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GC/MS Profiling, *In-Vitro* Cytotoxic and Antioxidant Potential of the Essential Oil of *Pulicaria Crispa* (Forssk) Growing in Egypt

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Research Article

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Abstract

The essential oil EO obtained by hydrodistillation from the aerial parts of *Pulicaria crispa* was profiled using GS/MS analysis. The oil was found to contain carvotanacetone as a major component (81.9%). Biological investigation revealed that the oil possessed EC_{100} value of12.8 µl/ml in the *in-vitro* cytotoxicity assay against peripheral blood mononuclear cells ($PBMC_S$). The oil showed no immune-stimulant activity. The antioxidant potential of the oil was assayed using DPPH radical inhibition resulting in inhibition percentage of 66.19% and trolox equivalent antioxidant capacity (TEAC) of 9.22µg/ml. The results indicate powerful antioxidant capacity of the oil, which makes it a good candidate to be used as a natural preservative or as a source of natural antioxidants to be incorporated in pharmaceutical applications. The cytotoxicity assay of the oil against colorectal"Caco-2" and hepatocellular "HepG-2" carcinoma cell lines showed IC_{50} values of 4.73 and 20.11 µl/ml, respectively. The obtained results spot the light on the colorectal anticancer potential of the oil, which should be subjected to future animal experimental study in form of enema.

Keywords: *Pulicaria Crispa*; GC/MS Analysis; Immune-Stimulant; Antioxidant; Anticancer Activity

Introduction

Herbs containing essential oils (EOs) and their components impressively demonstrate a wide array of potential activities in the fields of therapeutic products, cosmetics, perfumery, food industry and aromatherapy [1]. The genus *Pulicaria* , belongs to the *Inuleae* tribe (Asteraceae), encompasses around 100 species distributed worldwide. Different *Pulicaria* species' have been reported to possesses impressive biological activities and most of tribe species have been reported as traditional medicines [2]. Accordingly, various *Pulicaria* species have been thoroughly investigated phytochemically and biologically. *Pulicaria crispa* (Forrssk), is often synonymous with *Pulicaria undulata, Aster crispus, and Francoeuria crispa*. It is an annual herb locally known in Saudi Arabia as "Gethgath". Leaves are sessile, acute to obtuse, undulate to rarely toothed, Heads are hemispherical, heterogamous, radiate, and golden-yellow to orange, 0.5-1 cm across golden-yellow to orange in colour [3,4]. *P. crispa* is

traditionally used by people of southern Egypt and Saudi Arabia to treat inflammation and as an insect repellent .It is also used as an herbal tea [5]. Also, it was reported to be used in Saudi Arabia for bruises, skin infections and gastrointestinal disturbances [6]. Owing to its strong smelling EO, it has been used since ancient times for treatment of sinusitis and respiratory tract infections in the traditional medicine in the southern part of Iran [7].

Pulicaria crispa is distributed in Saudi Arabia, Kuwait, Iran, Iraq, Egypt, Afghanistan, Pakistan, India and many parts of north and west tropical Africa [8]. Many studies investigated the chemical composition of essential oil extracted from various *Pulicaria* species in the Middle East region [9-12]. However, to the best of our knowledge, the current study is the first one, performed to investigate the antioxidant, immune-stimulant and cytotoxic activities of the essential oil of *P.crispa* cultivated in Egypt.

Materials and Methods

Plant Material

The aerial parts of *Pulicaria crispa* were collected during the flowering stage, in March 2016 from their wild habitat in the western Egyptian desert, El- Sadat city, Al-Menoufia Governorate. The exact location of collection is 30.38182°N, 30.51159°E. The collected aerial parts were air-dried in shade for 7 days, and then pulverized to fine pieces. The plant was kindly verified by Dr. Mohamed El-Azazi; Assistant professor of Plant Ecology in the Environmental Studies and Research Institute, El-Sadat City University. Voucher specimen was kept in the herbarium of Faculty of Pharmacy, Damanhour University as (106P).

