The Effect of the Extraction Method on the Composition of Essential Oils of Bulruch (Scirpus Maritimus) and Antifungal Activity Against Fomitiporia Medirterranea

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Abstract

The chemical composition of essential oils from leaves and fruits of *Scirpus maritimus* collected in Eastern Algeria, was extracted by hydrodistillation (HS) and supercritical fluid extraction (CO2–SFE) followed by a GC-MS analyze. The chemical composition of essential oil present in leaves and fruits of *Scirpus maritimus* showed the presence of 80 chemical compounds. The essential oil obtained is particularly rich in olefinic, oxygenated monoterpenes, and sesquiterpene. Mainly quantitative differences were observed between the essential oils from leaves and fruits extracted by the two extraction methods. The major components identified in leaf extracts obtained by CO2–SFE and HS were the α-pinene (11.6% and 10.9%) and limonene (9.5% and 11.9%). For fruit, mycene is the major component (29.1% and 10%) obtained by CO2–SFE and by HS, respectively. Monoterpenes were dominant in both extracts (77.4 and 25.1%).

The study of antifungal activity of different extracts which was conducted on the fungal agent that caused wood rot of the vine, *Fomitiporia medirterranea*, revealed interesting properties that could be valued as biofungicide. The results obtained showed that the essential oil obtained by CO2–SFE and from fruits caused a relatively high rate of inhibition. This rate increased with duration and applied dose.

Keywords: antifungal activity, essential oil, CO2-SFE, hydrodistillation, *Scirpus maritimus*

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1. Introduction

Fungi reign as the primary microorganisms driving agricultural losses, responsible for over 80% of plant diseases. The scale of their impact becomes evident with approximately 8,000 distinct fungal identified species, collectively contributing to around 100,000 different plant diseases (Shuping and Eloff, 2017). Among these, Esca dieback emerges as particularly devastating to grapevines' woody tissues, resulting in a decline in vine health and overall productivity (Dubos, 2002). This issue extends beyond grapevines to impact other hosts, though specific instances are not delineated in the current discourse.

The challenge of controlling this fungal menace persists, especially since the prohibition of sodium arsenate in 2000, with effective measures remaining elusive. Historically, synthetic chemical fungicides have been the primary approach for disease management. However, their usage raises significant environmental concerns, and their indiscriminate application has led to the development of resistance in phytopathogenic fungi. Consequently, the ongoing research is dedicated to exploring antifungal molecules, playing a pivotal role in biological control against fungi. These sought-after molecules are often derived from the secondary metabolites of both cultivated and wild plants, with the intention of discovering novel and biologically active compounds. As of now, a conspicuous gap exists in the literature regarding the development of new molecules with efficacy in controlling dieback.

Bulrush (*Scirpus maritimus*), a perennial plant within the Cyperaceae family, is characterized by inflorescences producing achenes and the ability to remain dormant for several years (Clevering, 1995). While this species has garnered attention for its phytoremediation potential (Al-Baldawi et al., 2013), the antifungal activities of its essential oils (EO) have yet to be thoroughly investigated. Moreover, it is crucial to consider the potential impact of different EO extraction methods on the chemical composition of the plant material (Wang et al., 2012; Uquiche et al., 2015). In this context, supercritical fluid extraction (CO2-SFE) emerges as an environmentally friendly technology for obtaining essential oils from plants (Fornani et al., 2012).

This study aims to scrutinize the impacts of two essential oil extraction methods from *Scirpus maritimus* (hydrodistillation (HS) and supercritical fluid extraction (CO2-SFE) on the chemical composition of its leaves and fruits. Furthermore, it seeks to investigate the antifungal spectrum of action against detrimental fungi, such as *Fomitiporia mediterranea*. Through this rigorous examination, the research aspires to contribute valuable insights to the academic discourse on sustainable and effective approaches to fungal disease management in agriculture.

