Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2013; 5(1); 45-50

ISSN: 0975-4873

Research Article

Phytochemical Screening and Evaluation of Antimicrobial Activity of Crudes Extracts of Leaves Tetraclinis articulata (Vahl) Masters Algerian

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ABSTRACT

In this study, methanolic (MeOH), chloroform (Ch) and ethyl acetate (AcOEt) crudes extracts of leaves Tetraclinis articulata (Vahl) Masters obtained by cold maceration and heat maceration using the soxhlet extractor were screened for the presence of chemically active compounds by Standard methods and then were tested to evaluate their antimicrobial activity against twelve microbial strains using the disc diffusion method. Listeria monocytogenes was the most sensitive towards the different extracts, with diameters of inhibition ranging from 44.67 ± 1.53 mm and 53.00 ± 1.00 mm which is considered significantly different when comparing with the antibiotics used as positive control. However, the other results show the ineffectiveness of the extracts against Klebsiella pneumoniae, Fusariumculmorum, Aspergillus ochraceus, Aspergillus flavus and Candida albicans. The results revealed the presence of tannin Flavonoids and polyphenols in each extracts.

Keywords: Tetraclinis articulata (Vahl) Masters, crude extract, soxhlet, antimicrobial activity, Screening.

INTRODUCTION

Infectious diseases are considered the main causes of death worldwide. The clinical efficacy of existing antibiotics is threatened by the emergence of multidrug-resistant pathogens (1). These pathogens have evolved numerous defenses against antimicrobial agents. The growing failure of chemotherapy and antibiotherapy led to more screening of medicinal plants for their potential antimicrobial activity (2). There are several reports in the literature for the antimicrobial activity of crude extracts prepared from medicinal plants (3).

In Algeria, many plants are used traditionally to treat various diseases. Among these plants, Tetraclinis articulata (Vahl) Masters belongs to the Cupressaceous family. It is endemic of south western Mediterranean. Different parts of this tree are used in folk medicine for its multiple therapeutic effects. It is mainly used against childhood fevers, respiratory infections and intestinal, stomach pain, diabetes and hypertension (4-5-6). Studies on the biological activities of essential oils for their different parts show that these oils are antibacterial, antifungal and cytotoxic, antioxidant and anti-inflammatory (7,8,9,10). The aqueous extract of her wood can be used as natural biocides (11). The decoction of their leaves heals wounds, bruises and wounds.

The objectives of the present study were to determine the antimicrobial activity of methanolic, chloroform and ethyl acetate extracts of leaves Tetraclinis articulata (Vahl) Masters Algerian in vitro and the Phytochemical screening of these extracts.The antibacterial activity was carried out using the disc diffusion method and the determination of total polyphenols using Folin-Ciocalteu method.

MATERIALS AND METHODS

Plant material: Fresh leaves of Tetraclinis articulata (Vahl) masters were collected in January 2011 from district of Ain- Defla, area of El-Attaf from center western of Algeria, on trees taken randomly. The voucher specimen (INA/P/ No 105) was deposited in the herbarium of the institute of INA (institute national d'agronomie). These leaves were then dried in the dark at room temperature (25°C) for four weeks and then were sprayed to a fine powder.

Microbial strains: The antimicrobial activity of different extracts of leaves tetraclinis articulata (Vahl) masters was evaluated using seven bacteria (Salmonella enterica E32, Klebsiella pneumoniae CIP 8291, Listeria monocytogenes CIP 82110, Staphylococcus aureus CIP 7625, Pseudomonas aeruginosa CIP A22 Escherichia coli ATTC 10536, Agrobacterium N°2410) and four fungus

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Table 1: Preliminary phytochemical analysis of crude extracts of leaves Tetraclinis articulata (Vahl) Masters.

chemical groups extracts	tannin	flavonoids	Sterols and Terpenes	quinones	polyphenols	saponins	alkaloids
Chf	+	+	-	-	+	-	-
Chc	+	+	-	-	+	-	-
AcOEtf	+	+	-	+	+	+	-
AcOEtc	+	+	-	+	+	+	-
MeOHf	+	+	-	+	+	+	-
MeOHc	+	+	-	+	+	+	-

Ch: chloroform, MeOH: methanol; AcOEt: ethyl acetate
f: cold maceration; c: hot maceration
+: Presence of the compound; - : absence of the compound.