Extraction of the Essential Oil

According to the European Pharmacopoeia 2007, the air-dried finely ground aerial parts of *P.crispa* (200 g) were subjected to hydrodistillation for the extraction of its essential oil using a Clevenger- type distillation apparatus for 3h [13].

The collected essential oil was then dried by passing over anhydrous sodium sulphate and kept away from light, at 4^{o}C in a sealed glass vial, till subsequent analysis.

Compositional Analysis of Essential Oil by GC/MS

The essential oil of *P. crispa* was analyzed by HP-5890 Series II gas chromatograph, equipped with a flame ionization detector and a 15 m HP5 column, 0.32 mm ID, 0.25 µm film thicknesses.

GC/MS analysis was performed on Shimadzu GC-MS QP 2010. High purity helium was used as the carrier gas at a constant linear velocity of 43.8 cm/s. The constant flow was controlled by 82.5 KP a pressure giving a total flow of 19.2 ml/min, a column flow of 1.47 ml/min and a purge flow of 3.0 ml/min. The ion source and interface temperatures were 230°C and operated at ionization energy of 70 eV. The QP mass spectrometer was working with mass scan in a range of 40-700 m/z. The used column was Varian capillary column (TR5- CPSIL- 5CB, 5% poly-diphenyl 95% dimethylsiloxane), which is 50 m length, 0.32 mm diameter and 1.25 µm film thickness. Column oven temperature programmed from 40°C to 280°C in 10 min. Essential oil samples (20µl) were diluted with methanol (200µl). The injection volume was 1.0 µl. The split ratio was 10 and the injector temperature was 210°C.

Identification and Quantification of Essential Oil Composition

Identification of components was performed by comparing their relative retention indices (RI) determined with the reference of a homologous series of n-alkanes (C_8 to C_{24}) [14,15]. The fragmentation patterns of the mass spectra were compared with the WILEY (http://eu.wiley.com/WileyCDA/WileyTitle /productCd-04-70047852.html) and NIST 05 (http://www.nist.gov/srd/nist1a.htm) libraries. The linear temperature-programmed RIs of all the constituents were calculated based on the GC through the interpolation between bracketing n-alkanes as follow:

$$RI = 100\,[(t_{R(i)}+t_{R(z)})\times Z\,/\,(t_{R(z+1)} - t_{R(z)}]$$

Where Z was the number of carbon atoms in the smaller n-alkane, and $t_{R(i)}$,$t_{R(Z)}$

And $t_{R(Z\,+\,1)}$ were the retention time of the desired compound [16]. Quantitative data of the oil components were obtained from the electronic integration of the FID peak areas

Preparation of Oil Samples for the Bioactivity Study

Different concentrations of the prepared essential oil (12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.3906 µl/ml) were prepared using DMSO by serial dilution in a 96-well plate; these concentrations were screened for their immune-stimulant and anticancer potential against human

epithelial colorectal adenocarcinoma "Caco-2" and hepatocellular carcinoma "HepG-2" cell lines.

In-vitro Cytotoxicity Assay

In-vitro Cytotoxicity Assay on Normal Lymphocytes (Immune stimulant activity screening): In-vitro cytotoxicity assay was performed to evaluate the viability of normal Human Peripheral Blood Mononuclear cells (PBMCs) after incubation for 72 with the different concentrations of the *P. crispa* essential oil. Viability of the cells was measured using neutral red uptake assay to determine the maximum safe concentration of the oil on normal cells [17].

The results were interpreted to calculate the maximum safe concentration that keeps 100% cell viability (EC_{100}) for the essential oil using SPSS 15.0 for windows [18]. Data output was presented as Stimulation Index, which is the ratio of the optical density of lymphocytes stimulated by the tested material to the optical density of control unstipulated lymphocytes after removal of the effect of the blank. A value higher or equal to 1.5 indicates a significant immune-stimulant activity [19].

