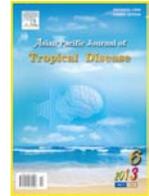


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Phytochemical screening and *in vitro* antimicrobial activity of *Thymus lanceolatus* Desf. from Algeria

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PEER REVIEW

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Comments

This is a good and an original study in which authors have valorised *T. lanceolatus*, an endemic medicinal plant from Algeria, for antimicrobial activity against pathogens responsible for many human diseases. The findings of this study are interesting, where authors report that *T. lanceolatus* contains some phytochemicals which provided a strong antimicrobial activity.

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ABSTRACT

Objective: To investigate the antimicrobial activity of an endemic *Thyme*, *Thymus lanceolatus* (*T. lanceolatus*), against a large number of pathogens.

Methods: Four solvent extracts were evaluated for antimicrobial activity using disc diffusion method and MIC determination on twenty-one strains.

Results: *T. lanceolatus* extracts showed a broad-spectrum antimicrobial activity, especially ethanol extract with inhibition zone diameters ranging from 14 to 32 mm, and MIC values from 0.052 to 0.500 mg/mL. Chloroform extract was more active against Gram-positive bacteria, since it has an inhibitory potency on *Staphylococcus aureus* and *Enterococcus faecalis* at only 31 µg/mL. While, hexane and water extracts were less effective since they were inactive against several strains.

Conclusions: The findings of this study indicate that *T. lanceolatus* has a strong antimicrobial potential, which justifies its use in folk medicine for treatment of infectious diseases. Since this species is poorly investigated, further refined studies on its pure secondary metabolites are needed and very important, in the perspective to identify new antimicrobial molecules from this endemic plant.

KEYWORDS

Antimicrobial activity, Endemic, *Thymus lanceolatus*, Solvent extracts

1. Introduction

Plant derivatives, such as infusions and decoctions, have since long been used in traditional medicine for prevention and treatment of several pathologies[1,2]. Including common infectious diseases, leading causes of morbidity and mortality[3], which are directly responsible for 26% of annual deaths worldwide[4], were also treated by those plants preparations since ancient times[5]. In modern medicine, the discovery of bioactive molecules from microbial origin and synthetic antimicrobial agents decreased the use of traditional plant preparations, especially in developed

countries. But in last decade, and besides their toxicities and side-effects[6], it has become clear that current antimicrobial drugs are losing their effectiveness[7], due to the emergence of resistance in pathogens to those antibiotics, which causes therapeutic failures and death of individuals[8]. Therefore, there is a continuing need to search for new antibiotics[3,7]. Since plant-derived antibacterials are always a source of novel therapeutics[9], many researchers in field of ethnopharmacology have concentrated in studies of those compounds in the past few decades[5] on a promising and wealthy source for safe and effective new antimicrobial agents.

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The genus *Thymus* L. which belongs to Lamiaceae family, rich in medicinal and aromatic species, is one of the well-known genera by their pharmaceutical properties. Used from antiquity as antimicrobial drugs, antiseptic, food preservative, as flavour in food, and cosmetic, *Thyme* species have thus a huge economic importance^[10]. *Thymus* genus has been extensively studied. Some native species in this genus remain poorly or not studied such as *Thymus lanceolatus* Desf. (*T. lanceolatus*) and *Thymus guyonii* de Noé which are endemic in Algeria^[11]. *T. lanceolatus*, an aromatic *Thyme* with long leaves, is a rare species which grows wild in Northwestern Algeria, precisely in mountains of Tlemcen and Tiaret. Apparently, no work has been published on the antimicrobial activity of different extracts from this plant. This plant is known locally, with the species *Thymus pallescens* de Noé, as “Zaater” and is traditionally used for the preparation of herbal teas and for flavoring and meat hygiene especially poultry.

