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Original Research Article

Phytochemical Screening and *In Vitro* Antibacterial Activity of Methanol Extract of *Thymelaea hirsuta* and *Anacyclus pyrethrum* from Algeria against Multi-Drug Resistant Bacteria Associated with Skin Infections

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ABSTRACT

Medicinal plants constitute a potential source of bioactive molecules which can provide a new source of antimicrobial agents. This study aims to evaluate the antibacterial activity against multi-drug resistant bacteria associated with skin infections and to screen the phytochemical composition of *Thymelaea hirsuta* and *Anacyclus pyrethrum* from Algeria. For this purpose, four multi-drug resistant bacteria associated with skin infections were isolated from pus samples compared to *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa 27853* were used. The antibacterial activity of the methanol extracts of both plants evaluated at two different concentrations (200 mg/mL and 100 mg/mL) using the agar disc diffusion method revealed that all strains tested showed susceptibility to *Thymelaea hirsuta* methanol extract with the values recorded for inhibition zone diameters ranging from 11.00 to 25.00 mm at both concentrations: 100 mg/mL and 200 mg/mL. However, the methanol extract of *Anacyclus pyrethrum* showed an antibacterial activity only against the clinical isolates of *Staphylococcus aureus* and *Escherichia coli* ATCC 25922 (*Staphylococcus aureus* 1 at the two concentrations 200 mg/mL, 100 mg/mL; *Staphylococcus aureus* 2 and *Escherichia coli* ATCC 259 at the concentration of 200 mg/mL). The phytochemical screening of methanol extracts revealed the presence of flavonoids, polyphenols, saponins, tannins, quinones and terpanoids; and the absence of alkaloids and anthraquinones in both extracts. However, coumarin was found only in methanol extract of *Thymelaea hirsuta*. This finding showed a high efficiency of *Thymelaea hirsuta* methanol extract against multi-drug resistant bacteria associated with skin infections mainly *staphylococcus aureus*

***Keywords*:** Phytochemical screening, antibacterial activity, *Thymelaea hirsuta*, *Anacyclus pyrethrum*, skin infections bacteria.

Introduction

Skin and soft tissue infections (SSTIs) represent a group of infections characterized by microbial invasion of the skin layers and underlying soft tissues. They have variable presentations, etiologies and severities.[1-3] These infections result in the production of pus, a white to yellow exudate, present at the site of inflammation formed by pyogenic bacteria, which can produce the accumulation of dead leukocytes and infectious agent. Both aerobic and anaerobic bacteria have been implicated in wound infections.

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The most common aerobic pus producing Gram positive bacteria are *Staphylococcus aureus* and *Streptococcus spp*. and Gram-negative bacteria are *Klebsiella*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter*, *Providencia*, *Acinetobacter*, in which *Staphylococcus aureus* is the most frequent bacteria. [4,5] These infections are being more acute with the appearance and the emergence of multidrug-resistance of bacteria strains which has been increasing for several decades due to the unnecessary antibiotic use, the improper prescription of antibiotic drug and the rapid and uncontrolled use of antimicrobials in agriculture and farming, causing difficulties in treating patients and an increase in the duration of treatment and the morbidity associated with infections.[6,7,8] Medicinal plants remain a potential source of bioactive molecules since they can produce a large variety of secondary metabolites, like alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins. Some biomolecules are highly efficient in the treatment of bacterial infections and can be alternatives to antibiotics.[9,10] *Thymelaea hirsuta* is a flowering medicinal plant species belonging to the family of *Thymelaeaceae* which is widely distributed in the Mediterranean region. The aerial parts of this species are largely used as remedy to treat inflammation, hypertension and as an antiseptic.[11] *Anacyclus pyrethrum* (L.) is one of the spontaneous plant species of the *Asteraceae* family, endemic to Algeria and Morocco and its root exhibits interesting therapeutic effects, they possess interesting pharmacological properties including anticonvulsive, aphrodisiac, anticancer, androgenic and fertilizing, bioinsecticide, antidiabetic, immunostimulant, antiparasitic, antifungal and antibiotic

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effects.[12] In traditional Algerian medicine, these two plants are used to treat skin abscesses and disinfect wounds. Therefore, the aim of this work is to evaluate the antibacterial activity of the methanol extract of *Thymelaea hirsuta* and *Anacyclus pyrethrum* collected from Khenchela (Algeria) against multi-drug resistant bacteria associated with skin infections.