Cancer cell lines and culture: Colorectal adenocarcinoma "Caco-2" (ATCC® Number: HTB-37™), and Hepatocellular carcinoma "HepG-2" (ATCC® Number: HTB-8065™) were ***obtained from the American*** type ***culture collection. The cells were maintained at 37 ºC in*** a humidified air incubator containing 5% CO_2***. The cells were grown in RPMI 1640 medium (MP Biomedicals Inc., Irvine, CA, USA) containing 10% foetal bovine serum (FBS, Logan, UT, USA) as well as 0.2% glucose, 2 mM glutamine***. Mitomycin C (MCC) (0.09 µg/ml) was used as a positive anti-cancer drug control (for HepG-2 cell line caused inhibition by 64% and for Caco-2 cell line caused inhibition by 57%).

The inhibitory effects of essential oil of *P.crispa* on the growth of Caco-2 and HepG-2cells were evaluated *in vitro* using neutral red uptake assay [17].

The half maximal inhibitory concentration (IC_{50}) values were determined from software SPSS 15.0 for windows [18].Cell inhibition percentage was calculated from the following equation:

$$\text{Cell inhibition (\%)} = 100 - [(E-B/C-E)] \times 100]$$

Where:
E: The mean absorbance of cells exposed to extract.
B: The mean absorbance of blank control.
C: The mean absorbance of control cells.

Antioxidant Activity of the Essential Oil

The antioxidant capacity of *P.Crispa* EO was determined using a test based on the redox potential of DDPH (Sigma–Aldrich, St. Louis, MO, USA). A 1 mM DPPH solution (0.394 mg/mL) in methanol was prepared and then diluted 1:10 to obtain a 100 µM solution (Abs at 515nm = 0.5-0.6). 500 µl of the sample and 500µl of 100 µM DPPH solution were mixed in a cuvette and a negative control with 500 µl of methanol and 500µl of DPPH solution was also prepared. Both solutions were incubated in the dark at room temperature for 15min. Absorbance was read at λ = 515 nm using methanol as blank.

The results were expressed as the percentage reduction in the radical absorbance [20].
(Abs max (negative control) – (Abs sample+ DPPH) x 100
Abs max

The oil concentrations used for the DPPH assay is chosen based on the cytotoxicity experiment on normal cells; where the maximum safe concentration (EC_{100}) used is 12.8 µl/ml.

The antioxidant potential of the oil was compared to a reference antioxidant agent which is trolox; the results will be expressed as trolox equivalent antioxidant capacity (TEAC). Calibration curve between inhibition percentage and concentrations of trolox12.5-0.3 µg/ml was constructed. The good linearity was proven by the high value of the correlation coefficient; which was found to be 0.9915. By substituting inhibition percentage of the tested essential oils in the regression equation of the calibration curve, TEAC of the oil was computed.

Results and Discussion

The Essential Oil Yield

The hydro distillation of 200gof the ground aerial parts of *P.crispa* yielded 1.2 ml EO with sweet odour, yellowish green colour with density lighter than water and a yield of 0.6 % (V/W).

Chemical Composition of Essential Oil of P.crispa

The GC/MS analysis of the essential oil of *P. Crispa* exhibited eight major components is represented in (Table 1). All the identified oil components were dominated by oxygenated monoterpenes type. The major component of *P. Crispa* EO was carvotanacetone, representing 81.99 % of

the total oil components. The relatively high percentage of carvotanacetone, was in a good agreement with previous studies on the EO of *P.crispa* collected from the Middle East region [7,9,10,21,22] but differs with those from Iran .The essential oils of *P.undulata* cultivated in Iran did not contain carvotanacetone at all [23-25].

No.	Compounds [a]	Relative area percentage (%)	RI*
1	Isothujol	1.60	1079
2	β-linalool	5.34	1082
3	Chrysanthenone	6.87	1119
4	Carvotanacetone	81.99	1158
5	α-Methyl-α-[4-methyl-3-pentenyl] oxiranemethanol	1.10	1182
6	2-(1-methyl-2-oxopropyl) cyclohexanone	0.69	1322
7	cis-Jasmone	0.35	1338
8	4-Oxatricyclo [4.3.1.1(3,8)]undecan-5-one	2.06	1349

Table 1: Chemical composition of *P. crispa* essential oil.

