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Comparative Study of Essential Oils from Tunisian *Pinus Halepensis* Mill. by Hydrodistillation and Microwave-Assisted Processes: Chemical Composition and Antioxidant and Cytotoxic Potential against Prostate and Cervical Cancer Cells

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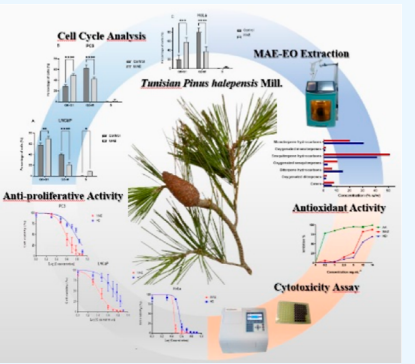
ABSTRACT: *Pinus halepensis* Mill. is a Mediterranean aromatic plant largely used, in addition to its nutritional value, in traditional medicine as antiseptic, antifungal, antituberculotic, and antirheumatic. Thus, the objective of this work was to appraise the antioxidant and cytotoxic activity of the essential oil (EO) of *P. halepensis* from Tunisia on cancer cell cultures, along with chemical composition evaluation by GC−MS. To attain the best yield and also highest quality in extraction of the EOs, conventional hydrodistillation (HD) and novel microwave-assisted extraction (MAE) methods have been performed and compared. The antioxidant activity was evaluated through the inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH)• radicals. The cytotoxic activity in prostate cancer cells (LNCaP and PC3) and cervical cancer cells (HeLa) of EO was evaluated by the MTT assay and effect on the cell cycle by flow cytometry analysis. A total of 38 and 37 components were identified from HD (HD-EO)- and MAE (MAE-EO)-extracted EOs, respectively, which were dominated by hydrocarbon compounds (HD-EO = 86.65%; MAE-EO = 77.36%), especially monoterpenes (HD-EO = 32.11%; MAE-EO = 21.55%) and sesquiterpenes (HD-EO = 44.29%; MAE-EO = 61.32%). Both extracted EOs showed significant antioxidant activity, as shown by the inhibition of DPPH• radicals [IC_{50} (HD-EO) = 4102.30 ± 159.73 μg mL^{-1} and IC_{50} (MAE-EO) = 3430.13 ± 78.46 μg mL^{-1}]. Also, the EOs exhibited substantial ($p < 0.001$) antiproliferative activities with G0-G1 arrest on PC3, LNCaP, and HeLa cells by yielding very low IC_{50} values more conspicuous in MAE-EO with respective IC_{50} values of 25.70 ± 6.58, 14.97 ± 3.21, and 14.55 ± 2.30 μg mL^{-1}. This finding points out for the first time that the EO of *P. halepensis* Mill. from Tunisia can be an effective natural antitumor agent with more pronounced activity when extracted with the MAE method that, after further in vivo studies, can be harnessed as a putative phytopharmaceutical for prostate and cervical cancer treatment.



INTRODUCTION

With 19.3 million new cases and about 10 million cancer deaths predicted for 2020, cancer is one of the leading causes of death worldwide. By 2040, there would be 28.4 million new cases, up 47% from 2020, according to predictions.[1] Cervical cancer ranks fourth among tumors in women's cancer incidence,[2] accounting for over 342.0 deaths in women in 2020.[1] Prostate cancer is the second most frequent type of cancer in men, accounting for over 375.0 deaths worldwide.[1]

Several in vivo and in vitro studies have pointed out putative anticancer activities for numerous essential oils (EOs) against many tumor cell lines. Hence, the prostate cancer PC3 cells are sensitive to EOs from *Pinus wallichiana*,[3] *Hypericum hircinum* L. *subsp. majus*,[4] and *Solanum erianthum*.[5] Likewise, the EO of *Machilus mushaensis* shows anticancer activity in cell culture, as cytotoxic activity was observed on oral, colon, liver, melanoma, leukemic, and lung cancer cells.[6] Other researchers have described cytotoxic activities of EO from *Plectranthus* stems on lung metastasis of melanoma tumors .[7] According to Bayala et al., the SF-767 cells, derived from a resistant glioblastoma, have their survival significantly decreased when exposed to *Lippia multiflora* or *Ocimum basilicum*.[8] These data indicate that EOs could be a significant source of new molecules. Also, isolation of these EOs and the characterization of their biological effects are necessary steps to develop new strategies to fight cancer.

Besides, recent advances in in silico techniques have significantly contributed not only to enhance the understanding of the mechanisms of action of EOs but also to aid in the design and development of more effective natural products

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for therapeutic use. Computational methods such as molecular docking, quantitative structure−activity relationship (QSAR) modeling, and molecular dynamics simulations are commonly used to predict the biological activity of EO components and to understand their interactions with biological targets. Aouf et al.[9] applied in silico methods to investigate the antimicrobial activity of EO and their components.

For example, molecular docking studies have been employed to predict the binding affinity of EO constituents for various enzymes and receptors involved in cancer, inflammation, and microbial infections. QSAR modeling helps in understanding the relationship between the chemical structures of EO components and their observed biological activities. Molecular dynamics simulations provide insights into the stability and behavior of these interactions at the molecular level. Jena Sudipta et al.[10] have used molecular docking and QSAR studies to explore the anticancer potential of EO compounds from various plants.

Aleppo pine, or *Pinus halepensis* Mill., is native to the Mediterranean region. It dominates forest forms in semiarid and arid regions, encompassing around 3.5 million hectares.[11]

Historically, this plant has been widely used in traditional medicine across various cultures. In Arab countries, the seeds of *P. halepensis* Mill. are traditionally used to prepare sweet pudding. Furthermore, its EO has been employed for its antiseptic, antituberculotic, and antirheumatic properties. The resin and extracts of *P. halepensis* Mill. have been used to treat wounds, respiratory ailments, and skin infections.[12−14] Moreover, various therapeutic properties have been identified for EO from *P. halepensis*, such as anticoagulant,[15] anti-inflammatory,[15−17] antimicrobial,[18,19] antibacterial,[14,20,21] antifungal,[22−25] larvicidal,[26] antioxidant,[15,20,27,28] and anticancer.[11,29]

The relevance of studying the EO of *P. halepensis* Mill. lies in its rich chemical composition, which includes a variety of bioactive compounds such as monoterpenes and sesquiterpenes. These compounds are known for their antioxidant, anti-inflammatory, and anticancer activities. Investigating the EO's biological activities can lead to the discovery of new natural compounds with potential therapeutic applications, particularly in the treatment of cancer and other serious diseases. Understanding the mechanisms by which these bioactive compounds exert their effects can contribute to the development of novel phytopharmaceuticals.[30]

Since EOs are concentrated hydrophobic solutions containing volatile chemical compounds from plants, the solvent used for the extraction and the chemical methods could modify the composition of the EOs. Currently, a variety of techniques, including solvent extraction, supercritical fluid extraction, and conventional hydrodistillation (HD), are used to extract EOs.[31] A number of drawbacks accompany these techniques, including intense heat, the need for comparatively large amounts of hazardous and polluting solvents, and issues with costly electrical equipment.[32]

On the other hand, novel microwave-assisted extraction (MAE) has been recognized as an important alternative in separation and extraction processes with several advantages compared to other extraction methods, mainly including reduction of extraction time, improvement of extract quality, support for low operating costs and energy consumption, and low CO_2 emissions.[33−36]

The goal of this study is to characterize and analyze the physical and chemical properties of the EOs of the *P. halepensis* Mill. needles obtained by the novel MAE method and to compare their yield and quality with those obtained by conventional HD. Furthermore, antioxidant and cytotoxic activities of *Pinus* EOs obtained from both HD and MAE methods have been enhanced and correlated to the quality of both obtained EOs. To our knowledge, no work has been performed for the anticarcinogenic activity of EO from *P. halepensis* Mill. needles growing in northern Tunisia, against prostate and cervical cancer cells. The present study serves as the first-hand evidence on the fact that this aromatic plant potentially serves as an effective alternative for prostate and cervical cancer future natural treatment.

RESULTS AND DISCUSSION

Physical Properties and Chemical Composition of *P. halepensis* Mill. EO. Table 1 shows, for each of the two extraction methods employed, the extraction yield as well as the physical characteristics (appearance and refractive index of the *P. halepensis* Mill. EO).[37]

Table 1. Yields and Physical Properties of EOs of *P. halepensis* Mill. Extracted by HD and MAE

	HD[a]	MAE[b]
material weight (g)	150	150
time of boiling (min)	30	5
time for extraction (min)	180	20
solvent or evaporation	water	water
volume of water (mL)	600	150
yield (%)	0.91	0.32
appearance	yellow	pale yellow
refractive index 20 °C	1.4923	1.4998

[a]HD, hydrodistillation extraction. [b]MAE, microwave-assisted extraction.

Extraction with MAE began much earlier when compared to HD, with 5 min versus 30 min, respectively. This is because microwaves require more efficient heat flow.[38,39] Furthermore, the extraction time is significantly decreased when MAE is used.[40]

The EO yields obtained from *P. halepensis* Mill. according to the various isolation techniques are 0.32% for MAE and 0.91% for HD, respectively. This yield was higher than the ones that Hmamouchi et al.[19] and Dob et al.[41] recorded (0.44 and 0.52%, respectively).

