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PHARMACOGNOSIE

# In vitro Antifusarial Activity of a Tar Extracted from the *Juniperus phoenicea* L. Wild in Southwest of Algeria

## Activité antifusarienne in vitro de goudron végétal extrait de *Juniperus phoenicea* L. sauvage du Sud-Ouest algérien

**B. Terfaya · A. Makhloufi · A. Mekboul · L. Benlarbi · D. Abdelouahid**



**Abstract** The biological activities of medicinal plants have been recognized for centuries. Among them, *Juniperus phoenicea* is very commonly used for its medicinal virtues (respiratory, hepatic and renal infections). Its wood is used for heating and manufacture of coal and its vegetable tar is used to treat certain cases of eczema. The aim of the present study is focused on the evaluation of the in vitro antifungal activity of a tar extracted from *Juniperus phoenicea* L. The physicochemical analysis was performed first; then, the evaluation of antifusarial activity of the *Juniperus phoenicea* L. was carried out by using the dilution and the direct contact method to determine the minimum inhibitory concentration (MIC). Results of the physicochemical analysis show a density value of 1.10, a refraction index of about 1.5112, an acid pH (4.02) and the dries matter ratio of 36, 34% for the tested tar. The antifungal activity of the tar under study against six strains of *Fusarium oxysporum f.sp. albedinis* (FOA; S1, S2, S3, S4, S5, S6) revealed that *Juniperus phoenicea* L. tar has a great antifungal activity against all the investigated strains. The antifungal activity results reveal that the fungal growth was inhibited by the tar. S3 proved to be the most sensitive strain with a minimal inhibition concentration value of 0.006 mg/ml. The present study indicates that *Juniperus phoenicea* L. tar has antifungal activity against the different tested fungal strains.

**Keywords** Tar · *Juniperus phoenicea* L. · Antifungal activity · Southwest Algeria

**Résumé** Des activités biologiques des plantes médicinales ont été identifiées pendant des siècles. Parmi elles, le genévrier de Phénicie qui est très recherché pour ses vertus médicinales (affections respiratoire, hépatique et rénale). Les bois sont utilisés pour le chauffage et la fabrication du charbon du bois et du goudron végétal pour traiter certains cas d'eczéma. Le but de la présente étude est concentré sur l'évaluation de l'activité antifongique in vitro d'un goudron végétal extrait à partir du *Juniperus phoenicea* L. L'analyse physicochimique a été effectuée premièrement ; puis l'évaluation de l'activité antifusarienne du *Juniperus phoenicea* L. par la méthode de dilution et le contact direct, et cela pour déterminer la concentration minimale inhibitrice (CMI). Les résultats d'analyse physicochimique de goudron examiné montrent une valeur de densité de 1,10, une indice de réfraction d'environ 1,5112, un pH acide de 4,02 et un rapport de matière sèche de 36,34 %. L'activité antifongique du goudron végétale a étudié contre six souches de *Fusarium oxysporum* f.sp *albedinis* (FOA) [S1, S2, S3, S4, S5, S6], a prouvé que le goudron de *Juniperus phoenicea* L. a une grande activité antifongique contre toutes les souches étudiées. Les résultats d'activité antifongique ont montré que les mycètes ont été empêchées par le goudron. (S3) s'est avéré être la souche la plus sensible avec une CMI de l'ordre de 0,006 mg/ml. La présente étude indique que le goudron de *Juniperus phoenicea* L. a une activité antifongique contre les différentes souches fongiques (FOA) examinées.

**Mots clés** Goudron · *Juniperus phoenicea* L · Activité antifongique · Sud-Ouest d'Algérie

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## Abbreviations

MIC: Minimal inhibitory concentration
PDA: Potato dextrose agar
PCR: Polymerase chain reaction
PDB: Potato dextrose broth
EDTA: Ethylenediaminetetraacetic acid

## Introduction

Medicinal plants have played an essential role in the development of human medicinal culture. These creatures have always virtually been at the forefront in disease fighting in all cultures and civilizations. Regarded as rich resources of traditional healing methods, many of the modern medicines are produced from them [1].