2. Materials and methods

2.1. Plant material

The bulrush (*Scirpus maritimus*) plant samples were gathered from a location situated in the semi-arid bioclimatic stage in the mountain range of the Wilaya Oum-El-Bouaghi (Eastern Algeria, 35° 52′ 39″ North and 7° 06′ 49″ East) during the spring of 2022. To preserve their integrity, leaves and fruits were air-dried and shielded from both light and moisture. Subsequently, the dried samples were finely powdered and stored in sealed, sterile bottles in dark conditions.

2.2. Extraction and identification of compounds

The essential oil extraction process employed two distinct methods: hydrodistillation and supercritical fluid extraction (CO2-SFE).

For hydrodistillation, 40 grams of air-dried leaves or fruits were combined with 500 mL of distilled water. The hydrodistillation procedure was conducted in a Clevenger-type apparatus for a duration of 3 hours. The resulting oil was separated from the liquid phase and subsequently dried over anhydrous sodium sulfate.

Conversely, supercritical extractions were conducted using a pilot-plant supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) with precise control over temperature and pressure. Each experiment involved packing the extraction vessel with 600g of milled leaves or fruits. Extractions were performed at 30 MPa and 40 °C with a CO2 flow rate of 60 g/min. The temperature was maintained at 40°C in both separators, and the extraction time was set to 5 hours.

The obtained essential oils were carefully stored in bottles covered with aluminum paper to shield them from light-induced oxidation. The oils were then refrigerated at a temperature of 4 °C. The extraction yield was expressed as grams of essential oil per 100 grams of dry plant material.

Finally, chromatographic analysis was conducted using gas chromatography coupled with mass spectrometry (GC/MS) to effectively separate and quantify the complex mixtures of volatile compounds in the essential oils.

2.3. Preparation of different doses for testing

The essential oils were previously solubilized in Tween 80 at a concentration of 3%. This was done due to the and to serve as a diluent for the creation of microemulsions, ensuring the homogenization of the essential oil solution. The control sample underwent testing using Tween 80 at a concentration of 3%.

2.4. Fungal material and antifungal activities

The fungi utilized in this study were isolated from infected wood of grapevines collected from a vineyard in the wilaya of Blida, Algeria during the agricultural campaign of 2020. Initial identification was conducted through morphological and cultural characteristics, subsequently

confirmed by molecular analysis of nucleotide sequences, particularly the internal transcribed spacer (ITS) (Ammad et al., 2014).

To assess the antifungal effects, the volatility method was employed. *Fomitiporia mediterranea*, the fungal strain under investigation, was cultured on Potato Dextrose Agar (PDA) medium at 25°C for 5 days. After this incubation period, a mycelia disc from a young fungal culture was positioned at the center of a Petri dish containing the culture medium. Sterilized Whatman paper discs, measuring 8mm in diameter, were impregnated and saturated with 30ml of each essential oil dilution and then placed on the lid of the Petri dish. These impregnated paper discs were covered with another disc soaked in Tween 80 (3%) for the control sample (Chutia et al., 2009). All Petri dishes were promptly closed and sealed with parafilm to prevent oil evaporation. Incubation took place for 5, 10, and 15 days at 25°C.

The antifungal activity of essential oils against the fungal strain was evaluated by recording the radial growth, expressed as the percentage inhibition of mycelial growth (PI). The calculation was performed using the formula described by Pandy et al. (1982):

This formula enabled the quantification of the inhibitory effect of essential oils on the mycelial growth of *Fomitiporia mediterranea* over the incubation period.

$$PI = (D_c - D_t)/D_c * 100$$

The percentage of inhibition of the growth of the tested fungus (PI) is calculated using the formula:

Where:

- PI: is the percentage of inhibition of growth of the fungus tested.

- DC: is the mean diameter of the mycelial growth of the untreated fungus (mm).

- DT: is the average diameter of the mycelial growth of the fungus in the presence of the treatment.