In the same line of research of new bioactive alternative products, this work aims to evaluate the antimicrobial activity of some aqueous and organic extracts from aerial parts and roots of the species *T. lanceolatus* harvested in Tlemcen region. The use of leaves of this plant for the preservation of food and in treatment of infectious diseases, such as festering wounds and foodborne disorders give an idea of their antimicrobial potential. In addition, traditionally known beneficial effects of its decoction on health require a qualitative study to determine the phytochemical families responsible for these effects.

2. Materials and methods

2.1. Plant material

The plant material of *T. lanceolatus* was collected in July 2012 during full inflorescence, from an elevated area called Terni (1300 m) located in the south of Tlemcen (west of Algeria) between 1°22'20"–1°22'30" West and 34°47'12"–34°47'35" North. The botanical identification of species was carried out by Laboratory of Ecology and Management of Natural Ecosystems, University of Tlemcen. A voucher specimen was deposited in our laboratory under code TL06121. All harvested plant materials consisting of roots, leaves, stems, flowers, and seeds were washed and dried by spreading in open air and away from sun light for 20 d.

2.2. Extracts preparation

All dried plant materials (100 g) were pulverized and extracted using Soxhlet apparatus separately by four solvents with different polarity, namely, hexane, chloroform, ethanol, and distilled water. After 6 h of extraction, until the used solvent turned pure and colorless^[12], extracts were filtered with Whatman No. 1 filter paper and then solvents were

removed by rotary evaporation. Then, residues obtained from each extract were recovered in dimethylsulfoxide (DMSO) at a concentration of 100 mg/mL. At last, resulting extracts were sterilized by filtration through syringe filter (0.2 µm) and conserved in dark at 4 °C.

2.3. Phytochemical tests

Screening of principal *T. lanceolatus* chemical families which have a pharmacological interest was performed according to standard methods^[13,14]. These tests involved detecting the different chemical families that existed in the plant by precipitation reactions or staining using specific reagents to each family of compounds.

2.4. Microbial strains

A large number of microbial species were selected for this study. A total of 21 reference strains including 19 bacterial and two fungal strains represented the principal sources of microbial infections for humans, for better evaluation of the antimicrobial potency of this medicinal plant. The fungal strains were yeasts which were *Candida albicans* ATCC 10231 (*C. albicans*) and *C. albicans* IPP 444. Among bacterial strains, 11 strains were Gram-negative which were *Acinetobacter baumannii* ATCC 19606 (*A. baumannii*), *Citrobacter freundii* ATCC 8090 (*C. freundii*), *Escherichia coli* ATCC 25922 (*E. coli*), *Enterobacter cloacae* ATCC 13047 (*E. cloacae*), *Klebsiella pneumoniae* ATCC 70603 (*K. pneumoniae*), *Proteus mirabilis* ATCC 35659 (*P. mirabilis*), *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Pseudomonas fluorescens* ATCC 13525 (*P. fluorescens*), *Salmonella enteritidis* ATCC 2453 (*S. enteritidis*), *Salmonella montevideo* ATCC 3581 (*S. montevideo*), and *Salmonella typhimurium* ATCC 13311 (*S. typhimurium*); and eight Gram-positive which were *Bacillus cereus* ATCC 11778 (*B. cereus*), *Bacillus subtilis* ATCC 6633 (*B. subtilis*), *Enterococcus faecalis* ATCC 29212 (*E. faecalis*), *E. faecalis* ATCC 49452, *Listeria monocytogenes* ATCC 19115 (*L. monocytogenes*), *Staphylococcus aureus* ATCC 25923 (*S. aureus*), *S. aureus* ATCC 29213, and *S. aureus* ATCC 33862.

2.5. Antimicrobial assay

2.5.1. Inoculum preparation

Previously conserved and revived strains were taken and inoculated in Mueller–Hinton broth (Conda Pronadisa™, Spain) for bacteria and Sabouraud broth (Fluka®, India) for fungi at 37 °C. After 24 h of incubation, suspensions were taken and shaken well using the vortex then diluted for standardizing. Inocula were set to 0.5 McFarland or an optical density from 0.08 to 0.13 at 625 nm wavelength, which corresponds to 10⁸ CFU/mL^[15].

