Chemical composition and evaluation of cytotoxic activity of *Achillea falcata* essential oil on HeLa and Caco-2 human cancer cell lines

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Abstract

Plants and their products, e.g. essential oils are mostly considered as safe medicine products. In recent years, medicinal plants have used as natural products that contribute to the prevention and treatment of disease. Medicinal plants play a crucial role in traditional medicine and in the maintenance of human health worldwide. The majority of *Achillea* species include highly bioactive compounds, so they have therapeutic applications. In the present study, the aim was to investigate *in vitro* anti-oxidant and cytotoxic effects of *Achillea falcata* essential oil. The essential oil was extracted from aerial parts using Cleverenger method and its components were determined using GC-MS analysis. The anti-oxidant potential of the essential oil was analyzed by DPPH and total phenolic content methods. MTT assay was used to detect cytotoxicity of the essential oil on human cervical cancer cells HeLa and colon cancer cells Caco-2. The results showed that essential oil exhibited a low DPPH scavenging activity, and 32.467 Gallic acid equivalents/g plant oil of total phenolic content. As concentration was increased, the essential oil exhibited a more powerful cytotoxic effect on HeLa and Caco-2 cancer cell lines indicating that *Achillea falcata* essential oil might include anti-cancer compounds triggering the cytotoxicity on HeLa and Caco-2 cancer cells.

Introduction

In the past few years, interest in the antioxidant and radical scavenging properties of medicinal herbs and their extracts has risen enormously. In fact, antioxidants contained in plants seem to be involved in the preservation of human health, e.g. by preventing cancer, slowing down the aging process and reducing the risk of cardiovascular and neurodegenerative diseases. Oxidative damage is a hallmark of all these physiological pathological conditions and plants, providing an exogenous source of antioxidants, may aid the natural defence systems of cells [1-3]. Therefore, the assessment of antioxidant and radical scavenging properties of traditionally widely used plants and plant extracts is an important issue in the quest both of new sources of natural antioxidants for functional foods, nutraceuticals [4,5] and of feasible and ‘natural’ alternatives to synthetic antioxidants in the food industry, since food-preserving compounds are being restricted due to their inherent risk of carcinogenicity [6]. *Achillea*, one of the most important genera of the Compositae (Asteraceae) family, comprises more than 100 species around the world, mainly distributed in Europe, Asia and North Africa [7]. In particular, *Achillea falcata* has been reported to have beneficial effects on internal hemorrhage, uterine hemorrhoid, stomach ailment, gastritis, and bladder stones [8]. In recent years, some pharmacological properties of this species were scientifically addressed. It has been demonstrated that some *A. falcata* sesquiterpene lactones are cytotoxic agents [9-12], its extracts/essential oil have antimicrobial, antioxidant and antiplatelet properties [8,13-16]. Botanical drugs based on or including *A. falcata* are regarded as completely safe for human use and non-toxic [8,17]. However, phytochemical, data regarding *A. falcata*, and the studies using the essential oil of *A. falcata* on cancer cell lines, are quite scarce [18,19]. The aim of this study was to evaluate antioxidant capacity and to investigate the effects of different concentrations of essential oil obtained from *A. falcata* on HeLa and Caco-2 cells and to assess the relationship with their total phenolic content, in the present study both chemical (free radical scavenging activity by DPPH) and MTT assay (cell viability of Hela and Caco-2 cell lines) were used.

Material and methods

Plant material

The aerial parts of the *A. falcata* plants was collected from the Karm Al-Maasara area in Lattakia in July 2016, and it was classified by plant taxonomist Dr. Emad Al-Qadi, Assistant Professor in the Department of Plant Biology at Damascus University. The plant was dried at room temperature during 1-week and then ground with an electric mill. The dried powdered material was stored at + 4°C.

