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Chemical Composition, *In vitro* Evaluation of Antioxidant Properties and Cytotoxic Activity of the Essential Oil from *Calamintha incana* (Sm.) Helder (Lamiaceae)

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ABSTRACT

Calamintha incana (Sm.) Helder (Lamiaceae) is an aromatic herb used in folk medicine in Jordan and neighboring countries. This study aimed to isolate and characterize the chemical composition of essential oils extracted from the aerial parts of *C. incana* and to evaluate the total phenolic and flavonoid contents, antioxidant activity, and cytotoxic potential. The essential oils from the aerial parts of *C. incana* (CIEO) were extracted by hydrodistillation, and GC/MS were performed for the chemical analysis of the oil. Total phenol and flavonoid contents were assessed using the colorimetric assay. The antioxidant activity of the oil was assessed using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reducing power ability. The cytotoxic activity of CIEO against MCF-7, T47D, Caco-2 cancer cell lines, and normal fibroblast cell line (MRC-5) was investigated by 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The results demonstrated that the main constituents of CIEO were benzenamine-4-methyl-3-nitro-(34.11%), and (2S,4R)-p-mentha-6,8-diene 2-hydroperoxide (31.48%). The phenolic content of CIEO was higher than the flavonoid content. With respect to the DPPH radical scavenging activity, the IC_{50} was 15.38 mg/mL, while for the reducing power ability the EC_{50} was 9.79 mg/mL). Moreover, CIEO was cytotoxic against cancerous and non-cancerous cells at 200 µg/mL. In conclusion, the essential oil extract of *C. incana* is characterized by its non-terpenoid aromatic compounds. Phenols were more abundant than flavonoids, and CIEO had a good antioxidant capacity and non-selective cytotoxic activity. Therefore, additional investigations are required to understand the mechanism of the cytotoxicity of this plant.

***Keywords*:** Antioxidant effect, Cytotoxicity, Essential oils, Lamiaceae, *Calamintha incana*.

Introduction

During the past decades, medicinal plants have played a significant role in drug discovery and development. Recently, scientists have attempted to study the biological effects of natural compounds on the human body to develop new treatments with high efficiency and low adverse effects compared with conventional drugs used against most destructive diseases, especially cancer.[1] Approximately 80% of people worldwide rely on medicinal plants for some part of primary healthcare.[2] However, little is known about the therapeutic potential of plants or cytotoxicity in most countries.[3] Safety is a significant issue for herbal therapies. Natural plant products need to be standardized, and preliminary investigations are performed to assess possible risks such as unwanted side effects, overdose, and toxicity.[2] Recently, the use of natural compounds, especially essential oils, in pharmaceutical medicine has expanded. Some essential oils show a broad spectrum of biological activities such as antioxidant, antimicrobial, analgesic, and anti-inflammatory, while others have anticancer activity.[4]

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The therapeutic potential of essential oils and their corresponding actions mainly depend on secondary metabolites and several other naturally occurring chemical constituents. Essential oils have numerous unique features useful for various applications, including the food industry, cosmetics, aromatherapy, and pharmaceutical medicines.[4]

In Jordan, medicinal plants represent approximately 20% of Jordan's total flora.[5] Several of which have been used in folk medicine and the pharmaceutical industry.[6] *Calamintha* is a herbaceous perennial plant belonging to the Lamiaceae family, distributed in Europe, the Eastern Mediterranean region, Central Asia, North Africa, and America.[7] Due of its aromatic features, leaves and aerial parts of this genus have a distinctive pleasant mint-like smell. In Jordan, *C. incana* (Sm.) Helder (Syn: *Thymus incanus* Sm.) was recorded in the rocky regions.[8] Traditionally, *C. incana* leaves are used as a spice and as herbal tea. In folk medicine, it is used to treat abdominal pain and general weakness, diaphoretic, expectorant, and antispasmodic.[6] Few studies have investigated the biological effects of *C. incana* essential oil, especially the potential cytotoxic activity.

Calamintha incana has also been reported to possess antimicrobial activities,[9] antioxidant activity,[10] and inhibitory activity of some enzymes (tyrosinase, α -glucosidase and α-amylase)[10] Despite the potential application of *C. incana*, no study on the cytotoxic effects of its essential oil has been conducted yet. Therefore, the chemical composition of the essential oil isolated from the aerial parts of *Calamintha incana* was investigated in this study. In addition to determining the total phenolic and flavonoid contents and evaluate the

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antioxidant capacity and cytotoxic potential against breast cancer and colorectal cancer cell lines and normal fibroblast cell lines.