Material and Methods

Collection of plant material

Thymelaea hirsuta (aerial party) and *Anacyclus pyrethrum* (roots) were collected in January 2023 from Khenchela (the East of Algeria). The identity of the plants was confirmed by Dr. Zeraib. A, Department of Agronomy, Abbes Laghrour Khenchela, University, Algeria and deposited with the voucher specimen number ZATH 00123 and ZAAP 00124 respectively. Plant material was air dried in the laboratory at room temperature for 15 days. The dried samples were ground well into a fine powder with laboratory grinder. The powder was stored in the refrigerator at 4°C for further processing.

Preparation of methanol extracts

About 20 g of powdered material of each plant were mixed with 200 mL of methanol (70%) and kept under agitation using electrical shaker for 72 h at room temperature. The extracts obtained from methanol were filtered through Whatmann filter paper No.1. The filtrates were then separately concentrated in vacuum using Rotary Evaporator at 30-40°C and residue water content was evaporated 40°C with drying oven. The obtained extracts were stored in refrigerator at 4°C and were dissociated in dimethyl sulfoxide (DMSO) for prior to use.[13]

Bacterial strains

A total of 07 microorganisms were used to assess the antimicrobial activity, it includes *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 obtained from the National Center for Biotechnology Research (Constantine, Algeria); and (*Staphylococcus aureus* 1, *Staphylococcus aureus* 2, *Pseudomonas aeruginosa* and *Proteus mirabilis*) clinical isolates from pus samples from skin (furuncles, pustules). The identification and characterization of isolates were performed on the basis of Gram staining, microscopic characteristics, colony characteristic, and biochemical tests using standard microbiological methods.[14]

Antibiotics susceptibility testing of clinical isolates

Antimicrobial susceptibilities of the isolates were tested by the agar disk diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[15]The method consists to prepare an inoculum for each bacterial isolate by adjusting the turbidity to a 0.5 McFarland standard and to spread it onto Muller-Hinton agar plates. After that, the antibiotic impregnated discs were dropped on agar plates. The incubation was carried out overnight at 37°C for 24 hours. The zones of inhibition were measured and the isolates were classified as sensitive, intermediate, and resistant.

Phytochemical screening

The extracts were subjected to phytochemical screening to test the presence of metabolites such as flavonoids, polyphenols, saponins, tannins, free quinones, coumarin, steroids, terpanoids, alkaloids and anthraquinone.[16,17]

Preparation of stock solutions

The extracts were prepared by reconstituting of the dried crude powder extracts in 100% (DMSO) at two different concentrations: 100 mg/ml and 200 mg/ml.

Antibacterial activity of the crude methanol extract

In vitro antibacterial activity of the crude methanol extracts was studied using the agar disc diffusion method. The bacterial strains collected in prepared slants of nutrients agar were sub-cultured into prepared nutrients agar and incubated at 37°C for 24 hours and standardized to 0.5 Mc-Farland in saline solution (0, 85%) before use. The Mueller Hinton agar plates were inoculated with test micro-organism, then, sterile filter paper discs (Whatman No1, 6 mm) was impregnated with 20µl of each concentration (200 mg/ml, and 100 mg/ml) using a micropipette. Disc impregnated with DMSO (100%) was used as a negative control. The Petri dishes were then incubated at 37 C for 24 h. After incubation, the diameter of the zone of inhibitions was measured in the units of a millimeter (mm). The experiments were carried out in triplicates and the mean values of three readings were recorded.[18]

Determination of minimal inhibition concentration (MIC)

Minimal inhibitory concentration (MIC) defines *in vitro* levels of susceptibility or resistance of specific bacterial strains to applied antibiotic or extract. Reliable assessment of MIC has a significant impact on the choice of a therapeutic strategy, which affects efficiency of an infection therapy. The MIC of each extract was determined by using the broth dilution method.[19] Five successive two-fold serial dilutions of methanol extracts were prepared in DMSO (50, 25, 12.5, 6.25 and 3.125 mg/mL). 5 mL of the MH broth was pipetted into each of 5 test tubes. 0.1 mL of the standardized inoculum of test pathogens was dispensed into each of the test tubes containing the MH broth, then, 100 µl of the prepared successive two-fold serial dilutions of the methanol extracts concentrations were mixed with the MH broth. Thereafter, all test tubes were incubated at 37ºC for 24 h. The MICs were read as least concentration that inhibited any visible growth (absence of turbidity) of the test organisms.

