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Phytochemical screening and antibacterial effect of phenolic extracts from two Mediterranean *Cupressus*

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Abstract

The objective of our research is to identify the chemical components and evaluate *in vitro*, the antibacterial activity of the extracts from the leaves of *Cupressus sempervirens* L. and *Cupressus arizonica* L. from northern Algeria against *Pseudomonas aeruginosa* ATCC 9027. The extraction was carried out by macerating the leaves in solvents of increasing polarity (chloroform, petroleum ether and aqueous methanol). A screening of the phenolic compounds was carried out for a qualitative characterization of the different extracts. The extracts obtained were put in contact with a strain of *P. aeruginosa* to determine their antibacterial potential. Phytochemical screening revealed the presence of several secondary metabolites: leucoanthocyanins, flavonols, flavonones, anthraquinones, catechin tannins, gallic tannins, steroids, triterpenes, saponin, cardiac glycosides, terpenoids, saponosides and free quinones. Aqueous methanol (high polarity) allows the extraction of most metabolites. The best extraction yield of the three solvents is chloroform, with an extraction yield of 61.23% (for *C. sempervirens*) and 52.27% (for *C. arizonica*), followed by the hydroalcoholic solvent 33.55% and the ethereal solvent with 0.39%. Hydroalcoholic extraction induces a very important sensitivity of *P. aeruginosa*, with a diameter of 16.2 mm for *C. arizonica*. Ethereal and chloroformic extracts induce weak inhibition. *P. aeruginosa* is extremely sensitive to the hydroalcoholic extract from *C. sempervirens*, the latter induces an inhibition zone with a diameter of 19.95 mm, which is statistically equal to that induced by Vancomycin. These results can be considered as a promising solution for the replacement of vancomycin with the hydroalcoholic extract from *C. sempervirens*.

Keywords: *Cupressus arizonica*, *Cupressus sempervirens*, *Pseudomonas aeruginosa*, hydroalcoholic extract.

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Introduction

In recent decades, scientific research has only confirmed the beneficial effects of plants on health. It has been suggested that their high content of bioactive agents, especially polyphenols, could be responsible for the prevention of many diseases and could be used as a natural alternative to synthetic drugs (Nostro *et al*., 2000). It is estimated that the active ingredients of plants represent about 25% of prescription drugs. That is a total of 120 natural compounds from 90 different plants (Djahra, 2014). Several scientific papers on polyphenols have been published to inform and sensitize the population about their interests. These molecules are recognized for their considerable antioxidant potentials, which are directly related to human health (Bouayed *et al*., 2008).

Polyphenols have the ability to trap free radicals, permanently generated by our body or formed in response to attacks from our environment or in case of infection. When ingested with food, they strengthen natural defenses by protecting cells and tissues against oxidative stress (Scalbert, 2004). Flavonoids prevent oxidative damage by different mechanisms of action: either by capturing hydroxyl, superoxide and peroxide radicals (Hodek *et al*., 2002). Either by chelation of metals (iron and copper), which are of great importance in the initiation of radical reactions or the inhibition of enzymes responsible for the generation of free radicals resulting from the infection process (Benavente-Garcia *et al*., 1997).

As for the antioxidant power of tannins, this property is very remarkable due to its phenolic nuclei (Zimmer and Cordesse, 1996). Many bacteria have developed resistance to most antibiotics, and it is a major health problem on a global scale (Lozniewski and Rabaud, 2010), which led us to study the efficacy of plants with therapeutic virtues in order to isolate the active ingredients. Cypresses (Cupressaceae) are medium-sized conifers, which contain more than 20 species distributed in the Mediterranean region, tropical Asia and North America (Liu *et al*., 2010). Cypress is considered a medicinal tree, widely used in traditional medicine, where its dried leaves are used for the relief of stomach pain, joint and muscle pains, malaria, cough, gout and rheumatism, as well as a hypoglycemic agent (Selim *et al*., 2014; Lakhdar *et al*., 2015).

