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Full Length Research Paper

Chemical composition and antifungal activity of volatile components from woody terminal branches and roots of *Tetraclinis articulate* (Vahl.) Masters growing in Tunisia

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The chemical composition of the volatile fractions obtained from distilled woody terminal branches and roots of *Tetraclinis articulata* (Vahl.) Masters was identified by Gas Chromatography- Flame ionization detectors (GC-FID) and Gas Chromatography-Coupled with Mass Spectrometry (GC-MS) . Twenty compounds represented 83.7% of the essential oil from woody terminal branches and 29.2% from the roots. Nonan-1-ol was the main constituent present in the second fraction of the essential oil from woody terminal branches with the highest percentage of 75.22. Monoterpene hydrocarbons viz: - terpinene (3.04), - carene (1.17) and -ocimene (1.09) were the main compounds. In the fourth fraction of the essential oil from roots, the major compounds detected were Bornyl acetate (16.63), Camphene (1.59) and -cadinene (2.86). This fraction was mainly rich in oxygenate compounds (20.25) and sesquiterpene hydrocarbons (6.84). Furthermore, the oils were tested for their antifungal activity against five phytopathogenic fungi. These oils from woody terminal branches and roots of *T. articulata* (Vahl.) Masters were found to possess antifungal activity.

Key words: Antifungal activity, Cupressaceae, essential oil, Nonan-1-ol, *Tetraclinis articulata* (Vahl.) Masters.

INTRODUCTION

Our study lies within the scope of the valorization of Tunisian medicinal and aromatic plants having the aim of discovering new bioactive natural products. Le Floc'h (1983) reported about their use in human and veterinary medicine in Tunisia and in other North African countries, in particular against intestinal and respiratory ailments (Buhagiar et al., 2000). *Tetraclinis articulata* (Vahl.) Masters belongs to the Cupressaceae family and is widespread throughout North Africa. In Tunisia, *T. articulata* is found in the north-east region of the country (Pottier-Alapetite, 1981).

The oil composition of some *T. articulata* organs has been studied by Buhagiar et al. (2000), Ait Igri et al. (1990) and Tekaya-karoui et al. (2007). In Tunisia, recent research has revealed that oils prepared from woody terminal branches contained more monoterpene hydrocarbons, representing 60.2% of the total identified compounds. These oils also contained a significant amount (18.8%) of non-terpenic compounds. Camphene (43.2%), *Z*- -ocimene (11.6%), nonan-1-ol (5.3%) and unidentified compounds (12%) were the major constituents of this oil. Root oils showed a greater variability in the percent- ages of their constituents compared to those from the aerial parts. Monoterpene hydrocarbons formed the most important fraction (74.1%) of all the identified components among which camphene (70.2%) was the main one. However, the amount varied from 43.2% in the woody terminal branches to 70.2% in

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Table 1. Fungal isolates used for assessing the antifungal activity of *T. articulata* essential oils.

Fungus	Plant source	Plant part sampled	Collection locality	Collection date
B. cinerea	Grapes	Fruit	Bou Argoub (North)	11/05/2000
F. oxysporum f. sp. *niveum*	Watermelon	Roots	Skhira (South)	23/05/2001
F. solani f. sp. *cucurbitae*	Watermelon	Roots	Skhira (South)	23/05/2001
R. solani	Watermelon	Roots and stems	Jebeniena (Center)	05/07/2001
A. solani	Tomato	Leaves	Chott Mariem (Center)	23/08/2005

the roots (Tekaya-karoui et al., 2007).

There are few available reports about antimicrobial activity of *T. articulata* in the world. Sandarac (resin) of *T. articulata* was tested for its antimicrobial activity against some microorganisms, viz: *Staphylococcus aureus, Streptococcus mutans, Streptococcus faecalis, Streptococcus durans, Streptococcus sanguis, Bacillus sub tilis*, and *Candida albicans* . The development of these microorganisms seemed to be significantly reduced in the presence of *T. articulata* sandarac (Al-qahtani and Al-Shawaf, 2005).