Essential-oil extraction

Essential oil was extracted from the plant samples using a Clevenger-type apparatus where the plant material is subjected to hydro distillation. Conditions of extraction were 100 g of samples, 1:5 plant material/water volume ratio, and a 2.5 hrs distillation. The oil was dehydrated with anhydrous sodium sulphate and immediately stored in airtight glassware in a refrigerator at + 4°C.

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GC-MS analyses
The essential oil was analyzed using a device chromatograph with a data-handling system. A Phenyl Methyl Silox HP-5MS% column (0.25 μm × 250 μm × 30 m) was used, with he as carrier gas (1 ml/min). The operating conditions were: injector and detector temperature, 260°C and 280°C, respectively; oven temperature program was started at 60°C, then it goes up 4°C/min to 200°C, subsequently rising at 8°C/min to 260°C, then held isothermally at 260°C for 7.5 min; injection mode, splitless (1 μl:1000 n-pentane solution). Linear retention indices were determined in relation to a homologous series of n-alkanes (C8–C22) under the same operating conditions. GC-MS analyses were performed employing the same chromatographic conditions as described above, using Agilent 7890 A.

Antioxidant activity
Evaluation of total phenolic compounds: The amount of total phenolics in the essential oil was determined with the Folin-Ciocalteu method [20], using gallic acid as a standard. Distilled water (450 µL) was combined and vortexed with 50 µL of sample and 250 mL of Folin-Ciocalteu's reagent. Then 2 mL of Na2CO3 (20%) was added, the mixture was vortexed. The absorbance of all samples was measured at 765 nm using a UV/VIS spectrophotomete after incubation at 40°C for 30 min. Quantification was done on the basis of the standard curve of gallic acid (solution of gallic acid 80% MeOH, 0.25–5 μg/mL). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight. Measurements were taken in triplicate and mean values calculated.

Radical scavenging properties assessment (DPPH assay): Antioxidant capacity was determined with DPPH reagent according to the method described by Mensor et al. [21]. The essential oil was diluted with ethanol to four different increasing concentrations between 0.2 and 2 mg/mL. The DPPH concentration in ethanol was 45 μg/mL, 0.3 mL of oil solution (sample) or ethanol (control) was mixed with 1 mL of DPPH and the absorption was recorded after 20 min in the dark, at 515 nm. Vitamin C was used as the referring substances. The following equation was used to calculate concentration of the DPPH radicals:

\[ \text{DPPH scavenging activity} \% = (A_0 - A_1)/A_0 \times 100\% \]

where, A0 is the absorbance of the control and A1 is the absorbance of the reaction mixture or standards. The values have been expressed as the mean of three replications.

Cell line and culture condition
HeLa (human cervical cancer cells) were Obtained from the Human cell culture laboratory in Atomic Energy Commission of Syria. MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma Chemical Co. The cells were cultured in RPMI medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were kept at 37°C in a humidified atmosphere containing 5% CO2. HeLa and Caco-2 cells were treated with different doses (0, 10, 15, 25, and 50 μg/mL) of A. falcata, and the Hela cultured cells were treated at two different time intervals (24 and 48 hrs). In addition, the DMSO alone was added to another set of cells as the solvent control (DMSO). The cells were then incubated for another 24 hrs prior to the addition of 20 μL of 2 mg/mL solution of MTT into each well. The incubation was continued for another 3 hrs before the media were removed. A mixture of DMSO (150 μL) was added to each well and mixed to ensure dissolving of the formazan crystals before the absorbance at 540 nm was measured. Each experiment was performed in triplicate and the 50% Hela cells inhibitory concentration (IC50) of the oil was calculated. The cell viability ratio was calculated by the following formula:

\[ \text{Inhibitory ratio} \% = (\text{OD}/\text{optical density contro} - \text{OD treated})/\text{OD control} \times 100\% \]

Cytotoxicity was expressed as the concentration of oil inhibiting cell growth by 50% (IC50 value).

Statistical study
The experiments were performed in triplicate. Statistical correlation of data was checked for significance by ANOVA and Student’s t-test. A p value<0.05 was considered to indicate a statistically significant difference.