The scale for estimating the antimicrobial activity, as provided by Mutai et al. (2009), classifies the diameters of inhibition zones (D) of microbial growth into five classes:

1. Very Highly Inhibition: D ≥ 30 mm.

2. Highly Inhibition: 21 mm ≤ D ≤ 29 mm.

3. Moderately Inhibition: 16 mm ≤ D ≤ 20 mm.

4. Slightly Inhibition: 11 mm ≤ D ≤ 16 mm.

5. No Inhibition: D < 10 mm.

This classification system enables the assessment of the effectiveness of the essential oils in inhibiting the growth of the fungal strain based on the observed diameters of the inhibition zones.

2.5. Statistical analyses

The results obtained from the assessment of the antifungal potency of bulrush essential oil underwent comprehensive statistical analysis with the following objectives: (*i*) to ascertain the antifungal efficacy, (*ii*) to substantiate the effectiveness of the extracts against the tested fungal strain, and (*iii*) to compare the effects of the extracts based on the utilized plant organs and extraction modes. For these analytical purposes, the R software was employed, utilizing the General Linear Model (GLM) to determine variance. Significance was established at a threshold of $P < 0.05$. Treatment means were calculated for each respective set of three replicates to provide a robust basis for comparison and inference.

3. Results

Yields of *Scirpus maritimus* essential oils vary depending on the targeted organ and the extraction method. The CO2-SFE extraction yield by supercritical was of about 0.37 and 0.29% respectively for leaves and fruits. This yield is more important than that obtained by the hydrodistillation process, which was 0.11 and 0.20% for leaves and fruits, respectively. The results of the quantitative and qualitative identification of chemical compounds by CG/SM, which are obtained by retention times, are summarized in Table (1). A total of 80 components were identified. They represent 99.6% (hydrodistillation) and 68.2% by CO2-SFE of the total leaf oil; 98.7% (hydrodistillation) and 83.6% by CO2-SFE of the total fruit oil. The chemical composition of the essential oils is specific to each organ. The essential oils from leaves and fruits mainly contain olefinic, oxygenated monoterpenes, and sesquiterpenes. Among the identified compounds in CO2-SFE leaf extracts obtained by and HS respectively, the α-pinene and limonene, representing each one more than 10%, were the major compounds (Table1). Similarly, β-caryophyllene, p-cymene, and sabinene were more present in essential oil extracted by CO2-SFE than by HS. For fruit, the mycene is the major component, and its content was three times higher by HS than by CO2-SFE. Monoterpenes in fruits were three times higher by CO2-SFE than by HS (Table1).

Volatility assays were performed against *F. mediterranea*. Three essential oil concentrations for each extract were used and tested against fungus culture for 5, 10, and 15 days. A significant inhibition action of the essential oil on the mycelia growth of the pathogen was observed by the appearance of a zone of inhibition after 5 days of incubation. The essential oils tested with the applied doses D2 (0.50%) and D1 (0.75%) showed a shock effect at the beginning of their application while achieving the high toxicity end of the test. While the D3 (0.25%) exhibited low toxicity during early application of treatments (5 days), it moderated toxicity at the end of the follow-up (10 days).

The essential oils exerted an inhibitory action on the growth of fungus (Table 2). Indeed, the mycelial growth of the fungus was reduced by at least 70%, using leaves the essential oil of and fruits obtained by both extraction methods at 0.75% to a 0.5% concentration. Furthermore, the fruit essential oils obtained by supercritical extraction fluid (CO2) were significantly more effective than the leaves (Table 2). A comparison among the rates of inhibition zones under the effect of different treatments on the tested strain showed a similarity in the evolution time of the mycelial growth with a decrease there of up to 15 days; The high dose (D1) treatment obtained by extraction of supercritical fruit recorded the highest percentage inhibition (Figure 1).