Materials and Methods

The following chemicals were obtained from commercial suppliers and used without further purification: Dulbecco's modified eagle medium (DMEM) (Lonza, Euro-clone), Fetal bovine serum (FBS) (Gibco), L-glutamine (Lonza, Euro-clone), Penicillin-streptomycin (Lonza), Trypsin-EDTA EDTA (Lonza, Euro-clone), Trypan blue stain (Sigma), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma), Folin-Ciocalteu phenol reagent, ascorbic acid, quercetin, gallic acid (analytical purity), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma). MCF-7, T47D, Caco-2, and MRC-5 cell lines were purchased from the American Type Culture Collection (ATCC®; USA). Other solvents and chemical reagents were purchased from commercial vendors and handled by standard procedures before use. Anhydrous sodium sulfate (Analar, England), N-hexane /GC grade (Tedia USA), and a hydrocarbon mixture of n-alkanes (C_8-C_{20}) was obtained from Fluka (Switzerland). Hydrodistillation was performed using a Clevenger apparatus (JSGW, India). Variant chrompack CP-3800 GC/MS/MS-200 (Satum, Netherlands) was used to analyze the chemical components of the oil extract.

Plant materials

The fresh aerial parts of the plant *C. incana* were collected during May, 2020 from its natural habitat in Ajloun county in Jordan. Plant materials were authentically identified by Prof. Sawsan Oran, Department of Biological Sciences, University of Jordan. A voucher specimen (voucher no. CI-UJ-14) was deposited at the herbarium of the Department of Biological Sciences, University of Jordan, Amman.

Isolation of essential oil

The aerial parts of *C. incana* were dried at room temperature in the dark and then ground into a fine powder. One hundred grams (100 g) of the dried plant was extracted by hydrodistillation using a Clevenger-type apparatus for 3 h.[11] The essential oil was collected and dehydrated using anhydrous sodium sulfate and stored in a dark vial at +4°C until chemical analysis and bioassays were performed. The essential oil content % (w/w) was calculated using the following equation (1)

$$The\ yield\ of\ oil\ = \frac{Net\ weight\ of\ extracted\ oil\ \ (g\ )}{weight\ of\ the\ dry\ matter\ (g)} x100 \quad \text{-------} \quad 1$$

GC-MS analysis

The analysis was performed using Varian Chrompack CP 3800 GC/MS/MS-200 (Satum, Netherlands) equipped with a split-splitless injector and a DB-5 capillary column (5% diphenyl, 95% dimethyl polysiloxane, 30 m×0.25 mm i.d., 0.25 µm film thickness). At a flow rate of 1 mL/min flow rate, helium (inert gas) was used as the carrier gas. A linear temperature program was used to separate the various oil components; and the column temperature was held constant at 60°C for 1 min before being raised to 240°C at a rate of 3°C/min and then held constant at 246°C for 3 min, for a total run time of 66 min. One milliliter (1 mL) of GC grade n-hexane was used to dilute 5 µL of *C. incana* oil. The oil sample was injected at a volume of 0.1 µL-split ratios of 1:30; the injector temperature was set at 246°C. The mass detector (FID) was programmed to scan ions in the 40-400 m/z range using a full scan mode electron impact (70 eV).

The essential oil components of *C. incana* were identified by comparing their recorded mass spectra with mass spectra from the databases NIST, Wiley, and Adam's libraries,[12] as well as by comparing their calculated arithmetic indices (KI calculated) with those in the literature, measured on columns with identical polarity.[13] According to Vandendool and Kratz,[14] the linear retention index (Arithmetic-Kovats index) was determined for each peak separated by GC-MS using the value of its retention time, and the retention times of the homologous n-alkanes (C_8-C_{20}) were injected after the essential oil in the same chromatographic conditions. The percentage of the oil component was determined by dividing the peak area of each constituent by the total peak area of all oil constituents and multiplying the result by 100.