The objective of the research is to identify the secondary metabolites that exist in the flakes of *Cupressus sempervirens* and *Cupressus arizonica* after grinding (phytochemical screening). Grinding allows increasing the contact surface of the sample with the solvent and better filtration of the solvent inside the plant material, which results in an increase in extraction yield. The objective of the extraction is to release the polyphenols present in the vacuolar structures by rupture of the plant tissue and by diffusion. These are extracted by solid-liquid extraction using different solvents with increasing polarity, namely: diethyl ether, chloroform and aqueous methanol. In the laboratory, a comparative analysis of the different extracts is carried out to study their bactericidal effect against *Pseudomonas aeruginosa* ATCC 9027.

Materials and methods

The study is carried out according to an experimental model in randomized complete blocks.

Plant material

The plants under study were chosen based on a meticulous literature search that showed that these plant species are very little studied. The leaves of *Cupressus sempervirens* L. and *Cupressus arizonica* used in this study were collected during April 2021, at the level of the Department of Biotechnologies and Agroecology of the University of Blida 1 (Blida-Algeria). The identification of the two species is made with the flora of Quezel and Santa (1963). In order not to damage the tree, we have chosen very green leaves that appear at the ends of the branches.

Bacterial strain

The bacterial strain used in this study is *Pseudomonas aeruginosa* ATCC 9027, pathogenic bacterium, Gram-negative category, clinically isolated, its collection and isolation were carried out in accordance with hygiene standards. The strain is stored and grown in Mueller-Hinton medium. It is part of the collection of the research laboratory in Biotechnology of Plant Production of the University of Blida 1 and was made available to us by the director Pr. Sid Ahmed SNOUSSI.

Polyphenol extraction

Drying

After harvesting, the samples were cleaned (cleaned of debris) and spread on paper. The aerial parts of the plant were dried in the dark at room temperature. We ventilate the samples every three days to prevent mold growth. Drying lasted 30 days on average (Debib *et al*., 2014).

Grinding

The dried leaves were crushed, sieved and stored in airtight bottles (room temperature) protected from moisture and light, until their use.

Preparation of raw extracts

For the extraction of polyphenols, we opted for the use of three solvents: petroleum ether, chloroform and aqueous methanol.

Hydroalcoholic extract

Ten grams of each plant material were put in contact with 100 ml of a mixture of methanol-water (70/30) (v/v). After 24 h of mechanical stirring at room temperature and protected from light, the mixture is vacuum filtered with a 0.4 μm Millipore membrane and evaporated to dryness under reduced pressure by a rotary evaporator (BUCHI R-215) at 45 °C to obtain the hydroalcoholic extract. The residues obtained are stored at 4 °C until their use (Romani *et al*., 2006).

Ethereal extract

First, 10 g of each plant material is macerated in 100 ml of petroleum ether. After 10 min of mechanical stirring at room temperature and protected from light. The mixture is vacuum filtered with a 0.4 μm Millipore membrane and concentrated in a rotary evaporator at a temperature of 30 °C to obtain the extract of diethyl ether. The residues obtained are stored at 4 °C until their use (Drissa *et al*., 2004).

Chloroformic extract

First, 10 g of each plant material is macerated in 100 ml of chloroform. After 10 min of mechanical stirring, at room temperature and protected from light, the mixture is vacuum filtered with a 0.4 μm Millipore membrane and concentrated in a rotary evaporator at a temperature of 40 °C to obtain one chloroformic extract. The resulting residues are stored at 4 °C until their use (Drissa *et al*., 2004). The heavy dry residues are captured by the solvents for the phytochemical study and by Dimethyl sulfoxide (DMSO) for the antibacterial activity (Debib *et al*., 2014).

Extraction yield

The extraction yield is calculated by the formula proposed by Falleh *et al*. (2008): $Y(\%)=\frac{M_{ext}}{M_{dry}}\times 100$. Where: Y= percentage of yield %; M_{ext}= mass of the extract after evaporation of the solvent in g; M_{dry}= dry mass of the plant sample in g.

