Original Article

In Vitro Investigation of the Effects of Certain Antibiotics and Plant Oils on the Clinical Isolates of *Streptococcus pneumoniae*

Mazen Safi[1]* , Marwa Khawajkiah[1], Nour Al-Sahar[1], Ayman Al-Mariri[1]

[1]Department of Molecular Biology and Biotechnology, Atomic Energy Commission, Damascus, Syria

Abstract
Background: With the serial trend toward antibiotic resistance in *Streptococcus pneumoniae*, novel drug development is needed to deal with pathogenic microorganisms that have developed widespread microbial resistance to antibiotics.
Methods: Eighty-seven clinical samples were collected from six hospitals in Damascus. A polymerase chain reaction (PCR) was performed to identify the bacterial genus and type. Minimum inhibitory concentrations on Luria-Bertani agar were conducted using several antibiotics and essential oils (EOs).
Results: Twenty-five isolates of *S. pneumoniae* were found, and amoxicillin and cephalosporins were the most effective antibiotics against 90% of *S. pneumoniae* bacteria. On the other hand, *Thymus syriacus* Boiss., *Origanum syriacum* L., *Rosmarinus officinalis* L., *Cinnamomum zeylanicum* L., and *Juniperus foetidissima* Willd were the most effective EOs.
Conclusion: Only *T. syriacus* Boiss., *O. syriacum* L., *R. officinalis* L., *C. zeylanicum* L., and *J. foetidissima* Willd oils had good inhibitory effects against *S. pneumoniae*.
Keywords: Antibiotics, Essential oils, MIC, *Streptococcus pneumoniae*

Introduction

Streptococcus is still the number one cause of lung diseases acquired through contact between people in adults and children (1). It is responsible for more than 1.6 million deaths annually, mostly in children and the elderly (2). There are increasing concerns around the world regarding high levels of resistance to antibiotics as well as a lack of efficacy in antimicrobial therapy, leading to the ineffectiveness of treatment and a decline in the usefulness of previous treatment programs. *Streptococcus pneumoniae* can be found in the upper part of the throat and causes most cases of community-acquired respiratory infections. Infections with such bacteria cause a huge increase in disease and mortality rates, such as serious meningitis and pneumonia (3-5). In many Asian countries, treatment options for antibacterial infections are normally experimental due to the lack of specialized laboratories to detect bacterial susceptibility tests, as well as its very elevated costs (6). High antibiotic resistance renders existing treatments ineffective and threatens future efforts to treat bacterial infections caused by these bacteria (7-9). Therefore, follow-up studies are a good tool for profiling antibiotic susceptibility and

guiding experimental therapy (10).

This study aims to isolate and characterize a few isolates of *S. pneumoniae* by using reliable serological diagnostic methods as well as the polymerase chain reaction (PCR) by applying specific primers in order to study their sensitivity against some antibiotics and essential oils (EOs). Considering that there are different resistance levels through different geographic regions, awareness about the presence of local resistance types is important before conducting experimental therapy *in vivo*.

Materials and Methods
Collection of Samples
Eighty-seven clinical samples were collected from six hospitals in Damascus between January and August 2019. These samples were distributed as 39 blood samples, 20 bronchial secretions, 18 pharyngeal swabs, and 10 oral swabs.

Isolation of Streptococcus pneumoniae
Briefly, the samples were cultured on blood agar media with 5% sheep blood and then incubated for 24 hours at

$37°C$ in the presence of 5% CO_2.

Biochemical Tests
An isolate was considered to be *S. pneumoniae* if it was alpha-hemolytic, bile soluble, optochin susceptible, and formed gram-positive cocci in chains. The fermentation of some sugars was tested using motion, catalase, and oxidase tests.

Extraction of DNA
The cetyltrimethylammonium bromide method was used to isolate DNA (11). Briefly, 2 μL of DNA extraction in the Tris-ethylenediamine tetra-acetic acid buffer was read using a Nanodrop machine in order to detect DNA concentration. This buffer was used as a blank. Then, the concentration of 100 ng/mL in each sample was prepared and stored at -20 °C.

Polymerase Chain Reaction
In the preparation of the single sample for the PCR, a set of chemical and molecular materials were utilized with a final volume of 25 μL (1.5 mM $MgCl_2$, 1X PCR buffer, 0.2 mM dNTPs, 1U DNA polymerase enzyme, 200 ng bacterial DNA, and 25 pmol of each set of forward and reversed primers). In addition, PCR amplification was performed using two sets of specific primer pairs (Table 1). PCR results were observed under UV light after electrophoresis in agarose gel pre-stained with ethidium bromide.

