EXPERIMENTAL PAPER

Antimicrobial activity of leaf and flowering cones of *Abies cilicica* ssp. *cilicica* (*Pinaceae*) crude extracts

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Summary

Introduction: *Abies cilicica* ssp. *cilicica* is an endemic species grown naturally in Syria. This taxon could be a potential and promising source of antimicrobial activity. **Objective:** Methanolic, ethanolic and acetonic extracts of leaf (LE) and flowering cones (FE) of *A. cilicica* ssp. *cilicica* plant were evaluated for their antimicrobial activity against 3 bacteria (*Enterococcus faecalis*, *Proteus vulgaris* and *Klebsiella pneumoniae*) and two fungi (*Candida albicans* and *Aspergillus niger*) pathogens. **Methods:** *A. cilicica* antimicrobial activity was evaluated by disc-diffusion method (zone of inhibitions-ZIs), activity index (AI) and minimum inhibitory concentrations (MICs) (Microdilution broth susceptibility assay). Similarly, ciprofloxacin (10 mg/ml) and nystatin (5 µg/ml) antibiotics were used as a standard for the antimicrobial activity of bacteria and fungi isolates, respectively. **Results:** Data revealed that the acetone FE with highest ZIs and lowest MICs showed the strongest antimicrobial activity against tested pathogens, while, ethanol LE was the lowest potent extract against all tested microorganisms. *P. vulgaris* bacteria was considered as the most sensitive isolate by showing the lowest MICs value of 0.52 µg/ml, whereas, *A. niger* fungus was pronounced as the most tolerant isolate by expressing the highest MICs value of 26.67 µg/ml. **Conclusion:** The current investigation proved the potential activity of *A. cilicica* extracts as an antibacterial and an antifungal source grown naturally in Syria.

Key words: antimicrobial activity, *Abies cilicica*, minimum inhibitory concentrations (MICs)

INTRODUCTION

Abies cilicica (Antoine et Kotschy) Carrière) subsp. *cilicica* belongs to *Abies* genus comprises about 50 species distributed in the highlands of Asia, Europe, North and Middle America and North Africa [1].

In Syria, it grows in nature reserve named “*Abies* and *Cedrus* reserve” located at 1500 m.a.s.l and occupies an area of 1350 hectares of a series of Syrian coastal mountains on eastern and western summit of the Prophet Mata. Within this reserve, approximately 142 plant species with 56 animals and birds species are in existence. Previously, it has been mentioned that different taxa of *Abies* were used as folk medicines against cold, stomach ache, indigestion, as well as vascular, pulmonary and venereal diseases [2-3].

A. cilicica is commonly known as Taurus Fir or Cilicia Fir. It is referred to several different names, e.g. Fir as a common English name, Le Sapin as a French name, Die Tanne as a German name, Goknar ağacı as a Turkish name, and as Ashuhu as a common Arabic name. Earlier, in 1853 it was known as *Pinus cilicica* Antoine et Kotschy. Two years later (1855), it was named as *Abies* (according to Élie-Abel Carrière); and this name is still in use. It is produced in areas of greater Syria and Asia Minor, this resin and its oil derivative were used in mummification, as an antiseptic, a diuretic, to treat wrinkles, extract worms and promote hair growth [4]. Their wood of most firs is considered unsuitable for general timber use, and is often used as a pulp or for the manufacture of plywood and rough timber.

Abies species have been successfully applied in pharmacological study and treatment due to their content of bioactive constituents. *Abies* spp. have been regarded to have antitumor, antimicrobial, anti-ulcerogenic, antiinflammatory, antihypertensive, antitussive, and central nervous system activities [5]. While, *A. pindrow* has been considered as an antioxidant due to its chemical constituents (total phenolic, flavonoid and flavonol) content [6].

Up to now, it has been reported that, 277 compounds have been isolated from 19 *Abies* species. In the previous study it was mentioned that terpenoids, flavonoids, and lignans, along with minor constituents of phenols, steroids, and others were the compounds of highest bioactive activities [7]. In recent study, the *A. webbiana* spasmolytic activity through possible blockade of Ca^{2+} channels showing its folkloric utility in gut and respiratory disorders in addition to antiplatelet activity was investigated [8]. Few investigations revealed the importance of *Abies* spp. *cilicica* as antimicrobial agent [9-13].