Phytochemical screening

Phytochemical screening is a qualitative test that allows highlighting the different chemical groups contained in a plant organ, the results are classified into very positive reaction= +++; positive reaction= ++; weak reaction= +; negative reaction= -; phytochemical tests were carried out on extracts prepared from *Cupressus* leaves. The detection of some compounds is achieved using the methods described by Harbone (1973); Trease and Evans (1989); Evans (1996) with some modifications.

Flavonoids

Bate-Smith test (flavan-3,4-diols test)

We put 1 ml of the extract in a tube to which we add 250 μl of concentrated HCl. The tube was subjected to a water bath for 30 min. The appearance of a red color indicates the presence of leucoanthocyanins, which are derivatives of flavan-3,4-diols (Karumi, 2004).

Wilstater test (tests for flavonols and flavonones)

In separate tubes, 1 ml of each extract is placed and 0.1 g of magnesium (Mg) is added. A drop of HCl is added and left to act under the hood. The appearance of a color that becomes purplish red (flavonols) or purplish red (flavonones) confirms the existence of flavonoids (Karumi, 2004).

Tannins

One hundred microliters of a 1% FeCl3 solution are added to a tube that contains 1 ml of the extract. In the presence of tannins, a greenish or blue-black color appears. The color turns blackish brown in the presence of gallic tannins (hydrolysable tannins) and greenish blue in the presence of catechetical tannins (condensed tannins) (Harborne, 1998).

Saponosides

Put 0.5 g of crushed leaves in 80 ml of distilled water and stir for a few minutes. The appearance of a foam in the medium proves the presence of saponosides (Trease and Evans, 2002).

Anthraquinones

We put 1 ml of the extract in a tube to which we add 500 µl of KOH. After stirring, the presence of anthraquinones is confirmed when the aqueous phase turns red (Ribérreau, 1968).

Saponins

Libermann-Burchard reaction: to 5 ml of extracts, we add 5 ml of acetic anhydride ($C_4H_6O_3$) and a few drops of concentrated H_2SO_4. Steroids give a red color with this reaction, while triterpenes give a green color (Bruneton, 1993).

Free quinones

The identification of free quinones in our extracts is carried out by adding a few drops of NaOH (1%) to 1 ml of the extract. Their presence is indicated by the appearance of purple color; yellow or red (Ribérreau, 1968).

Terpenoids

Five milliliters of extract were mixed with 2 ml of chloroform in a test tube. Three milliliters of concentrated sulfuric acid were carefully added along the wall of the test tube to form a layer. An interphase with a reddish-brown coloration indicates the presence of terpenoids (Bruneton, 1993).

Cardiac glycosides

One milliliter of concentrated sulfuric acid was poured into a test tube. Five milliliters of extract were mixed with 2 ml of glacial acetic acid that contained a drop of ferric chloride. This mixture was carefully added to 1 ml of concentrated sulfuric acid. The presence of cardiac glycosides was detected by the formation of a brown ring (Trease and Evans, 2002).

Evaluation of antibacterial activity

The bacterial sensitivity test is performed using the agar diffusion method, also called the disk method (Celiktas *et al*., 2007; Bssaibis *et al*., 2009).

Seeding

In sterile Petri dishes (Ø: 90 mm), 20 ml of Mueller-Hinton agar is poured and left to solidify for 20 min. In this culture medium, 1 ml of bacterial suspension of 10^8 CFU ml^{-1} was inoculated on the entire surface using a swab, the swab must be passed over the entire surface to obtain a homogeneous inoculation (Shunying *et al*., 2005). Sterile blank disks (Liofilchem®) of 6 mm in diameter are impregnated with a volume of 10 µl of extract and placed on the surface of the solidified medium (Ngameni *et al*., 2009). The Petri dishes were incubated for 18-24 h, at 37 °C. For the positive control, antibiotic disks (Penicillin and Vancomycin (Liofilchem®)) were used, while for the negative control, the sterile blank disk was soaked with Dimethyl sulfoxide (DMSO).