Results
Yield and Isolation of the essential oil
The yields of the oils obtained from the aerial parts of A. falcata (isolated after hydro distillation for 2.5 hrs from 100 g plant) was 0.367% (w/w), and the density of the essential oil was 0,92 gr.cm⁻³. The oil was limpid yellow and of agreeable smell.

GC-MS analyses
The GC-MS analysis of the essential oil obtained from the aerial parts of A. falcata allowed the identification of 23 compounds (Table 1), representing 92.902% of the oil. The oil was characterized by a high content of oxygen-containing compounds. The main components of the oil were Alpha Thujon (32.082%), trans-2,7-Dimethyl-4,6-octadien-2-ol (11.387%), Artemisia ketone (11.23%), 3-Thujanone (11.18%), Germacrene-D (8.373%).

Antioxidant activity
Evaluation of total phenols: Total phenols content was 32.467(mg GaEs/g plant oil).

DPPH assay: Antioxidant capacity of infusions from A. falcata oil measured with DPPH assay are shown in Table 2. The results of our DPPH analysis showed that A. falcata oil had low antioxidant activity (IC₅₀=2 mg/mL; at 2 mg/mL the ability to scavenge DPPH was 31.43%.

Cell viability
The anticancer activity of the oil against cancer cells (HeLa and Caco-2) was evaluated by MTT assay. Different concentrations of the oil (0, 10, 15, 25, and 50 μg/mL) were tested against the cancer cells. The cervical cancer cells and colon cancer cells showed substantial dose-dependent susceptibility to the treatment of different concentrations of the oil. The (Table 3) shows Height of cellular death percentage with increasing concentration of oil.

HeLa cells also treated with the oil in different time, at 24 and 48 hrs. The IC₅₀ value after 24 and 48 hrs intervals was 13.687 and 10.325 μg/ml respectively (Table 4).
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### Table 1. Composition of the essential oil from aerial parts of *Achillea falcata* by GC-MS analyses