Plants have been used as primary means of disease treatment from ancient times and until nowadays a significant number of species have been reported to possess various pharmacological properties [2–4]. Indeed, for thousands of years, medicinal plants have been used to treat health disorders, to add flavor and conserve food as well as prevent diseases epidemics [5]. Juniperus genus is a well-known source of cedar wood oil which is widely spread in the Northern Hemisphere and is used in folk medicine as well [6,7].

*Juniperus phoenicea* L., red juniper, or locally named "Araar" is a shrub belonging to Cupressaceae family, which is found in the Mediterranean area, including Algeria and which lies on the arid slopes and hills. It is more frequent in the forests of northern Algeria [8].

The *Juniperus phoenicea* L. is considered as an important medicinal plant, largely used in traditional medicine [9]. Its leaves are used in traditional medicine as a decoction to treat diarrhea, rheumatism and diabetes. The mixture of leaves and berries of this plant is used as an oral hypoglycemic agent, whereas the leaves are used against bronco-pulmonary disease and also as a diuretic [10].

According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with less adverse effect. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim [11]. To the best of our knowledge, clinical tests directed toward understanding the pharmacokinetics, bioavailability, efficacy, safety and drug interactions of newly developed bioactive compounds and their formulations (extracts) require a careful evaluation. Clinical trials are carefully planned to safeguard the health of the participants as well as to answer specific research questions by evaluating both immediate and long-term side effects and their outcomes are measured before the drug is widely applied to patients [12].

According to Stace (2010) [13], genus Juniperus L. (Cupressaceae) includes between 50 and 67 species, depending on taxonomic viewpoint. As an example; six species are more frequent: *Juniperus communis* L. (common juniper), *Juniperus oxycedrus* L. (prickly juniper), *Juniperus macrocarpa* S.S. (large-fruited juniper), *Juniperus nana* Willd. (dwarf juniper), *Juniperus phoenicea* L. (Phoenicean juniper) and *Juniperus sabina*. From a botanical point of view, these species are mainly shrubs or trees, and are widely distributed throughout the Northern Hemisphere [14].

*Juniperus phoenicea* L. is a monoecious small three of 3–5 m in height and can even reach up to 8 m, or sometimes it grows in the form of shrub, with scaly leaves and brown cones. It is spread in southern Europe, in the southwest Asia and North Africa, but most frequently in the western part of the Mediterranean region. The species occupies mountainous regions, reaching altitudes of up to 2,400 m in Morocco and 1,800 m in Spain. It grows in various types of Mediterranean forests and sometimes also in the pure stands [15,16].

*Juniperus phoenicea* L. was used since antiquity to produce oil which is rich on sesquiterpene, used in dermatology and cosmetology [17]. Otherwise, there are several studies reported on the chemical composition of solvent extracts from wood, berries and leaves of *Juniperus phoenicea* L. in which sesquiterpenes, diterpene acids and polyphenolic compounds have been isolated and characterized [18].

The use of tar is reserved mainly for patients with chronic stable, scalp psoriasis, atopic dermatitis and seborrhea dermatitis [19]. In the pharmaceutical and food industries, *Juniperus phoenicea* L. has excellent antioxidant and antibiological properties, due to some compounds (carnosol, carnosic acid, ursolic acid, betulinic acid) [15].

The present study is focused on the evaluation of the in vitro antifungal activity of a tar extracted from *Juniperus phoenicea* L. wood collected in Bechar Southwest of Algeria; against six strains of *Fusarium oxysporum f. sp. albedinis* (FOA) causing fusariosis (disease of date palm in Algeria).