Tropolone and hinokitiol from different Cupressaceous species has shown strong antifungal activity and broad antimicrobial spectrum which has led to their wide utilization in agriculture, clinical products, cosmetics and other areas. The mechanism of antimicrobial and insecticidal activity of tropolone and hinokitiol is unknown but it was well documented that tropolone greatly inhibited polyphenol oxidase (Saniewski et al., 2007).

The antimicrobial activity of oils of plants belonging to Cupressaceae family such as *Juniperus phoenicea* L. was tested. Minimum inhibitory concentration (MIC) of leaf and berry oils was determined and the oils were found to be `highly active against most of the tested strains (El-Sawi et al., 2007).

The antimicrobial activity of essential oils obtained from terminal woody branches and roots of *T. articulata* has not been investigated. Consequently, the main objective of this study was to identify by Gas Chromatography Phase coupled with Mass Spectrometry (CPG/SM); the bioactive principles of the essential oils from woody terminal branches and roots of *T. articulata* and characterize their activity against several phytopathogenic fungi.

MATERIALS AND METHODS

Plant material

T. articulata was identified according to the flora of Tunisia (Nabli, 1989). A specimen was collected, dried and a voucher specimen (Cupressaceae No.1) was deposited in the Laboratory of Chemistry of Natural Substances and Organic Synthesis, Faculty of Sciences of Monastir, Monastir University. The plant was gathered from the forestry station of Zaghouan in March 2005. The fresh plant was separated into two parts: Terminal woody branches and roots. Each part was divided into small pieces and weighed before hydrodistillation.

Essential oils preparation

Hydrodistillation of the fresh material was performed in a Clevenger -type apparatus for 4 h up to the point at which the oil contained in the matrix was exhausted .The oil obtained was collected and stored in sealed glass vials in a refrigerator at 4 - 5°C prior to the analysis. Yields based on fresh weight of the terminal woody branch and root samples were calculated.

Analyses of the essential oils

Gas chromatography (GC)

Gas Chromatograph was HP 5890-series II equipped with Flame ionization detectors (FID), HP-5 (5% phenyl 95% dimethylpolysiloxane) 30 m × 0.25 mm, 0.25 µm film thickness fused silica capillary column. The carrier gas was nitrogen with a flow rate of 1.2 ml/min. Oven temperature was programmed (50°C for 1 min, then 50 - 280°C at 5°C/min and held isothermal at 280°C for 1 min) .The injection port temperature was 250°C. Volume injected was 0.1 µl of 1% solution (diluted in hexane). Percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction.

Gas Chromatography-coupled with mass spectrometry (GC-MS)

The analyses of the volatiles were run on a Hewlett-Packard GC-MS system (GC: 5890 series II; MSD 5972). The fused-silica HP-5 capillary column (30 m × 0.25 mm, film thickness of 0.25 µm) was directly coupled to the MS. The carrier gas was helium, with a flow rate of 1.2 ml/min. Oven temperature was programmed (50°C for 1 min, then 50 - 280°C at 5°C/min and subsequently held isothermal for 20 min). The Injection port temperature was 250°C and that of detector 280°C. Split ratio was 1:50. Volume injected was 0.1 µl of 1% solution (diluted in hexane). Mass spectrometer: HP 5972 recording at 70 eV; scan time 1.5 s; mass range 40 - 300 amu. Software adopted to handle mass spectra and chromatograms was ChemStation.

Identification of essential oil components

Components of the oil were identified by comparison of their mass spectra with those of a computer library (Wiley 275). Further confirmation was done from retention index data generated from a series of alkanes retention indices (relative to C_9-C_{28} on the HP-5 column) (Shibamoto, 1987; Adams, 1995).

Antifungal activity

Fungal isolates

Fungi were collected from various locations in Tunisia. Table 1

lists the fungi, their hosts, plant parts from which they were isolated, location and date of collection. All fungal isolates were identified and samples of each fungus were deposited in the Collection Bank at the Plant Pathology Laboratory (Institut Supérieur Agronomique de Chott- Meriem, Tunisia). They included fungi causing soil-borne diseases such as: *Fusarium oxysporum* f. sp. *niveum* (Boughalleb and El Mahjoub, 2005) and *F. solani* f. sp. *cucurbitae* (Boughalleb et al., 2005b) and foliar, stem and fruit diseases such as *Botrytis cinerea* (Ben Ahmed, 2001).