So far, few studies have been carried out to clarify their utility in traditional folk medicine worldwide. Thereby, the current study was initiated to delineate the antimicrobial activities of *A. cilicica* subsp. *cilicica* grown naturally in Syria.

MATERIAL AND METHODS

Plant materials

A. cilicica ssp. *cilicica* leaves and flowering cone samples were collected from their natural habitats along Syrian coastal mountains (Slenfch-Lattakia) at 1500 m.a.s.l with 1400 mm annual rainfall. Sampling was performed in Autumn 2013 (35°36'21"N and 36°11'18"E) and plant parts were shade-dried for one week, powdered by special electric mill and stored separately in polyethylene bags until needed for analysis.

Extraction of plant material

Crude extracts from LE and FE of *A. cilicica* were prepared using methanol, ethanol and acetone solvents as recently reported [14].

Tested microorganisms and growth conditions

The pure clinical isolates of 3 bacteria (*E. faecalis*, *P. vulgaris* and *K. pneumoniae*) and two fungi (*C. albicans* and *A. niger*) pathogens were obtained from the Microbiology and Immunology Division, Department of Molecular Biology and Biotechnology of Atomic Energy Commission of Syria (AECS) in Damascus, Syria.

For bacteria pathogens: the cultures were maintained at 37°C on 2YT agar (peptone, 16 g/l; yeast extract, 10 g/l; NaCl, 5 g/l; agar, 13 g/l [Difco, BD, Sparks, MD]); and incubated for 24–48 h. Prior to antimicrobial sensitivity test, 0.2 ml of overnight culture of each organism was dispensed into 20 ml of sterile Mueller Hinton Broth (Hi-media Laboratory Pvt. Ltd., Mumbai, India) and then incubated for about 18–24 h to standardize the cultures to approximately 10⁶ CFU/ml [14]. The bacteria were suspended in a sterile phosphate-buffered saline (PBS). Bacteria abundance in PBS was monitored by recording the optical density (OD) at 590 nm. The exact counts were assessed retrospectively by viable counts on 2YT agar plates.

For fungal pathogens, the standardized culture of tested fungal were prepared by dissolving 3.9 g of PDA in 100 ml distilled water, autoclaving, pouring and inoculating into plates. The inoculated plates were incubated at 28±3°C for 2 days.

Antimicrobial activity assay

The disc-diffusion assay

The disc-diffusion method was adopted to test the antibacterial activity as described in some investigations [11, 14]. Ciprofloxacin (10 mg/ml) and nystatin

(5 µg/ml) were used as standard drug for antimicrobial activity of bacteria and fungi isolates, respectively. Filter paper discs (Whatman No.1) of 6 mm diameter were prepared and sterilized. The discs impregnated with 100 µl of extract dilutions (100 mg/ml) and reconstituted in minimum amount of examined solvents were applied over each of the culture plates previously seeded with the 10⁶ CFU/ml cultures of bacteria. Bacterial cultures were then incubated at 37°C for 18 h, while the paper discs impregnated with 20 µl of a solution of 10 mg/ml of ciprofloxacin were used as standard antimicrobials for comparison. Negative control was prepared using examined solvents (final concentration of the solvent in the highest concentration of plant extract was tested). Antimicrobial activity was determined by measurement of zone (in mm) of inhibition around each paper disc. For each extract, duplicate trials were conducted against each organism.

Activity Index (AI)

Activity index (AI) of *A. cilicica* ssp. *cilicica* plant extracts was calculated as previously reported, using the following formula [15]:

activity index (AI) = inhibition zone of sample/inhibition zone of standard.