Plant Sample Collection
The samples used in this study were bought from Damascus markets or collected during the flowering season from different Syrian regions (Table 2). They included *T. syriacus* Boiss., *O. syriacum* L., *L. stoechas L.*, *R. officinalis* L. *(Lamiaceae)*, *M. communis* L., *S. aromaticum* L. *(Myrtaceae)*, *C. zeylanicum* L. *(Lauraceae)*, *J. foetidissima* Willd *(Cupressaceae)*, *P. roseum* L. *(Geraniaceae)*, and *D. maritima* L *(Liliaceae)*. Leaves harvested during the pre-flowering stage were properly cleaned from all contaminants before the extraction procedure.

Isolation of Essential Oils
Before steam distillation, parts of the studied plants were finely chopped after drying them well using appropriate grinders. A water steam distillation device (Clevenger-type apparatus) was applied to isolate EOs. The European Pharmacopoeia method was followed (12), and the process of obtaining EOs was explained in detail in a previous article by the same researchers of this study (13). In brief, starting from 100 g of plant material, EOs are obtained using the distillation apparatus. The resulting oils are diluted in dimethyl sulfoxide and stored properly.

Essential Oil Susceptibility Assay
The susceptibility determination of EOs on sterile filter paper discs (6 mm in diameter) was explained in detail in a previous study conducted by the same researchers as this study (13). For this purpose, 50 μL of 5% concentration of each EO was added to each sterile paper, and 0.1 mL of the bacterial suspension was added with a final inoculum bacterial number of 10^6 CFU/mL.

Essential Oil Minimum Inhibitory Concentrations Determination
A broth susceptibility testing method was followed using three replicates for each EO (14). Ninety-six-well microtiter plates and Brucella° broth medium were used in this study. Concentration ranges of EOs were previously explained (13), and 1 x 10^6 CFU/mL of *S. pneumoniae* were added to each well. An average concentration of each EO has been adopted, ranging from 0.75 to 100 μL/mL. Positive (bacteria without EOs) and negative (without bacteria) controls were also performed with the same conditions. The plates were incubated for 24 hours at 37 °C. Minimum inhibitory concentration$_{50}$ (MIC) and MIC_{90} were determined as previously explained (13).

Minimum Inhibitory Concentrations Determination for Antibiotics
Recommendations of the Clinical Laboratory Standards Institute (15) were followed to determine the MICS of each antibiotic. The method of microdilution broth using 96 microwell plates was applied in antibiotic susceptibility estimation. The process of antibiotic susceptibility, MIC, and MIC_{90} estimation was explained in detail in a previous article by the same researchers of this study (16), using 10^6 CFU/mL of bacteria in each micro-well plate. The average of three tests for each antibiotic was taken into account. Investigated antibiotics were penicillin G, amoxicillin, cefixime, cefpodoxime, cefotaxime, ceftriaxone, cefazolin, cefuroxime, ofloxacin, cotrimoxazole, erythromycin, spiramycin, azithromycin, chloramphenicol, vancomycin, levofloxacin, tetracycline, rifampicin, trimethoprim-

Table 1. Polymerase Chain Reaction Primers

	Sequence 5`-3`	Gene	Size (bp)	Annealing (°C)
Streptococcus sp.	GTACAGTTGCTTCAGGACGTATC	*tuf*	761	55
	ACGTTCGATTTCATCACGTTG			
Streptococcus pneumoniae	ATTTCTGTAACAGCTACCAACGA	*ply*	348	52
	GAATTCCCTGTCTTTTCAAAGTC			
	TGGCAAGGTAAACTTCTAAAGCA			
	GCCCCTTCACAGTTGGTTAG			

Table 2. Sources and Characterizations of Studied Plants

Original Name	Family	Site	Hight (m)	Time of Collection	Used Part
T. syriacus Boiss.	*Lamiaceae*	Damascus mountain (Alsoja)	800	July	Aerial parts
O. syriacum L.	*Lamiaceae*	Kafr Nobol-Idlib	450	June	Aerial parts
L. stoechas L.	Lamiaceae	Countryside of Tartous	250	May	Aerial parts
M. communis L.	*Myrtaceae*	Latakia	350	June	Leaves
S. aromaticum L.	Myrtaceae	Market			Flowers
C. zeylanicum L.	*Lauraceae*	Market			Barks
R. officinalis L.	*Lamiaceae*	Jableh	350	May	Aerial parts
J. foetidissima Wild	*Cupressaceae*	Damascus Alsabourah	780	July	Leaves
P. roseum L.	*Geraniaceae*	Damascus Doummar	900	April	Aerial parts
D. maritima L.	*Liliaceae*	Countryside of Tartous	320	April	Bulbs

sulfamethoxazole, and sparfloxacin (Oxoid, UK).

Results

Bacteria Identification

Overall, 27 positive isolates (27/87, 31%) were obtained when culturing was performed on *S. pneumoniae* selective media. They were alpha-hemolytic, without motion, catalase-negative, oxidase-negative, bile soluble, and optichon -sensitive. In addition, they were all capable of fermenting galactose, raffinose, sucrose, inulin, and glucose. Finally, the arginine dihydrolase test was positive.