2.5.2. Disc diffusion method

We have used Kirby–Bauer's agar disc diffusion modified

method^[16], as preliminary antimicrobial potency evaluation of extracts. In short, plates of Mueller–Hinton agar for bacteria and Sabouraud agar for fungi were firstly inoculated by swabbing of standardized microbial suspension (10^8 CFU/mL), accordingly to Clinical And Laboratory Standards Institute 2006 recommendations^[15]. Then, Whatman No. 3 filter paper discs (6 mm of diameter), impregnated with 10 μ L of extracts at 100 mg/mL (1 mg per disc), were placed on the surface of agar. Four discs were placed peripherally in each plate. In addition, discs of gentamicin (10 μ g) and amphotericin B (100 μ g) were placed on the center of plate and served as positive controls for bacterial and fungal strains respectively. After incubation at 37 °C for 24 h, antimicrobial activity of *T. lanceolatus* extracts was determined by measuring the diameter of inhibition zones in millimeters (mm) by Vernier scale. All tests were performed in triplicate.

2.5.3. Determination of minimum inhibitory concentration

The MICs were determined by broth microdilution using 96–well microtiter plates method modified by Wiegand *et al.*^[17]. Briefly, 10 concentrations of each solvent extract ranged from 10.00 to 0.08 mg/mL were made by 1/2 dilutions in sterile glass tubes. Dilution was performed using a blank solution constituted by Mueller–Hinton broth or Sabouraud broth (for bacteria or fungi respectively) with 10% DMSO to keep the concentration of that emulsifier constant. Then, microtiter plates were prepared by filling 180 μ L of 5.10^5 CFU/mL inocula (prepared by 1/200 dilution of 10^8 CFU/mL inocula) with 20 μ L of each concentration. The final concentration in wells was ranging from 4.000 to 0.007 (mg/mL), and the final concentration of DMSO was 1% in each

well. After incubation at 37 °C for 24 h, MICs were determined as the lowest concentration of extract for which no microbial growth was observed by visual inspection of the media. At the same moment, susceptibility test according to Clinical And Laboratory Standards Institute 2006^[18] was performed with gentamicin for bacterial strains and amphotericin B for fungal strains and served as positive control. All tests were performed in triplicate.

3. Results

Screening of *T. lanceolatus* principal chemical families is presented in Table 1.

Table 1

Phytochemical screening of principal chemical families of *T. lanceolatus*.

Compositions	Solvents			
	Hexane	Chloroform	Ethanol	Water
Alkaloids	–	–	–	–
Anthocyanins	–	–	–	–
Anthraquinones	–	–	–	–
Coumarins	–	–	–	–
Emodins	–	–	–	–
Essential oils	+	++	++	–
Flavonoids	–	–	++	+
Iridoids	–	+	+	–
Saponins	–	–	–	++
Steroids	++	++	+	–
Tannins	–	–	+	++
Terpenoids	+	++	+	–
Xanthones	–	–	–	–

(++): Presence at high content; (+): Presence; (–): Absence.

Table 2

Antimicrobial activity of *T. lanceolatus* extracts using disc diffusion method.