The yield variability from needles, stems, and cones was determined by Amri et al.[22] to be 0.85, 0.60, and 0.60%, respectively. According to additional research, the production of EOs from fresh aerial parts of the *P. halepensis* Mill. ranged from 0.13 to 0.63% at ten locations in West and Northern Algeria.[14] Numerous other factors, such as the extraction method, time, temperature, type of solvent, and raw material treatment procedures (powder, drying, etc.), can also affect the yield of the *P. halepensis* Mill. oil extraction.[42]

P. halepensis Mill. EOs have a refractive index value of 1.3−1.7 at 20 °C, according to the European Pharmacopoeia [Ph. EUR., 2012]. The refractive index determines the oil quality on a global scale. Higher refractive index EOs are known to be of greater quality than lower refractive index EOs.[43] The refractive index and the quality of the extracted EOs are unaffected by the ensemble of extraction techniques.

Based on their qualitative chemical structure, a comparative analysis of the two *P. halepensis* Mill. EOs isolated from the

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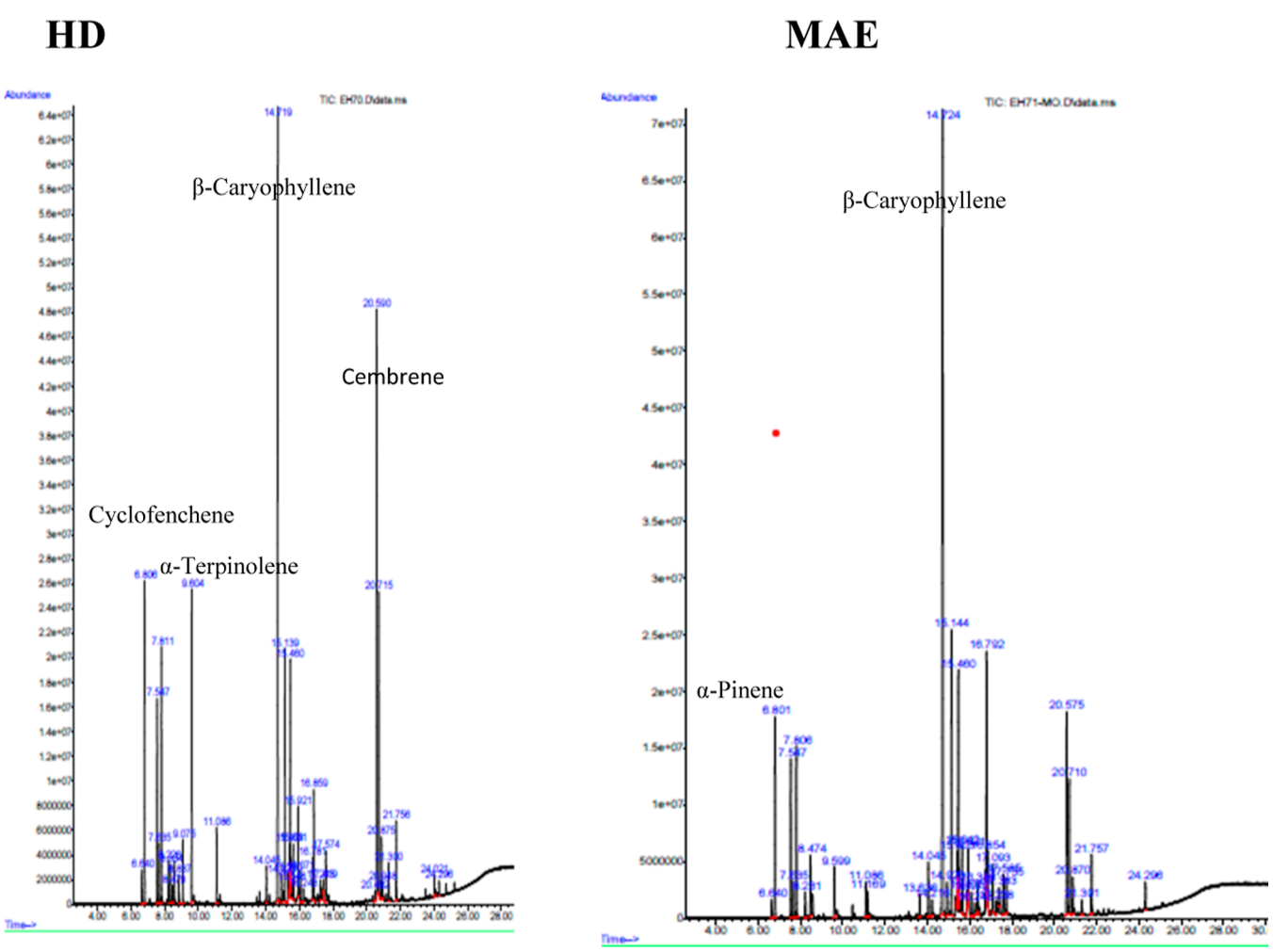

Figure 1. GC−MS chromatograms of the *P. halepensis* Mill. EOs extracted by HD and MAE.

needles was also carried out. Figure 1 displays the chromatograms, and Table 2 lists the chemical compositions.

With 100 and 99.99% of the total EOs, *P. halepensis* Mill. EOs extracted by HD and MAE revealed 38 and 37 volatile chemicals, respectively. The main components of the EO recovered using the HD technique were *β*-caryophyllene (22.20%), cembrene (13.24%), and cyclofenchene (8.05%). Nevertheless, *β*-caryophyllene (31.50%), *α*-caryophyllene (7.80%), and phenethyl isovalerate (6.29%) were the dominant constituents of the obtained EO using the MAE method.

The primary volatile component in both EOs which was from *P. halepensis* Mill. grown in Tunisia is *β*-caryophyllene, with a greater concentration seen in the MAE method. Samples taken from Tunisia,[44,45] Algeria,[14,46] Marocco,[20,47] France,[48] and Greece[26] have all shown similar results. Furthermore, *α*-terpinolene (6.78%) is present in EO extracted by HD; this quantity is reduced with MAE (1.42%). Conversely, HD-EO has a lower content of caryophyllene oxide (0.74%) compared with MAE (5.77%).

Eleven compounds, including camphene (8.05%), 4-carene (0.75%), *β*-ocimene (0.55%), *γ*-terpinene (1.22%), *γ*-muurolene (0.70%), *β*-cadinene (0.28%), *β*-bisabolene (0.24%), *α*-gurjunene (1.50%), andien-beta (0.24%), cembrene A (0.30%), and methyl 8,15-pimaradien-18-oate (0.40%) are the only compounds found in the EO isolated by HD. Conversely, only ten constituents are specifically present in EO isolated by MAE such as *α*-pinene (6.01%), *p*-cymenol (0.64%), *α*-cubebene (0.53%), *β*-cubebene (0.44%), cubebol (0.43%), elemol (0.68%), *α*-humulene epoxide II (1.11%), caryophyllenol II (0.39%), *cis*-*α*-bisabolene (1.50%), and *α*-cadinol (0.89%). Consequently, a change of the extraction method may cause certain compounds to disappear or modify the abundances of some compounds.[49]

A comparison of the compound chemical classes obtained in both EOs is shown in Figure 2. Significant differences can be observed. MAE gave an EO more concentrated in oxygenated compounds such as terpinen-4-ol, phenethyl isovalerate, caryophyllene oxide, and guaiol (22.11%) compared to the HD method (12.46%).

Besides, MAE induced the highest percentage of sesquiterpenes (61.32%) compared to HD (44.29%). However, EO extracted by the HD method was found to be richer in monoterpene (33%) than MAE-extracted EO (21.98%).

Previous studies showed that the concentration of oxygenated compounds and sesquiterpenes increased with the MAE technique. According to the current results, there was also a decrease in monoterpene content as compared to the HD approach.[50−52] Contrarily, Wang et al.[53] found that the oxygenated compounds of the EO obtained by HD were higher than that MAE method.

When comparing the EO extracted by MAE to that extracted by the HD method, the largest concentration of oxygenated

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Table 2. Chemical Composition of *P. halepensis* Mill. Needle EOs Obtained by HD and MAE Processes[a]