Fungal isolates were maintained on Potato Dextrose Agar (PDA, Difco Laboratories, Inc., Detroit, MI, USA), stored at room temperature and sub-cultured once a month (Dorman and Deans, 2000). The isolates were grown for 7 - 10 days.

Antifungal screening

A 1 ml conidial suspension (10^5 spores/ml) of each fungus was added into each Petri dish. Thereafter, 15 ml of PDA amended with streptomycin sulphate was added to the dishes containing the spore suspension. Once the substrate solidified, four discs of Whatman no. 5 filter paper (6 mm in diameter) were soaked with 2 µl of crude extract, kept for drying and placed on inoculated Petri dishes. In the case of the control, the small discs were moistened with methanol. After being kept at 4°C for 2 h, inoculated plates were incubated at 25°C for 8 days. Antifungal activity was evaluated by measuring the zone of inhibition for each tested organism. All tests were performed three times.

Disc diffusion method

The qualitative and quantitative antifungal assay of essential oil of woody terminal branches and roots and their fractions was carried out by the disc diffusion method (Marmonier, 1987). A suspension of each tested microorganism (500 µl) was spread on Petri dishes cont- aining cooled specific sterile M-H agar medium (pH 7.2).

Paper discs (5 mm diameter) were impregnated with 5 µl of the crude oils and their fractions diluted in chloro- form at 1 mg/ml, kept for drying, and placed on the inoculated Petri dishes, which were, after being kept at 4°C for 2 h, incubated at 37°C for 24 h.Chloroform and a standard antibiotic (Ampicillin) were used as controls. The developing inhibition zones were measured in millim- etres and compared with those of control discs. All tests were performed three times.

RESULTS AND DISCUSSION Chemical

composition of essential oils

The yield of oils from the roots and woody terminal branches of *T. articul*ata was 0.09 and 0.13% (w/wx100), respectively. The oils were light yellow, liquid at room temperature and their odours were agreeable.

Essential oils from roots and woody terminal branches were resolved by silica gel Column Chromatography (sds, 70 - 200 µm/2100027), so that 150 × 5 ml sub-fractions were collected and regrouped into 10 fractions (T_1 to T_{10}) for woody terminal branches and into 11 fractions (R_1 to R_{11}) for roots (Figures 1 and 2).

The fractions T_2 and R_4 which had maximum weight and exhibited an antifungal effect, were resolved by silica gel column into six sub-fractions each for woody terminal branches ($T_{2.1}$ to $T_{2.6}$) and roots $R_{4.1}$ to $R_{4.6}$ (Figures 1 and 2).

The composition of the essential oils from the fractions T_2 and R_4 which showed an activity along with their retention indices (RI) are reported in Table 2. The preponderant constituents are arranged according to their elution on the apolar HP- 5 capillary column. Nonan-1-ol, was the main constituent present in the fraction T_2 from terminal branches with a percentage of 75.22. Other main compounds detected were monoterpene hydrocarbons viz: -Terpinene (3.04), -3 carene (1.17)), and - ocimene (1.09) but no sesquiterpenes were detected (Table 2).

In R_4 fraction, Bornyl acetate (16.63), Camphene (1.59) and -cadinene (2.86) were the major compounds. This fraction was found to be mainly rich in oxygenate compounds (20.25) and sesquiterpene hydrocarbons (6.84) (Table 2).

Antifungal activity of essential oils

Antifungal activity of essential oils from terminal woody branches

The best antifungal activity was exhibited by T_1 against *Alternaria solani* (29.8 ± 2.0 mm) (Table 3).

On the other hand, *A. solani* and *B. cinerea* exhibited largest inhibition zones of 25.0 ± 0 mm and 18.3 ± 0.6 mm, respectively in the presence of T_6 essential oil. *A. solani* was the most affected in the presence of T_7, T_8 and T_9 essential oils with an inhibition zone that ranged from 21.0 ± 2.0 - 27.3 ± 2.1 mm. *F. oxysporum niveum* and *A. solani* were most influenced by $T_{2.6}$ with an inhibition zone of 16.7 ± 1.5 and 19.3 ± 1.2 mm respectively (Table 3).