Determination of minimum inhibitory concentrations (MICs)

Microdilution broth susceptibility assay was used to estimate MICs values [16]. Three replicates of serial dilutions of extract (50 mg/ml) were prepared in LB broth medium in 96-well microtiter plates, using a range of concentrations for LE and FE extracts from *A. cilicica* from 0.166 to 40 µl per well. One hundred microlitres of freshly grown bacteria standardized 10⁶ CFU/ml in LB broth were added to each well. Positive control was achieved in the same conditions but without extract. Negative control was also made with the same conditions but without the addition of bacteria. The plate was incubated with shaking for 24 h at 37°C. The lowest concentration that completely inhibited visual growth was recorded and interpreted as the MICs.

Statistical analysis

All tests were performed in triplicate and values are presented as mean ± SD. Variance analysis among examined pathogens, solvents and plant parts was calculated separately using STAT-ITCF program and Student-Newman-Keuls test [17].

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

Antimicrobial activity of methanolic, ethanolic and acetonetic crude extracts from LE and FE of *A. cilicica* ssp. *cilicica* have been investigated against 5 pathogens.

Our data presented herein, revealed the all *A. cilicica* extracts showed a highest inhibitory effect against all tested microorganisms compared to the tested applied antibiotic standard expect against *E. faecalis* (tab. 1).

It was noticed that ZIs value for bacteria ranged between 15 and 25 mm in ethanol LE against *E. faecalis* and methanol FE against *P. vulgaris* isolate, respectively (tab. 1). As for fungi, this ZIs value varied between 14 and 19 mm for ethanol LE against *C. albicans* and acetone FE against *A. niger*, respectively (tab. 1).

Table 1.

Antimicrobial activity of *A. cilicica* ssp. *cilicica* expressed as ZIs [mm]

Microorganisms	Zone of inhibition [mm]						Main effect	Control
	Methanol		Ethanol		Acetone			
	LE	FE	LE	FE	LE	FE		
<i>E. faecalis</i>	16±0.1	18±0.13	15±0.13	17±0.10	17±0.19	19±0.15	16.78 B	22±0.12
<i>P. vulgaris</i>	19±0.23	25±0.19	17±0.13	21±0.19	19±0.08	23±0.24	20.33 A	19±0.12
<i>K. pneumoniae</i>	16±0.09	17±0.12	15±0.12	17±0.08	18±0.17	19±0.13	16.42 C	18±0.09
<i>C. albicans</i>	16±0.15	18±0.17	14±0.08	16±0.13	16±0.19	17±0.08	15.93 D	10±0.21
<i>A. niger</i>	17±0.19	18±0.09	15±0.11	16±0.12	17±0.15	19±0.18	16.77 B	11±0.13
Main effect of solvents	17.78 B		15.83 C		18.13 A			
Main effect of plant parts	16.23 B	18.26 A	16.23 B	18.26 A	16.23 B	18.26 A		

Observations are expressed as mean ± standard deviation (SD), n=3.

LE: leaf extracts; FE: flowering cones extracts

As shown in table 1, the growth of bacteria and fungi isolates were inhibited by methanolic, ethanolic and acetonetic extracts from LE and FE of *A. cilicica* on different degrees, according to the tested organisms, plant fraction and examined solvent. This observation was in accordance with previous data [9].

Activity index (AI) was also evaluated against the tested microorganisms (tab. 2). It was found that AI value ranged between 0.68 and 1.32 for ethanol LE against *E. faecalis* and methanol FE against *P. vulgaris*, respectively (tab. 2). Whereas, for fungi this value varied between 1.36 and 1.80 for ethanol LE against *A. niger* and methanol FE against *C. albicans*, respectively.

Analysis of variance for ZIs and AI was highly significantly different ($p<0.001$) among the examined pathogens, solvents and plant parts, separately (tab. 1, 2).

Table 2.

Activity index (AI) of *A. cilicica* ssp. *cilicica* against tested pathogens.

