Effect of the Method of Preparation on the Composition and Cytotoxic Activity of the Essential Oil of *Pituranthos tortuosus*

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The aerial parts of *Pituranthos tortuosus* (Desf.) Benth and Hook (Apiaceae), growing wild in Egypt, yielded 0.8%, 0.6%, and 1.5% (v/w) of essential oil when prepared by hydrodistillation (HD), simultaneous hydrodistillation-solvent (*n*-pentane) extraction (Lickens-Nickerson, DE), and conventional volatile solvent extraction (preparation of the "absolute", SE), respectively. GC-MS analysis showed that the major components in the HD sample were *β*-myrcene (18.81%), sabinene (18.49%), *trans-iso*-elemicin (12.90%), and terpinen-4-ol (8.09%); those predominent in the DE sample were terpinen-4-ol (29.65%), sabinene (7.38%), *γ*-terpinene (7.27%), and *β*-myrcene (5.53%); while the prominent ones in the SE sample were terpinen-4-ol (15.40%), dill apiol (7.90%), and *allo*-ocimene (4*E*,6*Z*) (6.00%). The oil prepared in each case was tested for its cytotoxic activity on three human cancer cell lines, *i.e.* liver cancer cell line (HEPG2), colon cancer cell line (HCT116), and breast cancer cell line (MCF7). The DE sample showed the most potent activity against the three human cancer cell lines (with IC_{50} values of 1.67, 1.34, and 3.38 μg/ml against the liver, colon, and breast cancer cell lines, respectively). Terpinen-4-ol, sabinene, *γ*-terpinene, and *β*-myrcene were isolated from the DE sample and subjected to a similar evaluation of cytotoxic potency; significant activity was observed.

Key words: Pituranthos tortuosus, Cytotoxic, Terpinen-4-ol

Introduction

An extremely promising strategy of cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents to block the development of cancer in humans (Gupta *et al*., 2004). Numerous bioactive constituents have been hypothesized to act as cancer-preventing agents by inhibiting the activation of procarcinogens, enhancing the detoxification of carcinogens, preventing them from interacting with critical target sites or stopping the progression of carcinogenesis (Wattenberg, 1990, 1992; Hursting *et al*., 1999). Plants, vegetables, and herbs used as folk and traditional medicines have been currently accepted as one of the main sources of cancer chemopreventive agents in drug discovery and development (Abdullaev *et al*., 2000).

Essential oils exhibit a very interesting chemotherapeutic potential; several essential oil constituents have been described as cytotoxic agents comprising *β*-caryophyllene, *β*-elemene, *δ*-elemene, *α*-humulene and others (Wang *et al*., 2005; Sylvestre *et al*., 2006; Hou *et al*., 2006; Tao *et al*., 2006; Xiao *et al*., 2006). Concerning essential oil-bearing species of the Apiaceae, many of these have been shown to exert unique cytotoxic and antileukaemic activities (Babu *et al*., 1995; Pae *et al*., 2002; Yim *et al*., 2005). The genus *Pituranthos* (Apiaceae) is represented in Egypt by two species of wild growing desert shrubs, *P. tortuosus* (Desf.) Benth and Hook and *P. triradiatus* (Hochst.) Asch. (Täckholm, 1974). Previous reports on the chemical composition of *P. tortuosus* included the investigation of its phenolic, hydrodistilled essential oil and sesquiterpene lactone contents (Mahran *et al*., 1989; Abdel-Mogib *et al*., 1992; Abdel-Ghani and Hafez, 1995; Singab *et al*., 1998; Abdel-Kader, 2003). Also the effect of the time of collection on the chemical composition and antimicrobial activity of its essential oil was reported (Abdelwahed *et al*., 2006).

Selection of the suitable method for preparation of essential oils is a tedious process and

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relies on several factors. Hydrodistillation is the most common and cheapest method for isolation of essential oil from plant material, but it is well known that this process may affect the composition of the oil by isomerization, saponification or polymerization of the more labile constituents (Koedam *et al*., 1979).

In the present study, three different techniques were adopted for extraction of the essential oil from the flowering aerial parts of *P. tortuosus*. A comparative investigation of the prepared samples was performed to emphasize the influence of the preparation techniques on both the chemical composition and cytotoxic efficacy. Furthermore, the cytotoxicity of each of the major components of the oil was individually evaluated using doxorubicin as reference.

Material and Methods

Plant material

The aerial parts of *P. tortuosus* (Desf.) Benth and Hook were collected at Wadi Hagol-Suiz Road, Egypt, during April 2007. Authentication of the plant was established by Sherif El-Khanagry, Agriculture Museum, El-Dokki, Cairo, Egypt. A voucher specimen (Reg. No. P-1) is kept in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Preparation of the essential oils

Fresh flowering aerial parts of *P. tortuosus* were subjected to hydrodistillation (HD) in a Clevenger-type apparatus. The oil was also prepared by simultaneous hydrodistillation-solvent (*n*-pentane) extraction (Lickens-Nickerson, DE). Another aliquot was prepared by the conventional volatile solvent extraction method (preparation of the floral absolute, SE) (1 kg for each). The oil obtained in each case was dried over anhydrous sodium sulfate and stored in a refrigerator till analysis.