## Materials and Methods

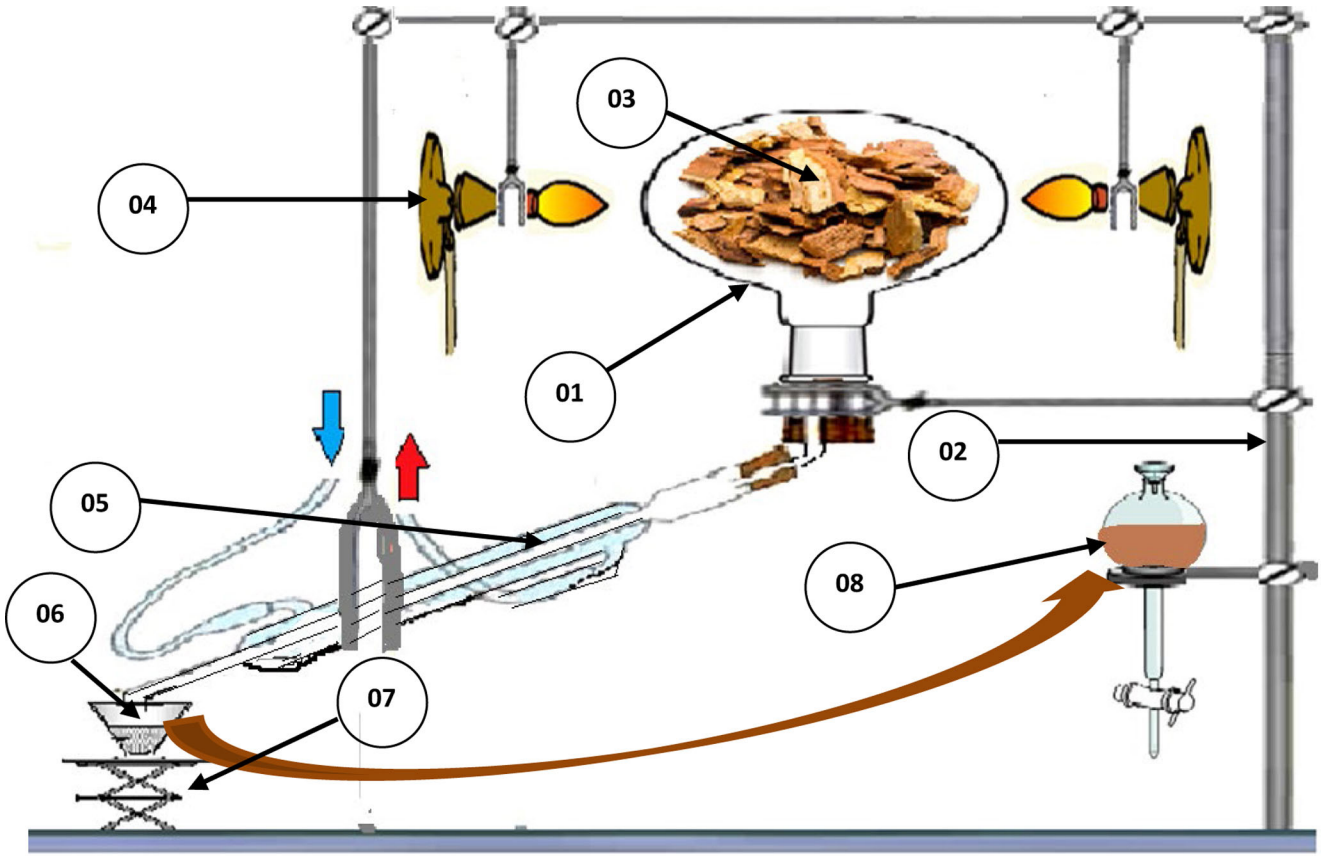
### Plant Material

The selected samples of "*Juniperus phoenicea* L." were collected from Bechar mountains, southwest of Algeria; and from an area in Beni Ounif known as "Rosfa Tayba," north of Bechar, during January and February 2016. This collected vegetal material was dried for 15 days in the dark at ambient laboratory temperature (20 °C–28 °C).

### Distillation of Tar

Tar extraction was performed in the VRVSA "research laboratory" at Tahri Mohamed University of Bechar. The wood of the aerial parts "stems and branches" was cut before the operation, using a special device described below (Fig. 1).