Statistical analysis

For the antibacterial activity, the experiments were carried out in three different experiments. The data were expressed as arithmetic mean ± standard deviation, evaluated with excel (2016).

Results and Discussion

Phytochemical screening

The phytochemical screening of methanol extracts revealed the presence of flavonoids, polyphenols, saponins, tannins, quinone and terpanoids; while Alkaloids and Anthraquinone were absent in both extracts. However, coumarin was found only in methanol extract of *Thymelaea hirsuta* (Table 1). Secondary metabolites of plants exert antimicrobial activity in different mechanism. The antibacterial activity of flavonoids may have multiple cellular targets rather than a specific site of action. The formation of complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation is one of their molecular actions. Thus, their mode of antimicrobial action may be related to their ability to inactivate cell envelope transport proteins, microbial adhesins, enzymes, and so forth. Lipophilic flavonoids may also disturb microbial membranes.[20] Polyphenols have also been demonstrated potential antibacterial, antifungal and antiviral activities. Antimicrobial effects of the wood-associated polyphenolic compounds were tested against both Gram-negative (*Salmonella*) and Gram-positive bacteria (*Listeria monocytogenes*). Polyphenols were able to suppress a number of microbial virulence factors, such as, inhibition of biofilm formation, neutralization of bacterial toxins, and reduction of host ligands adhesion and show synergism with antibiotics.[21] Saponins show antimicrobial activity by inhibiting the growth of Gram-negative or Gram-positive microorganism. However, some saponins are not efficient against Gram-negative microorganisms because they can't penetrate into the cell membranes of the microorganisms.[22] The antimicrobial effects of tannins have been widely recognized. Tannins can exert the inhibition of cell protein synthesis since they found to react with praline-rich protein to from irreversible complexes. Herbs that have tannins as their major components are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery.[23]

Antibiotic sensitivity pattern of clinical isolates strains

The examination of the data listed in Table 2 has shown that both clinical isolates *Staphylococcus aureus* 1 and *Staphylococcus aureus* 2 were resistant to penicillin, cefoxitin, oxacillin and erythromycin; *Proteus mirabilis* was resistant to fosfomycin, furan and colistin, while *Pseudomonas aeruginosa* showed a resistance to gentamicin, ofloxacin, trimethoprim/sulfamethoxazole, chloramphenicol, imipenem, ceftriaxone, cefipime and ceftazidime. According to the literature, when the bacteria is non-susceptible to at least one agent in three or more antimicrobial categories, it is regarded as MDR bacteria which remains

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a significant problem for humanity as it is associated with morbidity, mortality, and financial consequences.[24,25] Therefore, all clinical isolates strains were recorded as multi-drug resistant bacteria strains.

Antibacterial activity

The results of the antibacterial activity and the MIC of methanol extracts from *Anacyclus pyrethrum* (roots) and *Thymelaea hirsuta* (aerial party) against multi-drug resistant bacteria associated with skin infections were shown in table 05 and Figure1 respectively. The obtained data revealed that all strains tested showed susceptibility to *Thymelaea hirsuta* methanol extract with the values recorded for inhibition zone diameters ranging from 11.00 to 25.00 mm at the two different concentrations: 100 mg/mL and 200 mg/mL and a minimal inhibition concentration which correspond the lowest concentration of plant extracts that completely inhibited the growth of the organism in the broth medium (inhibition of 100%) ranging from 25 mg/mL −100 mg/mL. However, the methanol extract of *Anacyclus pyrethrum* showed an antibacterial activity only with both clinical isolates of *Staphylococcus aureus* and *Escherichia coli* ATCC 25922 (*Staphylococcus aureus* 1 at the two concentrations 200 mg/mL, 100 mg/mL; *Staphylococcus aureus* 2 and *Escherichia coli* ATCC 25922 at the concentration of 200 mg/mL) and a minimal inhibition concentration corresponding to 50 mg/mL with *Staphylococcus aureus* 1.