Determination of total phenolic content (TPC)

The total phenolic content of *C. incana* oil extract was determined using the Folin-Ciocalteu procedure reported by Bouyahya *et al*.[15] One hundred microliters of oil extract (1mg/mL methanol) was combined with 500µL of Folin-Ciocalteu reagent (formerly diluted 10-fold with distilled water), and 400 µL of sodium carbonate (7.0% w/v). After 40 min of incubation in darkness at room temperature, the absorbance was measured at 760 nm (UV-vis spectrophotometer), and the values of triplicate were averaged. The linear equation of calibration curve prepared with various concentrations of 0–120 µg/mL of gallic acid was applied to calculate the total phenolic content. TPC was displayed as gallic acid equivalent (mg of gallic acid/ g of oil extract).

Determination of total flavonoid content (TFC)

The total flavonoid content of the *C. incana* oil extract was assessed using the Aluminum chloride colorimetric assay described by Bouyahya *et al*.[15] One milliliter of oil extract (mg/ mL in methanol) was added to 1 mL of (2% w/v) aluminum chloride in methanol. After incubation for 40 min at room temperature, the absorbance of the mixture was measured at 430 nm using the spectrophotometer. Quercetin (0-120 µg/mL) was used to prepare the calibration curve. TFC was represented as mg quercetin equivalent (QE)/g of oil extract. All values were determined three times.

Antioxidant activity

DPPH free radical scavenging activity

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) antiradical scavenging assay was conducted following the method of Polatoglu *et al*.[16] In brief, 100 µL of DPPH solution (0.1 mM, in methanol) was combined with 20 µL of the samples at varying concentrations (5-30000 µg/mL). The mixture was then shaken and incubated in the dark at room temperature for a half-hour. The absorbance at 517 nm was measured against a blank (methanol). Ascorbic acid (5-100 µg/mL) was used as a positive control. The inhibition of the DPPH radical by the oil extract was calculated based on equation (2)

$$DPPH\ radical\ scavenging\ activity\ \% = \frac{(absorbance\ of\ blank - absorbance\ of\ the\ sample)}{(absorbance\ of\ blank)} x100 \quad \text{----------} \quad 2$$

The IC_{50} value (the concentration of the essential oil required to scavenge 50% of DPPH free radicals), had determined from the calibration curve of the free radical scavenging activity % versus the oil extract concentrations.

Reducing power ability

The method described by Ekin *et al*.[17] was used to estimate the reducing power ability of *C. incana* oil extract. Briefly, 1mL of each concentration (50-1000 µg/mL) of oil extract was combined with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. After incubating at 50°C for 20 min, the reaction mixture was rapidly cooled, mixed with 2.5 mL of 10% trichloroacetic acid (TCA), and centrifuged at 3000 rpm for 10 min. Following that, 2.5 mL of supernatant was taken and thoroughly mixed with 2.5 mL of D. H_2O and 0.5 mL of 0.1% ferric chloride before allowing to stand for 10 min. The absorbance of the mixture was measured at 700 nm against the blank (Phosphate buffer at pH 6.6) by a visible/UV spectrophotometer. The experiment was performed in a triplicate, and the findings were denoted as mean ± standard deviations. Further, the reducing power of the *C. incana* oil extract was compared with the ascorbic acid (standard).

Cell culture

The following cells (MCF-7: mammary gland carcinoma; T47D: human ductal breast epithelial cancer; Caco-2: colorectal adenocarcinoma; MRC-5: normal human fibroblast) were cultured in

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Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 5 mL of penicillin-streptomycin (100 µg/mL), and 5mL of 1% L-glutamine (2 mM). Then they were incubated in 25 cm^2 culture flasks at 37°C in a 5% CO_2 atmosphere with 95% humidity. Moreover, the cells were washed in a triplicate with phosphate buffer saline (PBS) and harvested by the trypsin-EDTA solution after it reached 75-80% of confluency. The cells were then re-suspended in a 3 mL growth medium to get a single-cell suspension. The trypan blue dye exclusion assay was used to count the number of viable cells.[18]

Cell viability assay (MTT)

