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Inhibitory Effects of *Juniperus oxycedrus* Essential Oils Against Some Pathogens

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Abstract: In the current study, in vitro antimicrobial activity of *Juniperus oxycedrus* essential oils was screened against *Staphylococcus aureus*, Streptococcus sp, Bacillus sp, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Candida albicans* by using disc and well diffusion, minimal inhibition concentration (MIC) and minimum microbiocidal concentration (MBC) methods. *Juniperus oxycedrus* essential oils have moderate inhibition activity with diameters ranging from 7 mm to 19 mm, when *S. aureus* was the most sensitive strain then *P. aeruginosa* was the most resistant. The minimum inhibitory concentrations (MIC) were ranging from 250 µl/ml to >500 µl/ml while minimum bactericidal concentrations (MBC) were ≥ 500 µl/ml. This study shows that *Juniperus oxycedrus* could be a good candidate in the search for new active compounds based on herbal against these common pathogens.

Keywords: Antimicrobial Activity, *Juniperus oxycedrus* Essential Oils, Pathogens

1. Introduction

The rapid development of antimicrobial drug-resistant pathogens and their spread around the world are amongst the most serious threats to public health and to successful antibacterial treatment. In recent years, the emergence of bacterial resistance against multiple antibiotics has accelerated dramatically (Lai et al., 2011; Grundmann et al., 2011). Efforts are being made to discover new antimicrobial agents from sources like micro organisms, animals and plants, which are either less toxic or not toxic at all (Bhat et al., 2014). Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active chemical compound as antimicrobial agent. They are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemical entitled for synthetic drugs (Hammer et al., 1999). *Juniperus oxycedrus* (*Cupressaceae* family and the *Gymnosperrnae* division) is chiefly a Mediterranean species that grows from Morocco, Algeria, and Tunisia in north Africa into Portugal, Spain, France, Italy, Greece, the Balkans, Turkey and eastward into Iran (Dahmane et al. 2015). In folk medicine, the plant is prescribed as a panacea for every kind of diseases, including, against infectious and inflammatory disorders of upper respiratory system and gastro-intestinal complaints or as a hypotensive remedy (Akkol et al., 2010). *Juniperus oxycedrus* oil is widely employed in human and veterinary dermatology to treat chronic eczema and other skin diseases (Bouhlal et al., 1988) and rectified oil is used as a fragrance component in soaps, detergents, creams, lotions, and perfumes (Leung and Foster, 1996). The boiled fruit of *Juniperus oxycedrus* is widely used in the treatment of gastrointestinal disorders, common colds, as expectorant in cough, to treat calcinosis in joints, as diuretic to pass kidney stones, against urinary inflammations, hemorrhoids, and as antidiabetic, while the resin was used for wound healing (Yesilada et al., 1993; Abdou et al., 2011).

In the present study, the aim was to evaluate the antimicrobial activity of the oils of *J. oxycedrus* obtained from plants growing in the northwest of Algeria.

2. Materials and Methods

2.1. Plant Material and Essential Oil Extraction

The leaves of *Juniperus oxycedrus* were collected from Sidi Mansour beach of Mostaganem Province in the north-

Western of Algeria in March 2016. Plant identification was carried out by Dr Belgherbi Benamar, botanist at the Biology Department, University of Mascara (Algeria). Plant material was dried in the dark, at room temperature.

The leaves were dried at room temperature and submitted to hydrodistillation for 3 h with 500 ml distilled water using a Clevenger-type apparatus. The extracted oil was collected and dried over anhydrous sodium sulfate, then stored in sealed glass vials in a refrigerator at 4°C prior to analysis.

2.2. Tested Microorganisms

The microorganisms used in this study consisted of three Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Streptococcus sp* and *Bacillus sp*; three Gram - negative bacteria *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumonia* (ATCC 700603); and one yeast, *Candida albicans* (ATCC 10231). The reference bacterial strains, American Type Culture Collection (ATCC) were obtained from Microbiology Laboratory, Tlemcen University (Algeria), the two clinical bacterial isolates (*Streptococcus sp* and *Bacillus sp*) were collected from Medicinal Analysis Laboratory, Hospital of Mohammadia (Province of Mascara, Algeria), and the candidal strain (*C. albicans*) was supplied by Microbiology Laboratory of Oran University (Algeria).

2.3. Antimicrobial Assay

The essential oil from *J. oxycedrus* was tested for antimicrobial activities using agar disc diffusion and Agar-well diffusion techniques to determine the diameter of growth inhibition zones while broth dilution method was used to determine the MIC and MBC/MFC.