It is a simple method that is similar to the traditional method described by Gast (1999) [20]. Vegetal tar extraction




**01:** Metal container
**02:** Metal support
**03:** Shrub woods
**04**: Bunsen burner
**05**: Cooling coil
**06:** Distillate
**07:** lifting support
**08:** Tar after 2 hours

**Fig. 1** Device of vegetable tar extraction similar to traditional method described by Gast (1999)

in the laboratory is done using a device made up of a metal container that contains the wood of the shrub, related to a support. Above and around this metal container; a Bunsen burner was placed and is heated in order to carbonize its contents, it required 6 h of heating. The cooling agent is placed with the metal support by a pump and a crystallizer to recover the tar. The extraction continues until the exhaustion of the raw material.

At the end, the gross product obtained is left for 2–3 h at rest after it is spread in three layers: water, tarry mud (part the oily one) and vegetable tar. The distilled tar was stored at ambient temperature until it was used.

### Physicochemical Characterization of *Juniperus phoenicea* Tar

Methods in conformity with AFNOR (1986) have been used to determine specific density at 20 °C. Refractive index (RI) was measured with a refractometer at 20 °C. The methods used for the determination of acid index are also determined by the titration method using reagents standards (phenolphthalein indicator–sodium hydroxide titrant and ethanol–ether solution) [21].

## In vitro Antifungal Activity

### Fungal Strains

The antifungal activity was evaluated by both direct contact and dilution methods on six selected strains of fungus *Fusarium oxysporum f. sp. albedinis*(1) [FOA(1)], FOA(2), FOA (3), FOA(4), FOA(5), FOA(6) that were isolated from date palm from Beni ounif area infected by the fusariosis disease. Fungal spores were prepared by growing mold on potato dextrose agar (PDA) at 27 °C for 7 days, and spores were

suspended in sterile 1% tween-80. Spore count was performed by using a hemocytometer and adjusted to obtain $10^5$–$10^6$ spores/ml with potato dextrose broth (PDB) [22].

### Fungal DNA Isolation, Purification and Quantification

The total DNA of dried mycelium of each strain was extracted and purified as described by Kumar et al. [23], in the Universal method: A single protocol universally used for plant, algae, blood, bacteria, fungus will be more demanding than those suited specific to a particular biological material. The extraction buffer used in this case included 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1.5 M NaCl, 2% CTAB, β-mercaptoethanol, PVP (in case of plant sample). The method utilizes the classical protocol for homogenization by liquid nitrogen, incubation in water bath at 65 °C, deproteinization was performed by chloroform–isoamyl alcohol followed by ethanol precipitation and washing. Alternative to the above, other universal methods termed as "nuclei method" and "protoplast method" may be successfully used to prepare high quality megabase-sized DNA [24].

The RNA was eliminated by adding proteinase K (RNase; 10 mg/ml). The DNA was dissolved in 200 µl TE buffer (10 mM Tris-HCl, pH 6.0, 1 mM EDTA; pH 8.0), quantified and diluted to an approximate concentration of 5 ng/µl for PCR reactions [25].

### PCR Amplification of Fungal DNA

The used primers were preselected among several primers because they permitted to reveal polymorphism on small DNA samples used for preliminary trials. The PCR techniques were optimized according to primers. Reaction was achieved by 1 cycle consist of 4 min of denaturation at 95 °C followed by 30 cycles consisting of 1 min at 94 °C, 1 min at 36 °C and 1 min 30 s at 72 °C. One cycle for 15 min at 72 °C was conducted at the end. PCR reactions were performed in a total volume of 25 µl, containing 25 ng genomic DNA, 10X Taq buffer, 10 mM dNTP, 25 mM $MgCl_2$, 1 unit Taq DNA polymerase (Promega) and 10 mM primer.