Table 1: phytochemical screening of the methanol extracts

	Thymelaea hirsuta	*Anacyclus pyrethrum*
Flavonoids	+	+
Polyphenols	+	+
Saponins	+	+
Tannins	+	+
Free quinone	+	+
Coumarin	+	-
Steroids	-	-
terpanoids	+	+
Alkaloids	-	-
Anthraquinone	-	-

+ : Presence, - : Absence

Table 2: Antibiotic sensitivity pattern of clinical isolates of *S. aureus* 1 and *S. aureus* 2

	S. aureus 1	***S. aureus 2***
Penicillin	R	R
Cefoxitin	R	R
Oxacillin	R	R
Gentamicin	S	S
Amikacin	S	S
Erythromycin	R	R
Clindamycin	S	S
Pristinamycin	S	S
Vancomycin	S	S
Ofloxacin	S	S
Trimethoprim/Sulfamethoxazole	S	S
Fosfomycin	S	S
Fusidique Acid	S	S
Chloramphenicol	S	S

S: sensitive; R: resistant

Antibacterial activity of *Thymelaea hirsuta* methanol extract was found mainly against both Gram negative and Gram positive bacteria. In contrast, other studies were not consistent with our results, Kang *et al*.[26] reported that the Gram positive bacteria are more resistant to methanol extracts of medicinal plants than the Gram negative bacteria, this sensibility due to their hydrophilic out membrane owing to the consist of lipopolysaccharide molecular, thus, small hydrophilic molecules pass the outer membrane, on the other hand, this outer membrane have property passing the lipophilic compounds and macromolecules and permeating outer membrane of the microorganisms is prerequisite condition for any solute having antibacterial activity. However, Al-Bakri and Afifi.[27] reported that the antimicrobial activity of the tested plant extracts against the Gram negative bacteria was either low or inactive, and Gram negative bacteria show higher resistance towards antimicrobial agents. Trigui *et al*.[28] indicated that *Thymelaea hirsuta* from Tunisia revealed stronger antibacterial and antifungal activities against all the tested microorganisms using the ethyl acetate crude extract while, the acetone extract showed a strong antibacterial activity but a weak antifungal activity. Hexane, chloroform, ethanol and water extracts were found to be inactive within the tested concentration range. Previous studies also reported that *Thymelaea hirsuta* extracts had antibacterial activity.[29,30] Felhi *et al*.[31] revealed that the essential oil isolated by the hydro-distillation of aerial parts of *Thymelaea hirsuta* exhibited a moderate-to-potent antimicrobial activity against all the microorganisms tested. Gram-positive bacteria were noted to be more sensitive to the oil than gram-negative bacteria and yeasts. Bounab *et al*.[32] also reported that essential oil of *Thymelaea hirsuta* from Algeria has antibacterial activity against eight tested bacteria. These differences may be due probably to the phytochemical composition of the plants used and the extraction process (water or solvents). The known antibacterial mechanisms of medicinal plants against microorganisms include inhibition of cell wall synthesis or interference in the permeability of cell membrane which had a consequence mutation, cell damage, and death of cells due to the increase of permeability and loss of cellular constituents, membrane disruption and change of the structure and the function of key cellular constituents.[26] Moreover, no antimicrobial activity was recorded for *Anacyclus pyrethrum* methanol extract against *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 at both tested concentrations (200 mg/ml and100 mg/ml). Furthermore, Jalayar *et al*.[33] reported that the methanol extract of *Anacyclus pyrethrum* has an inhibitory effect on *Escherichia coli* at the concentrations from 300 to 1000 mg/mL with a value of MIC and MBC corresponding to 800 mg/ mL. According to previous study [19,34], the absence of antimicrobial activity does not mean that the bioactive compounds are not present in the plant or the plant has no antimicrobial activity against microorganisms since various factors such as the dose of solvent used in the extraction of metabolites, the extraction time and rotary evaporator used during extraction of crude also trigger influence on the activities of crudes (the temperature used)