Results of Polymerase Chain Reaction

Electrophoresis of PCR products on the agarose gel confirmed that 25 out of the 27 (92.5%) isolates confirmed by biochemical tests belonged to *S. pneumoniae* (Figure 1).

Sensitivity of Streptococcus pneumoniae Isolates to Essential Oils

Based on the primary screening results, the most effective EOs were *T. syriacus* Boiss. (19-23 mm), *O. syriacum* L. (18-24 mm), and *R. officinalis* L. (17-223 mm), whereas *P. roseum* L. and *D. maritima* L. EOs were ineffective against *S. pneumoniae* isolates used in this study.

Essential Oil Minimum Inhibitory Concentrations

The results of Table 3 demonstrate the MICs of several EOs against 25 isolates of *S. pneumoniae*. Based on the data, the most effective EOs against these isolates, depending on the inhibition of 90% of bacteria, were *T. syriacus* Boiss., *O. syriacum* L., *R. officinalis* L., *C. zeylanicum* L., and *J. foetidissima* Willd. Conversely, *O. syriacum* L. EO presented the most MIC_{50} potent inhibition against *S. pneumoniae* (MIC_{50}: 0.0625–0.125 µL/mL). However, *P. roseum* L. and *D. maritima* L. EOs produced the highest MIC_{50} and MIC_{90} values in the range of 1–2 µL/mL and 4–6 µL/mL, respectively.

Antibiotic Minimum Inhibitory Concentration Test

Table 4 provides MIC_{50} and MIC_{90} of several antibiotics on 25 isolates of *S. pneumoniae*. The results revealed that the most effective antibiotics against these isolates,
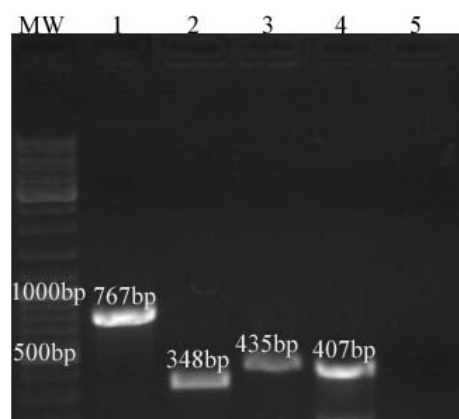

Figure 1. PCR on a 1% Agarose Gel Where: (1) *Streptococcus* spp. (Sample From Damascus Hospital), (2) *Streptococcus pneumoniae* Type (Bronchial Secretions), (3) *Streptococcus mutans* Type (Oral Swab, Positive Control), (4) *Streptococcus pyogenes* Type (Pharyngeal Swab, Positive Control), and (5) Negative Control Without DNA; MW: DNA Molecular Weight Marker

depending on the inhibition of 90% of bacteria, were cefixime, cefpodoxime, cefuroxime, ceftriaxone, ofloxacin, vancomycin, levofloxacin, rifampicin, and sparfloxacin. Contrarily, spiramycin, penicillin G, amoxicillin, cefazolin, and erythromycin were the least effective. Vancomycin and Sparfloxacin showed the highest effect (MIC_{90} = 0.125–0.25 µg/mL).

Discussion

Global interest is increasing regarding finding alternatives to traditional treatments for *S. pneumoniae* due to the increasing resistance of these bacteria to almost all these therapies. Zafar et al indicated that almost 29.7% of *S. pneumoniae* isolates represented significantly low sensitivity to erythromycin (6), with a resistance of 28% of the isolates. In another study of the same group, the resistance rate of *S. pneumoniae* to erythromycin increased to 54.8% in 2016 (17).

Therefore, when selecting the appropriate antibiotic protocol used to treat diseases caused by *S. pneumoniae* (e.g., pneumonia and meningitis), the researchers should keep in mind the high number of strains resistant to penicillin and macrolides (penicillin-intermediate *S. pneumoniae*). Several clinical studies have shown the necessity of accurately calculating the doses of penicillin

Table 3. MIC$_{50}$ and MIC$_{90}$ (µL/mL) Ranges of Some Essential Oils on 25 Isolates of *Streptococcus pneumoniae*

Essential Oils	MIC$_{50}$	MIC$_{90}$
T. syriacus Boiss.	0.125-0.25	0.25-0.5
O. syriacum L.	0.0625-0.125	0.125-0.25
L. stoechas L.	1-2	4-8
M. communis L.	0.5-2	4-6
S. aromaticum L.	0.5-1	2-4
C. zeylanicum L.	0.125-0.25	0.125-
R. officinalis L.	0.25-0.125	0.5-1
J. foetidissima Wild	0.25-0.5	0.5-2
P. roseum L.	1-2	4-6
D. maritima L.	1-2	4-6

Note. MIC: Minimum inhibitory concentration.