Table 2: polyphenol content of crude extracts of leaves Tetraclinis articulata (Vahl) Masters expressed as mg of gallic acid equivalent per gram of extract.

extraits	extracts polyphenol content (mg EAG / g extract)
Chf	42,49±0,54
Chc	84,14±1,03
AcOEtf	127,65±1,28
MeOHf	133,5±1,15
AcOEtc	145,94±0,78
MeOHc	156,63±0,79

(Fusarium culmorum C.L.M, Aspergillus flavus C.L.M, Aspergillus ochraceus C.L.M, Botrytis cinerea C.L.M) and a yeast (Candida albicans IPA 200).These micro-organisms are known by their high pathogenic capacity and their resistance to various antibiotics and antifungal.

Extraction : The different extracts of leaves Tetraclinis articulata (Vahl) Masters are prepared by cold maceration of 20 g of powder of these leaves at room temperature in each 100ml solvents: ethyl acetate (AcOEt), chloforme (Ch) and methanol (MeOH) at a rate of 20% (w / v) for 24 hours with agitation. The solutions obtained are carefully screened repeatedly cotton wool and Whatman paper N°1, the filtrate is evaporated using a rotary evaporator (Evaporator E100).

For the extracts obtained by hot maceration, using the Soxhlet extractor, this allows for continuous solvent extraction of a solid. It consists of a glass body which is placed a thick filter paper cartridge, a tube and a siphon tube supply. The body of the extractor is placed in a flask containing 100 ml for each extraction solvent used: ethyl acetate (AcOEt), chloforme (Ch) and methanol (MeOH). 20g of powder to be extracted are placed in the extractor fitted with a condenser. When the balloon is heated, the solvent vapors pass through the tube adductor condense in the cooler and fall in the body of the adductor, thereby soak the plant powder in the solvent. The condensed solvent accumulates in the extractor up to the top of the siphon tube, which then causes the return fluid in the balloon, accompanied by the substances extracted. The solvent in the flask becomes progressively enriched with soluble compounds. After soxhlet extraction, organic solvents are recovered and concentrated by evaporation using a rotary evaporator (Evaporator E100). Green viscous extracts are then obtained.

The final residue obtained from the three maceration either hot or cold is kept at+4 °C in vials sealed after they are weighed to calculate the yield of extraction.

Phytochemical screening of different extracts: This study helps to highlight the main chemical groups contained in our extracts.

Test for tannins: The presence of tannins has been demonstrated in the different extracts by adding a few drops of ferric chloride (FeCl3) of 2%. The response to FeCl3 causes the appearance of brownish green or a blue-black coloration (12).

Test for flavonoids: The presence or absence of flavonoids in an extract can be highlighted by a quick and simple test called reaction Shinoda. It consists to add a 1 ml of the extract, a few drops of HCl (2N) and about 0.5 g of magnesium metal with agitation for 3 minutes. The presence of flavonoids was confirmed by the red, orange, pink or purplish red color (13).

Test sterols and terpenes: In a test tube, dissolve the extract in 5 ml of chloroform are added 2 ml of acetic anhydride and 3 to 4 drops of sulfuric acid, a violet-purple and a fleeting blue color, which gives way gradually to a green color (12).

Test for quinones substances: The presence of quinones can be obtained by alkalizing with a few drops of soda 2 ml of the extracts .We obtain with quinones a color going from the red to purple (14).

Test for polyphenols: A 2 ml of our extracts, add a drop of alcoholic solution of ferric chloride of 2%. The presence of polyphenolics drift causes the appearance of a blue or dark green color (14).

Test for saponosides: we introduce into a vial 5 ml of 0.1 N HCl, in a second vial 5 ml of 0.1 N NaOH, then add in each one 2 to 3 drops of extract. The formation of foam indicates the presence of saponins (12).