MTT is water-soluble tetrazolium salt (yellow color), and it can be reduced by mitochondrial dehydrogenase in metabolically active cells to generate purple lipophilic formazan crystal, which can be quantified upon solubilization by spectrophotometer. The total amount of formazan crystals formed is directly proportional to the number of viable cells in the culture.[19] The cytotoxic activity of *C. incana* oil extract on the tested cell lines was assessed by MTT colorimetric assay (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay) according to the procedure described by Bustanji *et al*.[18] Briefly, a total of $1x10^4$ cells per well were seeded in a 96-well plate and kept at 37°C, 5% CO_2 humidified incubator for 24 h, before the addition of treatments. Then the cells were treated with 100 µL escalating doses of the oil extract (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL). After 72 h, MTT was typically prepared in phosphate-buffered saline at 5 mg/mL, then added 20 µL directly to cells in culture and incubated for three hours. After that, the supernatant containing MTT was removed carefully, and 200 µL DMSO (dimethyl sulfoxide) per well was added to dissolve the formed formazan crystals. The absorbance was measured at 570 nm and 630 nm using a microplate (ELISA) reader, and the difference between readings was used for the analysis of results. Doxorubicin (0.05-100 µg/mL) was used as a positive control. Negative control wells (medium only), untreated cells were used as assay control (cells with 0.01% DMSO, culture medium). The mean absorbance of medium control was blanked and was subtracted. The absorbance of assay control cells was taken as 100% viability, and the values of treated cells were calculated as a percentage of control. Equation (3) was applied to calculate the percentage of cell viability.

$$Cell\ viability\ (\%) = \frac{absorbance\ of\ the\ sample}{absorbance\ of\ control} x100 \quad \text{----------} \quad 3$$

The relation between the percentage of cell viability and extract concentration was schemed to get the viability curve of the cancer cell lines with the oil extract. The IC_{50} value was detected as a *C. incana* oil extract concentration, which displayed 50% inhibition of growth on any tested cell line.

Statistical analysis

All assays were performed in three replicates, and the data were presented as mean± standard deviation. The IC_{50} values for different assays were calculated from the linear regression analysis. The comparison between IC_{50} of oil extract against the cancerous cell lines versus the IC_{50} of the non-cancerous cell line was evaluated using one-way ANOVA followed by Dunnett's multiple comparison test using GraphPad In-Stat software (GraphPad Software, San Diego, CA, USA). The results were considered to be statistically significant at $p < 0.05$.

Results and Discussion

Oil extraction and phytochemical analysis

The percentage yield of the oil extract obtained from dried aerial parts of *C. incana* using hydrodistillation for extraction was 0.50% (w/w). The chemical composition of *C. incana* essential oil was determined using gas chromatography coupled to mass spectrometry (GC/MS). A total of thirty-one compounds comprising 99.85% of the oil was characterized in the aerial parts. Benzenamine,4-methyl-3-nitro (34.11%), (2S,4R)-p-Mentha-6,8-diene 2-hydroperoxide (31.48%), cis- piperitone oxide (7.72%), menthone (4.05%), and azulene (3.57%) were identified as the major components of the oil. The composition of the oil is reported in Table 1. The non-terpenoid aromatic compounds represented the highest essential oil components (78.76%). Regards to the terpenes, monoterpenes accounted for 10.88%. However, oxygenated diterpenes were the least components of the essential oil (Table 1).

Considerable variability was noted in the chemical composition and the biological properties of *C. incana* essential oil. The essential oil composition can vary from one plant to another, even in the same species. These variations accompany several aspects that may critically influence the yield, composition, and biological activities of the essential oils, such as genetic background, environmental conditions, habitat, developmental stage, time of harvesting, and extraction methods.[20] The phytochemical profile of the essential oil isolated from the aerial parts of *C. incana* (Sm.) Boiss found in Turkey showed that the significant constituents included oxygenated monoterpenes, trans-piperitone oxide (41.37%), piperitenone oxide (34.47%), piperitenone (6.67%), and monoterpene phenol thymol (3.37%).[10] Also, a study was conducted on the same species in 1995 by Tümen and his colleagues. They identified thirty-one compounds that represented 94.75% of the oil. The main constituents were piperitenone oxide (66.60%), limonene (6.22%), and piperitone oxide (5.91%).[21] Regards to the other species of *Calamintha*, *C. officinalis* Moench essential oil, about thirty-four components had been identified, where the major constituents of this oil were β-bisabolene (9.9%), germacrene D (7.6%), β-bourbonene (7.4%), and piperitenone (5.3%).[22] The *C. nepeta* oil collected from different regions in Italia, pulegone, piperitenone oxide, and piperitenone were the main components (64.4–39.9%; 2.5–19.1%; 6.4–7.7%). In contrast, the oil extracted from Portuguese *C. nepeta* is predominantly composed of isomenthone (35.8–51.3%), 1,8-cineole (21.1–21.4%), and trans-isopulegone (7.8–6.0%).[23]