Microorganisms	Activity index (AI)						Main effect
	Methanol		Ethanol		Acetone		
	LE	FE	LE	FE	LE	FE	
<i>E. faecalis</i>	0.73±0.11	0.82 ±0.13	0.68±0.14	0.77±0.11	0.77±0.19	0.86±0.06	0.77 E
<i>P. vulgaris</i>	1.00±0.23	1.32 ±0.16	0.89±0.014	1.11±0.11	1.00±0.08	1.21±0.25	1.08 C
<i>K. pneumoniae</i>	0.89±0.09	0.94 ±0.10	0.83±0.12	0.95±0.04	1.00±0.17	1.06±0.07	0.91 D
<i>C. albicans</i>	1.60±0.15	1.8 ±0.06	1.40±0.09	1.60±0.08	1.60±0.19	1.70±0.06	1.62 A
<i>A. niger</i>	1.55±0.20	1.64±0.07	1.36±0.011	1.45±0.09	1.55±0.15	1.73±0.09	1.51 B
Main effect of solvents	1.21 B		1.08 C		1.24 A		
Main effect of plant parts	1.12 B	1.24 A	1.12 B	1.24 A	1.12 B	1.24 A	

Observations are expressed as mean ± standard deviation (SD), n=3.

LE: leaf extracts; FE: flowering cones extracts

Based upon data presented herein, it worth noting that the acetone FE showed the strongest antimicrobial activity against tested pathogens with highest ZIs and lowest MICs values. The highest observed potent of acetone FE against microorganisms could be attributed to the fact that this extract exhibited the most bioactive constituents (alkaloids, flavonoids, saponins, terpenoids and steroid compounds, data not shown here). These secondary bioactive compounds have been previously reported as the most effective compounds known for their antimicrobial activity [18]. Other research indicated the pharmacological effect of carotenoids, alkaloids, vitamins, minerals and polyphenols as antioxidants [6].

Other study demonstrated that the essential oil of rosin cones of *A. cilicica* ssp. *cilicica* had an antimicrobial effects compared to those obtained from leaves of the same species [9].

Previously, different degree of antimicrobial effect of essential oil from 4 species of *Abies* against 12 microorganisms has been reported [10]. The previous study mentioned that the inhibitory effect of *Abies* spp. against microorganisms could vary according to the ecological conditions, plantation and natural form of *Abies*.

Whereas, in previous investigation the antimicrobial activity of different parts (leaves, resins, bark, cones and fruits) of 5 forest species grown in Turkey, against 14 microorganisms was described [11]. The previous study indicated that no antifungal effect was found in all tested extracts (chloroform, acetone and ethanol). The growth of *E. coli* was not affected by different plant parts extracts except by chlorophorm and acetone *A. cilicica* leaves extracts with ZIs of 16–18 mm, respectively.

Furthermore, in other study the antimicrobial activity of different amount (1 μ l/disc, 2 μ l/disc and 4 μ l/disc) of essential oil cones rosin's *A. cilicica* ssp. *cilicica* against 14 microorganisms (8 Gram-positive, 3 Gram-negative bacteria and 3 yeast strains) was described [13]. The previous investigation revealed low inhibitory activity at 1 and 2 μ l/disc. While, at 4 μ l/disc, the ZIs was ranged between 8 mm (*S. aureus* and *M. luteus*) and 12 mm (*M. smegmatis* and *C. xerosis*) in the case of Gram-positive bacteria. While, it was varied between 8 mm (*P. aeruginosa*) and 13 mm (*K. pneumoniae*) with no effect observed against *E. coli* in the case of Gram-negative bacteria. Whereas, it was ranged between 7 mm (*K. fragilis*) and 11 mm (*S. cerevisiae*) for yeast microorganisms.

While, in our study, ZIs for bacteria varied between 15 mm (*E. faecalis* and *K. pneumoniae*) and 25 mm (*P. vulgaris*). As for fungi, estimated ZIs value ranged between 14 mm (*C. albicans*) and 19 mm (*A. niger*). Other investigation, however, demonstrated that ZIs for 5 bacteria isolates ranged between 7.2 mm (*P. aeruginosa*) and 10.7 mm (*E. coli*) as revealed by DMSO oil dilution 1/1000 of Numidia (*A. numidica*) needles collected from Seraidi, Algeria [19].