Reading

The determination of antibacterial activity was estimated by measuring the diameter (in millimeters) of the inhibition zone around the disks, induced by the extracts. The calculation is performed using the processing and measurement software Digimizer™. The results are symbolized by signs that depend on the sensitivity of the strains to the extract (Ponce *et al*., 2003), (Table 1).

Table 1. Sensitivity and degree of activity according to the diameter of inhibition.

Diameter of the inhibition halo (Ø)	Degree of susceptibility to germs
Ø < 8 mm	Non-sensitive/resistant
8.1 > Ø > 14 mm	Sensitive
14.1 > Ø >19 mm	Very sensitive
Ø > 19.1 mm	Extremely sensitive

Ponce *et al*. (2003).

Statistical data treating

The statistical analysis of the results obtained was carried out using the SPSS© software version 20.0.0 for Windows™. The experiments were repeated ten times to verify the normality of the statistical distribution. The results show the same trends. An analysis of variance (Anova) is performed, followed by a post-hoc Tukey test at the 5% threshold and a Student's test to see the existence of significant differences between the extraction methods according to the plant tested, taking into account counts, extract yield and inhibition of the development of *P. aeruginosa* ATCC 9027.

Results and discussion

Extract yield

For the two plants *Cupressus sempervirens* and *Cupressus arizonica*, the best extraction yield of the three solvents used is chloroform, with an average extraction yield of 61.23% and 52.27% respectively, followed by the hydroalcoholic solvent 33.55% and the ethereal solvent with 0.39% (Figure 1). The extraction yields shown in the figure reveal a significant difference (*C.*

sempervirens: F= 52.38; *p*= 0 and *C. arizonica*: F= 27.24; *p*= 0) compared to the solvent used. The Anova test reveals three homogeneous groups (Table 2, Table 3). Statistically and according to Student's t-test, the three solvents (chloroform, hydroalcoholic, ethereal) differ significantly for the two plants (Table 4).

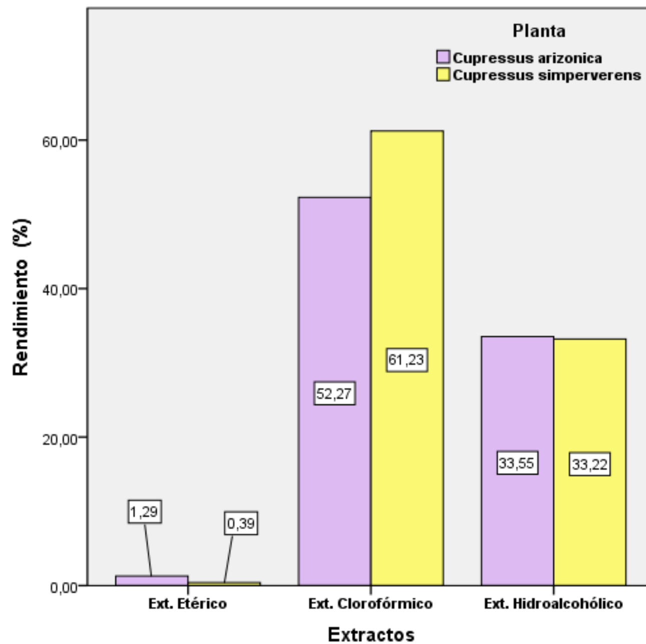

Figure 1. Extraction yield of phenolic extracts.

Table 2. Distribution of extract yield in homogeneous groups for *C. arizonica* (Tukey's test).

Extracts	N	Subset for alpha = 0.05		
		1	2	3
Ethereal extracts	10	1.29		
Hydroalcoholic extracts	10		33.56	
Chloroformic extracts	10			52.28
Intragroup meaning		1 000	1 000	1 000

Table 3. Distribution of extract yield in homogeneous groups for *C. sempervirens* (Tukey's test).

Extracts	N	Subset for alpha = 0.05		
		1	2	3
Ethereal extracts	10	0.4		
Hydroalcoholic extracts	10		33.23	
Chloroformic extracts	10			61.26
Intragroup meaning		1 000	1 000	1 000

Table 4. Student's t-test for the comparison of the extracts according to the plants.