Determination of Total phenolic Content (TPC) and Total Flavonoid Content (TFC)

Plants hold thousands of phytochemical compounds, such as phenolic acid and flavonoid components. The effect of these phytochemicals is currently of great interest due to their antioxidative and possible anticarcinogenic activities. Also, phenols and flavonoids function as free radical scavengers, reducing agents, and singlet oxygen quenchers, as well as their metal-chelating abilities. The phenolics or polyphenols are significant plant secondary metabolites because of their antioxidant activities by chelating redox-active metal ions, halting lipid-free radical chains, and revoking the hydroperoxide conversions into reactive oxyradicals.[24] The result was obtained from a calibration curve ($y = 0.0062 x + 0.0807$, $R^2 = 0.9984$) of gallic acid (0–120 µg/mL) (Figure 1) and expressed in mg gallic acid equivalents (GAE) per gram oil extract weight. The content of the phenolic compounds in the oil extract of *C. incana* was 24.33 ± 0.034 mg GAE/ g of oil extract. Furthermore, the rich flavonoid plants are an excellent antioxidant source that helps increase the overall antioxidant power of an organism and protect it against lipid peroxidation.[25] The total amount of phenols (TPC) and flavonoids (TFC) of oil extract for *C. incana* was calculated. In the current study, the total flavonoid content (TFC) of the oil extract was1.03 ± 0.002 mg QE/g oil extract, and it was computed using the calibration curve of quercetin, $y = 0.0268x + 0.0397$, $R^2 = 0.999$ (Figure 2). The content of total flavonoids was represented as mg quercetin equivalent QE/ g of oil extract. The value is the mean of three analyses ± standard deviation.

The essential oil from *C. nepeta* exhibited lower phenol content (3.36±0.23 mg GAE/g oil) than *C. incana* essential oil (24.33±0.034 mg GAE/g oil).[26] The total phenol and flavonoid contents of methanolic extract of *C. origanifolia* were 199.90 ±0.03 mg GAE/L, 26.50 ± 0.07 mg QE/L, respectively.[27] In the methanolic extract of *C. vulgaris*, was contained a significant amount of phenols 39.41±0.18 mg/g (GAE/g) and flavonoid 12.03 ± 0.23 mg/g (QE/g).[28]

The total phenol and flavonoid contents of methanolic extract of *C. origanifolia* were 199.90 ±0.03 mg GAE/L, 26.50 ± 0.07 mg QE/L, respectively.[27] In the methanolic extract of *C. vulgaris* was contained a significant amount of phenols 39.41±0.18 mg/g (GAE/g) and flavonoid 12.03 ± 0.23 mg/g (QE/g).[28]

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Table 1: The chemical composition of the essential oil of *Calamintha incana*