| #  | Compounds                   | RI  | RT   | %    | Method of identification |
|----|-----------------------------|-----|------|------|--------------------------|
| 1  | 2-methyl-(CAS)              | 862 | 4.776| 0.2094| MS/RI                   |
| 2  | yomogi alcohol              | 1000| 9.5432| 3.6262| MS/RI/ST                |
| 3  | Cymene                      | 1019| 10.514| 0.4286| MS/RI/ST                |
| 4  | 1,8-Cineole                 | 1027| 10.867| 3.0419| MS/RI/ST                |
| 5  | 2-Methyl-2,4-hexadiene      | 1036| 11.3626| 2.6074| MS/RI                   |
| 6  | Artemisia ketone            | 1063| 12.699| 12.573| MS/RI/ST                |
| 7  | Artemisia alcohol           | 1092| 14.1549| 0.6155| MS/RI/ST                |
| 8  | alpha.-Thujon               | 1114| 15.367| 35.9224| MS/RI/ST               |
| 9  | Ethyl 3-hydroxy hexanoate   | 1120| 15.836| 12.3931| MS/RI                  |
| 10 | L-trans-Pinocarveol         | 1136| 16.664| 0.2502| MS/RI                   |
| 11 | Nerol                       | 1148| 17.357| 0.2573| MS/RI/ST                |
| 12 | 2,7-dimethyl-2,6-octadien-4-ol| 1174| 18.891| 1.8989| MS/RI                   |
| 13 | p-Cymen-2-ol                | 1183| 19.4411| 0.2153| MS/RI                   |
| 14 | Propanal, 2-methyl-3-phenyl- | 1236| 22.6114| 0.2434| MS                      |
| 15 | (3RS,4SR)-2,5-Dimethyl-4-vinyl-5-hexene-1,4-diol | 1244| 23.1544| 1.1808| MS          |
| 16 | Lavandulol, acetate         | 1292| 26.0939| 0.6132| MS/RI/ST                |
| 17 | Carvacrol (CAS)             | 1303| 26.772| 0.4239| MS/RI/ST                |
| 18 | 1,5,5-Trimethyl-6-methylene-cyclohexene | 1332| 28.544| 0.4007| MS                      |
| 19 | alpha.-Cubebene             | 1369| 30.852| 0.2351| MS/RI                   |
| 20 | Jasmone                     | 1396| 32.5902| 0.2831| MS/RI                   |
| 21 | Methyl eugenol              | 1405| 33.025| 0.304| MS/RI                   |
| 22 | Germacrene D                | 1411| 33.432| 0.4979| MS/ST                   |
| 23 | beta.-Cubebene              | 1421| 34.029| 0.1724| MS                      |
| 24 | GERMACRENE-D                | 1477| 37.734| 9.2307| MS/RI/ST                |
| 25 | trans.-beta.-Farnesene      | 1479| 37.498| 0.483| MS/RI                   |
| 26 | Bicyclogermacrene           | 1489| 38.103| 1.2591| MS/ST                   |
| 27 | beta.-Sequiphellandrene (CAS)| 1522| 40.092| 3.6455| MS/RI                   |
| 28 | 1,5-epoxysalvial-4(14)-ene  | 1559| 42.4477| 0.3332| MS/RI                   |
| 29 | Spathulenol                 | 1572| 43.2013| 1.4255| MS/RI/ST                |
| 30 | 3-Phenylpropionic acid, 2-methylbutyl ester | 1599| 44.9188| 0.2686| MS/RI                   |
| 31 | beta.-Eudesmo               | 1643| 47.2473| 0.8102| MS/RI/ST                |
| 32 | Methyl jasmonate            | 1646| 47.444| 0.2655| MS/RI                   |
| 33 | 2,6,11,15-Tetramethyl-hexadeca-2,6,8,10,14-pentaene | 1841| 56.561| 1.0348| MS/ST                   |

RI: Retention index identical to bibliography; MS: Identification based on comparison of mass spectra; ST: Standard time retention time identical to authentic compounds.

### Table 2. Concentrations of the essential oil from aerial parts of *Achillea falcata* by DPPH analysis

| Concentration mg/ml | 0.2 | 1 | 1.5 | 2 |
|---------------------|-----|---|-----|---|
| DPPH%               | 11.13 | 20.495 | 27.159 | 31.43 |
| C. Vitamin 39:24    |       |     |     |     |

### Table 3. The cellular death ratios of HeLa and Caco-2 cells treated with graded concentrations of essential oil

| Concentration mg/ml | 0.2 | 1 | 1.5 | 2 |
|---------------------|-----|---|-----|---|
| DPPH%               |     |   |     |   |
| C. Vitamin 39:24    |     |   |     |   |

### Table 4. The cellular death ratios of HeLa cells treated at 24 and 48hrs with essential oil

| Concentration mg/ml | 24hrs | 48hrs |
|---------------------|-------|-------|
| DPPH%               |       |       |
| C. Vitamin 39:24    |       |       |

### Statistical study

The cervical cancer and colon cancer cells showed substantial dose-dependent susceptibility to the treatment of different concentrations of the oil, and the Statistical study showed that *A. falcata* oil affects HeLa cells more than Caco-2 cells, The HeLa cells did not showed time-dependent susceptibility to the treatment of the oil at different times.