compounds	formula	RT	RI	relative area (%)	
				HD	MAE
β-thujene	$C_{10}H_{16}$	6.640	930	0.73	0.48
α-pinene	$C_{10}H_{16}$	6.801	937		6.01
camphene	$C_{10}H_{16}$	6.806	952	8.05	
sabinene	$C_{10}H_{16}$	7.547	975	4.60	4.47
β-pinene	$C_{10}H_{16}$	7.635	979	1.17	0.92
β-myrcene	$C_{10}H_{16}$	7.806	991	5.70	4.62
α-phellandrene	$C_{10}H_{16}$	8.226	1004	0.83	0.64
4-carene	$C_{10}H_{16}$	8.334	1011	0.75	
p-cymene	$C_{10}H_{14}$	8.479	1025	0.33	1.50
β-ocimene	$C_{10}H_{16}$	8.837	1038	0.55	
γ-terpinene	$C_{10}H_{16}$	9.075	1060	1.22	
α-terpinolene	$C_{10}H_{16}$	9.604	1078	6.78	1.42
terpinen-4-ol	$C_{10}H_{18}O$	11.086	1176	1.40	0.85
p-cymenol	$C_{10}H_{14}O$	11.169	1183		0.64
α-cubebene	$C_{15}H_{24}$	13.636	1351		0.53
α-copaene	$C_{15}H_{24}$	14.045	1376	0.70	1.29
β-cubebene	$C_{15}H_{24}$	14.216	1389		0.44
β-caryophyllene	$C_{15}H_{24}$	14.719	1405	22.20	31.50
β-sesquiphellandrene	$C_{15}H_{24}$	14.926		0.52	0.76
α-caryophyllene	$C_{15}H_{24}$	15.139	1454	5.28	7.80
γ-muurolene	$C_{15}H_{24}$	15.330	1464	0.70	
phenylethyl pivalate	$C_{13}H_{18}O_2$	15.403		0.59	0.82
phenethyl oxalate		15.460		4.84	6.29
β-cadinene	$C_{15}H_{24}$	15.548		0.28	
α-muurolene	$C_{15}H_{24}$	15.641	1499	1.20	2.08
cubebol	$C_{15}H_{26}O$	15.875	1515		0.43
δ-cadinene	$C_{15}H_{24}$	15.921	1511	1.60	1.04
α-cedrene	$C_{15}H_{24}$	16.071	1410	0.57	0.53
elemol	$C_{15}H_{26}O$	16.243	1548	0.25	0.37
geranylgeraniol	$C_{20}H_{34}O$	16.388			0.68
caryophyllene oxide	$C_{15}H_{24}O$	16.781	1581	0.74	5.77
guaiol	$C_{15}H_{26}O$	16.859	1597	2.07	1.46
α-humulene epoxide II	$C_{15}H_{24}O$	17.093	1602		1.11
cadine-1,4-diene	$C_{15}H_{24}$	17.243		0.36	0.35
caryophyllenol II	$C_{15}H_{24}O$	17.383	1655		0.39
cis-muurola-3,5-diene	$C_{15}H_{24}$	17.419		0.24	
α-gurjunene	$C_{15}H_{24}$	17.574	1409	1.50	
eremophilene	$C_{15}H_{24}$	17.585			1.50
isoshyobunone	$C_{15}H_{24}O$	17.735			0.89
andien-beta	$C_{19}H_{28}O$	20.492		0.24	
cembrene	$C_{20}H_{32}$	20.590	1921	13.24	5.23
β-gurjenene	$C_{15}H_{24}$	20.715	1430	6.08	3.43
3E-cembrene A	$C_{20}H_{32}$	20.870	1954	1.17	0.91
cembrene A	$C_{20}H_{32}$	20.948	1962	0.30	
trace		21.300		0.89	0.43
isocembrol	$C_{20}H_{34}O$	21.756	1928	1.65	1.72
methyl 8,15-pimaradien-18-oate	$C_{21}H_{34}O_2$	24.021		0.40	
methyl dehydroabietate	$C_{21}H_{30}O_2$	24.296		0.28	0.69
total	100	99.99			
hydrocarbon compounds	86.65	77.36			
oxygenated compounds	12.46	22.11			
monoterpene hydrocarbons	30.71	20.06			
oxygenated monoterpenes	1.40	1.49			
sesquiterpene hydrocarbons	41.23	50.9			
oxygenated sesquiterpenes	3.06	10.42			
diterpene hydrocarbons	14.71	6.40			
oxygenated diterpenes	1.89	2.40			

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Table 2. continued

compounds	formula	RT	RI	relative area (%)	
				HD	MAE
esters	6.11	7.80			

[a]RT, retention time; RI, retention index; HD, hydrodistillation extraction; and MAE, microwave-assisted extraction,

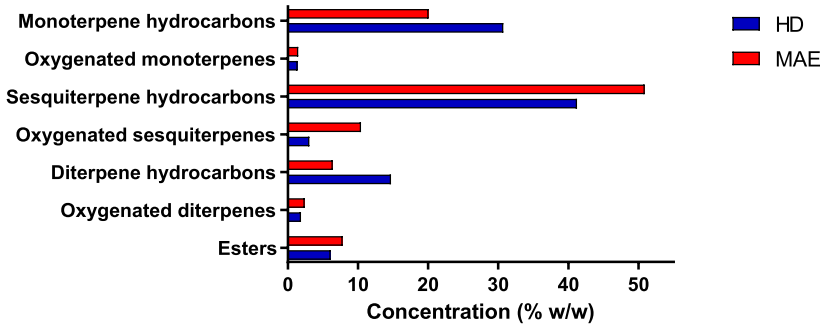

Figure 2. Comparison the chemical classes of compounds in EO of *P. halepensis* Mill. leaves obtained by HD and MAE extraction methods.

molecules is found in the former. This is most likely because MAE includes potential cost savings and efficiency improvements for industrial economic perspectives as the MAE process requires less water than HD in order to decrease degradation of the volatile potent compounds due to oxidation or hydrolysis, which are considered undesirable chemical reactions.[36] Additionally, Filly et al.[54] described that oxygenated compounds have more dipole moments compared to monoterpene hydrocarbons. This causes them to interact more strongly with microwaves and thus they are easier to extract than monoterpene hydrocarbons.

As shown in previous works,[55] the EO's chemical composition varies and is dependent on various factors, including plant part, maturity, geographic location, climatic conditions, harvesting period, and especially extraction process methods.

Antioxidant Potential of *P. halepensis* Mill. EO. The antioxidant capacities of *P. halepensis* Mill. EOs extracted by MAE or HD were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assays. The percentage of inhibition as the EO concentration increases is displayed in Figure 3.

Both EOs were able to reduce DPPH$^{\bullet}$ in a concentration-dependent manner. The results obtained were compared to those of standard ascorbic acid (Table 3).

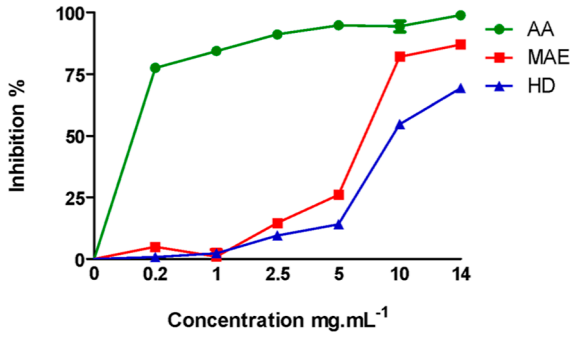

Figure 3. Radical scavenging ability of *P. halepensis* Mill. EOs was assessed by HD and MAE using different concentrations and ascorbic acid (AA) as control. MAE, microwave extraction; HD, hydrodistillation; AA, ascorbic acid.

Table 3. Antioxidant Activity Values of EOs Obtained by MAE and HD and Ascorbic Acid[a]

	DPPH radical scavenging assay activity, IC_{50} (μg mL^{-1})
MAE	3430.132 ± 78.464
HD	4102.301 ± 159.730
ascorbic acid	1.501 ± 1.698

[a]DPPH: 2,2-diphenyl-1-picrylhydrazyl. IC_{50} μg mL^{-1}. Data is shown as the mean ± standard deviation (SD) and N = three independent experiments.

The results given in Table 3 showed that the EO obtained by MAE presented a higher antiradical effect as calculated by IC_{50} = 3430.13 ± 78.46 μg mL^{-1} compared to that obtained by the HD method IC_{50} = 4102.30 ± 159.73 μg mL^{-1}. Similar results regarding the EO's antioxidant activity were also reported by Guo et al.[56] and Araujo et al.,[57] which reported that the DPPH$^{\bullet}$ free radical scavenging capacity of the EO obtained using microwave extraction was higher than that of the conventional technique.

Similar findings were noticed by Chouchan for the extraction of *Mentha spicata*, who demonstrates that the EO using SFMAE has better biological activity in terms of antioxidant potential when compared to the EO extracted by the HD method. Consequently, microwave extraction preserves the biological integrity of the product, alleviating concerns that electromagnetic waves could compromise its medicinal value.[58] The antioxidant activity not only could be attributed to the presence of the oxygenated compounds which are directly proportional to the antioxidant capacity of the EO[59] but also could be related to the amount of sesquiterpene β-caryophyllene as earlier described.[60] Indeed, previous studied showed that the antioxidant activity of β-caryophyllene in two test methods was 1.25 μM for DPPH and 3.23 μM for FRAP.[61]

Cytotoxic Activity of *P. halepensis* Mill. EO. Several studies have investigated the chemical composition and biological activities of EOs extracted from *P. halepensis* Mill. and related species. For instance, Bouzenna et al.[44] found that the EO of *P. halepensis* Mill. from Tunisia contained significant amounts of β-caryophyllene and α-pinene which exhibited strong antioxidant and anti-inflammatory activities. Similarly, research by Djerrad et al.[46] highlighted the presence of