Table 4. MIC$_{50}$ and MIC$_{90}$ (µg/mL) of Some Antibiotics on 25 Isolates of *Streptococcus pneumoniae*

Antibiotic	MIC$_{50}$	MIC$_{90}$
Penicillin G	0.125-0.25	2-4
Amoxicillin	0.25-1	2-4
Cefixime	0.125-1	0.5-1
Cefpodoxime	0.062-0.25	0.25-0.5
Cefotaxime	0.125-0.25	0.5-1
Ceftriaxone	0.062-0.125	1-2
Cefazolin	0.25-1	2-4
Cefuroxime	0.125-0.25	0.25-1
Ofloxacin	0.25-0.5	0.5-1
Cotrimoxazole	0.5-1	1-2
Erythromycin	1-2	2-4
Spiramycin	1-2	4-6
Azithromycin	0.5-1	1-2
Chloramphenicol	0.5-1	1-2
Vancomycin	0.062-0.125	0.125-0.25
Levofloxacin	0.125-0.25	0.5-1
Tetracycline	0.25-0.5	1-2
Rifampicin	0.062-0.125	0.125-0.25
Trimethoprim-Sulfamethoxazole	0.25-0.5	1-2
Sparfloxacin	0.062-0.125	0.125-0.25

Note. MIC: Minimum inhibitory concentration.

and erythromycin if reduced sensitivity of isolates to these antibiotics is suspected (18). Clinical guidelines in many countries suggest elevated doses of some beta-lactam groups because penicillin-intermediate *S. pneumoniae* strains might not be susceptible to doses that have been used since more than thirty years ago. However, some studies investigating antibiotic resistance in Pakistan confirmed the full efficacy of the amoxicillin/clavulanic acid and cefuroxime combination, increasing the confidence in their continued use as a good regimen therapy against *S. pneumoniae* infections (17).

Some microbiological assay results revealed the potent efficacy of some EOs (including peppermint, cinnamon bark, thyme, and the like). Accordingly, further research should be conducted to estimate their suggested role as alternatives or supported therapies to traditional agents. Its multi-ingredient formula expresses its antibacterial benefit.

In our study, the most effective EOs on *S. pneumoniae* were *T. syriacus* Boiss., *O. syriacum* L., *R. officinalis* L., *C. zeylanicum* L., and *J. foetidissima* Willd. Our results are consistent with those of De Aguiar et al, indicating that MICs for several EOs, including *Origanum*, *Cinnamomum*, *Thymus*, and *Rosmarinus*, were low and ranged from 0.312 µg/mL to 0.625 µg/mL (19). Inouye et al also found that thyme and cinnamon bark were highly effective against these types of bacteria (20). In addition, a previous study showed similar results for MIC and the potent antibacterial activity of cinnamon bark against these bacteria (21).

EOs commonly consist of a combination of substances (22). The major constituents of cinnamon bark and thyme are cinnamaldehyde and carvacol, respectively, and those of rosemary oils include α-pinene, 1,8-cineole, and camphor, indicating that the activity of these oils was due to their major constituents.

Conclusion

The current study evaluated the antibacterial effect of 20 widely used antibiotics against 25 isolates of *S. pneumoniae*. Penicillin appeared to be relatively effective,

and amoxicillin and cephalosporins were highly effective. Azithromycin and erythromycin were also highly influential and could be used as a first-line alternative treatment. Interestingly, vancomycin and levofloxacin were also highly effective and could be potential alternative treatment options for streptococcal infections. In this study, it was found that most of the EOs had a moderate effect on these bacteria, and only the EOs of *T. syriacus* Boiss., *O. syriacum* L., *R. officinalis* L., *C. zeylanicum* L., and *J. foetidissima* Willd. had a good effect on the isolates tested in this study.

Authors' Contribution
Conceptualization: Ayman Al-Mariri and Mazen Safi.
Data curation: Mazen Safi and Ayman Al-Mariri.
Formal analysis: Mazen Safi.
Funding acquisition: Atomic Energy Commission of Syria.
Investigation: Mazen Safi and Marwa Khawajkiah.
Methodology: Mazen Safi and Ayman Al-Mariri.
Project administration: Mazen Safi and Ayman Al-Mariri.
Resources: Mazen Safi and Ayman Al-Mariri.
Supervision: Mazen Safi and Ayman Al-Mariri.
Validation: Mazen Safi and Ayman Al-Mariri.
Visualization: Mazen Safi, Marwa Khawajkiah, and Nour Al-Sahar.
Writing-original draft: Mazen Safi.
Writing-review and editing: Mazen Safi.

Competing Interests
There are no competing interests to be declared.

Ethical Approval
This study was conducted *in vitro*. There is no experiment conducted on humans or live animals.

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