Antifungal activity of essential oils from roots

Essential oils obtained from *T. articulata* roots demonstrated an antifungal effect against some plant pathogenic fungi. The strongest one was exhibited for R_4 and R_{11} against *A. solani* (26.0 ± 1.0 and 26.7 ± 2.1 mm, respectively) (Table 4).

The largest inhibition zone (26.7±2.1mm) for *A. solani* was seen in the presence of R_{11} extracted from roots of *T. articulata*. *B. cinerea* was inhibited only by R_8 essential oil (inhibition zone 24.8 ± 0.9 mm). *F. oxysporum niveum* was inhibited by most of the fractions of the essential oil obtained from roots (Table 4).

However, practically no effect of root essential oils against *B. cinerea* and *Rhizoctonia solani* was observed. In the light of the high activity of R_4 fraction against *A. solani*, the study further test its active sub-fractions. All the R_4 sub-fractions showed antifungal effects againstsome of the plant pathogenic fungi tested. *R. solani* was inhibited only in the presence of $R_{4.1}$ fraction, which also reduced the *in vitro* growth of *F. solani cucurbitae, A. solani* and *F. oxysporum niveum*, with

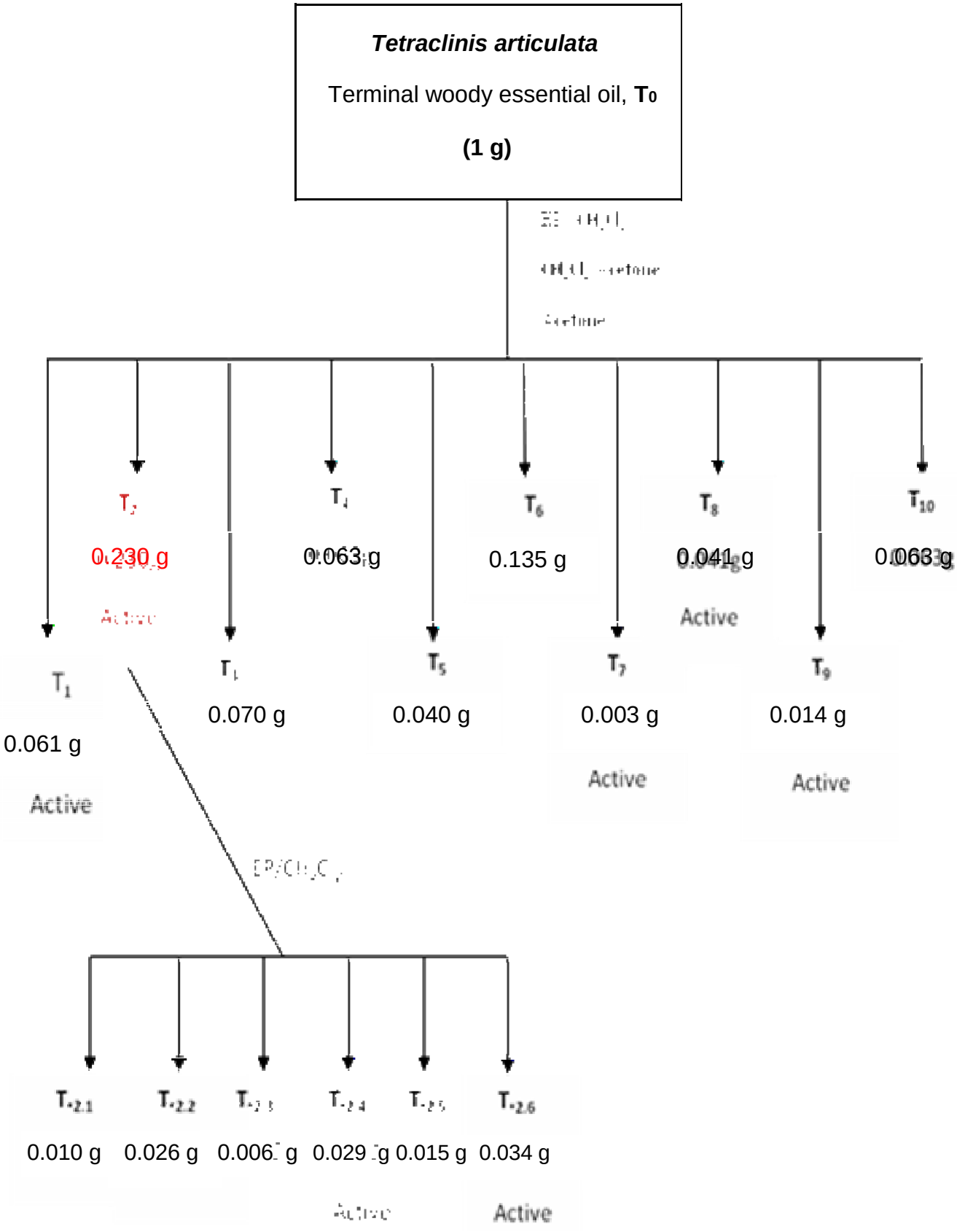

Figure 1. Fractionation of *T. articulata* woody terminal branches essential oil fractions. T_1 - T_2- T_7 - T_8- T_9- $T_{2.4}$ and $T_{2.6}$ exhibited an antifungal effect.