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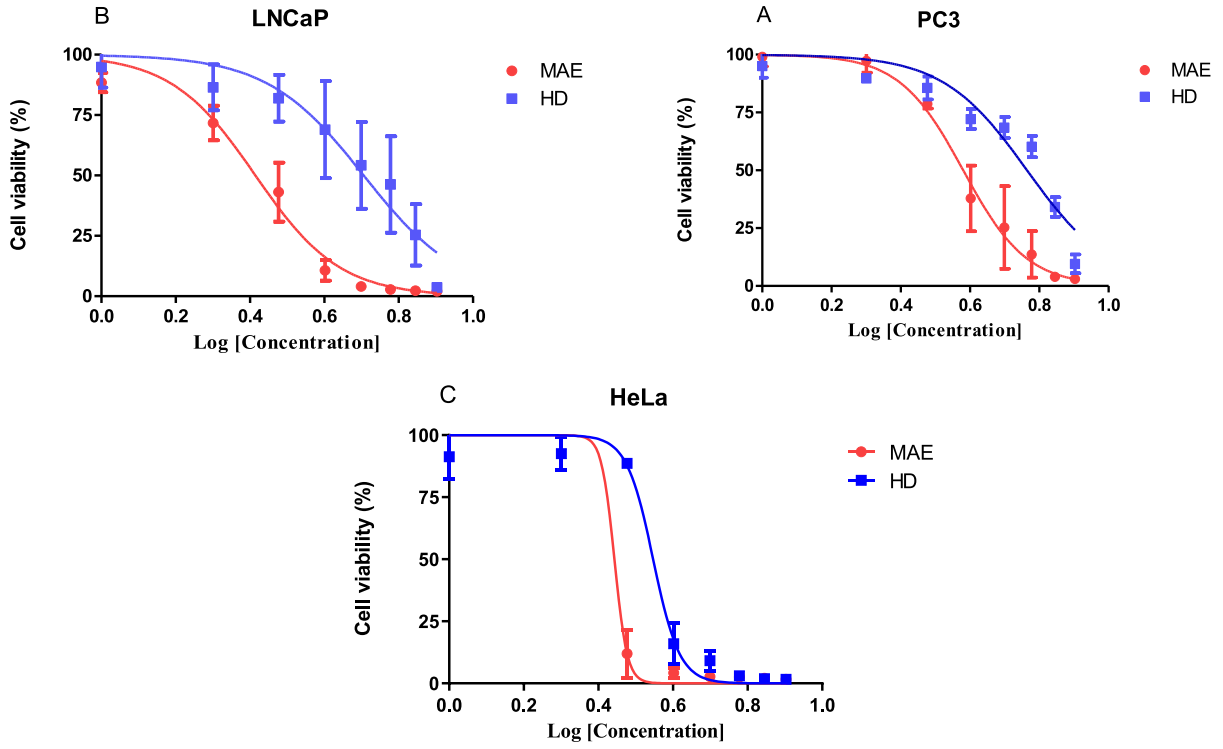

Figure 4. Dose-dependent cytotoxic activity of *P. halepensis* Mill. EO was assessed by HD and MAE on various cell lines. (A) PC3, (B) LNCaP, and (C) HeLa. The cell lines were treated for 48 h at indicated concentrations expressed in log (μg mL^{-1}). Values are expressed as mean ± SD and N = three independent experiments and assays are carried out in octuplicate.

monoterpenes and sesquiterpenes in the EO, which demonstrated notable antimicrobial and cytotoxic effects against various cancer cell lines.

Currently, there has never been any research done on the cytotoxic activity of EO from *P. halepensis* Mill. needles against the human cancer cells PC3 (prostate), LNCaP (prostate), and HeLa (cervical). To assess the antiproliferative effects of the EOs obtained by MAE and HD, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test was performed on these cell lines (Figure 4). Cell viability experiments pointed out that both EOs are potent cytotoxic agents inducing dose-dependent cell cytotoxicity in three tumor cell lines. The median inhibitory concentration IC_{50} values for each cancer cell line are displayed in Table 4.

Table 4. IC_{50} (Mean ± SD, n = 3) Values of *P. halepensis* Mill EO on Three Tumor Cell Lines[a]

	PC3	LNCaP	HeLa
HD	65.19 ± 2.61	49.34 ± 19.49	23.05 ± 2.68
MAE	25.70 ± 6.58	14.97 ± 3.21	14.55 ± 2.30

[a]IC_{50} (μg mL^{-1}): half maximal inhibitory concentration.

Based on the three cancer cell lines tested, the data show that the MAE-EO was more potent than the HD one, with IC_{50} values ranging from 14.55 ± 2.30 to 25.70 ± 6.58 μg mL^{-1} versus 23.05 ± 2.68 to 65.19 ± 2.61 μg mL^{-1}, respectively (Table 4). According to the National Central Institute of the United States, plant extracts with a cytotoxicity showing an IC_{50} < 30 μg mL^{-1} could be considered as potential active ingredients for the development of anticancer drugs.[62] Therefore, EO obtained from *P. halepensis* Mill. obtained by MAE with lower IC_{50} can be considered for further analyses for identifying promising agents for anticancer therapy. The cytotoxicity of this EO can be attributed to the main compound β-caryophyllene, which exhibited cytotoxic activity against PC3, HCT 116, HT-29, ME-180, K562, and PANC-1 cancer cell lines in a previous study.[61]

Three lipid fractions obtained from *P. halepensis* Mill. seeds (neutral lipids, glycolipids, and phospholipids) were studied against human cancer cells A549 (lung), HCT 15 (umbilical), and HL60 (myeloma) by the MTT assay at three concentrations (1, 10, and 100 μg mL^{-1}). An absence of inhibition was noted in the three cancer cells. A concentration of 100 μg mL^{-1} and a low inhibition effect of 10 to 15% cell proliferation were observed in A549 cells.[11] These results are in agreement with a previous study, effect of seeds oil of *P. halepensis* Mill. against U78 cells.[29]

To decipher how EOs obtained by MAE could decrease the cell viability, flow cytometry analysis was performed on the 3 cell lines derived from human tumors. The results are shown in Figure 4 and Table S1.

In the 3 cell lines, *P. halepensis* Mill. EO increases the proportion of cells in the G0-G1 phase when comparing treated versus untreated cells (70 versus 59% for LNCaP cells, p < 0.01; 50 versus 30% for PC3 cells, p < 0.0001; and 59 versus 20%, p < 0.001 for HeLa cells). Conversely, EO decreases the percentage of cells in the G2-M phase when comparing treated versus untreated cells (21 versus 39% for LNCaP cells, p < 0.0001; 42 versus 64% for PC3 cells, p < 0.0001; and 38 versus 80%, p < 0.0001 for HeLa cells) (Figure 5). Our results indicated that the EO of *P halepensis* Mill. induces the cytotoxic effect by stopping the cell cycle in the G0-G1 phase in human prostate and cervical cancer cell lines. β-Caryophyllene isolated from *Chrysanthemum boreale* (Korea) was characterized with anticancer properties which induce the

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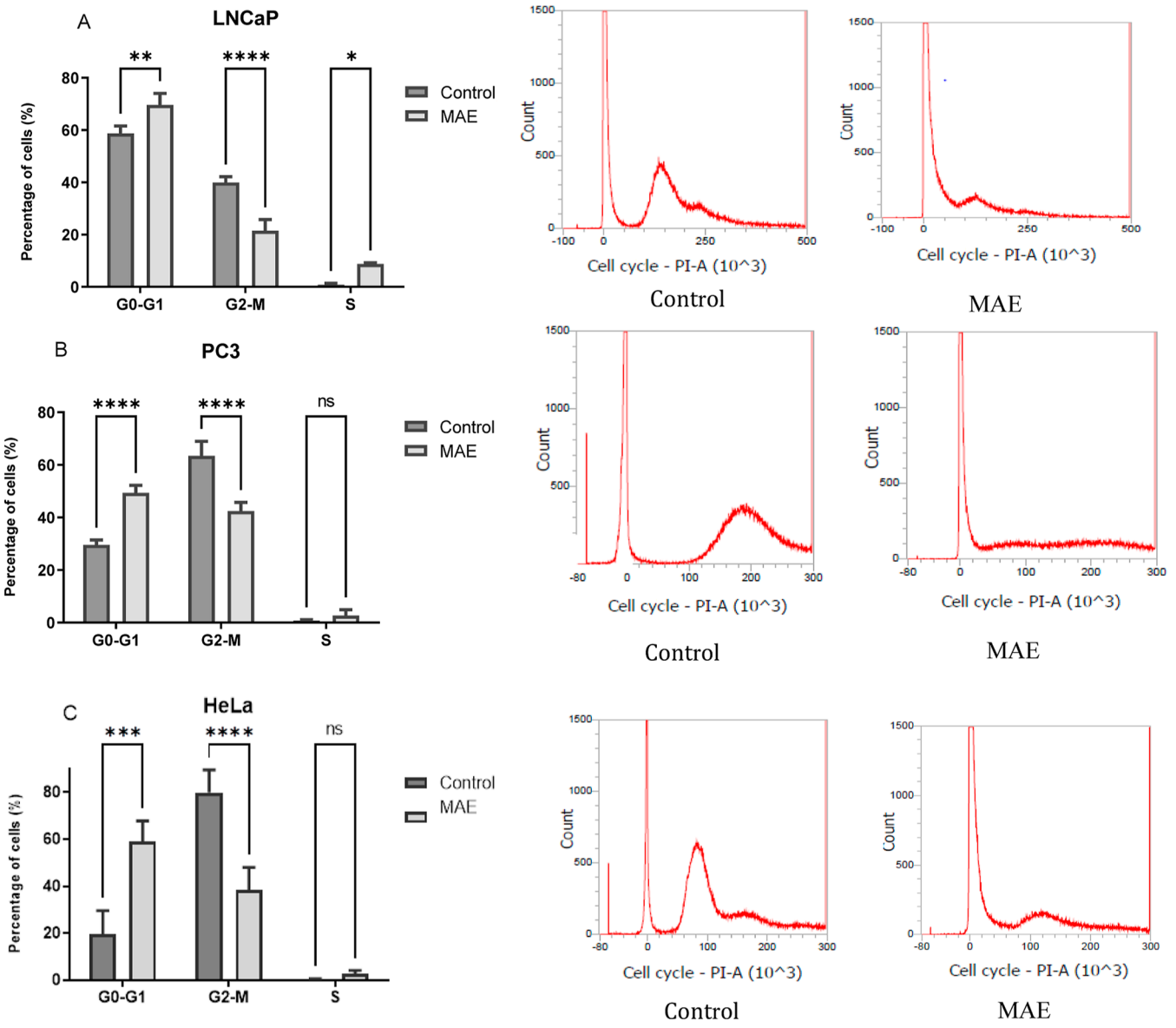