RI* Kovats retention indices calculated relative to homologous series of n-alkanes determined by GC-MS QP2010 on a TR5- CPSIL- 5CB column.

The oil also contained chrysanthenone (6.87%), β-linalool (5.34%), 4-oxatricyclo [4.3.1.1(3,8)]undecan-5-one (2.06%), isothujole (1.6%), α-methyl-α-[4-methyl-3-pentenyl] oxiranemethanol (1.1%), 2-(1-methyl-2-oxopropyl)cyclohexanone (0.69%) and cis-jasmone (0.35%).

However, the study by Ross et al. (1997) showed some qualitative and quantitative differences in the oil composition than what obtained in our study [21]. This was attributed to the difference in geographical origin of the plant which was collected from Elba Mountain in the South Eastern Egyptian coast.

Bioactivity Study of the Essential Oil

Immune Stimulant Activity Screening: The results of *in-vitro* cytotoxic activity of *P.crispa* oil on human peripheral blood mononuclear cells (Table 2) were expressed in terms of maximum safe concentration that keeps 100% cell viability (EC_{100}), which was found to be 12.8 µl/ml; however, the effective concentration that kills 50 % of the cells (EC_{50}) was 38.61 µl/ml.

	Concentration (µl/ml)				
	25	12.5	6.25	3.125	1.5625
Average absorbance	1.0335	1.2505	1.4805	1.6785	1.776
Cell viability percentage	64.9864	78.6313	93.0937	105.544	111.675
Actual cell viability percentage	80.2243	93.8692	108.332	120.782	126.913

Table 2: Cytotoxic activity of different concentrations of essential oil on PBMCs.

The essential oils of *P. crispa* showed stimulation indices lower than 1.5 for all the tested concentrations as shown in Figure 1. Accordingly, the essential oil of *P.crispa* cultivated in Egypt proved to exhibit no immune-stimulant activity.

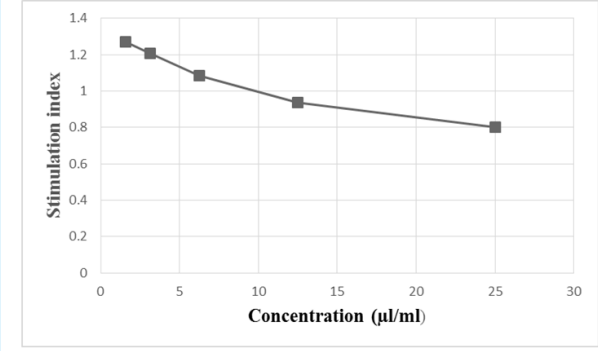

Figure 1: Stimulation indices for different concentrations of *P. crispa* essential oil.

Antioxidant activity: The percentage reduction in the absorbance of the DPPH radical based on the highest safe dose (EC_{100}) is computed to be 66.193% which indicates a powerful antioxidant capacity. The percentage scavenging of DPPH radical is then expressed as "trolox equivalent antioxidant capacity (TEAC) and found to be about 9.220086546 µg/ml from the constructed calibration curve (Figure 2).This means that the highest percentage scavenging activity obtained by the oil was found to be equivalent to the antioxidant potential of 9.220086546 µg/ml of trolox.

This finding indicates that the essential oil of *P. crispa* can be regarded as a powerful antioxidant agent.

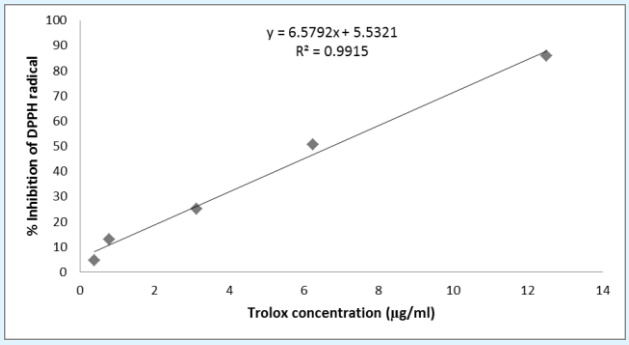

Figure 2: Calibration curve of the % inhibition of DPPH radical bytrolox versus the applied concentration range (12.5-0.3µg/ml).