Table 3: Antimicrobial activity of crude extracts of leaves Tetraclinis articulata (Vahl) Masters obtained by hot maceration.

	Diameters of zone inhibition (mm)					
extracts / strains	MeOHc		AcOEtc		Chc	
Concentration mg / l / bacteria	5	50	5	50	5	50
Listeria monocytogenes	51,33±1,53 a	61,00±1,00b	44,67±1,53c	60,00±1,00b	53,00±1,00 d	59,00±1,00b
Staphylococcus aureus	14,50±0,50f	18,50±0,50e	13,50±0,50f	15,50±0,50f	14,33±0,29f	18,67±0,58e
Salmonella enterica	10,50±0,50i	12,50±0,16h	10±0,16i	12,50±0,50h	14,43±0,29f	16,57±0,58g
Escherichia coli	12,50±0,16h	14,50±0,16f	10,50±0,16i	12,50±0,16h	-	-
Klebsiella pneumoniae	-	-	-	-	-	-
Pseudomonas aeruginosa	14,50±0,50f	18,50±0,16e	-	-	-	-
Agrobacterium tumefaciens	14,50±0,29f	17,50±0,29e	13,17±0,29f	18,19±0,50e	13,50±0,29f	17,18±0,50e
fungus	5	50	5	50	5	50
Fusariumculmorum	-	-	-	-	-	-
Aspergillus ochraceus	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-
Botrytis *cinerea*	12,0±0,29h	14,50±0,29f	12,17±0,50h	14,19±0,29f	12,50±0,29h	14,19±0,29f
Yeast	5	50	5	50	5	50
Candida albicans	-	-	-	-	-	-

Table 4: Antimicrobial activity of crude extracts of leaves Tetraclinis articulata (Vahl) Masters obtained by cold maceration.

	Diameters of zone inhibition (mm)					
Extracts / strains	MeOHf		AcOEtf		Chf	
Concentration mg / l / bacteria	5	50	5	50	5	50
Listeria monocytogenes	46,67±1,53c	55,00±1,00d	51,67±1,53a	56,00±1,00d	52,67±2,52a	61,67±1,53b
Staphylococcus aureus	10,00±0,50i	12,50±0,50h	10,17±0,29i	10,17±0,29i	10,50±0,50i	12,17±0,29h
Salmonella enterica	10,50±0,29i	12,17±0,50h	10,17±0,29i	12,17±0,29h	12,50±0,29h	14,17±0,50f
Escherichia coli	10,50±0,50i	12,00±0,16h	12,19±0,29h	14,17±0,05f	-	-
Klebsiella pneumoniae	-	-	-	-	-	-
Pseudomonas aeruginosa	10,50±0,16i	14,50±0,50h	-	-	-	-
Agrobacterium tumefaciens	14,17±0,50f	18,00±0,29e	12,17±0,29h	17,17±0,16e	14,50±0,16f	16,17±0,50g
fungus	5	50	5	50	5	50
Fusariumculmorum	-	-	-	-	-	-
Aspergillus ochraceus	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-
Botrytis *cinerea*	10,0±0,50i	12,50±0,29h	12,17±0,29h	14,19±0,29f	10,50±0,50i	12,19±0,50h
Yeast	5	50	5	50	5	50
Candida albicans	-	-	-	-	-	-

Table 5: Antimicrobial activity of antibiotics reference.
Diameters of zone inhibition (mm)