RI^{exp}	RI^{lit}	Compound	Peak area %	IM
993.04	988	3-Octanol	0.12	MS
1024.23	1024	dI-Limonene	0.60	MS
1026.58	1026	1,8-cineole	0.57	MS
1138.31	1122	3-cyclopentene-1-acetaldehyde, 2,2,3-trimethyl	0.22	MS
1159.16	1148	Menthone	5.51	MS
1162.23	1165	Borneol	0.76	MS
1170.15	1167-1177	Isopulegone	0.30	MS
1237.49	1233-1243	Benzenamine, 4-methyl-3-nitro	34.11	MS
1250.02	1250-1254	Cis- piperitone oxide	7.72	MS
1257.30	1366	Piperitone oxide	0.14	MS
1265.91	1266	(+)-Isopiperitenone	0.72	MS
1280.78		Unknown	0.15	
1294.16	1289	Thymol	0.66	MS
1303.41	1298	Carvacrol	0.13	MS
1334.62		Benzenaminium,3-hydroxy-n,n,n-trimethyl,hydroxide, inner salt	1.23	MS
1341.74	1362-1392	Phenol, 2-methoxy-3-(2-propenyl)	0.19	MS
1365.40	1365-1381	(2S,4R)-p-Mentha-6,8-diene 2-hydroperoxide	31.48	RRI
1394.27		Cinerolon	0.18	MS
1412.57	1407	Longifolene	2.88	MS, RRI
1446.33	1452	Humulene<alpha-> db5-1528	0.31	MS, RRI
1455.77	1493	Muurola-14 (14), 5-diene<trans->	0.14	MS, RRI
1473.84	1374	(-)-Isoledene	1.26	MS, RRI
1489.68	1475	Azulene	3.57	MS, RRI
1514.65	1521	Calamenene< trans->	0.17	MS
1570.04	1577	Spathulenol	1.23	MS, RRI
1574.26	1668	14-Hydroxy-9-epi-(E)-caryophyllene	0.34	MS, RRI
1583.77	1439	Aromadendrene	0.23	MS
1630.77	1578-1584	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar (1aα,4aα,7β,7aβ,7bα)]-	0.20	MS
1944.82	1977	Bifloratriene	4.05	MS
	2106	Bicyclo[9.3.1]pentadeca-3,7-dien-12-ol,4,8,12,15,15-pentamethyl-,[1R-(1R*,3E,7E,11R*,12R*)]-	0.15	MS
	1728	(Z)-γ-Curcumen-12-ol	0.68	
Monoterpenes (MT)	10.88			
Hydrocarbons MT	1.47			
Oxygenated MT	9.41			
Sesquiterpenes (ST	6.01			

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Hydrocarbons ST	4.99
Oxygenated ST	1.02
Diterpenes (DT)	4.20
Hydrocarbons DT	4.05
Oxygenated DT	0.15
Non-terpenoid non-aromatic compounds	0
Non-terpenoid aromatic compounds	78.76
Total identified %	99.85

The numbers in bold represent the major components of CIEO.
RI ^{exp}: retention indices calculated against n-alkanes (C_8-C_{20}) on DB-5 capillary column.
RI ^{lit} retention indices for the volatile compounds from literature.
Peak area percentage was determined by calculating the peak area of the FID chromatogram in GC analysis.
IM: Identification method based on the retention indices (RI) of authentic compounds on the DB-5 capillary column; MS, identified based on computer matching of the mass spectra with those on database NIST, Wiley, and Adam's libraries and comparison with literature data.

Antioxidant activity

DPPH free radical scavenging activity

Antioxidants are significant materials that possess the power to protect the body from damage caused by free radical-induced oxidative stress. α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging assay is one of the most common methods for evaluating the potential antioxidant extract or other biological sources. Oil extract's free radical scavenging activity was analyzed using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay when DPPH (free radical) reacts with antioxidant compounds that can give hydrogen to the DPPH and reduced it to a more stable form. This reaction is triggering a transformation in color (from deep purple to light yellow). The absorbance was read at 517 nm. The result showed that the oil extract and positive control (ascorbic acid) had free radical scavenging activity in a dose-dependent manner. The data revealed that the inhibition of the DPPH radical scavenging assay of the essential oil (IC_{50} was 15.38 mg/mL) was lower than the ascorbic acid with IC_{50} of 21.24 µg/mL as illustrated in (Figure 3).

Reducing power ability

The reducing power ability is based on the principle that antioxidant compound which has reduction potential, react with potassium ferricyanide ($K_3Fe^{3+}(CN)_6$) to form potassium ferrocyanide ($K_4Fe^{2+}(CN)_6$), which then reacts with ferric chloride (yellow color) to create a ferric-ferrous complex (green or blue color) depending on the reducing power of the sample.[17] The reducing power potential for *C. incana* oil extract compared with ascorbic acid at 700 nm is elucidated (Figure 4). The data showed that the oil extract had antioxidant activity in a dose-dependent manner; reducing potential was enhanced with increasing concentration of oil extract. At (1 mg/mL) concentration, the reducing power activity of *C. incana* oil was 0.154 ± 0.003 compared to the ascorbic acid standard, which gave a reducing ability of 2.657±0.016. The EC_{50} value defines the concentration of the sample, which is required to reduce 50% of Fe^{+3} ions at 700 nm. A lower EC_{50} means higher reducing power and antioxidant activity. The EC_{50} value of the oil extract (9787.5 µg/mL) was significantly higher than ascorbic acid (90.63 µg/mL).

