures were centrifuged for 5 minutes at 5000 rpm and the supernatants were evaporated by rotary evaporator then by freeze-drying. The extracted powder of each plant species was dissolved in 10% dimethyl sulfoxide (DMSO) to obtain 200 mg/ml final concentration.

Media preparation

Thirty percent of LSB (lysed sheep blood) media was prepared. Five freeze-thaw cycles were performed using sheep blood (to obtained lysed blood) by complete freezing the blood at -20°C and then full thawing the blood at room temperature. LSB was prepared using sheep lysate diluted in distilled water to obtain final concentration 50% and centrifuged at 20,000 rpm for clarification. Six ml of supernatant was added to 94 ml nutrient broth supplemented with Ca^{++} and Mg^{++} ions at 1 µg/ml final concentration of each ion [46].

Antibacterial activity against *Streptococcus pneumoniae*

The in-vitro antibacterial activities of the plant extracts were evaluated using clinical isolate of Gram-positive *S. pneumoniae*. Minimum inhibitory concentrations (MIC) were determined using micro-broth dilution method [46]. Extracts were serially diluted two folds in LSB broth medium in microtiter plates. Then duplicates of each dilution (100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391 and 0.195 mg/ml) were inoculated with 1µl of 5×10^7 (CFU/ml) of *S. pneumoniae*. The last two wells were used as positive and negative controls. Also, Zamzam water control was used to study its effect on bacterial growth. The inoculated microtiter plates were incubated at 35°C for 48 h using CO_2 incubator. The lowest extract concentration (highest dilution) that inhibited the growth of tested microorganisms was considered as MIC.

RESULTS

The tested clinical isolate *S. pneumoniae* had showed susceptibility to four antibiotics, which are Chloramphenicol, Tetracycline, Streptomysin and Gentamicin. However, it was resistant to the other screened antibiotics in this study, which are Vancomycin, Erythromycin, Clindamycin, Bacitracin, Penicillin G, Amoxicillin, Oxacillin and Methicillin (Table 1), which represents 67 % resistance of the examined antibiotics.

Table 1: Susceptibility of *Streptococcus pneumoniae clinical strain to 12 conventional antibiotics**

Antibiotic	Inducibility	Antibiotic	Inducibility
Vancomycin (30)	R	Penicillin G (10)	R
Chloramphenicol (2)	S	Amoxicillin (30)	R
Erythromycin (15)	R	Oxacillin (1)	R
Clindamycin (30)	R	Methicillin (5)	R
Tetracycline (30)	S	Streptomysin (10)	S
Bacitracin (10)	R	Gentamicin (10)	S

*** *Streptococcus pneumoniae* tested on blood agar medium, R: Resistant; S: Sensitive.**

The antibacterial activity for the five plant species in this study was recorded by measuring their minimum inhibitory concentrations (MIC) (Table 2). The MIC results of the conducted experiment showed that both aqueous extracts (distilled water and Zamzam water) were more effective than the ethanol extracts for all plants under investigation with the exception of distilled water extract of *E. adenocaulos* (Figure 1). The lowest MIC was for the distilled water extract of *V. fruticulosum* indicating its highest antibacterial activity among the studied plant species. Of great concern, there was a remarkable difference in the bioactivity of both aqueous extracts of *E. adenocaulos* and *V.agnus-castus* as Zamzam water extract have had lower MIC values than distilled water extracts. However, an opposite phenomenon was recorded in the distilled water extract of *P. judaica*, *U. urens* and *V. fruticulosum* as they have had lower MIC range (0.195-6.25 mg/ml)

Table 2: Antibacterial activity of the tested plant extracts against *S. pneumoniae*.

Plant species / Extract type	*Echinops adenocaulos* (MIC) mg/ml	*Paritaria judaica* (MIC) mg/ml	*Urtica urens* (MIC) mg/ml	*Verbascum fruticulosum* (MIC) mg/ml	*Vitex agnus-castus* (MIC) mg/ml
Distilled water	-	3.125	6.25	0.195	25
Zamzam water	0.781	50	12.5	50	12.5
Ethanol	100	100	12.5	50	-