2.4. Agar Diffusion Test

The disc diffusion method was employed for the determination of antimicrobial activities of *J. oxycedrus* essential oil. Filter paper discs (6 mm in diameter) were soaked with 10 µl of the different dilutions of the essential oils (Pure, 1/2, 1/4, 1/8, 1/16, and 1/32), and placed on the seeded plates. A standard inoculums of young cultures, 18-24 h old bacteria and yeast was spread on Mueller Hinton agar or Sabouraud dextrose agar medium respectively. A negative control was prepared using the solvent (DMSO) employed to dissolve the oil. Gentamicin (10 µg/disc) and Amphotericin B (20 µg/disk) were used as positive reference drugs for bacteria and *C. albicans* respectively. The plates were incubated at 37°C (24 hrs) for bacterial strains, and 30°C (24 hrs) for yeast. Antimicrobial activity was evaluated by measuring the diameters of inhibition zones (Mazari et al., 2010).

2.5. Agar-Well Diffusion Technique

This method was carried out according to Reddy et al. (2013) with modification. The overnight culture of the microorganisms cultures were inoculated on Mueller-Hinton agar plates for cultivation of bacteria, and Sabouraud plates for cultivation of yeast using sterilized cotton swabs. After media were solidified, the holes were made by using a sterilized cork borer each hole was filled with one of the different dilutions of the essential oils (Pure, 1/2, 1/4, 1/8, 1/16, and 1/32). Plates of bacteria were incubated at 37°C, while those of yeast at 30°C for 24 hours. Negative and positive controls were used. After incubation, all plates were examined for the presence of zone of inhibition around the wells.

2.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Microbiocidal Concentration (MBC) of Essential Oil

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined for all microorganisms strains by broth dilution method.

Bacterial strains were cultured overnight at 37°C in nutrient agar and *C. albicans* was cultured overnight at 30°C in Sabouraud dextrose agar (SDA) to obtain working culture (10^6 viable cells/ml). Stock solutions of known concentrations of the tested essential oil were prepared and serial dilutions were plated at the plate with the aid of automatic micropipette in order to represent 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128 of the initial concentration. Test and control plates were incubated at 37°C for 24 h for bacterial strains, 48 h for yeast at 30°C and the MICs and MBCs were determined. After incubation, bacterial growth was evaluated by the presence of turbidity and a pellet on the well bottom. The MIC was defined as the lowest concentration of the compounds to inhibit the microorganism growth. The MBC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no turbidity development and without visible growth. (Almeida et al., 2013; Riahi et al., 2013).

3. Result and Discussions

The *in vitro* antimicrobial activity of *J. oxycedrus* essential oils estimated by the diameter of inhibition varied according to essentials oils dilutions and microorganisms strains were summarised in Table 1. The essential oils displayed a variable degree of antimicrobial activity against the different strains tested. Gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus sp* and *Bacillus sp*) the most sensitive microorganisms, followed by *C. albicans*, while Gram-negative bacteria were most resistant than the other test organisms, showing a low sensitivity especially with low dilutions (1/4, 1/8, 1/16 and 1/32). Among microorganisms, *S. aureus* was the most sensitive strain then *P. aeruginosa* was the most resistant. The diameter inhibition zones of *J. oxycedrus* essential oils for test organisms were in the range of 11-12 mm for *K. pneumoniea,* 8-12 mm for *E. coli,* 7-8 mm for *P. aeruginosa,* 8-15 mm for *S. aureus,* 7-12 mm for *Streptococcus sp,* 7-18 mm for *Bacillus sp* and 8-19 mm for *C. albicans.*

Table 1. *The zone of inhibition diameter exhibited by various doses of oil of J. oxycedrus against various pathogens using well and Disc diffusion methods.*

Strains	Method	Gent	DMSO	Growth inhibition diameter (mm)					
				pure	1/2	1/4	1/8	1/16	1/32
K. pneumoniea	Disc	26	NI	11	NI	NI	NI	NI	NI
	Wells	28	NI	12	11	NI	NI	NI	NI
E. coli	Disc	19	NI	11	11	NI	NI	NI	NI
	Wells	23	NI	12	8	NI	NI	NI	NI
P. aeruginosa	Disc	17	NI	7	7	NI	NI	NI	NI
	Wells	17	NI	8	7	NI	NI	NI	NI
S. aureus	Disc	24	NI	11	9	9	9	8	NI
	Wells	26	NI	15	13	10	10	NI	NI
Streptococcus sp	Disc	28	NI	10	9	7	7	NI	NI
	Wells	24	NI	12	12	NI	NI	NI	NI
Bacillus sp	Disc	23	NI	14	15	10	8	7	NI
	Wells	20	NI	18	10	NI	NI	NI	NI
C. albicans	Disc	25	NI	17	NI	NI	NI	NI	NI
	Wells	NI	NI	19	10	8	NI	NI	NI