Figure 5. Effect of the *P. halepensis* Mill. EO extracted by MAE treatment on the cell cycle in cancer for 48 h. (A) LNCaP, (B) PC3, and (C) HeLa. All data are statistically significant (mean ± SD, N = three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ versus the control group).

cytotoxic effect in human lung cancer cell lines by stopping the cell cycle in the G1 phase by downregulating cyclin D1, cyclin E, cyclin-dependent protein kinase (CDK)-2, -4, and -6, and RB phosphorylation and by upregulating $p21^{CIP1/WAF1}$ and $p27^{KIP1}$.[63]

EOs of *P. halepensis* Mill. are mainly enriched in *β*-caryophyllene. Evidence points out the cytotoxic potential of *β*-caryophyllene to several cancer cells; this dose-dependent effect ranges from antiproliferative to lethal cytotoxicity. *β*-Caryophyllene is a "dietary cannabinoid" by the interaction with cannabinoid receptor type 2 (CB2).[64] Indeed, the modulation of CB2 has been associated with the cytotoxic potential of BCP (Anticancer Potential of Cannabinoids, Terpenes, and Flavonoids Present in Cannabis). Other mechanisms described include the regulation of different pathways as ROS generation and MAPK activation via JAK1/STAT3, NF-kB, and PI3K/AKT/mTOR/S6K1 and the regulation of apoptotic signaling.[65] In addition, the mechanisms of *β*-caryophyllene cytotoxicity on cancer cells include membrane swelling and remodeling by upregulation of cholesterol and lipid biosynthesis pathway genes (*β*-caryophyllene enhances the transcriptional upregulation of cholesterol biosynthesis in breast cancer cells).

Figure 6 shows representative photos depicting the effect of MAE- and HD-extracted *P. halepensis* Mill. EOs on PCa cell lines.

The EO of *P. halepensis* Mill. has been extensively studied for its chemical composition and biological activities, with various studies highlighting its rich content of monoterpenes and sesquiterpenes, such as *β*-caryophyllene and *α*-pinene, which correlated with its biological activities. Likewise, the obtained finding was in accordance with the recently published research indicating that the EO of Tunisian *P. halepensis* Mill. needles is rich in *β*-caryophyllene (41.28%) and *α*-pinene (14.52%) and possesses a significant antiproliferative effect against human breast cancer (MCF-7) and human colon adenocarcinoma

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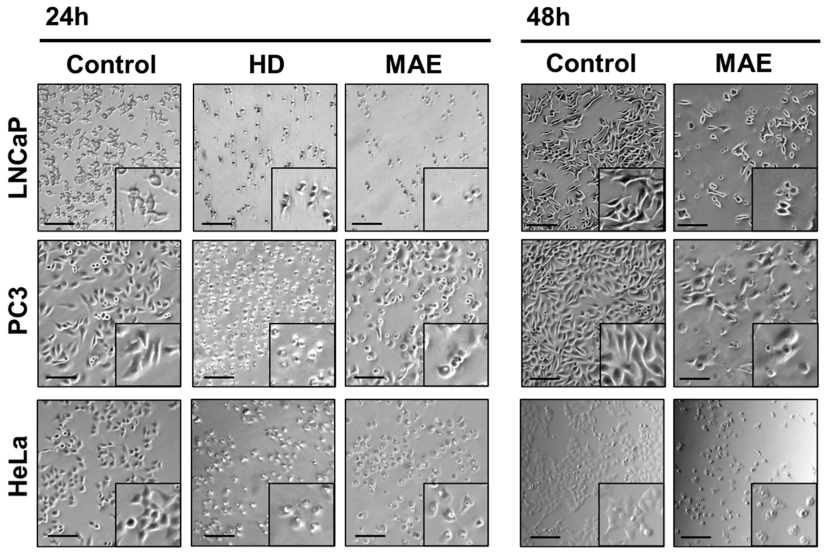

Figure 6. Representative images of PCa cell lines treated with ethanol (control) and EOs of *P. halepensis* Mill. extracted by HD and MAE at IC_{50} value and incubated for 24 or 48 h (scale bars, 50 μm).

(HT-29) cell lines with IC_{50} values of 30 ± 2.26 and 26.67 ± 1.31 μg mL^{-1}, respectively.[30]

In a similar manner, a study by Fekih et al.[14] on the EO of *P. halepensis* Mill. from Algeria reported a similar composition to that found in Tunisian samples, with significant amounts of β-caryophyllene and α-pinene, which exhibited strong antimicrobial and antioxidant activities.

Comparing these findings with other aromatic plants, the EOs of *P. wallichiana* and *Pinus sylvestris* have also shown substantial bioactivity. *P. wallichiana* EO, rich in α-pinene and β-pinene, exhibited strong antiproliferative effects on human prostate cancer cells.[66] Moreover, the EO of *P. sylvestris*, containing compounds like limonene and β-caryophyllene, demonstrated significant antioxidant and anti-inflammatory properties.[67]

The consistency in the presence of certain bioactive compounds such as β-caryophyllene and α-pinene across different species of Pinus highlights the potential of these oils as sources of natural therapeutic agents. However, the specific composition and concentration of these compounds can vary significantly depending on the geographic location, environmental factors, extraction method, and part of the plant used. These factors underscore the importance of comprehensive studies.

In conclusion, our contribution gives for the first time a comprehensive evaluation of the chemical composition and antioxidant and cytotoxic activities of *Pinus* EOs extracted with conventional HD and novel MAE methods. The chemical composition points out the richness of both EO extracts in piperitone and terpenes. MAE-EO exhibited a significant antiproliferative effect in human cancer cell lines (LNCaP, PC3, and HeLa), more pronounced than that of HD-EO. The antioxidant capacity of both EOs showed a significant inhibition of DPPH• radicals and the cytotoxic activities on prostate (LNCaP and PC3) and cervical (HeLa) cancer cells are significant. Besides, EOs also prevent the migration of grown cancer cells and lead to the arrest of their cell cycle in the G0-G1phase. Overall, the in vitro screening of antioxidant and cytotoxic activities of EOs of *P. halepensis* Mill. from Tunisia constitutes a scientific basis to illuminate the mechanisms of action of phytochemicals on human cancer cells in vivo for future application of EOs of *P. halepensis* Mill. from Tunisia in tumor treatment.

EXPERIMENTAL SECTION

Plant Material and EO Extraction. *Plant Material.* In January 2018, fresh *P. halepensis* Mill. needles, which are not endangered or protected, were collected from Rimel forest in Bizerte located in northern Tunisia (latitude 37° 17′ 48″ N; longitude 10° 0′ 2″ E). The taxonomical identification was confirmed by Pr BOUGHANMI Naziha of the Faculty of Sciences of Bizerte, Tunisia, at a regional INRGREF (National Research Institute of Rural Engineering, Water, and Forests) station.

HD Extraction. European Pharmacopeia states that a 1000 mL round-bottom flask of a traditional Clevenger-type apparatus was filled with 600 mL of distilled water and 150 g of fresh needles. After that, HD was done for 3 h or until no more EO could be reused. The extraction temperature (100 °C) is the same as the boiling point of water. The obtained EO was conserved at 4 °C and protected from light until analysis.

Microwave-Assisted Extraction. Microwave extraction was carried out in a Milestone microwave laboratory oven (NEOS, Milestone, Italy). It is a multimode 2.45 GHz reactor that can output power in increments of 10 up to a maximum of 900 W. The software controlled the power, temperature, time, and stirring rate during the experiment. 150 mL of distilled water and 150 g of fresh needles were combined in a 2 L Pyrex glass cylinder. The MAE process was split into two stages: the sample was heated in the first step to almost the boiling point (100 °C), and the oil was then distilled in the second step. A 500 W microwave power and a 250 rpm stirring rate were maintained during the 20 min microwave extraction. At the end of the experiment, EO was gathered and kept in a brown glass vial at 4 °C for further chemical and biological analysis.

Yield Extraction. Using both methods, the extraction yield of EOs from *P. halepensis* Mill. was determined to be as follows (eq 1)

$$\text{yield}(\%) = \frac{\text{mass of extracted EO}}{\text{initial fresh mass of plant}} \times 100 \quad (1)$$

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Refractive Index. The AOAC methods[68] were used to determine the EO's refractive index obtained by the two methods from the needles of *P. halepensis* Mill., and the temperature was 20 °C.

Chemical Composition. *Gas Chromatography–Mass Spectrometry Analysis.* Mass spectrometry analyses of EO were performed on an Agilent model 6890N gas chromatograph, coupled with an Agilent MSD 5973N model. This setup was equipped with an Agilent J&W Scientific capillary HP-5MS column (30 × 0.25 mm, a film thickness of 0.25 μm). The temperatures of the injector and detector were maintained at 250 and 300 °C, respectively. The volume of 1 μL of the EO solution, diluted in *n*-hexane at a ratio of 1/10 (v/v), was injected in split mode with a split ratio of 1/10. Helium (He) was carried as the carrier gas at a flow rate of 1 mL min^{-1}. Temperature programming of the oven was initiated with a 3 min hold at 60 °C followed by a gradual increase to 300 °C and held at 300 °C for 37 min. The temperatures of source and quadrupole remained fixed at 200 and 150 °C, respectively. Mass scanning was conducted from 50 to 500 m/z with an ionizing voltage of 70 eV.