For FOA-specific amplification, PCR primers included two primer pairs; FOA1 (CAGTTTATTAGAAATGCCGCC) coupled with BIO3 (GGCGATCTTGATTGTATT GTGGTG), and FOA28 (ATCCCCGTAAAGCCCTGAAGC) coupled with TL3 (GGTC GTCCGCAGAGTATACCGGC) [26]. FOA-specific PCR reactions were performed according to Fernandez et al. (1998) [24] as follows: 1 cycle for 4 min at 95 °C followed by 30 cycles for 30 s at 92 °C, 30 s at 60 °C and 30 s at 72 °C for the FOA1-BIO3 primer pair; and 30 cycles for 30 s at 92 °C, 30 s at 62 °C and 45 s at 72 °C for the FOA28-TL3 primer pair. Thereafter, a cycle of 15 min at 72 °C was conducted. PCR reactions were performed in a total volume of 20 µl, containing 10–100 ng genomic DNA, 10X Taq buffer, 0.2 mM of dNTP, 1.5 mM MgCl2, 1 unit Taq DNA polymerasc (Promega) and 1 µM primer, as previously described by Fernandez et al. (1998). The PCR reactions were incubated in a TC3000 Thermocycler (Progene, Techne England). All amplification products were separated in stained agarose gels (1.8% w/v; 15 × 10 cm, W × L) with ethidium bromide in TAE buffer electrophoresed at 100 V for 1 h 30 min [27]. The DNA weight marker used was λ (lambda) digestive by enzymes Hind III and EcoR1. At the end of electrophoresis, the gels were visualized by UV illumination and photographed using a Bioprint System 3000WL X-PRESS computer assisted machine (software BIO-1D).

### Dilution Method (MIC)

Antifungal tests were performed according to the method reported by Hassikou et al. [28], the antifungal activity evaluated via direct contact method was performed in Petri dishes containing PDA, a culture media and tar, at different concentrations (0.0006, 0.001, 0.002, 0.003, 0.005, 0.012, 0.024 and 0.039 mg/ml) which were inoculated with fungus suspension obtained from pure culture, and incubated at 25 °C for 7 days. The diameter of the fungus colony was obtained by calculating the average of two perpendicular diameters compared with the witness petri dishes.

## Results

### Physicochemical Results

*Juniperus phoenicea* L. tar is a dark, viscous liquid, with a smoky odor and acrid, slightly aromatic taste with the extraction yield of 1.20% (v/w) on dry weight basis. It is soluble in the following solvents: very slightly soluble in water, soluble in ether, chloroform, amyl alcohol, ethyl acetate, alcohol and partly soluble in petroleum ether [29,30].

The physicochemical index of *Juniperus phoenicea* L. tar are illustrated in table 1.

### Antifungal Activity

#### *Analysis of the Population of F. o. f. sp. albedinis by the Specific PCR Technique*

The amplification of genomic DNA by specific primer couple to Foa, TL3-FOA28, revealed a specific band which has the size of 400 bp (Fig. 2). However, this band has also been amplified in the case of six strains of *Fusarium oxysporum* which one was isolated from the Beni ounif palm grove. For amplification of a specific band of size 200 bp using BIO3-FOA1 coupled primer revealed a specific band. The assembly of the two types of bands revealed by two primer couples

(TL3-FOA28 and BIO3-FOA1) only gave 30% of reliability. The dendrogram based on recombined results obtained by two primer couples permitted to distinguish two big distinct groups (data not shown): the groups 1 and 2 are composed of a mixture of FOA strains from different origins and *Fusarium oxysporum* strains [31].

**Table 1** Physicochemical index of *Juniperus phoenicea* L. tar

| Refractive Index | Specific Density at 20 °C | Acid Index | Rate of Matter Dries |
|---|---|---|---|
| 1.5112 | 1.10 | 4.02 | 36.34% |

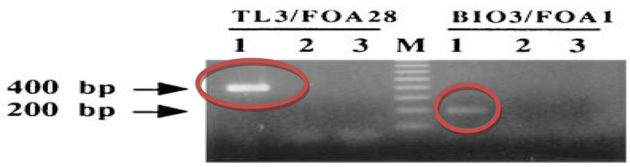



**Fig. 2** Amplification of genomic DNA of FOA by PCR method (Source: original, 2018)

***Dilution Method (MIC)***

The inhibition growth zones measured by dilution method (MIC) are presented in table 2.

The antifungal activity of studied tar against six strains of *Fusarium oxysporum f. sp. albedinis* showed that *Juniperus phoenicea* L. tar has a great antifungal activity against all the investigated strains. The antifungal activity results revealed that the fungi growths were inhibited by the tested tar. Nevertheless, S3 was the most sensitive strain with minimal inhibition concentration value 0.006 mg/ml (Fig. 3).