Moreover, MICs was also determined as preliminary screening of antimicrobial activity of *A. cilicica* extracts (tab. 3). In this regards, estimated MICs values ranged between 0.52 and 10.0 μ g/ml for acetone FE against *P. vulgaris* and ethanol LE against *E. faecalis*, respectively for tested bacteria. As for fungi, this MICs values varied between 8.33 and 26.67 μ g/ml for methanol and acetone FE against *C. albicans* and ethanol LE against *A. niger*, respectively. Variance analysis of MICs revealed highly significant different ($p < 0.001$) only among the examined pathogens (tab. 3).

Table 3.

Minimum inhibitory concentrations (MICs) *A. cilicica* ssp. *cilicica* against tested pathogens.

Microorganisms	Minimum inhibition concentrations (MICs) [μg/ml]						Main effect
	Methanol		Ethanol		Acetone		
	LE	FE	LE	FE	LE	FE	
<i>E. faecalis</i>	8.33±2.89	3.33±1.44	10.00±0.00	5.83±3.82	6.67±2.89	3.33±1.44	6.25 C
<i>P. vulgaris</i>	2.08±0.72	0.73±0.48	2.5 ±2.17	1.67 ±0.72	1.67±0.72	0.52±0.18	2.15 D
<i>K. pneumoniae</i>	5.83±3.82	5.83± 3.82	6.67± 2.89	8.33± 2.89	5.00± 0.00	4.17±1.44	6.81 C
<i>C. albicans</i>	11.67±7.62	8.33±2.89	13.33±5.78	10.00±0.00	11.67±7.64	8.33±2.89	11.11 B
<i>A. niger</i>	16.67±5.78	13.33±5.77	26.67±11.55	16.67±5.78	16.67±5.78	13.33±5.78	16.21 A
Main effect of solvents	8.28 A		9.33 A		7.9 A		
Main effect of plant parts	8.99 A	8.02 A	8.99 A	8.02 A	8.99 A	8.02 A	

Observations are expressed as mean $\pm$ standard deviation (SD), n=3.

LE: leaf extracts; FE: flowering cones extracts

In previous, the antibacterial effect of essential oil extracts for *A. holophylla* and *A. koreana* has been reported [12]. This investigation indicated that *A. koreana* oil was more potent as antibacterial and antifungal agent than *A. holophylla*. Both essential oils showed great antibacterial effect against tested bacteria in the range of 2.2–8.8 $\mu\text{g}/\text{disc}$ with MICs values of 5.5–21.8 mg/ml. Moreover, the previous study revealed the antifungal potential against *C. glabrata* with MICs value of 0.5–2.2 mg/ml. Whereas, in other study the antimicrobial activity of essential oil of nine *Abies* species growing in Turkey against 8 bacteria and 2 yeast strains was investigated [9]. The previous study showed different inhibitory activity level of oil against tested microorganisms. In this regards, yeast were the most sensitive pathogens. Whereas, *E. coli* and *S. aureus* were the most resistant bacteria expect for *A. koreana* and *A. cilicica* ssp. *isurica* and little for *A. alba* and *A. firma* essential oil. Whereas, in other investigation it was demonstrated that MICs value of essential oil cones rosin's *A. cilicica* ssp. *cilicica* ranged between 0.5 $\mu\text{g}/\text{ml}$ (*M. smegmatis*) and 3.5 $\mu\text{g}/\text{ml}$ (*S. aureus* and *M. luteus*) for the Gram-positive bacteria [13]. Whereas, it varied between 0.5 $\mu\text{g}/\text{ml}$ (*K. pneumoniae*) and >10 $\mu\text{g}/\text{ml}$ (*E. coli*). It ranged between 0.5 $\mu\text{g}/\text{ml}$ (*S. cerevisiae*) and 1.75 $\mu\text{g}/\text{ml}$ (*K. fragilis*). It was noticed that the previous investigation stated that *S. cerevisiae*, *K. pneumoniae* and *M. smegmatis* were the most sensitive microorganisms showing the lowest MICs value of 0.5 $\mu\text{g}/\text{ml}$ [13].