Extracts	t	dof	*p*	Mean difference	STD difference
Ethereal extracts	83.415	1 515	0.001	0.895	0.011
Chloroformic extracts	33.212	1 312	0	8.972	0.027
Hydroalcoholic extracts	13.98	1 984	0.005	0.331	0.024

Similarities are common in the leaves of trees harvested in the same place. Environmental conditions have an important influence on the production of metabolites, so the extraction yields of plants growing in the same place can be expected to be very similar (Graglia *et al*., 1996). Our results refute this hypothesis and clearly show that the yield of the species is not related to environmental conditions because the two trees are in the same area at a distance of 2.45 m.

These results correlate with those of Farhat *et al*. (2009), who explain that the variable yield of the extracts is reduced to the differential solubility of the various phenolic compounds in solvents and that this solubility is a function of their polymerization degrees. The interaction with the other components and the nature and physicochemical characteristics of the solvents used and in particular, their polarity. In fact, the solubility of the substances contained in the plant material in a certain solvent depends on these properties. As a result, extraction yields and the composition of extracts vary from solvent to solvent and plant to plant (Falleh *et al*., 2008).

Phytochemical screening

Phytochemical screening of *C. arizonica* and *C. sempervirens* indicates that the two species have phenolic compounds whose presence differs depending on the type of solvent used (hydroalcoholic, ethereal and chloroformic extract) (Table 5).

Table 5. Phytochemical screening of *Cupressus arizonica* and *Cupressus sempervirens* according to the nature of the solvent.

		Cupressus arizonica			*Cupressus sempervirens*		
		Ext (A)	Ext (E)	Ext (C)	Ext (A)	Ext (E)	Ext (C)
	Saponosides		+ + +			+ + +	
Flavonoids	Leucoanthocyanins	+ +	-	-	+ +	+	-
	Flavonols	+ +	-	-	+ +	-	-
	Flavonones	+	-	-	+	-	-
	Anthraquinones	+ +	-	-	+ +	-	-
Tannins	Catechetical tannins	+ +	-	-	+ +	-	-
	Gallic tannins	-	-	+ +	-	-	+ +
Saponin	Steroids	+	-	-	+	+	-
	Triterpenes	-	+	+	-	+	+
	Cardiac glycosides	+	-	-	+ +	-	-
	Terpenoids	+	-	-	+	-	+ +
	Free quinones	+ +	-	-	+ +	-	-

Ext (A)= hydroalcoholic extract; Ext (E)= ethereal extract; Ext (C)= chloroformic extract; +++= very positive reaction; ++= positive reaction; += weak reaction; -= negative reaction.

Chloroform allows the extraction of gallic tannins, while the extraction with alcohol allows the identification of catechetical tannins. Saponosides are strongly present in the leaves of *C. arizonica* and *C. sempervirens*. The extraction with petroleum ether from the leaves of *C. sempervirens* shows a weak reaction to leucoanthocyanins and steroids. On the other hand, the use of this solvent for *C. arizonica* leaves results in a negative reaction.

Terpenoids are strongly observed when using chloroform extract for *C. sempervirens* leaves. This is not the case with *C. arizonica* leaves. From these results, it can be deduced that the use of a hydroalcoholic solvent allows a better extraction of phenolic compounds, except for gallic tannins, which, as mentioned above, are available through the use of chloroform in the extraction process.

These results corroborate with those of Azzaz *et al*. (2019), they reported that the composition of extracts from *C. sempervirens* leaves varies depending on the extraction solvent and the method used. In addition, no studies have been conducted on extracts from *C. arizonica*. This study was conducted for the first time in *C. arizonica* in order to prospect the various secondary metabolites existing in this plant. Compounds such as flavonoids, terpenes, quinones, tannins, and saponins in plants are known to be responsible for protection against biotic and abiotic stress, antifungal activity and numerous biological activities (Hiermann *et al*., 1998; Kanwal *et al*., 2010).