Identification of Components. The main compounds in the EO of *P. halepensis* Mill. were performed on the basis of retention indices determined by coinjection with reference to a homologous series of *n*-alkanes, under identical experimental conditions. The identification was performed by comparing of their mass spectra with those from commercial libraries NIST17 and with data from mass spectrum literature; the oil constituents were determined.[69]

Antioxidant Activity. Based on the radical scavenging effect of the stable DPPH (Sigma-Aldrich, USA) free radical activity, as reported by Brand-Williams et al.,[70] the total antioxidant property of the EOs of *P. halepensis* Mill. was evaluated. The samples were first diluted in methanol (1 mg mL^{-1}). After the DPPH solution was added to the samples and properly mixed, the reaction was allowed to occur for 30 min. The absorbance at 517 nm was measured after 30 min incubation time at room temperature.

Additionally, the DPPH solution in methanol was also tested in the absence of any antioxidant (control). The formula below was used to determine the radical scavenging activity (eq 2)

$$\text{DPPH scavenging (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100 \quad (2)$$

with $A_{control}$ as the absorbance of the DPPH solution in methanol and A_{sample} as the absorbance of the test sample.

By measuring the effective concentration at which free radicals are scavenged by 50%, or the IC_{50} value, the scavenging activity was expressed. Nonlinear regression was used to calculate the various IC_{50} values from graph drawing. The positive control in this experiment was ascorbic acid.

Cell Culture. We have selected three human cancer cell lines that represent common models for prostate and cervical cancers, allowing for the evaluation of the EO efficacy across different cancer types. The human cancer cell lines used were PC3 (derived from bone metastasis of prostate cancer, androgen-insensitive),[71] LNCaP (derived from lymph node metastasis of prostate, androgen-responsive),[72] and HeLa (derived from a cervical tumor).[73] The culture medium used for LNCaP and PC3 cells was RPMI 1640 (Invitrogen, Carlsbad, CA). DMEM was used as a culture medium for HeLa cells (Invitrogen, Carlsbad, CA). The Genetics, Reproduction, and Development Institute possessed all of these cell lines. The cells were cultured as monolayer adherent cultures in 75 cm^2 tissue culture flasks at 37 °C in a humidified atmosphere with 5% CO_2. The medium was supplemented with 10% fetal calf serum (FCS, Biowest, Nuaillé, France), 1% streptomycin (Invitrogen, Oslo, Norway), and 1% penicillin.

Cytotoxicity Assay. The cytotoxicity and IC_{50} values of the EOs were ascertained by the MTT assays (Sigma-Aldrich, USA).[74] In 96-well plates, cancer cell lines were plated at a density of 2 × 10^4 cells per well. The cell lines were treated with the EOs at varying concentrations for 48 h after the first 24 h, with ethanol (max 1/1000) serving as the solvent.[75] Following treatments, cancer cells were incubated for 4 h at 37 °C with 5 mg mL^{-1} of MTT solution. Utilizing a Multiskan GO (Thermo Scientific, USA) microplate reader spectrophotometer, the absorbance at 570 nm was measured to investigate the viability of the cells. The proportion of cell viability relative to the 100% control group was used to express it.

The MTT assay was performed in triplicate for each concentration of EO, and each experiment was repeated three times independently for each cell line to ensure the reliability and reproducibility of the results.

Cell Cycle Analysis. Cancer cells were initially placed into 6-well plates, incubated at a concentration of 35 × 10^4 for a duration of 24 h at 37 °C. Prior to the addition of the test compound, EO was processed and applied at a concentration corresponding to the IC_{50} value. The treated cells were then allowed to incubate for 48 h. After the treatment period, the cell lines were trypsinized, centrifuged, fixed in 4% (v/v) formaldehyde solution in PBS for 15 min at room temperature, and subsequently washed with PBS to prepared them in suspension as described by Bayala et al.[76] This step allows for the centrifugation of cancer cells and the removal of supernatant. After that, 0.2 mL of the FxCycle PI/RNase staining solution (Invitrogen, Carlsbad, CA) was added to each tube and thoroughly mixed. The samples were then incubated for 30 min at room temperature and protected from light. Cancer cells were then subjected to flow cytometry for cell cycle analysis using FACS with excitation at 488 nm, and the emissions were collected through a 585/42 bandpass filter. Flow cytometry analysis was performed at GReD.

Statistical Analysis. GraphPad Prism was used for all statistical analyses, with each experiment's mean ± SD calculated from a minimum of three separate experiments. Two-way RM ANOVA was used to analyze the differences between the control and treated groups and they were considered significant, and the differences were $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05123.

Table of the effect of MAE-extracted *P. halepensis* Mill EO on the cancerous cell cycle (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca-Cancer J. Clin.* **2021**, *71* (3), 209−249.

(2) Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R. L.; Torre, L. A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca-Cancer J. Clin.* **2018**, *68* (6), 394−424.

(3) Yousuf Dar, M.; Shah, W. A.; Mubashir, S.; Rather, M. A. Chromatographic analysis, anti-proliferative and radical scavenging activity of Pinus wallichina essential oil growing in high altitude areas of Kashmir, India. *Phytomedicine* **2012**, *19* (13), 1228−1233.

(4) Quassinti, L.; Lupidi, G.; Maggi, F.; Sagratini, G.; Papa, F.; Vittori, S.; Bianco, A.; Bramucci, M. Antioxidant and antiproliferative activity of Hypericum hircinum L. subsp. majus (Aiton) N. Robson essential oil. *Nat. Prod. Res.* **2013**, *27* (10), 862−868.

(5) Essien, E.; Ogunwande, I.; Setzer, W.; Ekundayo, O. Chemical composition, antimicrobial, and cytotoxicity studies on S. erianthum and S. macranthum essential oils. *Pharm. Biol.* **2012**, *50* (4), 474−480.

(6) Su, Y.-C.; Ho, C.-L. Composition, in-vitro anticancer, and antimicrobial activities of the leaf essential oil of Machilus mushaensis from Taiwan. *Nat. Prod. Commun.* **2013**, *8* (2), 273−275.

(7) Manjamalai, A.; Grace, V. M. B. The chemotherapeutic effect of essential oil of Plectranthus amboinicus (Lour) on lung metastasis developed by B16F-10 cell line in C57BL/6 mice. *Cancer Invest.* **2013**, *31* (1), 74−82.

(8) Bayala, B.; Bassole, I. H. N.; Gnoula, C.; Nebie, R.; Yonli, A.; Morel, L.; Figueredo, G.; Nikiema, J.-B.; Lobaccaro, J.-M. A.; Simpore, J. Chemical composition, antioxidant, anti-inflammatory and anti-proliferative activities of essential oils of plants from Burkina Faso. *PLoS One* **2014**, *9* (3), No. e92122.

(9) Aouf, A.; Bouaouina, S.; Abdelgawad, M. A.; Abourehab, M. A.; Farouk, A. In silico study for Algerian essential oils as antimicrobial agents against multidrug-resistant bacteria isolated from pus samples. *Antibiotics* **2022**, *11* (10), 1317.

(10) Panda, P. C.; Jena, S.; Ray, A.; Sahoo, A.; Das, P. K.; Kamila, P. K.; Kar, S. K.; Nayak, S. Anti-proliferative activity of Piper trioicum leaf essential oil based on phytoconstituent analysis, molecular docking and in silico ADMET approaches. *Comb. Chem. High Throughput Screening* **2023**, *26* (1), 183−190.

(11) Kadri, N.; Khettal, B.; Adjebli, A.; Cresteil, T.; Yahiaoui-Zaidi, R.; Barragan-Montero, V.; Montero, J.-L. Antiangiogenic activity of neutral lipids, glycolipids, and phospholipids fractions of Pinus halepensis Mill. seeds. *Ind. Crops Prod.* **2014**, *54*, 6−12.

(12) Macchioni, F.; Cioni, P.; Flamini, G.; Morelli, I.; Maccioni, S.; Ansaldi, M. Chemical composition of essential oils from needles, branches and cones of Pinus pinea, P. halepensis, P. pinaster and P. nigra from central Italy. *Flavour Fragrance J.* **2003**, *18* (2), 139−143.

(13) Cheikh-Rouhou, S.; Hentati, B.; Besbes, S.; Blecker, C.; Deroanne, C.; Attia, H. Chemical composition and lipid fraction characteristics of Aleppo pine (Pinus halepensis Mill.) seeds cultivated in Tunisia. *Food Sci. Technol. Int.* **2006**, *12* (5), 407−415.

(14) Fekih, N.; Allali, H.; Merghache, S.; Chaïb, F.; Merghache, D.; El Amine, M.; Djabou, N.; Muselli, A.; Tabti, B.; Costa, J. Chemical composition and antibacterial activity of Pinus halepensis Miller growing in West Northern of Algeria. *Asian Pac. J. Trop. Dis.* **2014**, *4* (2), 97−103.