## Discussion

Our returns of the physicochemical index of *Juniperus phoenicea* L. tar (Table 1) are low compared to those obtained by Benlarbi et al. (2014) and Terfaya et al. (2017). This difference in tar content is related to several factors, such as the geographical area of collection, climate, stage of development and the season [32].

According to the direct contact method (Table 2), the vegetal tar of *Juniperus phoenicea* L. has a great and good inhibitory antifungal activity against all the investigated fungi. The concentration inhibition of the tar is between 0.003 and

**Table 2** MIC of tar (mg/ml) on fungal strains

| Strains | Witness | 0.0006 | 0.001 | 0.002 | 0.003 | 0.005 | 0.006 | 0.012 | 0.024 | 0.039 |
|---|---|---|---|---|---|---|---|---|---|---|
| **S1** | + | + | + | + | + | + | **MIC** | – | – | – |
| **S2** | + | + | + | + | + | + | **MIC** | – | – | – |
| **S3** | + | + | + | + | + | + | **MIC** | – | – | – |
| **S4** | + | + | + | + | + | **MIC** | – | – | – | – |
| **S5** | + | + | + | + | **MIC** | – | – | – | – | – |
| **S6** | + | + | + | + | **MIC** | – | – | – | – | – |

–: inhibition; +: growth

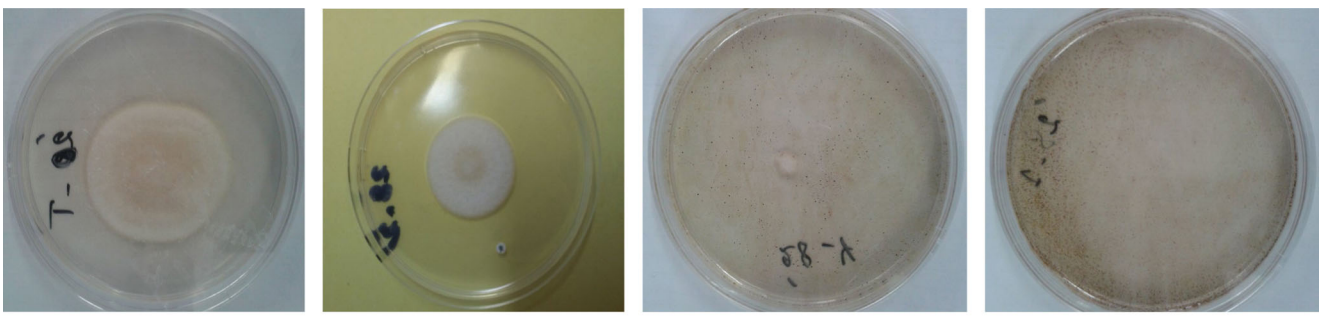



**Fig. 3** Effect of *Juniperus phoenicea* L. tar with various concentrations on strain FOA (S3) (Source: original, 2018)

0.006 mg/ml. The diameters of inhibition growth zone ranged from 15 to 50 mm with the highest inhibition zone values observed against the strain of *Fusarium oxysporum f. sp. Albedinis*(3) FOA(3) (50 mm). However, the studied fungi did not show the same sensitivity against vegetable tar, the same inhibited concentration with S1, S2, S3 which is 0.006; 0.005 for S4, and finally 0.003 for S5 and S6. In the literature, the tar derived from *Juniperus phoenicea* L. is used in folk medicine of North Africans countries [33]. The tar is accessible to consumers without a prescription and is known to be used externally for skin disorders in dermatology and hair care, and more essentially for scalp care, eczema, scale affections, hair loss and psoriasis, it is also used against "evil eye," abdominal pain and diarrhea, psychiatric disorder, cancer, fever, cephalgia, angina, weight loss, the common cold and hypotonia, without any scientific evidence to support these uses despite such common use [34].

## Conclusion

The *Juniperus phoenicea* L. tar has a good antifungal activity. The strong antifungal activity of *Juniperus phoenicea* L. against an array of filamentous fungi strains is an indication of the broad spectrum of the antifungal potential related to this type of tar. This could make tar a promising element of natural compounds for the development of safer antimicrobial agents.

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