Organisms	Extracts (1 mg per disc)				Antibiotics	
	Hexane	Chloroform	Ethanol	Water	GEN1	AMB2
<i>A. baumannii</i> ATCC 19606	9±1	14±1	20±1	8±0	18±1	NA
<i>C. freundii</i> ATCC 8090	8±0	13±1	14±1	–	18±1	NA
<i>B. cereus</i> ATCC 11778	–	19±1	16±1	–	20±1	NA
<i>B. subtilis</i> ATCC 6633	–	20±1	18±1	–	20±1	NA
<i>E. coli</i> ATCC 25922	10±1	10±1	16±1	8±0	21±1	NA
<i>E. cloacae</i> ATCC 13047	–	9±1	20±1	9±1	20±1	NA
<i>E. faecalis</i> ATCC 29212	11±0	18±1	15±1	9±0	19±1	NA
<i>E. faecalis</i> ATCC 49452	12±1	17±1	14±1	11±1	20±1	NA
<i>K. pneumoniae</i> ATCC 70603	–	7±0	16±1	–	20±1	NA
<i>L. monocytogenes</i> ATCC 19115	8±0	14±1	15±1	9±1	21±1	NA
<i>P. mirabilis</i> ATCC 35659	–	9±0	25±1	12±1	22±1	NA
<i>P. aeruginosa</i> ATCC 27853	–	8±0	17±1	–	17±1	NA
<i>P. fluorescens</i> ATCC 13525	–	8±0	22±1	8±0	18±1	NA
<i>S. enteritidis</i> ATCC 2453	8±0	7±0	18±1	7±0	19±1	NA
<i>S. montevideo</i> ATCC 3581	9±1	9±1	19±1	9±0	18±1	NA
<i>S. typhimurium</i> ATCC 13311	10±1	9±1	20±1	9±0	19±1	NA
<i>S. aureus</i> ATCC 25923	–	33±2	32±1	15±1	21±1	NA
<i>S. aureus</i> ATCC 29213	–	30±1	30±1	14±1	20±1	NA
<i>S. aureus</i> ATCC 33862	–	31±1	30±1	15±1	20±1	NA
<i>C. albicans</i> ATCC 10231	–	11±0	15±1	–	NA	19±1
<i>C. albicans</i> IPP 444	–	12±1	16±1	–	NA	20±1

Data are expressed as mean±SD. (–): no activity. NA: not applicable. GEN1: Gentamicin (10 μ g per disc), AMB2: Amphotericin B (100 μ g per disc).

These phytochemical analyses showed that Algerian *Thyme* was richly contains essential oils, saponins, and polyphenols, namely flavonoids and tannins. While, these tests have shown absence of many secondary metabolites, such as alkaloids, anthraquinones, xanthones, and coumarins. Yields of *T. lanceolatus* extracts were 6.3% by hexane, 16% by chloroform, 14% by ethanol, and 9.8% by water.

The antimicrobial activity of *T. lanceolatus* extracts evaluated by disc diffusion method is summarized in Table 2.

From these preliminary results, we could notice clearly that Algerian *Thyme* had an interesting antimicrobial activity. Among active *T. lanceolatus* extracts, ethanol and chloroform extracts have shown a broad-spectrum antimicrobial activity on both bacterial and fungal strains. Ethanol extract was the most active, with inhibition zones large than 14 mm, up to 32 mm against *S. aureus* strains. Chloroform was more active against Gram-positive bacteria. Concerning the rest of solvents, hexane and water extracts were inactive against some strains, such as *Bacillus* spp., *P. aeruginosa*, and *K. pneumoniae*.

Quantitative evaluation of *T. lanceolatus* extracts antimicrobial potency through MIC determination is summarized in Table 3. As disc diffusion method results, ethanol and chloroform revealed a strong antimicrobial

activity. From which, ethanol extract has shown a broad-spectrum activity against all studied strains, with low MIC values ranging from 0.052 to 0.500 mg/mL whether for Gram-positive, Gram-negative bacteria or fungi. *S. aureus* and *E. faecalis* strains were the most sensitive to *T. lanceolatus* extracts, which were inhibited at 0.031 mg/mL by chloroform extract. While, hexane extract was the less active since 4 mg/mL had no effect on several strains.

4. Discussion

Since microbial resistance to antibiotics reduces the possibility of treating infectious diseases and increases mortality^[19], the discovery of new antimicrobial agents is very important to improve mankind's future health^[3]. In this report, we have evaluated the antimicrobial potency of *T. lanceolatus* extracts as potential source for new antimicrobial agents, since this species is not studied. As expected, antimicrobial activity evaluation against large number of microbial strains, by two recommended methods showed that *T. lanceolatus* extracts had a strong effect against microorganisms, especially ethanol and chloroform extracts with board-spectrum activity. Comparison of *T. lanceolatus* antimicrobial activity with other medicinal plants well known by their strong antimicrobial activity, such as *Allium*

Table 3

Minimum inhibitory concentrations for *T. lanceolatus* extracts.