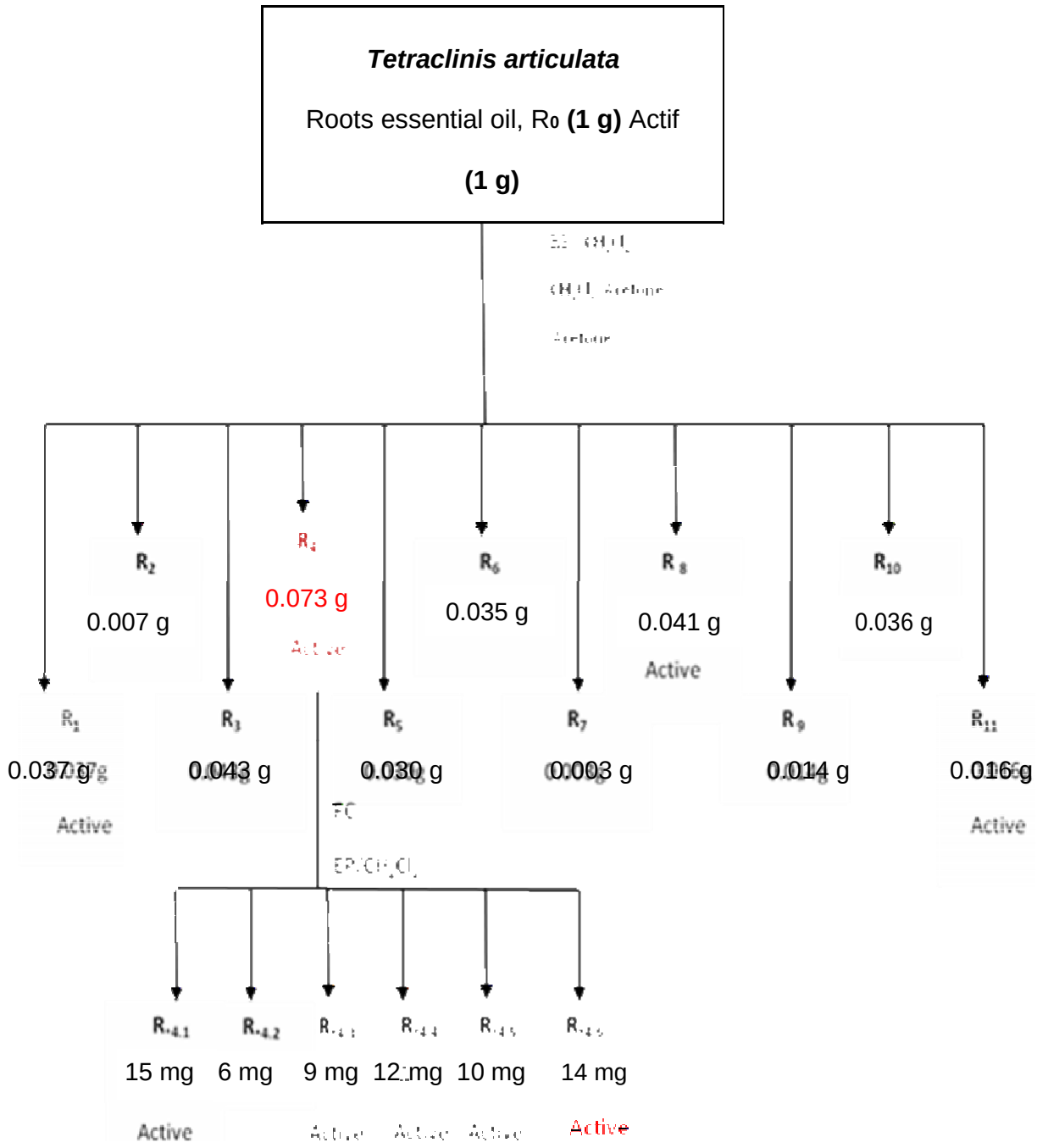

Figure 2. Fractionation of *T. articulata* roots essential oil fractions. R_1- R_4- R_8- R_{11}-$R_{4.1}$- $R_{4.3}$- $R_{4.4}$- $R_{4.5}$- and $R_{4.6}$ exhibited an antifungal effect.

inhibition zones of 11.7 ± 0.5, 19.3 ± 0.6 and 20.7 ± 2.9 mm, respectively (Table 4). The results obtained demonstrated that the essential oil fractions $R_{4.3}$ and $R_{4.5}$ exhibited highest antifungal activities against *A. solani* with inhibition zones 28.3 ± 0.6 and 21.3±2.3 mm, respectively (Table 4). It was also seen that the crude oil from roots was less effective than its fractions R_1, R_2, R_3, R_9, R_{10} and R_{11} against *F. oxysporum niveum*. Boughalleb et al. (2005a) has earlier reported the antifungal effects of volatile components extracted from stems and flowers of *Lantanacamara*, *Malvaviscus arboreus* and *Hibiscus rosasinensis,* but no inhibition is reported for the volatile com- ponents obtained from leaves. The pathogens tested were *A. solani*, *B. cinerea*, *F. solani cucurbitae* and *F. oxysporum niveum*. The metabolites responsible for such activity could be certain fatty acids as mentioned by