Cytotoxic Activity on Cancer Cell Lines

The essential oil of *P.crispa* showed high anticancer efficacy against human colorectal adenocarcinoma (Caco-2) with high IC_{50}valueof 4.73 µl/ml; whereas a slightly lower anticancer efficacy against hepatocellular cancer cell line (HepG-2) was observed with IC_{50} values of 20.11 µl/ml (Figure 3). These interesting activities, of the investigated oil may be attributed to its high content of carvotanacetone, according to previous reports [26,27].

Moreover, the percentage inhibition of the maximum safe concentration (EC_{100}=12.8 µl/ml) of *P.crispa* EO on Caco-2 and HepG-2 cell lines were computed to be 97.224 and 33.3022 %.

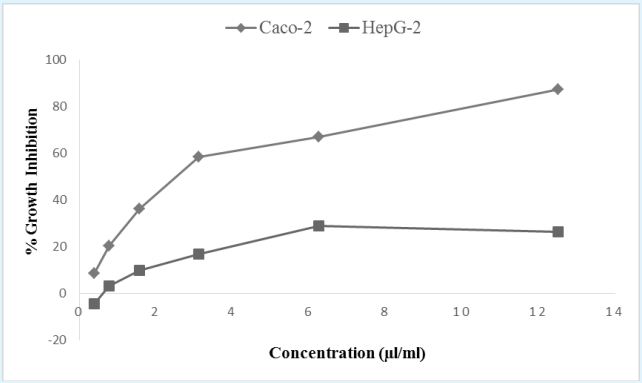

Figure 3: Cytotoxic activity of the essential oil of *P. crispa*onCaco-2 and HepG-2 cell lines.

Safety and selectivity of *P. crispa* oil as candidate anticancer drug: It is important to establish that the candidate anticancer drug has cytotoxic activity at concentrations that can be achieved *in-vivo* without inducing toxic effects to normal cells. The relative effectiveness of the candidate anticancer drug in inhibiting cancerous cells compared to inducing normal cell death is defined as the "Therapeutic or Selectivity Index" and can be calculated using the following expression [28].

S.I. = EC50 value/IC50 value

It is desirable to have a high therapeutic index giving maximum anticancer activity with minimal normal cell toxicity. Studies determining cytotoxicity and therapeutic indices should be conducted before the initiation of phase (I) clinical studies. Candidate drugs with greater SI values are considered to be better lead compounds than those with relatively lower values [28, 29].

P.crispa oil was found to have SI of 8.163 against colorectal adenocarcinoma, which is higher than that against hepatocellular carcinoma (SI=1.92). Accordingly, this oil may providea more sufficient therapeutic activity with a comfortable safety margin in the treatment of colorectal cancer than hepatocellular carcinoma. *P. crispa* EO can be considered to be a potential anticancer candidate against colorectal cancer. Future investigation on the use of the oil as an enema for the treatment of colorectal cancer in rats is worth experiment.

Conclusion

The composition of *P. crispa* essential oil was investigated using GC/MS analysis, which revealed that the oil was characterized by a high content of oxygenated monoterpenes with carvotanacetone as the major component. Regarding the biological investigation of the oil, the essential oil of *P.crispa* showed high anticancer efficacy against human colorectal adenocarcinoma (Caco-2) and a slightly lower anticancer efficacy against hepatocellular cancer cell line (HepG-2). These interesting effects, of the investigated oil may be attributed to its high content of carvotanacetone. The oil was also found to possess a strong antioxidant capacity but showed no immune-stimulant activity. Thus, the oil can be regarded as a potential anticancer candidate against colorectal cancer, the finding which necessitates further future experimentations.

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