### Discussion

Many of the *Achillea* species are widely used in traditional medicine of several cultures due to many therapeutic properties, such as antioxidant, antispasmodic, anti-inflammatory, antimicrobial, stomachic, emmenagogue and antiseptic [14,15]. Contemporary studies have revealed that many *Achillea* species possess antioxidant and anticancer properties as well [16,17]. In particular, *A. falcata* has been described to have useful effects on internal hemorrhages, stomach ailments, gastritis, and bladder stones [18]. Many other reports have shown anti-proliferative activity of isolated constituents from *A. falcata* [19-21]. Infusion of *A. falcata* has been proved to possess antioxidant activities [22]. Phytochemically, *A. falcata* has been reported to contain a diversity of chemical constituents, most of them being volatile organic compounds present in its essential oil. The various chemical constituents that have been reported in *A. falcata* are monoterpenoids.
like cineole, camphor and borned 92.902%. Flavonoids have also been reported from the plant. Sesquiterpene lactones including 3-β-methoxy-iso-seco-tanapartholide (β-tan) which exhibit potent antitumor properties have also been reported from A. falcata. β-tan which was purified from A. falcata, differentially inhibited the growth of the epidermal human HaCaT cells at non-cytotoxic concentrations to primary epidermal keratinocytes. However, there are no reports on the Anti-oxidant properties of an A. falcata oil, and nor is there any report on the antitumor effect of the oil of A. falcata on cervical cancer and colon cancer cells. Therefore, our aim was to evaluate the antioxidant properties of the oil extract of A. falcata with demonstrating its effect on HeLa human cervical cancer cells and Caco-2 human colon cancer cells. In our study antioxidant activities for A. falcata essential oil was studied by two methods, the first one was evaluation of total phenols which was (32.467 mg GaEs/g), and the second method was by DPPH analysis (IC50 >2 mg/mL), and the results of these methods showed that A. falcata oil had low antioxidant activity.

The GC–MS analyses of the essential oil obtained from the aerial parts of A. falcata allowed the identification of 23 compounds (Table 1), representing 92.902% of the oil. The oil was characterized by a high content of oxygen-containing compounds. The main components of the oil were Alpha Thujon (32.082%), trans-2,7-Dimethyl-4,6-octadien-2-ol (11.387%), Artemisia Ketone (11.23%), 3-Thuajanone (11.18%). These compounds were reported in the other studies of the aerial parts of the A. falcata oil, but In different concentrations, Where the Alpha Thujon compound was the most abundant in our study, and his concentrate was reached to 35.9224%, followed him Artemisia Ketone compound and his concentrate was 12.573%, and This is different from their concentration in the oils obtained from A. falcata extracted from plants grown in Jab. Kneisse (Lebanon), which Alpha Thujon was (3.0%), and Artemisia Ketone (5.2%), [16] and the Ceneole 1,8 compound which was the main compound in the A. falcata oil extracted from Antalya (Turkey) (14-24%) [22] which that in our oil are present in lower concentration (3.0419%). These differences may be attributed to the various environmental factors as different geographical, the nature of the soil, Climate difference, or harvest time, In addition to the genetic factor.

Cell viability was assayed after 24 h of treatment for Caco-2 cells,24 and 48 h for HeLa cells, using MTT assay, which the method measures the ability of metabolically active cells to convert tetrazolium salt into a blue formazan product, the absorbance of which is recorded at 540 nm using an ELISA microplate reader. Viability results were showed the oil exhibited one dose-dependent growth inhibitory, and don't time-dependent effects on the HeLa cancer cells, and a variation in the effect of oil on the HeLa and Caco-2 cell lines. The IC50 on HeLa cells was 13.687 µg/ml, while the value of IC50 on Caco-2 cells was not reached, even with the highest concentration used in this study which was 50 µg/ml, so that need to additional experiments using higher concentrations, or prolonging the treatment time with this oil to accurately determine this value. In general, may be the different effect of the oil on the different cell lines due to the receptors sensitivity of the cells types to the same oil components, or to the ability of the different oil components to activate different signaling pathway by the cell type.

Conclusion

In conclusion, our study provides the evidence that the oil of A. falcata has antioxidant and antitumor properties, whereas, previous studies extracted specific compounds from A. falcata essential oil and studied their effect many fields, and the present investigation using the achillea falcata oil supported the traditional use of this plant in the Syrian folk medicine.

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None.

Conflict of interest

Authors declare nothing to disclose.

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