Compared to the other studies, the total antioxidant potential of *C. incana* essential oil from Turkey was examined using DPPH radical scavenging assay with IC_{50} 19.28 ± 0.74 mg Trolox Equivalents/g oil and FRAP assay was 53.63 ± 0.10 mg Trolox Equivalents/g oil.[10] Viewed to the oil extract from *C. origanifolia*, DPPH data revealed that oil extract had the highest radical scavenging activity (0.05 ± 0.01 mmol TE/L) in comparison to methanolic and ethanolic extracts; even higher than that of the positive control (BHT), used at the same dose. The reducing power capacity against the ferric ion for *C. origanifolia* oil extract (0.87 ± 0.06 mmol TE/L), and the hexane extract (0.73 ± 0.06 mmol TE/L), while in the chloroform extract (0.48 ± 0.06 mmol TE/L).[27]

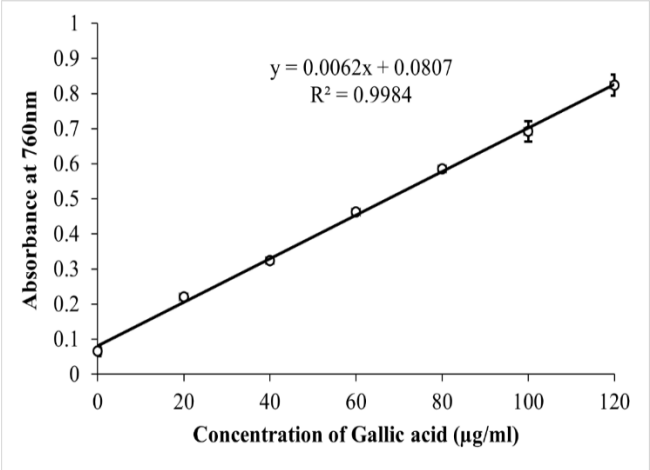

Figure 1: Standard calibration curve of Gallic acid for estimating total phenolic content in *C. incana* oil extract. Values are mean of three replicates ±SD. The regression equation was obtained by plotting the absorbance values at 760 nm against the concentration of Gallic acid (0-120 µg/mL).

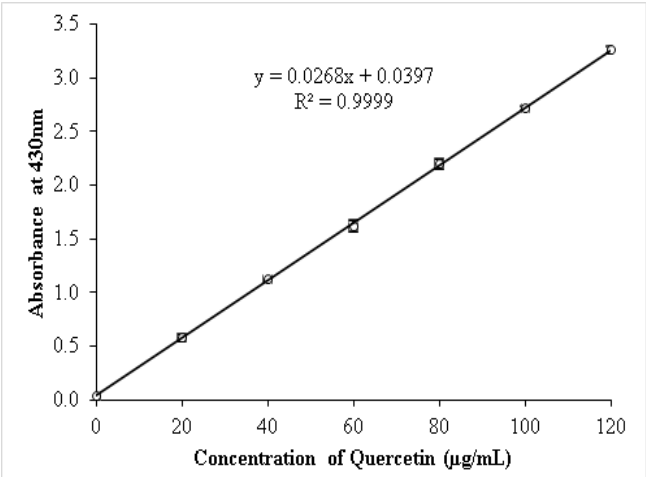

Figure 2: Standard calibration curve of Quercetin for estimating total flavonoid content in *C. incana* oil extract. Values are mean of three replicates ±SD. The regression equation was obtained by plotting the absorbance values at 430 nm against the concentration of Quercetin (0-120µg/mL).

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In vitro cytotoxic activity of C. incana oil extract

The cytotoxic effect of essential oil from *C. incana* against MCF-7, T47D, Caco-2, and MRC-5 cell lines was evaluated by (MTT) assay. The results show that the *C. incana* oil extract exhibited cytotoxic activity against the tested cell lines in a dose-dependent pattern. At 200 µg/mL extract concentration, the percentage of growth inhibition in both Caco-2 and T47D (100%) while in MCF-7 (79.84%) compared to non-treated cells (control). Concerning the IC_{50} values of the oil extract against the tested cell lines, the Caco-2 cell line was the lowest value, followed by the MCF-7 cell line, and the highest value was noticed in the T47D cell line. The data showed that *C. incana* oil extract exerted a significant cytotoxic effect on the Caco-2 cell line and no significant inhibition in both breast cancer cell lines (MCF-7, T47D). The cytotoxicity was also noticed on a normal fibroblast cell line. The results were demonstrated in Table 2.