(15) Abbou, A.; Kadri, N.; Debbache, N.; Dairi, S.; Remini, H.; Dahmoune, F.; Berkani, F.; Adel, K.; Belbahi, A.; Madani, K. Effect of precipitation solvent on some biological activities of polysaccharides from Pinus halepensis Mill. seeds. *Int. J. Biol. Macromol.* **2019**, *141*, 663−670.

(16) Basholli-Salihu, M.; Schuster, R.; Hajdari, A.; Mulla, D.; Viernstein, H.; Mustafa, B.; Mueller, M. Phytochemical composition, anti-inflammatory activity and cytotoxic effects of essential oils from three Pinus spp. *Pharm. Biol.* **2017**, *55* (1), 1553−1560.

(17) Kotroni, E.; Simirioti, E.; Kikionis, S.; Sfiniadakis, I.; Siamidi, A.; Karalis, V.; Vitsos, A.; Vlachou, M.; Ioannou, E.; Roussis, V.; et al. In Vivo evaluation of the anti-inflammatory activity of electrospun micro/nanofibrous patches loaded with pinus halepensis bark extract on hairless mice skin. *Materials* **2019**, *12* (16), 2596.

(18) Badawy, M. E.; Kherallah, I. E.; Badawy, M. E.; Salem, M. Z.; Yousef, H. A. Chemical composition and activity of bark and leaf extracts of Pinus halepensis and Olea europaea grown in AL-Jabel AL-Akhdar region, Libya against some plant phytopathogens. *J. Appl. Biotechnol. Bioeng.* **2017**, *3*, 331−342.

(19) Hmamouchi, M. Chemical and antimicrobial properties of essential oils of five Moroccan Pinaceae. In *The Antimicrobial/ Biological Activity of Essential Oils*; Allured Publishing Corporation, 2005.

(20) Bouyahya, A.; Belmehdi, O.; Abrini, J.; Dakka, N.; Bakri, Y. Chemical composition of Mentha suaveolens and Pinus halepensis essential oils and their antibacterial and antioxidant activities. *Asian Pac. J. Trop. Med.* **2019**, *12* (3), 117.

(21) Raho, G. B. Antibacterial potential of essential oils of the needles of Pinus halepensis against Staphylococcus aureus and Escherichia coli. *J. Coastal Life Med.* **2014**, *2* (8), 651−655.

(22) Amri, I.; Hamrouni, L.; Hanana, M.; Gargouri, S.; Fezzani, T.; Jamoussi, B. Chemical composition, physico-chemical properties, antifungal and herbicidal activities of Pinus halepensis Miller essential oils. *Biol. Agric. Hortic.* **2013**, *29* (2), 91−106.
(23) Hamrouni, L.; Hanana, M.; Amri, I.; Romane, A. E.; Gargouri, S.; Jamoussi, B. Allelopathic effects of essential oils of Pinus halepensis Miller: chemical composition and study of their antifungal and herbicidal activities. *Arch. Phytopathol. Plant Prot.* **2015**, *48* (2), 145−158.
(24) Amri, I.; Lamia, H.; Gargouri, S.; Hanana, M.; Mahfoudhi, M.; Fezzani, T.; Ezzeddine, F.; Jamoussi, B. Chemical composition and biological activities of essential oils of Pinus patula. *Nat. Prod. Commun.* **2011**, *6* (10), 1531−1536.
(25) Mohamed, A. A.; Behiry, S. I.; Ali, H. M.; EL-Hefny, M.; Salem, M. Z.; Ashmawy, N. A. Phytochemical compounds of branches from P. halepensis oily liquid extract and S. terebinthifolius essential oil and their potential antifungal activity. *Processes* **2020**, *8* (3), 330.
(26) Mitić, Z. S.; Jovanović, B.; JovanovićStojanović-RadićMihajilov-KrstevJovanovićNikolić, S. Č.Z. Z. T. N. M. B. M.; Marin, P. D.; Zlatković, B. K.; Stojanović, G. S.; Nikolić, B. M.; Marin, P. D.; Zlatković, B. K.; Stojanović, G. S. Essential oils of Pinus halepensis and P. heldreichii: Chemical composition, antimicrobial and insect larvicidal activity. *Ind. Crops Prod.* **2019**, *140*, 111702.
(27) Salim, H.; Rimawi, W. H.; Shaheen, S.; Mjahed, A. Phytochemical analysis and antibacterial activity of extracts from Palestinian Aleppo pine seeds, bark and cones. *Asian J. Chem.* **2019**, *31* (1), 143−147.
(28) Meziti, H.; Bouriche, H.; Kada, S.; Demirtas, I.; Kizil, M.; Senator, A.; Garrido, G. Phytochemical analysis, and antioxidant, anti-hemolytic and genoprotective effects of Quercus ilex L. and Pinus halepensis Mill. methanolic extracts. *J. Pharm. Pharmacogn. Res.* **2019**, *7*, 260−272.
(29) Rigane, G.; Jebali, J.; Ghazghazi, H.; Riguene, H.; Laarbi Khouja, M.; Ben Salem, R. Chemical composition and biological activities of pinus halepensis Mill. oil. *Rev. Roum. Chim.* **2019**, *64* (11), 999−1006.
(30) Dakhlaoui, S.; Bourgou, S.; Bachkouel, S.; Ben Mansour, R.; Ben Jemaa, M.; Jallouli, S.; Megdiche-Ksouri, W.; Hessini, K.; Msaada, K. Essential oil composition and biological activities of Aleppo pine (Pinus halepensis Miller) needles collected from different Tunisian regions. *Int. J. Environ. Health Res.* **2023**, *33* (1), 83−97.
(31) Riela, S.; Bruno, M.; Formisano, C.; Rigano, D.; Rosselli, S.; Saladino, M. L.; Senatore, F. Effects of solvent-free microwave extraction on the chemical composition of essential oil of Calamintha nepeta (L.) Savi compared with the conventional production method. *J. Sep. Sci.* **2008**, *31* (6−7), 1110−1117.
(32) Vinatoru, M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason. Sonochem.* **2001**, *8* (3), 303−313.
(33) Kaufmann, B.; Christen, P. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. *Phytochem. Anal.* **2002**, *13* (2), 105−113.
(34) Wang, L.; Weller, C. L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **2006**, *17* (6), 300−312.
(35) Ferhat, M. A.; Meklati, B. Y.; Smadja, J.; Chemat, F. An improved microwave Clevenger apparatus for distillation of essential oils from orange peel. *J. Chromatogr. A* **2006**, *1112* (1−2), 121−126.
(36) Lucchesi, M. E.; Chemat, F.; Smadja, J. Solvent-free microwave extraction of essential oil from aromatic herbs: comparison with conventional hydro-distillation. *J. Chromatogr. A* **2004**, *1043* (2), 323−327.
(37) de l'Europe, C. *Pharmacopée Européenne*; Conseil de l'Europe, 1996.
(38) Golmakani, M.-T.; Rezaei, K. Comparison of microwave-assisted hydrodistillation withthe traditional hydrodistillation method in the extraction of essential oils from Thymus vulgaris L. *Food Chem.* **2008**, *109* (4), 925−930.
(39) Moradi, S.; Fazlali, A.; Hamedi, H. Microwave-assisted hydro-distillation of essential oil from rosemary: Comparison with traditional distillation. *Avicenna J. Med. Biotechnol.* **2018**, *10* (1), 22.
(40) Bustamante, J.; van Stempvoort, S.; García-Gallarreta, M.; Houghton, J. A.; Briers, H. K.; Budarin, V. L.; Matharu, A. S.; Clark, J. H. Microwave assisted hydro-distillation of essential oils from wet citrus peel waste. *J. Cleaner Prod.* **2016**, *137*, 598−605.
(41) Dob, T.; Berramdane, T.; Chelghoum, C. Essential oil composition of Pinus halepensis Mill. from three different regions of Algeria. *J. Essent. Oil Res.* **2007**, *19* (1), 40−43.
(42) Argo, B.; Hermanto, M.; Andriani, D.; Rosadhani, J. The effect of ginger oil extraction using Microwave Assisted Hydro-distillation (MAHD) method on zingiberene content. In *IOP Conference Series: Earth and Environmental Science*; IOP Publishing: 2020; p 012002.
(43) Wibowo, S.; Komarayati, S. Sifat Fisiko Kimia Minyak Cupresus (Cupressus Benthamii) Asal Aek Nauli, Parapat Sumatera Utara. *Jurnal Penelitian Hasil Hutan* **2015**, *33* (2), 93−103.
(44) Bouzenna, H.; Hfaiedh, N.; Bouaziz, M.; Giroux-Metges, M.-A.; Elfeki, A.; Talarmin, H. Cytoprotective effects of essential oil of Pinus halepensis L. against aspirin-induced toxicity in IEC-6 cells. *Arch. Physiol. Biochem.* **2017**, *123* (5), 364−370.
(45) Ameur, E.; Sarra, M. E.; Takoua, k.; Mariem, K.; Nabil, A.; Lynen, F.; Larbi, K. M. Chemical composition of five Tunisian Pinus Species' essential oils and effect of their blends on Otitis infection. *Ind. Crops Prod.* **2022**, *180*, 114688.
(46) Djerrad, Z.; Djouahri, A.; Kadik, L. Variability of Pinus halepensis Mill. Essential oils and their antioxidant activities depending on the stage of growth during vegetative cycle. *Chem. Biodiversity* **2017**, *14* (4), No. e1600340.
(47) Postu, P. A.; Sadiki, F. Z.; El Idrissi, M.; Cioanca, O.; Trifan, A.; Hancianu, M.; Hritcu, L. Pinus halepensis essential oil attenuates the toxic Alzheimer's amyloid beta (1−42)-induced memory impairment and oxidative stress in the rat hippocampus. *Biomed. Pharmacother.* **2019**, *112*, 108673.
(48) Santonja, M.; Bousquet-Mélou, A.; Greff, S.; Ormeño, E.; Fernandez, C. Allelopathic effects of volatile organic compounds released from Pinus halepensis needles and roots. *Ecol. Evol.* **2019**, *9* (14), 8201−8213.
(49) Cardoso-Ugarte, G. A.; Juárez-Becerra, G. P.; SosaMorales, M. E.; López-Malo, A. Microwave-assisted extraction of essential oils from herbs. *J. Microw. Power Electromagn. Energy.* **2013**, *47* (1), 63−72.
(50) Ferhat, M. A.; Meklati, B. Y.; Chemat, F. Comparison of different isolation methods of essential oil from Citrus fruits: cold pressing, hydrodistillation and microwave 'dry'distillation. *Flavour Fragrance J.* **2007**, *22* (6), 494−504.
(51) Lucchesi, M. E.; Smadja, J.; Bradshaw, S.; Louw, W.; Chemat, F. Solvent free microwave extraction of Elletaria cardamomum L.: A multivariate study of a new technique for the extraction of essential oil. *J. Food Eng.* **2007**, *79* (3), 1079−1086.
(52) Bendahou, M.; Muselli, A.; Grignon-Dubois, M.; Benyoucef, M.; Desjobert, J.-M.; Bernardini, A.-F.; Costa, J. Antimicrobial activity and chemical composition of Origanum glandulosum Desf. essential oil and extract obtained by microwave extraction: Comparison with hydrodistillation. *Food Chem.* **2008**, *106* (1), 132−139.
(53) Wang, Z.; Ding, L.; Li, T.; Zhou, X.; Wang, L.; Zhang, H.; Liu, L.; Li, Y.; Liu, Z.; Wang, H.; et al. Improved solvent-free microwave extraction of essential oil from dried Cuminum cyminum L. and Zanthoxylum bungeanum Maxim. *J. Chromatogr. A* **2006**, *1102* (1−2), 11−17.
(54) Filly, A.; Fernandez, X.; Minuti, M.; Visinoni, F.; Cravotto, G.; Chemat, F. Solvent-free microwave extraction of essential oil from aromatic herbs: from laboratory to pilot and industrial scale. *Food Chem.* **2014**, *150*, 193−198.
(55) Farhat, A.; Ginies, C.; Romdhane, M.; Chemat, F. Eco-friendly and cleaner process for isolation of essential oil using microwave energy: experimental and theoretical study. *J. Chromatogr. A* **2009**, *1216* (26), 5077−5085.