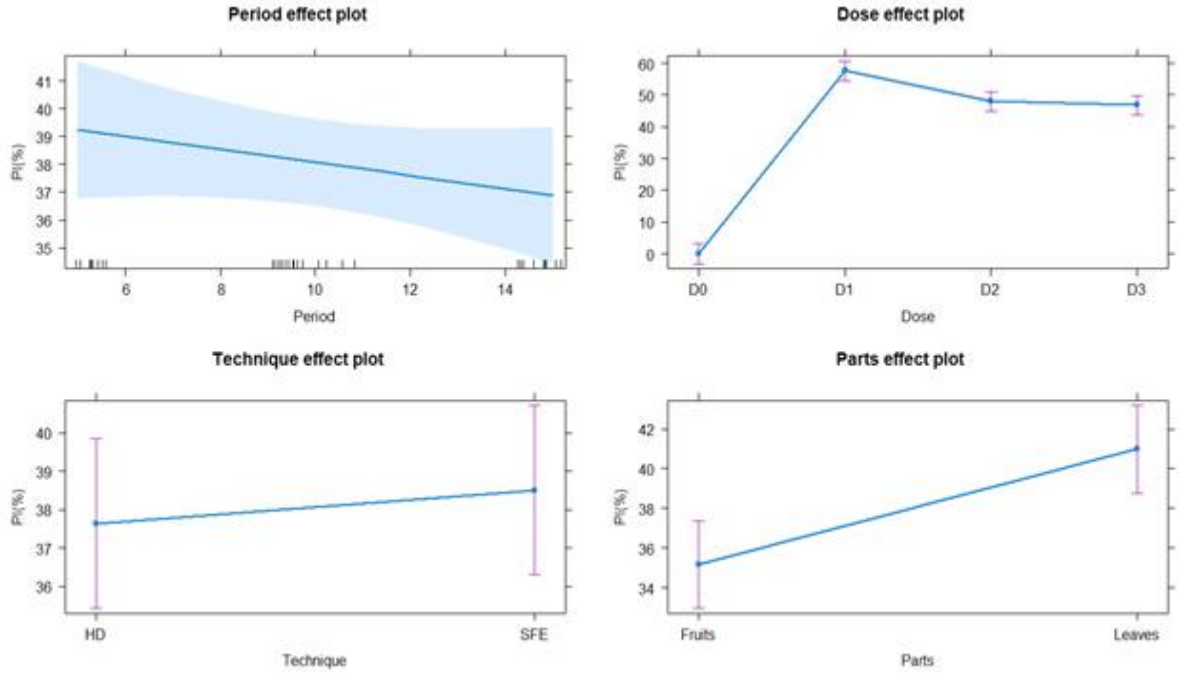

Fig1. The effect of time, dose, extraction methods

and part of *S. maritimus* plant on antifungal activity.

4. Discussion

The oil yield obtained from hydrodistillation and supercritical extraction fluid CO2-SFE of the *Scirpus maritimus* was different quantitatively and qualitatively. A higher yield was observed for CO2-SFE. Similar results were already observed in coriander (Pavlic et al., 2015). The difference in the chemical composition of essential oils of plant species depends on several factors, such as origin, phenological stage, environmental influences, genetics, and extraction mode (Russo, 1998; Thompson, 2003; Gil et al., 2012; Dobrovalkyte et al., 2013; Ammad et al, 2018). The quantitative and qualitative analysis of the essential oil showed the presence of the majority chemical compound (active ingredient) with specificity for each organ (leaves and fruits). This analysis revealed the richness of oxygenated, olfenic monoterpenes and sesquiterpenes. Studies that emphasized the quantitative variability of the composition of essential oils according to the organ

or species involved have concerned other species of the family Cyperaceae or other botanical families (Gil et al., 2002, Wang et al. 2012; Ngankeu-Pagning et al., 2016; Peirera et al., 2016). Regarding the chemical composition, Table 1 indicated that the essential oils of leaves were characterized by the relatively high content of α-pinene and limonene, and the second oil from the fruit contained mycene as the major component, and the monoterpenes were dominant. This family is known to possess an important antifungal activity (Sokovic and Van Griensven, 2006). Many authors have attributed the antifungal capacity of the essential oils from different Juniperus, Calocedrus, Pistacia, and Cupressus species to the presence of α-pinene, Z-caryophyllene, and other components. Our results showed that the α-pinene and limonene were the major components of the essential oils of *Scirpus* obtained from leaves.