Our data presented herein was in accordance with previous study findings. The estimated MICs value ranged between 0.52 $\mu\text{g}/\text{ml}$ (*P. vulgaris*) with FE acetonetic extract and 10 $\mu\text{g}/\text{ml}$ (*E. faecalis*) with LE ethanolic extract.

In other investigation phytochemical components of oil from rosin of *A. cilicica* ssp. *cilicica* cones collected in Turkey were reported [20]. The latter investigation revealed that α -pinene followed by β -pinene and myrcene were essential chemical compounds of the cones of rosin. Whereas, it has been previously investigated in the essential oil composition of Numidia (*A. numidica*) needles collected from Seraidi, Algeria [19]. The previous study indicated that wealth in terpene compounds were the major components in oil. Indeed, the same investigation revealed that oil had good inhibitory effects, varying according to the examined origin.

CONCLUSION

Overall, *A. cilicica* ssp. *cilicica* FE was found to be the most potent against all tested microorganisms compared to the LE. Acetone FE with highest ZIs and lowest MICs showed the strongest antimicrobial activity against tested pathogens. Ethanol LE was the lowest active extract against all tested organisms. Otherwise, fungi were resistant to *A. cilicica* extracts with MICs of 26.67 $\mu\text{g}/\text{ml}$ compared to bacteria where MICs was found to be 10 $\mu\text{g}/\text{ml}$. In this regards, *A. niger* fungi could be considered as the most resistant pathogen showing the highest MICs value.

Thereby, *A. cilicica* spp. *cilicica* crude extracts were potent against bacteria and fungi with different degree. It is worth to test their purified bioactive compounds to investigate their biological effect separately.

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DZIAŁANIE PRZECIWDROBNOUSTROJOWE SUROWEGO WYCIĄGU Z IGIEŁ I PĄKÓW KWIATOWYCH *ABIES CILICICA* SSP. *CILICICA* (PINACEAE)

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Streszczenie

Wstęp: *Abies cilicica* ssp. *cilicica* jest gatunkiem endemicznym dziko rosnącym w Syrii. Roślina ta może być wartościowym źródłem związków o działaniu przeciwdrobnoustrojowym. **Cel:** Badania miały na celu określenie aktywności przeciwbakteryjnej (*Enterococcus faecalis*, *Proteus vulgaris* i *Klebsiella pneumoniae*) oraz przeciwgrzybiczej (*Candida albicans*, *Aspergillus niger*) wyciągów metanolowych, etanolowych i acetonowych z igieł (LE) i pąków kwiatowych (FE) *Abies cilicica* ssp. *cilicica*. **Metody:** Do oceny działania przeciwdrobnoustrojowego zastosowano metodę krążków bibułowych (ZIs), oraz metodę rozcieńczeń seryjnych w podłożu płynnym (MICs). Określano również indeks aktywności przeciwdrobnoustrojowej (AI). Jako wzorcowe substancje antybiotyczne użyto ciprofloksacyny (10 mg/ml) i nystatyny (5 µg/ml). **Wyniki:** Stwierdzono, że najsilniejsze działanie

wobec badanych bakterii i grzybów chorobotwórczych wykazywał wyciąg acetonowy z liści *Abies cilicica* ssp. *cilicica*, natomiast wyciąg etanolowy działał na te drobnoustroje najsłabiej. Spośród badanych bakterii największą wrażliwością na wyciąg acetonowy z igieł omawianego gatunku odznaczał się *P. vulgaris* (MICs = 0.52 µg/ml), a spośród badanych grzybów najwyższą wrażliwość wykazywał *A. niger* (MICs = 26.67 µg/ml). **Wnio-**
ski: Przeprowadzone badania wskazują na silne działanie przeciwbakteryjne i przeciwgrzybicze wyciągów otrzymanych z *A. cilicica* rosnącego w Syrii.

Słowa kluczowe: właściwości przeciwdrobnoustrojowe, *Abies cilicica*, minimalne stężenie hamujące (MICs)