https://doi.org/10.1021/acsomega.4c05123
ACS Omega 2024, 9, 34128−34139

(56) Guo, Y.; Li, Y.; Li, Z.; Jiang, L.; Cao, X.; Gao, W.; Wang, J.; Luo, D.; Chen, F. Deep eutectic solvent-homogenate based microwave-assisted hydrodistillation of essential oil from Litsea cubeba (Lour.) Pers. fruits and its chemical composition and biological activity. *J. Chromatogr. A* **2021**, *1646*, 462089.
(57) Araujo, A. R.; Périno, S.; Fernandez, X.; Cunha, C.; Rodrigues, M.; Ribeiro, M. P.; Jordao, L.; Silva, L. A.; Rodilla, J.; Coutinho, P.; et al. Solvent-free microwave extraction of Thymus Mastichina essential oil: Influence on their chemical composition and on the antioxidant and antimicrobial activities. *Pharmaceuticals* **2021**, *14* (8), 709.
(58) Singh Chouhan, K. B.; Tandey, R.; Sen, K. K.; Mehta, R.; Mandal, V. A unique model of gravity assisted solvent free microwave based extraction of essential oil from mentha leaves ensuring biorefinery of leftover waste biomass for extraction of nutraceuticals: Towards cleaner and greener technology. *J. Cleaner Prod.* **2019**, *225*, 587−598.
(59) Meullemiestre, A.; Petitcolas, E.; Maache-Rezzoug, Z.; Ginies, C.; Chemat, F.; Rezzoug, S.-A. Isolation of volatils from maritime pine sawdust waste by different processes: Ultrasound, microwave, turbo hydrodistillation, and hydrodistillation. *Wood Mater. Sci. Eng.* **2014**, *9* (2), 76−83.
(60) Dar, M. Y.; Shah, W. A.; Rather, M. A.; Qurishi, Y.; Hamid, A.; Qurishi, M. Chemical composition, in vitro cytotoxic and antioxidant activities of the essential oil and major constituents of Cymbopogon jawarancusa (Kashmir). *Food Chem.* **2011**, *129* (4), 1606−1611.
(61) Dahham, S. S.; Tabana, Y. M.; Iqbal, M. A.; Ahamed, M. B.; Ezzat, M. O.; Majid, A. S.; Majid, A. M. The Anticancer, Antioxidant and Antimicrobial Properties of the Sesquiterpene β-Caryophyllene from the Essential Oil of Aquilaria crassna. *Molecules* **2015**, *20* (7), 11808−11829.
(62) Suffness, M. Assays related to cancer drug discovery. In *Methods in Plant Biochemistry: Assays for Bioactivity*; Academic Press, 1990; Vol. *6*, pp 71−133.
(63) Chung, K.-S.; Hong, J. Y.; Lee, J.-H.; Lee, H.-J.; Park, J. Y.; Choi, J.-H.; Park, H.-J.; Hong, J.; Lee, K.-T. β-Caryophyllene in the Essential Oil from Chrysanthemum Boreale Induces G1 Phase Cell Cycle Arrest in Human Lung Cancer Cells. *Molecules* **2019**, *24* (20), 3754.
(64) Gertsch, J.; Leonti, M.; Raduner, S.; Racz, I.; Chen, J.-Z.; Xie, X.-Q.; Altmann, K.-H.; Karsak, M.; Zimmer, A. Beta-caryophyllene is a dietary cannabinoid. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (26), 9099−9104.
(65) Fidyt, K.; Fiedorowicz, A.; Strządała, L.; Szumny, A. β-caryophyllene and β-caryophyllene oxide—natural compounds of anticancer and analgesic properties. *Cancer Med.* **2016**, *5* (10), 3007−3017.
(66) Bayala, B.; Bassole, I. H.; Scifo, R.; Gnoula, C.; Morel, L.; Lobaccaro, J.-M. A.; Simpore, J. Anticancer activity of essential oils and their chemical components-a review. *Am. J. Cancer Res.* **2014**, *4* (6), 591.
(67) Mitić, Z. S.; Jovanović, B.; JovanovićMihajilov-KrstevStojanović-RadićCvetkovićMitrović, S. Č.T. Z. Z. V. J. T. L.; Marin, P. D.; Zlatković, B. K.; Stojanović, G. S.; Mitrović, T. L.; Marin, P. D.; Zlatković, B. K.; Stojanović, G. S. Comparative study of the essential oils of four Pinus species: Chemical composition, antimicrobial and insect larvicidal activity. *Ind. Crops Prod.* **2018**, *111*, 55−62.
(68) Bates, J. *AOAC. Official Methods of Analysis*; Association of Official Analytical Chemists: Virginia, USA, 1994.
(69) Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*; Allured Publishing Corporation Carol Stream: Illinois, 2007; Vol. *456*.
(70) Brand-Williams, W.; Cuvelier, M.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Food Sci. Technol.* **1995**, *28*, 25−30.
(71) Kaighn, M.; Narayan, K. S.; Ohnuki, Y.; Lechner, J. F.; Jones, L. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest. Urol.* **1979**, *17* (1), 16−23.
(72) Horoszewicz, J. S.; Leong, S. S.; Chu, T. M.; Wajsman, Z. L.; Friedman, M.; Papsidero, L.; Kim, U.; Chai, L. S.; Kakati, S.; Arya, S. K.; Sandberg, A. A. The LNCaP cell line-a new model for studies on human prostatic carcinoma. *Prog. Clin. Biol. Res.* **1980**, *37*, 115−132.
(73) Culliton, B. J. HeLa (for Henrietta lacks). *Science* **1974**, *184* (4143), 1268.
(74) Sylvester, P. W. Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. In *Drug Design and Discovery*; Springer, 2011; pp 157−168.
(75) Legault, J.; Pichette, A. Potentiating effect of β-caryophyllene on anticancer activity of α-humulene, isocaryophyllene and paclitaxel. *J. Pharm. Pharmacol.* **2010**, *59* (12), 1643−1647.
(76) Bayala, B.; Nadembega, C.; Guenn&eacu, S.; Buñ, J.; Mahouk&egr, T.; Wendkuuni, F.; Yonli, A.; Baron, S.; Figueredo, G.; A Lobacca, J. M.; et al. Chemical Composition, Antioxidant and Cytotoxic Activities of Hyptis suaveolens (L.) Poit. Essential Oil on Prostate and Cervical Cancers Cells. *Pak. J. Biol. Sci.* **2020**, *23* (9), 1184−1192.

https://doi.org/10.1021/acsomega.4c05123
ACS Omega 2024, 9, 34128−34139