The tested essential oil has shown high toxicity against studied fungal strains. These results vary depending on the doses, the time of incubation, the tested organ, and the method of extraction. All tested doses showed fungicidal activity as seen in Table 2. The essential oils exhibited different degrees of inhibition on the growth of test fungi. The essential oil tested was highly toxic after only 5 days of incubation. Then the inhibition rate was increased after 10 days to reach its maximum. The doses D1 and D2 showed the highest inhibitory effect compared to D3. In contrast, fungi were not inhibited by the control sample, whatever the time of incubation. Expectedly, a higher inhibitory effect was achieved by EO obtained from CO2-SFE. Indeed, extraction method allowed to extract richer in monoterpene hydrocarbons (EO)(Table 1). The latter are known to have, which are known to have a fungicidal effect. All tested doses showed a fungicidal activity, with the highest effect observed for D1 and D2, whatever the extraction method. The chemotype of the essential oils obtained in this study is particularly rich in hydrocarbon monoterpenes, such as α -pinene, limonene, and p-cymene (Table1).

Furthermore, several studies have demonstrated that the antifungal activity of the essential oil

was attributed to the major components (Sokovic and Van Griensven, 2006; Olugbenga-Akinkunmi et al., 2016; Camele et al., 2012). Monoterpene hydrocarbons totally inhibit the growth of several fungi and plants. They can pass through cell membranes by establishing pores. Therefore, altering the membrane fluidity and integrity by the insertion of monoterpenes within the membrane of the fungi causes perturbation of the cellular functions. Extract components would act on the mycelium's hyphae, causing output components of the cytoplasm, loss of rigidity and integrity of the hyphae's cell wall, causing disintegration and mycelial death (Al-Ja'fari et al., 2011, Ben Ghnaya et al., 2016). As a result, the plants' secondary compounds possess several modes of action against the fungal strain. But generally, their action takes place in three phases. The first phase is the attack of the wall by the plant extract, causing permeability and provoking the loss of cellular constituents. The second phase is the acidification of the inside of the cell, which blocks the production of cellular energy and synthesis of structural components. Finally, the third phase is the destruction of genetic material, leading to the death of fungi (Camele et al., 2012). According

to our results, we can conclude that the strong antifungal activity observed in essential oils tested can be attributed only to the major components. It can be the result of synergies between the differentconstituents of these oils (El Ajjouri et al., 2008). The results of this study indicate that the higher concentration of the essential oil (D1) was the most effective dose. The colony diameter is reduced each time we increase the dose. The use of natural substances (plant extracts, essential oils) as alternatives to chemical agents remains a promising solution. These products are the subject of several studies for their possible use as an alternative to pesticides.

Conclusion

Our study demonstrated the effect of extraction method on the yield and composition of *Scirpus* essential oil, as reported for several other species. Essential oil yield was higher when CO2-SFE was used. These essential oils were rich in monoterpene hydrocarbons regardless of the extraction method. To our knowledge, this study is the first report on the fungicidal activity of essential oils of *Scirpus maritimus*. The essential oils of this species provided evidence of the antifungal activity against *F. mediterrania*. These results add information to consolidate our findings about the inhibitory properties of *S. maritimus* extracts on the growth of dieback fungal pathogens. Further investigations are needed to ascertain the effects of essential oils and each component against dieback pathogens.

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Table 1. Chemical composition of essential oil from leaves or flowers of *Scirpus maritimus* extracted by CO2- SEF and hydrodistillation.

Compound	Ik	Hydrodistillation		CO_2-FES	
		Leaves	Fruits	Leaves	Fruits
Epi-bicyclosesquiphellandrene	1485	tr*	0.1	0.3	tr
Bicyclogermacrene	1490	tr	-	0.3	tr
α-muurolene	1499	0.9	0.5	0.5	0.3
b-bisabolene	1502	0.2		0.3	-
γ-cadinene	1511	0.6	0.5	0.3	0.5
Cubebol	1518	-		0.6	0.2