Table 2. Chemical composition of bioactive fractions of essential oil from woody terminal branches (T_2) and roots (R_4) of *T. articulata.*

No.	Compound	RI (HP-5)	T_2	R_4
1	Camphene	953	0.07	1.59
2	Sabinene	967	0.06	0.32
3	Myrcene	990	-	0.33
4	-3 carene	1006	1.17	1.27
5	-Terpinene	1010	3.04	-
6	-Ocimene	1035	1.09	-
7	ä-Terpinene	1059	3.04	-
8	Camphor	1122	-	0.27
9	Nonan-1-ol	1150	75.22	-
10	Myrtenal	1171	-	3.37
11	Terpineol	1185	0.17	0.43
12	Bornyl acetate	1270	-	16.63
13	á-Ylangene	1370	-	0.38
14	Caryophyllene	1415	-	1.27
15	Aromadendrene	1440	-	0.53
16	á-Humulene	1453	-	1.10
17	ã-Cadinene	1515	-	2.86
18	á-Calacorene	1542	0.02	0.37
19	â-Calacorene	1548	-	2.58
20	Caryophyllene oxide	1576	-	0.89

The major components and their percentages are listed in order of their elution on apolar column HP-5; retention indices measured an apolar column, (-) compound absent in the fraction. Bold types indicate major compound.

Table 3. Inhibition zone (mm) of fungal growth due to essential oil and its fractions from *T. articulata* woody terminal branches.