According to the results given in Table 2, MIC of essential oil was found more effective against *S. aureus* (250 µl /mL) followed by *Bacillus sp* (500 µl /mL) and *C. albicans* (>500 µl/ml). The MBC value was 500 µl/ml for *S. aureus* and>500µl/ml for *Bacillus sp.*

Table 2. *MIC and MBC values of J. oxycedrus essential oils on test organisms.*

	MIC	MBC
E. coli	No tested	No tested
S. aureus	250 µl/ml	500 µl/ml
Streptococcus sp	No tested	No tested
Bacillus sp	500 µl/ml	>500µl/ml
C. albicans	>500 µl/ml	-

Generally, the Gram-negative bacteria are more resistant to essential oils or antibacterial compounds than Gram-positive bacteria, which is in a good agreement with previous reports (Russell, 1995; Smith-Palmer et al., 1998; Dorman and Deans, 2000; Burt, 2004; Shan et al., 2007). This resistance could be ascribed to the structure of the cellular walls of Gram-negative bacteria, mainly with regard to the presence of lipoproteins and lipopolysaccharides that form a barrier to restrict entry of hydrophobic compounds (Russell, 1995; Cox and Markham, 2007).

Many workers have reported about the antimicrobial activity of *J. oxycedrus* oil against a diverse range of organisms comprising gram positive and gram negative organism, yeast and fungi. The essential oil from the heartwood of Sardinian *J. oxycedrus* showed activity against gram-positive bacteria and most of the screened blastomycetes (Bonsignore et al., 1990). Stassi et al. (1998) reported that the essential oils from leaves and berries of *J. oxycedrus* ssp. *oxycedrus* from Greece exhibited activity against several microorganisms. Digrak et al. (1999) demonstrated the activity of different solvent extracts of leaves bark and fruits of *J. oxycedrus.* The essential oil extracted from leaves of Italian *Juniperus oxycedrus* ssp. *oxycedrus* exhibited rather good or weak activity against *C. albicans* and *S. aureus* (Angioni et al., 2003). In another study, Ateş and Erdoğrul (2003) reported that *Juniperus oxycedrus* seed extracts showed a 7-15 mm/20 µl inhibition zone against 13 bacterial species, but the ethyl acetate extracts showed no inhibition zone against *E. faecalis*, while acetone and chloroform extracts did not inhibit *P. aeruginosa*. Aqueous and methanol extracts of the leaves of *Juniperus oxycedrus* were investigated by Karaman et al. (2003) for their in vitro antimicrobial properties and they observed that the aqueous extract of *J. oxycedrus* had no antimicrobial effect against the test microorganisms whereas the methanol extract had inhibitory effects on the growth of 57 strains of 24 bacterial species. Cavaleiro et al. (2003) the oil from leaves of *J. oxycedrus* ssp. *oxycedrus* is most active on *Candida*, *Aspergillus* and dermatophytes, with MIC and MLC values ranging from 0.08– 0.16 µl ml^{-1} to 0.08–0.32 µl ml^{-1}, respectively.

Medini et al. (2012) showed that *E. coli* was found to be extremely resistant to leaf essential oil of *J. oxycedrus* from Tunisia while *S. aureus* was the most sensitive strain with MIC ranged from 600 to 650 mg/ml. Sela et al. (2013) tested the essential oils of *J. oxycedrus* against 16 bacterial isolates of Gram positive and Gram negative bacteria and one strain of *C. albicans.* The most sensitive bacteria was *Haemophilus influenzae* (MIC=125 ml/ml). The essential oils showed moderate antimicrobial activity against *S. pneumoniae*, *S. aureus*, *S. agalactiae, S. pyogenes*, *Corynebacterium* spp., *E. coli* and *C. jejuni* (MIC>500 ml/ml) and no activity against *C. albicans, S. epidermidis, Acinetobacter* spp.*, S. enteritidis, S. flexnery, K. pneumonia, P. aeruginosa, Enterococcus* and *P. mirabilis.*

Biological activity of an essential oil is related to its chemical composition. The relation between composition and bioactivity of the essence from the aromatic plants may be attributable both to their major components (alcoholic, phenolic, terpenic or ketonic compounds) and the minor ones present in the oil. It may act together synergistically or antagonistically to contribute to some activity of the tested oil (Pandey et al., 2014). The chemical composition of the

essential oil of *J. oxycedrus* was previously investigated in different region in the world and the most of these studies indicated that α-pinene was the major component (Derwich and Chabir, 2011) which is known to possess an important antimicrobial activity (lsmail et al., 2011)

4. Conclusion

The current study not only confirmed the antimicrobial capacity recognized at *J. oxycedrus* but also to show that the essential oils of this plant could be used in the treatment of infections caused by these pathogens. A chemical fractionation might be previously performed to isolate and identify the compound responsible for this activity.

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