MIC: Minimum Inhibitory Concentration in mg/ml. (-): no inhibition of growth

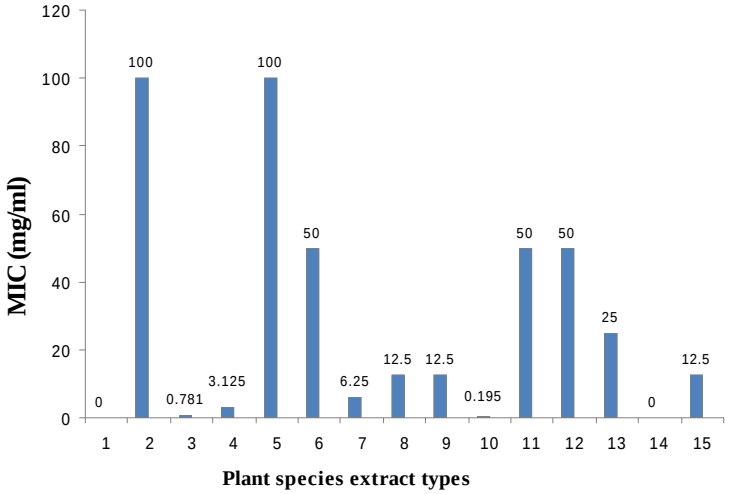

Figure 1. Minimum Inhibitory Concentration (mg/ml) of selected plant extracts against *S. pneumoniae*. (1) *E. adenocaulos* d.H_2O,(2) *E. adenocaulos* EtOH, (3) *E. adenocaulos* Zamzam, (4) *P. judaica* d.H_2O, (5) *P. judaica* EtOH, (6) *P. judaica* Zamzam, (7) *U. urens* d.H_2O, (8) *U. urens* EtOH, (9) *U. urens* Zamzam, (10) *V. fruticulosum* d.H_2O, (11) *V. fruticulosum* EtOH, (12) *V. fruticulosum* Zamzam, (13) *V. agnus-castus* d.H_2O, (14) *V. agnus-castus* EtOH, (15) *V. agnus-castus* Zamzam

than Zamzam water extract (12.5-50 mg/ml). Among the ethanol extracts, *V. agnus-castus* have no effect on *S. pneumoniae*, while the other species have antibacterial effect with MIC values ranging from 12.5 to 100 mg/ml. The significant finding in this experiment was that all Zamzam extracts of the five plants showed antibacterial activity against *S. pneumoniae*.

DISCUSSION

The infection incidence by multidrug-resistant Gram-positive bacteria is increasingly causing the treatment of the diseases caused by them by the currently available antibacterial agents is extremely difficult [3]. Similar case was recorded in *S. pneumoniae* antibacterial resistance, which became widespread in many parts of the world during 1980s. Previously pneumococcal infections are treated with penicillin as the first choice drug, and erythromycin is also frequently used [47]. Since then, resistance of pneumococci to a variety of antimicrobial agents has evolved from an ominous medical curiosity to a worldwide health problem. Previous investigations considering the antibacterial resistant profile of *S. pneumoniae* indicated no fixed pattern [3, 48-52]. For example, antimicrobial medications classified as macrolides (e.g. erythromycin) and lincosamides (e.g. clindamycin) show strong activity against *Streptococci* making them the recommended alternatives for patients who cannot tolerate Beta-lactams [53]. However, its resistance has increased to a point that it is clinically relevant in the following classes of antibiotics: Beta-lactams (penicillins, cephalosporins and carbapenerns), Macrolides (erythromycin, azithromycin, clarithromycin and clindamycin), Tetracyclines and folate inhibitors (trimethoprim-sulfamethoxazole) and Fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin) [54]. The resistance rate of *S. pneumoniae* varies with the locality or region studied the frequency and intensity of utilization and empirical use frequency of the antimicrobial drugs [49]. Owing to the continued clinical pressure for novel approaches to combat antibiotic-resistant bacterial infections, there has been a resurgence of interest in the search for new antibacterial agents that are able to overcome multidrug-resistant mechanisms emanating the approach of screening natural products from plants. Plants have already successfully yielded compounds with activities suggesting that they inhibit efflux pumps of Gram-positive bacteria, which may be referred to the presence of alkaloids, flavonoids, tannins, polyphenols and many other bioactive chemicals [48]. Therefore, in this study we sought to identify medicinal plants that could provide compounds for further antimicrobial drug development.

This study was conducted on a clinical multidrug-resistant isolate of *S. pneumoniae*. The studied strain of *S. pneumoniae* performed an antibiotics resistance, which coincides with or differs from the recorded antimicrobial agents resistance profile of *S. pneumoniae* in the literature [3, 48-54]. Out of the twelve-screened antibiotics, only four have had effect against the studied *S. pneumoniae* representing 67% resistance to the tested antibiotics, most of which are commonly used to treat *S. pneumoniae* infections. Hence, this spring the examination of five wild plants extracts in Palestine. In addition to that, due to unique properties of the Zamzam water, which have been discussed previously, it spouts the idea of the examination of the Zamzam water extract antibacterial efficacy of the studied plant species. The recorded results confirmed the difference in using Zamzam water from distilled water as extract solvent, which has been illustrated via the higher antibacterial effect of Zamzam water extract of *V. agnus-castus* than its water extract.