Samples	*F. solani cucurbitae*	*F. oxysporum niveum*	*A. solani*	*B. cinerea*	*R. solani*
T_O[a]	12.2[b] ± 0.76	15 ± 0	-	-	-
T_1	10.7 ± 0.6	16 ± 1.7	29.8 ± 2.0	-	-
T_2	11.3 ± 0.6	16.6 ± 1.7	-	-	-
T_3	-	-	19.3 ± 0.6	-	-
T_4	11.0 ± 1.0	-	16.0 ± 1.0	-	-
T_5	-	15.0 ± 1.0	-	-	-
T_6	-	-	25.0 ± 0.0	18.3 ± 0.6	-
T_7	10.5 ± 0.9	13.3 ± 0.8	21.0 ± 2.0	19.0 ± 0.5	-
T_8	12.2 ± 1.6	16.0 ± 1.7	27.3 ± 2.1	-	-
T_9	-	15.0 ± 1.0	25.3 ± 1.5	-	-
T_{10}	12 ± 1.7	15.0 ± 1.5	-	-	-
$T_{.2.1}$	-	-	-	-	-
$T_{.2.2}$	-	-	-	-	-
$T_{.2.3}$	-	-	-	-	-
$T_{.2.4}$	-	18.0 ± 1.0	21.0 ± 2.0	-	-
$T_{.2.5}$	-	15.7 ± 0.3	-	-	-
$T_{.2.6}$	-	16.7 ± 1.5	19.3 ± 1.2	-	-

[a] Crude essential oil of *T. articulata* woody terminal branches.
[b] size of inhibition is the mean ±standard error of five determinations per fungus and per volatile component.
- Absence of any activity.

Table 4 Inhibition zone (mm) of fungal growth with essential oil and its fractions from *T. articulata* roots.

Samples	*F. solani cucurbitae*	*F. oxysporum niveum*	*A. solani*	*B. cinerea*	*R. solani*
R_0[a]	-	-	31.82b ± 2.1	-	-
R_1	-	16.7 ± 1.57	20.3 ± 1.5	-	-
R_2	-	15.2 ± 0.3	-	-	-
R_3	-	15.0 ± 1.4	-	-	-
R_4	-	-	26.0 ± 1.0	-	-
R_5	-	-	-	-	-
R_6	-	-	-	-	-
R_7	-	-	-	-	-
R_8	11.5 ± 1.5	-	-	24.8 ± 0.9	-
R_9	-	14.7 ± 2.9	-	-	-
R_{10}	-	14.3 ± 2.9	-	-	-
R_{11}	11.0 ± 0.9	15.0 ± 0.0	26.7±2.1	-	-
$R_{.4.1}$	11.7 ± 0.5	20.7 ± 2.9	19.3±0.6	-	22.7 ± 1.57
$R_{.4.2}$	-	16.0 ± 1.0	-	-	-
$R_{.4.3}$	-	16.7 ± 2.57	28.3±0.6	-	-
$R_{.4.4}$	11.7 ± 1.5	20.0 ± 2.6	18.3±1.5	-	-
$R_{.4.5}$	15.3 ± 0.6	-	21.3 ± 2.3	20.0 ± 1.0	-
$R_{.4.6}$	11.3 ± 1.5	14.0 ± 1.0	-	-	-

[a]Crude essential oil of *T. articulata* roots. [b] Inhibition size percent is the mean ± standard error of five determinations per fungus and per volatile component. - Absence of any activity.

Car-balleira and Cruz (2000).

On the whole, the synergetic effects of the chemical constituents of the essential oils were not proven for *T. articulata* root oils. Activity of R_4 against *A. solani* could also be attributed to the presence of high concentration of Bornyl acetate (16.63%). El- Sawi et al. (2007) have shown that berry oil is inactive against *F. oxysporum* and *Aspergillus niger*. In contrast, Stassi et al. (1996) have found that the antimicrobial activity of *Juniperus oxycedrus* berry oil is due mainly to its content of terpineol. In our case the antifungal activity could have been the result of the presence of the ester Bornyl acetate as mentioned by Tzakou et al. (1998).

The results of the bioassays with crude woody terminal branches and its fraction T_2 against *F. solani* f. sp. *cucurbitae* (11.3 ± 0.6 mm) and *F. oxysporum* f. sp. *niveum* (16.6 ± 1.7 mm) could be related to the high percentage of aliphatic alcohols, especially to the nonan-1-ol. These results are in agreement with those of studies conducted on other essential oils (Knobloch et al., 1989) which are rich in terpineol and aliphatic alcohols.

Conclusively, it appears from this study that volatile components from woody terminal branches and roots of *T. articulata* may be promising sources of antimicrobial compounds to be used as non-conventional pesticides in the control of plant diseases.

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