Antibiotic strains	Amoxicillin		Clarithromycin		Ciprofloxacin	
Concentration mg / l bacteria	5	50	5	50	5	50
Listeria monocytogenes	21,17±1,53j	31,33 ±1,04k	12,5 ±0,5h	19±0,5j	37,17±0,76m	40,83±0,76n
Staphylococcus aureus	16,5±0,5g	16,83±0,76g	16,50±0,50g	17,33±0,29e	24,50±0,50o	26,50±0,50o
Salmonella enterica	11,5±0,5h	22,67±0,58j	19,50±0,50j	21,83±0,29j	33,50±0,50p	37,50±0,50m
Escherichia coli	20,33±0,58j	28,50±0,50o	14,17±0,29f	24,33±0,58o	27,17±0,29o	34,33±0,58p
Klebsiella pneumoniae	38,50±0,50m	40,17±0,29n	19,17±0,29j	44,50±0,50c	42,50±0,50n	44,33±0,58c
Pseudomonas aeruginosa	10,33±0,58i	29,33±0,58o	13,17±0,29f	25,17±0,76o	33,50±0,50p	35,17±0,29p
Agrobacterium tumefaciens	10,33±0,58i	15,67±0,29g	18,50±0,50e	30,50±0,50o	26,00±1,00o	32,50±0,50o
fungus	5	50	5	50	5	50
Fusariumculmorum	-	-	-	-	-	-
Aspergillus ochraceus	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	-
Botrytis *cinerea*	-	-	-	-	-	-
Yeast	5	50	5	50	5	50
Candida albicans	-	-	-	-	-	-

Each value represents the average ± standard deviation
-: absence of zone of inhibition
A, B, C, D, E, F, G, H, I, J, K, L, M, N, O and P is letters carried in index of the averages for the test of comparison, thus the averages which carry the same letter do not present a significant difference at the threshold of 5%

Test for alkaloids: the residues are taken again by 5 ml of hydrochloric alcohol in test tubes. Add some drops of the reagent of Dragendorff. The appearance of an orange precipitate indicates the presence of alkaloid (14).

Determination of total polyphenols: The content of total phenols in different extracts was determined using Folin-Ciocalteu method described by Singleton et al (14). This assay is based on the quantification of the total concentration of hydroxyl groups present in the extract. The Folin-Ciocalteau is a yellow acid solution containing a complex ion polymer (heteropolyacids). In alkaline medium, the reagent of Folin-Ciocalteau oxidizes phenols in ions phenolates and reduces its hétéropolyacides partially, which form a blue complex. Briefly, a 0.2 ml of extract prepared in distilled water is added to 0.8 ml of solution of Na2CO3 (75 mg / ml distilled water), after stirring, 1 ml of Folin-Ciocalteu solution is added to the Overall, after 2 h of incubation, the absorbance at 765 nm is measured. The results are expressed as mg Gallic acid equivalent per gram of extract.

Antibacterial activity: The antimicrobial activity of extracts was determined using agar diffusion method quoted by Kablan et al (15), with some modifications. The different culture media used were nutrient agar and potato dextrose Agra (PDA) to study the sensitivity of strains to different extracts.

The methanol extracts (MeOHc, MeOHf), chloroform (CHC, Chf) and ethyl acetate (AcOEtc AcOEtf) are solubilized in absolute methanol. We thus prepared two solutions for each extract with concentration of 50 mg / ml and 5 mg / ml. Blotting paper discs of 6 mm of diameter are impregnated with 50 ml of each solution and absolute methanol as a negative control. These are incubated in an oven at 37°C for 24 hours. Disks of amoxicillin, clarithromycin and ciprofloxacin were also used as positive control.

After 18h of incubation at 37°C, microbial suspensions were prepared in the physiological water to give a final density of 5,106 cfu/l. This inoculum is inoculated by plating on Petri dishes containing nutrient agar for bacteria and potato dextrose Agra (PDA) for fungi. The antibacterial activity was determined by measuring the diameter of zone inhibition around each disk. The antimicrobial activity is manifested by the appearance of a halo of inhibition of microbial growth around the impregnated discs. The result of this activity is expressed by the diameter of zone inhibition. The strain with a diameter D ≤8mm, D ≤ 14mm, D ≤ 19mm D> 20 mm is

considered respectively as a resistant strain, sensitive, very sensitive, extremely sensitive.

STATISTICAL ANALYSIS

Statistical analysis was performed by statistical software Graph Pad Prism. The results were expressed as mean ± SD (standard deviation) (n = 3). The results were analyzed by univariate ANOVA tests (one-way ANOVA) and (two-way ANOVA) followed by Tukey test for the comparison of results of extracts with the negative controls and comparison between them. The difference was considered statistically significant when the p value is less than 0.05%.