Effect of phenolic extracts from *C. sempervirens* and *C. arizonica* on the development of *P. aeruginosa*

The calculation of the inhibition diameter of *P. aeruginosa* induced by the different extracts and by the antibiotics (Penicillin and Vancomycin) is shown in Figure 2. *P. aeruginosa* is extremely sensitive to the positive control, vancomycin, where an inhibition zone diameter of 24.25 mm is observed; the same occurs with the hydroalcoholic extract from *C. sempervirens*, where the test organism is considered extremely sensitive. With an inhibition zone of the order of 19.95 mm. The results indicate that the hydroalcoholic extraction induces a very pronounced sensitivity of *P. aeruginosa* with a diameter of 16.2 mm for *C. arizonica*. Ethereal and chloroformic extracts induce relatively weak inhibition (Table 6).

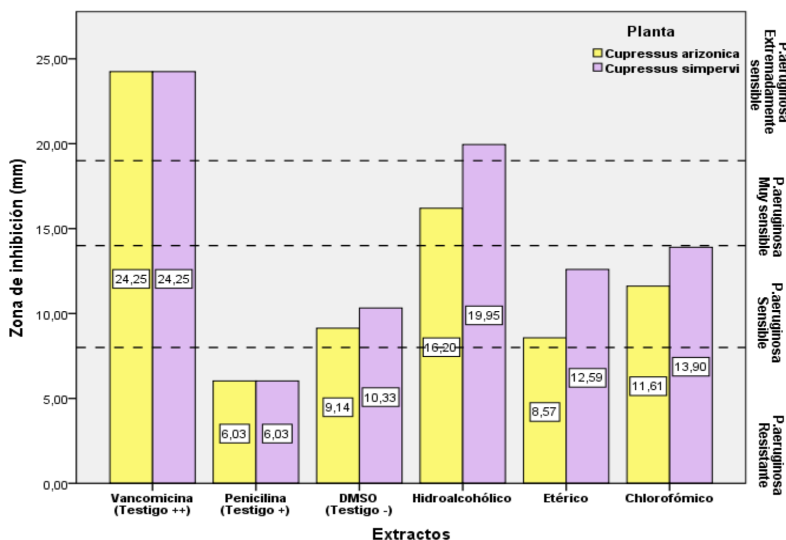

Figure 2. Variation of the diameter of the inhibition zone (mm) depending on the plant and the solvent.

Table 6. Sensitivity of *P. aeruginosa* to phenolic extracts from *C. sempervirens* and *C. arizonica*.

Plant	Extract	Interpretation of the inhibition zone
C. arizonica	Hydroalcoholic	Very sensitive
	Ethereal	Sensitive
	Chloroformic	Sensitive
C. sempervirens	Hydroalcoholic	Extremely sensitive
	Ethereal	Sensitive
	Chloroformic	Sensitive
Controls	DMSO (control -)	Resistant
	Penicillin (control +)	Resistant
	Vancomycin (control ++)	Extremely sensitive

C. arizonica shows low numbers of the inhibition zone compared to *C. sempervirens*. Student's test (t-test) at the 5% level indicates that this difference is not statistically significant (p> 5%) (Table 7).

Table 7. Student's t-test for the comparison of the inhibition zones of the extracts according to the plants.

Extracts	t	dof	p	Mean difference	STD difference
Ethereal extracts	-1.962	3.752	0.13	-4.02	2.05
Chloroformic extracts	-0.597	3.701	0.58	-2.29	3.84
Hydroalcoholic extracts	-2.284	4	0.08	-3.75	1.64
DMSO	-0.946	2.215	0.44	-1.19	1.26
Penicillin	0	4	1	0	0.02
Vancomycin	0	4	1	0	1.81

An analysis of variance using Anova at the level of 5% followed by a post-hoc Tukey test allows dividing the inhibition diameter into homogeneous groups (F= 24.09; p= 0). In *C. arizonica*, the distribution of inhibition diameters in three homogeneous groups was observed. The first is represented by Penicillin, ether extract, DMSO and chloroform extract. The second is represented by chloroform and hydroalcoholic extract. Vancomycin is isolated in group 03 (Table 8).