δ-cadinene	1522	1.9	1.3	0.7	1.1
Cadina-1,4-diene	1529	0.5	0.3	tr	tr
α-cadinene	1532	-	0.1	tr	tr
α-calacorene	1537		0.2		-
Elemol	1540	-	0.1	tr	-
GermacreneB	1548	0.3	-		
Germacrene D-4-ol	1566	-	-	0.5	0.2
Spathulenol	1568	1.0	0.2	0.2	-
Oxide de caryophyllene	1573	1.5	0.3	0.7	tr
Globulol	1576	0.8	0.1	tr	-
Epoxyde de humulene II	1597	0.4	0.1	tr	-
Fonenol	1547	0.6	0.2	0.1	-
1-epi-cubenol	1607	0.7	0.3	1.2	-
τ-cadinol	1614	1.4	0.6	0.5	tr
τ-muurolol	1627	2.7	0.8	0.4	tr
α-muurolol	1630	1.0	0.3	tr	tr
α-cadinol	1636	4.9	1.8	1.2	tr
α-bisabolol**	1659	1.0	0.2	0.2	
Epi-α-bisabolol**	1662	tr	tr		1.3
Cembrene	1910	-	-	0.9	
C20H30	1932	-	0.6	0.3	1.3
Hexadecanoicacid	1960	tr		2.4	7.7
Manoyl oxide	1965	tr	0.4	1.2	-
Epi-13-oxyde de manoyl	1980	tr			Tr
Linoleicacid	2115	-		-	11.6

Oleicacid	2122	-		-	5.1
C19H28O	2209	-		-	3.1
3-pentadecyl phenol (304)	2350	-		-	12.0
Phenol -3-n-alkyl (330)	2479	-		-	8.3
Squalen	2556	-		1.1	0.7
Total		99.6	98.7	68.2	83.6
OlefinicMonoterpenes		52.2	77.4	38.1	25.1
OxigenatedMonoterpenes		4.8	4.5	4	0.6
OlefinicSesquiterpenes		21.3	8	11.2	5.7
OxigenatedSesquiterpenes		16	4.9	5.6	1.5
Fattyacid		-	-	2.4	24.4
Others		5.3	3.8	6.9	26.3

*tr: Traces.

- : Not found.

Table 2: Antifungal activity of *Scirpus maritimus* essential oils extracted using two methods against *Fomitiporia mediterranea.*

Incubation (Days)	Dose	HD				SFE			
		Leaves		Fruits		Leaves		Fruits	
		D (mm)	PI(%)	D (mm)	PI(%)	D (mm)	PI(%)	D (mm)	PI(%)
5	D1	19.0±0.4	57.8	22.5±0.4	50.0	17±0.41	62.2	20.0±0.4	55.55
	D2	19.3±0.4	57.2	27.5±0.3	38.9	18.2±0.36	59.5	22.0±0.4	51.11
	D3	21.0±0.3	51.1	23.8±0.4	47.2	19.1±0.37	57.6	22.1±0.4	50.88

	Control	45.0±0.4	0.0	45.0±0.4	0.0	45.0±0.4	0.0	45.0±0.4	0.0
10	D1	22.3±0.3	70.3	37.5±0.3	50.0	28±0.43	62.7	31.0±0.4	58.66
	D2	30.4±0.4	59.5	47.2±0.3	37.1	42±0.44	44.0	42.0±0.4	44.0
	D3	40.0±0.4	46.7	51.2±0.4	31.7	42±0.45	42.7	45.0±0.3	40.0
	Control	75.0±0.3	0.0	75.0±0.3	0.0	75.0±0.3	0.0	75.0±0.3	0.0
15	D1	41.8±0.4	58.3 ±	43.0±0.4	52.22	39±0.44	56.6	40±0.45	55.6
	D2	55.0±0.4	45.0 ±	52.0±0.4	42.22	42±0.44	53.3	51±0.47	43.33
	D3	44.3±0.4	55.8 ±	43.0±0.4	52.22	51±0.35	43.3	52±0.43	42.22
	Control	90.0±0.5	0.0	90.0±0.5	0.0	90.0±0.5	0.0	90.0±0.5	0.0

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