Although *C. incana* oil extract can inhibit cancer cells' proliferation at low concentrations (200 µg/mL), it also inhibits human nonmalignant cells' proliferation at similar concentrations. The nonspecific activity explains that this essential oil is a complex compound and may have synergistic effects. In this case, more than one chemical component in this oil may be contributed to the cytotoxic activity. Therefore, further studies are required to reveal whether the oil candidate inhibits cancer cell growth selectively.

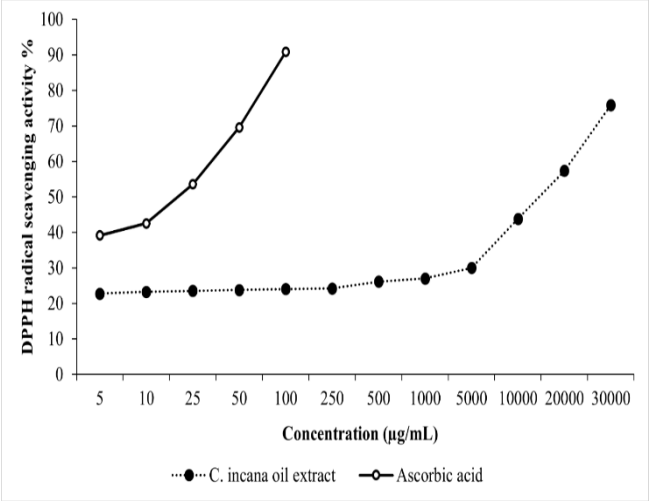

Figure 3: Comparative DPPH radical scavenging activity of oil extract of *C. incana* and ascorbic acid (standard).

Table 2: IC_{50} values (µg/mL) of *C. incana* oil extract, in comparison to the positive control (doxorubicin) against the tested cancer cell lines and healthy fibroblast cells. Values are mean of three replicates ± SD

	MCF-7	T47D	Caco-2	MRC-5
C. incana oil extract	51.57 ± 1.67	106.78 ± 5.1	33.05 ± 3.45[a]	58.48 ± 6.54[a]
Doxorubicin	0.146 ± 0.008[a]	0.125 ± 0.005[a]	0.059 ± 0.017[a]	0.932 ± 0.048[a]

[a] *p*-value < 0.05

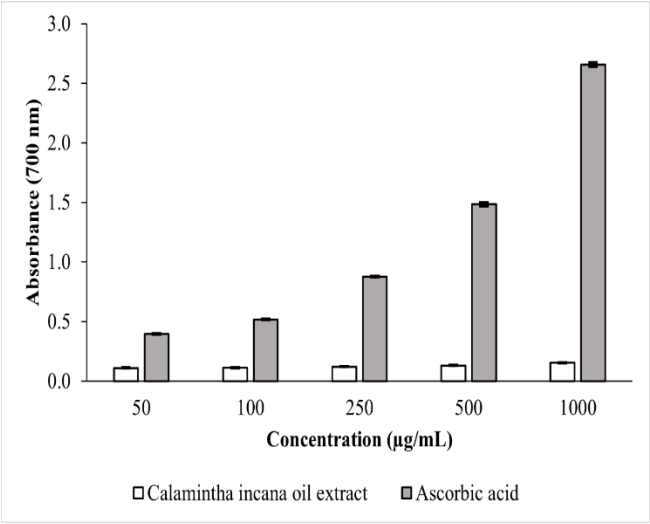

Figure 4: Comparative reducing power ability of *C. incana* oil extract at different concentrations at 50-1000 µg/mL compared with ascorbic acid (standard).

Conclusion

For the first time, the essential oil composition and chemical profile of *C. incana*, which grows wild in Jordan, were studied. The oxygenated diterpenes were minor components of the essential oil, while the non-terpenoid aromatic compounds were the most abundant. The phenolic content of this essential oil, on the other hand, was higher than the flavonoid content. Furthermore, the findings revealed that *C. incana* essential oil has good antioxidant properties. Additionally, at a concentration of 200 g/mL concentration, the oil extract of *C. incana* demonstrated cytotoxicity on both cancerous and non-cancerous cell lines. As a result, more research is needed to understand the action of the chemical compounds in the tested oil and their pharmacological effects.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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