Table 8. Distribution of *C. arizonica* extracts in homogeneous groups according to their effects on the inhibition of *P. aeruginosa*.

Extracts	N	Subset for alpha = 0.05		
		1	2	3
Penicillin	10	6.03		
Ethereal extracts	10	8.57		
DMSO	10	9.14		
Chloroformic extracts	10	11.61	11.61	
Hydroalcoholic extracts	10		16.2	
Vancomycin	10			24.25
Intragroup meaning	10	0.104	0.231	1 000

Tukey's test shows four homogeneous groups in *C. sempervirens* (F= 15.12; *p*= 0). The first is represented by penicillin and DMSO. The second group is represented by DMSO, the ethereal extract. The third group is represented by ethereal, chloroformic and hydroalcoholic extracts. The effect of the hydroalcoholic extract is similar to the effect of vancomycin isolated in group 04 (Table 9).

Table 9. Distribution of *C. sempervirens* extracts in homogeneous groups according to their effects on the inhibition of *P. aeruginosa.*

Extracts	N	Subset for alpha = 0.05			
		1	2	3	4
Penicillin	10	6.03			
DMSO	10	10.32	10.32		
Ethereal extracts	10	12.59	12.59	12.59	
Chloroformic extracts	10		13.9	13.9	
Hydroalcoholic extracts	10			18.31	18.314
Vancomycin	10				24.24
Intragroup meaning		0.118	0.644	0.207	0.18

From the results obtained, it is deduced that the diameters of the inhibition zones vary depending on the type of solvent studied. The antibacterial activity of these extracts is mainly due to their chemical profile. It should be noted that the difference between the antimicrobial activities of the extracts can be explained by the difference in the active compounds obtained. Therefore, this activity is related to the polarity of the extracted bioactive substances and extraction solvents (Machiex *et al*., 2005).

These results are better than those obtained by Hayouni *et al*. (2007) with chloroform extracts from the fruits of *Juniperus phoenicea* (Cupressaceae) against *P. aeruginosa* ATCC 9027, where they found halos of inhibition zones of 3.03mm ±0.21. The variable yield of the extracts is reduced to the differential solubility of the different phenolic compounds in the solvents and that this solubility is a function of their degree of polymerization, the interaction with the other constituents and the type of solvent used (Falleh *et al*., 2008; Debib *et al*., 2014).

By combining the results of phytochemical screening, we were able to show that antibacterial activity is linked to the presence of phenolic molecules in the leaves of both plants. These secondary metabolites are responsible for various biological activities. Therefore, great attention has been paid to natural products derived mainly from plants (Chaudhary *et al*., 2012; Chaitra *et al*., 2015).

Conclusions

The present work shows that *C. sempervirens* and *C. arizonica* contain high-quality secondary metabolites, which constitutes the scientific basis for the therapeutic use of the studied leaves. This study is, therefore, a phytochemical contribution to the knowledge of the two species of cypress of great interest in the field of pharmacology. Phytochemical screening revealed the presence of

several secondary metabolites: leucoanthocyanins, flavonols, flavonones, anthraquinones, catechin tannins, gallic tannins, saponin (steroids and triterpenes), cardiac glycosides, terpenoids, saponosides and free quinones, which are closely related to the polarity of the solvents used.

The best extraction yield of the three solvents used is chloroform, with an average extraction yield of 61.23% (for *C. sempervirens*) and 52.27% (for *C. arizonica*). The results indicate that the hydroalcoholic extraction induces a very pronounced sensitivity of *P. aeruginosa* with a diameter of 16.2 mm for *C. arizonica*. However, the study is limited by the clinical response of the bacterium in a hospital environment. In fact, a clinical case study could give more visibility to the effect of cypress extracts on the development of *P. aeruginosa*. In addition, a suitable formulation should be considered to minimize the number of leaves and optimize the bactericidal effect of the hydroalcoholic extract from *C. sempervirens*.

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