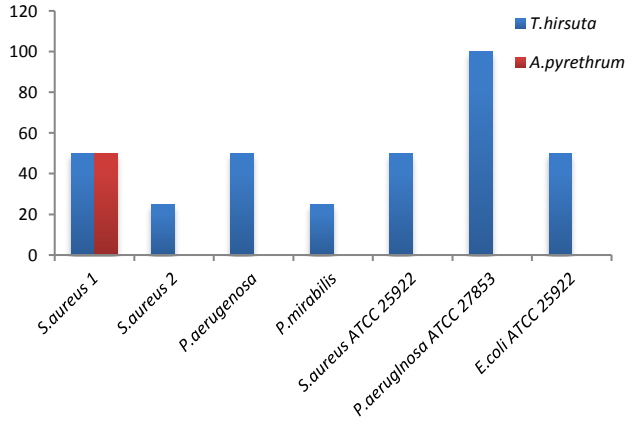

Figure 1: Minimal inhibitory concentrations (MIC) of methanol extracts against selected strains bacteria

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Table 3: Antibiotic sensitivity pattern of clinical isolate of *Proteus mirabilis*

Antibiotic	Sensitivity pattern
Cefoxitin	S
Gentamicin	S
Amikacin	S
Trimethoprim/Sulfamethoxazole	S
Fosfomycin	R
Amoxicillin	S
Amoxicillin+ Clavulaniqu Acid	S
Ticarcillin	S
Piperacillin	S
Cefazolin	S
Cefotaxim	S
Imipenem	S
Ciprofloxacin	S
Nalidixic Acid	S
Furan	R
Colistin	R

Table 4: Antibiotic sensitivity pattern of clinical isolate of *Pseudomonas aeruginosa*

Antibiotic	Pattern sensitivity
Gentamicin	R
Ofloxacin	R
Trimethoprim/Sulfamethoxazole	R
Chloramphenicol	R
Ticarcillin	S
Piperacillin	S
Imipenem	R
Colistin	S
Ticarcilin+ Clavulanic Acid	S
Ceftriaxone	R
Cefipime	R
Ceftazidime	R
Aztreonam	S

Table 5: Inhibition diameter (mm) of methanol extracts against bacteria strains tested

Test organism	*Thymelaea hirsuta*		*Anacyclus pyrethrum*		Control
	200 mg/ml	100 mg/ml	200 mg/ml	100 mg/ml	DMSO
S. aureus 1	25.00* ± 00 mm	20.6 ± 0.57 mm	11.00 ± 00	10 ± 00 mm	00 mm
S. aureus 2	23.66 ± 0.57 mm	21 ± 01 mm	09.00 ± 00	00.00 mm	00 mm
P. aeruginosa	15.33 ± 0.57 mm	14.33 ± 0.57 mm	00.00 mm	00.00 mm	00 mm
P. mirabilis	12.33 ± 0.57 mm	10.00 ± 00 mm	00.00 mm	00.00 mm	00 mm
S. aureus ATCC 25922	12.66 ± 0.57 mm	11.00 ± 00 mm	00.00 mm	00.00 mm	00 mm
P. aeruginosa ATCC 27853	14.66 ± 0.57 mm	14.00 ± 00 mm	00.00 mm	00.00 mm	00 mm
E. coli ATCC 25922	17.33 ± 1.15 mm	15.00 ± 1.73 mm	09.00 ± 00	00.00 mm	00 mm

* Strains with inhibition zones <8mm were classified as negative (00 mm)
Results are means of three different experiments

Conclusion

The present study shows that methanol extract of *Anacyclus pyrethrum* has antibacterial effect against both clinical isolates of *Staphylococcus aureus* and *Escerichia coli* ATCC 25922, while *Thymelaea hirsuta* methanol extract exhibited significant antibacterial activity against all of the 7 bacteria used in this study. This supports the traditional usage mainly of *Thymelaea hirsuta* for therapeutics to treat skin abscesses and disinfect wounds and would be used as alternative to antibiotics.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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