RESULTS AND DISCUSSION

The different crude extracts of leave powder Tetraclinis articulata (Vahl) Masters were obtained by performing two types of maceration: cold maceration and hot maceration using the soxhlet extractor, using three solvents in each maceration: methanol, chloroform and ethyl acetate. These six crude extracts: methanol extract of cold and hot maceration (MeOHf, MeOHc), chloroform extract of cold and hot maceration (Chf, Chc) and ethyl acetate extract of cold and hot maceration (AcOEtf, AcOEtc) had yields, respectively (35.9%, 30.22%), (22.25%, 18.42%), (25.80%, 21.51%). All these extracts were then subjected to qualitative phytochemical analysis whose results are reported in Table 1 and a quantitative analysis of polyphenols whose results are reported in Table 2.

The phytochemical analysis of the different extracts gave positive tests for tannins, flavonoids and polyphenols, and negative tests for alkaloids, sterols and terpenes. For Quinones and saponins, the results show their presence in the methanol extracts and ethyl acetate and their absence in the chloroform extracts.

The rate of total polyphenols in our extracts was calculated from the calibration curve, established with precise concentrations of Gallic acid (0-200mg/ml) as the reference standard, under the same conditions. The results are expressed as mg Gallic acid equivalent per g of extract (EAG mg / g extract). The results show that these extracts are rich in phenolic compounds and the extracts obtained by soaking hot (Soxhlet) contain a significant concentration of these compounds compared to extracts obtained by cold maceration

The antimicrobial activity of crude extracts and antibiotics were evaluated in vitro against twelve microbial strains, the results are reported in Tables 3, 4 and 5.

The bacterial strains were more sensitive than fungal strains, particularly Listeria monocytogenes, which was the most sensitive species to these six extracts, with inhibition diameters ranging of 44, 67±1,53mm to 53, 00±1, 00 mm around the discs of 5 mg/ml, while around the discs of 50 mg/ml, the diameters range from 55.00 ± 1.00 mm to 61.67 ± 1.53 mm, although this effect is significantly different ($p < 0.05$) than the positive control antibiotic.

The results show that Escherichia coli is sensitive to methanolic and ethyl acetate extracts with a zone inhibition ranging from 10.50 ± 0.50 mm to 14.17 ± 0.05 mm, but it proved to be resistant to the chloroform extracts, for the Pseudomonas aeruginosa has proved to be resistant to the chloroform and ethyl acetate extracts and has proved to be sensitive to the methanolic extract with diameters of inhibition ranging from 10,50±0,16mmt to 14,50±0,50mm around the discs of 5 mg/ml and from 50 ± 0.50 mm to 18.50 ± 0.16 mm around the disks of 50 mg/ml, indicating that the antimicrobial activity depends the solvent used for extraction.

The results of antibiotics have proven ineffective all antibiotics against all fungal strains tested and effective against all bacterial strains tested. Klebsiella pneumoniae, Fusariumculmorum, Aspergillus ochraceus and Aspergillus flavus and the yeast Candida albicans have proved to be resistant to the extracts tested.

CONCLUSION

This work shows that the antimicrobial extracts of medicinal plants depends several factors: the concentration of the extract, the solvent extraction and the method of extraction. The natural extracts of plants contain a variety of phenolic compounds which are attributed the inhibitory microorganisms.

Preliminary studies chemicals were made, they show that our extracts contain flavonoids, polyphenols, tannins, quinones and saponins and absence of sterols, terpenes and alkaloids.

The antimicrobial activity of different extracts was evaluated in vitro against twelve pathogenic microbial strains using agar diffusion method. The bacterial strains were more sensitive than fungal strains, their inhibitory effect on Listeria monocytogenes has been dramatic, it is much more important than the positive control antibiotic. Generally, the methanolic extracts were slightly more effective than chloroform or ethyl acetate extract.

Our results show that all crude extracts tested possess antimicrobial activities in vitro. Further studies will be considered, as the in vivo study, Isolation and characterization of active compounds in different extracts in order to determine the compounds responsible for the antimicrobial activity of the extracts tested.

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