Moreover, Zamzam water extract of *E. adenocaulos* has showed antibacterial effect in contrast to the water extract, which has had no antibacterial effect at all. Therefore, we can recommend that using Zamzam water as extract solvent may reveal different antibacterial agents with higher activity potency taking into account the observed growth of *S. pneumoniae* in Zamzam water alone. All studied plant species extract types showed antibacterial effect against *S.pneumoniae* except for *E. adenocaulos* water extract and *V. agnus-castus* ethanol extract. Nonetheless, water extract of *V. fruticulosum* and Zamzam water extract of *E. adenocaulos* have had very low MIC values, 0.195 and 0.781 mg/ml, respectively.

Findings in this study confirmed the observations of some other researchers, namely that some *Verbascum* species contain substances with antimicrobial activity against Gram-positive bacteria [38-40, 55-57]. However, this is the first study to provide data that *V. fruticulosum* possess potential antibacterial activity against *S. pneumoniae*. Different species of *Verbascum* have been known for containing biologically active compounds such as flavonoids, phenylethanoid, neolignan glycosides, saponins and iridoid glycosides [58]. Therefore, further studies are required in order to clarify the bioactive principles of these activities in *V. fruticulosum*.

Moreover, the antibacterial efficacy of *E. adenocaulos* against *S. pneumoniae* emphasizes the medicinal use of different species of *Echinops* in several countries [27]. Therefore, the results from the present study have proved the scientific basis of the traditional uses of *Echinops* in the treatment of ailments such those caused by *S. pneumoniae*. This antibacterial activity may be due to the bioactive components in *Echinops* such as flavonoids and thiophene acetylenes [28, 59]. Additionally, it is suggested that further investigations must be carried out to determine possible toxicities of *E. adenocaulos* to ensure the safety of the extracts to be used in traditional medicine as it has not been previously used medicinally in our country.

In spite of the negative antibacterial effect of *U. pilurifera* against *S. pneumoniae* [60], all three extract types of *U. urens* in our study showed inhibitory effect against *S. pneumoniae*. Moreover, the negative antibacterial effect of three plant extract types of *U. urens* against Gram-negative bacteria [34] may suggest its specificity against Gram-positive bacteria. While, *V. agnus-castus* has antibacterial activity against Gram-negative bacteria [41]. However, in this research both aqueous extracts of *V. agnus-castus* showed inhibitory effect against *S. pneumoniae*, which is a Gram-positive bacterium. This out finding add a scientific base for the traditional use of *V. agnus-castus* in the treatment of pulmonary diseases caused by *S. pneumoniae* in Palestine as it has been consumed locally for other diseases treatment [1].

Parietaria judaica was not previously detected for its antibacterial effect in spite of its herbal value in the treatment of many diseases [30]. Therefore, it was studied in this research. The results of the antibacterial effect of the three extract types of *P. judaica* against *S. pneumoniae* confirm scientifically our interest in that plant to be a source for potential antibacterial agents.

CONCLUSSIONS

Plants have not been completely investigated, nevertheless, data from previous literatures as well as our results revealed the great potential of plants for therapeutic treatment. Therefore, more studies need to be conducted to search new compounds. Moreover, once they are extracted and before being used in new therapeutic treatments, they should have their toxicity tested in vivo. However, these plant extracts were active against the multidrug-resistant *S. pneumoniae* under very low concentrations recording MIC values up to 0.195 mg/ml in some of them, thus minimizing the possible toxic effects. Adding to that, some of these plant extracts were more effective than the traditional antibiotics to combat the pathogenic tested microorganism.

Success in isolating compounds from plants materials is highly dependent on the type of the solvent used in the extraction procedure [61], which is proved by the overall evaluation of the present study results in which all extracts types of the same plant species showed varying degrees of antibacterial activity against *S. pneumoniae* in combination to the variations among the studied plant species.

Therefore, the results suggest the presence of either good antibacterial potency or high concentration of an active principle in the extracts. In conclusion, this study supports to a certain degree the traditional medicinal uses of the plants in diseases therapy and reinforces the concept that ethno botanical approach to screen plants as potential sources of bioactive substances is successful.

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