Organisms	Extracts (mg/mL)				Antibiotics (µg/mL)	
	Hexane	Chloroform	Ethanol	Water	GEN1	AMB2
<i>A. baumannii</i> ATCC 19606	4.000±0.000	0.125±0.000	0.125±0.000	4.000±0.000	2.000±0.000	NA
<i>C. freundii</i> ATCC 8090	2.000±0.000	1.000±0.000	0.500±0.000	–	2.666±1.154	NA
<i>B. cereus</i> ATCC 11778	–	0.083±0.036	0.125±0.000	–	4.000±0.000	NA
<i>B. subtilis</i> ATCC 6633	–	0.125±0.000	0.250±0.000	–	4.000±0.000	NA
<i>E. coli</i> ATCC 25922	4.000±0.000	1.000±0.000	0.500±0.000	–	2.000±0.000	NA
<i>E. cloacae</i> ATCC 13047	–	1.000±0.000	0.250±0.000	4.000±0.000	2.000±0.000	NA
<i>E. faecalis</i> ATCC 29212	2.000±0.000	0.031±0.000	0.104±0.036	4.000±0.000	8.000±0.000	NA
<i>E. faecalis</i> ATCC 49452	4.000±0.000	0.041±0.018	0.063±0.000	–	8.000±0.000	NA
<i>K. pneumoniae</i> ATCC 70603	–	1.000±0.000	0.500±0.000	–	1.333±0.577	NA
<i>L. monocytogenes</i> ATCC 19115	–	0.031±0.000	0.250±0.000	4.000±0.000	2.000±0.000	NA
<i>P. mirabilis</i> ATCC 35659	–	1.000±0.000	0.208±0.072	2.000±0.000	>8.000	NA
<i>P. aeruginosa</i> ATCC 27853	–	2.000±0.000	0.250±0.000	–	1.000±0.000	NA
<i>P. fluorescens</i> ATCC 13525	–	1.000±0.000	0.250±0.000	–	1.000±0.000	NA
<i>S. enteritidis</i> ATCC 2453	–	1.333±0.577	0.500±0.000	4.000±0.000	2.000±0.000	NA
<i>S. montevideo</i> ATCC 3581	–	1.000±0.000	0.250±0.000	4.000±0.000	2.000±0.000	NA
<i>S. typhimurium</i> ATCC 13311	4.000±0.000	0.500±0.000	0.250±0.000	4.000±0.000	2.000±0.000	NA
<i>S. aureus</i> ATCC 25923	–	0.041±0.018	0.063±0.000	1.000±0.000	0.416±0.144	NA
<i>S. aureus</i> ATCC 29213	–	0.031±0.000	0.052±0.018	1.000±0.000	0.333±0.144	NA
<i>S. aureus</i> ATCC 33862	–	0.031±0.000	0.063±0.000	0.833±0.288	0.250±0.000	NA
<i>C. albicans</i> ATCC 10231	–	0.125±0.000	0.104±0.036	–	NA	1.000±0.000
<i>C. albicans</i> IPP 444	–	0.125±0.000	0.063±0.000	–	NA	1.000±0.000

Values are expressed as mean±SD. All tests were performed in triplicate (mg/mL). (–): not determined. NA: not applicable. GEN 1: Gentamicin; AMB 2: Amphotericin B.

sativum (*A. sativum*) and *Camellia sinensis*, it shows that Algerian *Thyme* has an effectiveness close to these herbs. For example, the MIC of *A. sativum* methanol extract was 0.1 mg/mL on *B. subtilis*, while MIC of *T. lanceolatus* ethanol extract was 0.25 mg/mL on this Gram-positive bacillus. In the other hand, *T. lanceolatus* ethanol extract was active on all strains tested contrary to *A. sativum* methanol extract where 2 mg/mL of this extract was ineffective against *S. aureus* and *C. albicans*[20]. Furthermore, inhibition zone diameters of *C. sinensis* ethanol extract were up to 16 mm, 17 mm, 17 mm against *B. subtilis*, *E. coli*, and *S. aureus* respectively, as the strongest antimicrobial activity[21]. When compared with *T. lanceolatus* ethanol extract, we note clearly that Algerian *Thyme* is more active since inhibition zone diameters of ethanol extract on *S. aureus* were large than 30 mm.

Since implied in protection from insects and microbial infections, some secondary metabolites are the natural defense system of plants[22,23], which explains the antimicrobial potency of those substances. The principally chemical families responsible for antimicrobial activity in medicinal plants are polyphenols, terpenoids, alkaloids, iridoids, saponins, coumarins, and xanthoness[7]. The genus *Thymus*, which belongs to Lamiaceae family, is well known by its high content of terpenoids[10] and polyphenols[24]. Phytochemical screening of *T. lanceolatus* has confirmed that this species has the same phytochemical profile, a high content of polyphenols and essential oils, while alkaloids and coumarins are absent. Therefore, the antimicrobial activity seen in *T. lanceolatus* extracts is principally due to those secondary metabolites, or possibly synergistic effect between them, specifically noted in ethanol extract, which contains iridoids, flavonoids, tannins, and essential oils, and was the most active among all extracts. *T. lanceolatus* chloroform extract was more active against Gram-positive bacteria mainly due to its richness in essential oils, since those volatile secondary metabolites are effective against Gram-positive bacteria than Gram-negative ones[25].

As findings of this study, evaluation of *T. lanceolatus* antimicrobial potency on large number of microorganisms has shown that Algerian *Thyme* has a strong antimicrobial activity against all strains selected for this study, which justifies its wide use by local population. Since it is rich in terpenoids and flavonoids, which can be used as new pharmacologically acceptable antibiotics in the future[7,26], *T. lanceolatus* can be a source of new antibiotics. In the other hand, because of the absence of data for the chemical composition, further refined studies on extracts obtained from this plant are interesting.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Assessment and evaluation of unstudied plants, which are often endemic and rare, is scientifically very important. This line of research has an importance in pharmaceutical industry for valorization of those plants, especially species with biological activities, in the perspective to discover new molecules with pharmacological interest.

Research frontiers

The study was carried out to investigate the antimicrobial activity of *T. lanceolatus*, which is, up to date, an unstudied species since there are no studies published online on its chemical and biological properties. The evaluation of the antimicrobial activity of this plant against a large number of microbial species is among the strongest points of this work.

Related reports

Apparently, no study was performed on the antimicrobial activity of *T. lanceolatus*. While other antimicrobial studies performed in some species of the genus *Thymus* showed that their extracts have commonly a strong antimicrobial activity, for example methanol extract of *Thymus vulgaris*, as reported by Al-Bayati *et al.*, 2008.

Innovations & breakthroughs

This study is the first academic research that highlights *T. lanceolatus* as medicinal plant in providing a very interesting antimicrobial activity, which opens the field of in-depth research on this endemic species.

Applications

Actually, multidrug resistance present a real public

health problem, especially in developed countries, where discovery of new antimicrobial drugs is an urgent necessity. In this study, authors have demonstrated that endemic species *T. lanceolatus* has some phytochemicals with an interesting antimicrobial activity, which can be a source of new antimicrobial molecules. Further studies are needed for investment of these findings.

Peer review

This is a good and an original study in which authors have valorised *T. lanceolatus*, an endemic medicinal plant from Algeria, for antimicrobial activity against pathogens responsible for many human diseases. The findings of this study are interesting, where authors report that *T. lanceolatus* contains some phytochemicals which provided a strong antimicrobial activity.

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