Analysis of the oils

The GC mass analysis was conducted on an Agilent (Houston, TX, USA) GC-MS system, model 6890, fitted with an Agilent mass spectroscopic detector (MSD), model 5937, as well as a 30 m long, cross-linked 5% phenyl polysiloxane (HP-5MS, Hewlett Packard, Palo Alto, CA, USA) fused silica column (0.25 mm i.d., 0.25 μm film thickness). The initial temperature was 80 °C, kept isothermal for 3 min, then increased to 260 °C at 8 °C/min; the final temperature was kept isothermal for 15 min. The ion source temperature was 230 °C and the quadrupole temperature was 150 °C. The carrier gas was helium adjusted at a flow rate of 0.1 ml/min. Ionization energy was 70 eV, and scan range was 40–500 *m/z* at 3.62/scan. The identification of the oil components was based on the Wiley MS Data Library (6th ed.), followed by comparison of MS data with data published in the literature (Adams, 2004). The results of GC-MS analysis are recorded in Table I.

Isolation of the major constituents from the DE sample

The essential oil sample prepared by the simultaneous hydrodistillation-solvent extraction (DE) method was monitored on TLC plates precoated with silica gel 60 GF_{254} (20 x 20 cm) using the solvent system *n*-hexane/ethyl acetate (95:5, v/v) and vanillin/sulfuric acid (Wagner *et al*., 1984) as spray reagent. The bands corresponding to sabinene, terpinen-4-ol, γ-terpinene, and β-myrcene (R_f = 0.14, 0.27, 0.32, and 0.94) were located by comparison with authentic samples (obtained from Sigma-Aldrich, St. Louis, MO, USA). These bands were separately scraped and eluted with *n*-hexane. The solutions were filtered, and the solvent evaporated under reduced pressure. The process was repeated until 8–10 mg from each compound had been obtained.

Determination of LD_{50}

The LD_{50} values of the three oil samples were calculated according to Karber's (1931) procedure.

Cytotoxicity assay

The cytotoxicity of the essential oils prepared by each of the three methods as well as that of the isolated compounds was measured using the sulforhodamine B assay (SRB) (Skehan *et al*., 1990) on three human cancer cell lines: liver cancer cell line (HEPG2), colon cancer cell line (HCT116), and breast cancer cell line (MCF7). The IC_{50} (dose which reduces the survival to 50%) and IC_{10} (dose which reduces survival to 10%) values for each test sample were calculated and compared to the reference drug doxorubicin.

Table I. Composition of the essential oil of the aerial parts of *P. tortuosus* (Desf.) Benth and Hook prepared by hydrodistillation (HD), hydrodistillation-solvent extraction (DE), and conventional solvent extraction (SE).

Retention index	Compound	Content (%)		
		HD	DE	SE
700	*n*-Heptane	-	-	3.46
800	*n*-Octane	-	-	2.71
936	*α*-Thujene	0.44	0.31	4.28
977	Sabinene	18.49	7.38	1.48
998	*β*-Myrcene	18.81	5.53	4.61
1006	*α*-Phellandrene	-	1.16	3.51
1019	*α*-Terpinene	-	3.83	2.61
1027	*p*-Cymene	1.17	-	-
1035	*β*-Phellandrene	-	1.06	-
1040	*cis*-*β*-Ocimene	1.53	1.50	2.01
1052	*trans*-*β*-Ocimene	-	1.29	0.50
1061	*m*-Cymene	3.94	0.78	-
1065	*γ*-Terpinene	2.55	7.27	2.33
1095	*α*-Terpinolene	0.50	3.59	1.49
1100	*trans*-Sabinene hydrate	0.48	4.56	-
1101	Linalool	1.21	2.00	1.24
1133	*allo*-Ocimene *(4E,6Z)*	4.70	1.72	6.00
1138	1-Terpineol	-	1.61	0.69
1180	Terpinen-4-ol	8.09	24.65	15.40
1188	*p*-Cymen-8-ol	0.76	0.90	0.51
1192	*α*-Terpineol	-	1.99	2.50
1200	*cis*-Piperitol	0.64	1.07	0.42
1210	*trans*-Piperitol	1.28	1.82	1.51
1230	*β*-Citronellol	-	1.47	-
1232	*cis*-Carveol	0.16	0.21	0.49
1239	Thymol methyl ether	-	0.64	-
1241	Ascaridole	1.06	2.13	1.01
1245	*cis*-Terpinen-3-ol	-	1.38	0.76
1249	Carvacrol methyl ether	2.49	0.52	0.41
1291	Bornyl acetate	0.96	0.99	0.91
1293	Thymol	-	2.28	0.78
1298	Carvacrol	-	0.72	-
1301	*p*-Cymen-7-ol	0.59	0.64	-
1361	Eugenol	2.60	0.95	2.09
1485	Germacrene-D	-	0.86	0.41
1493	*β*-Selinene	1.31	0.42	1.92
1510	*β*-Bisabolene	3.28	2.70	3.78
1520	Myristicin	0.89	-	-
1531	*trans*-*iso*-Elemicin	12.90	5.03	1.69
1581	Spathulenol	2.28	1.60	5.01
1624	Dill apiol	2.08	0.53	7.90
1655	*β*-Eudesmol	2.37	1.30	1.21
1629	Citronellyl pentanoate	0.95	1.28	1.15
1945	Phytol	-	-	0.88
Non-oxygenated compounds				
	Monoterpene hydrocarbons	47.02	35.45	28.82
	Sesquiterpene hydrocarbons	4.59	3.98	6.11
	Aromatic hydrocarbons	5.11	-	-
	Aliphatic hydrocarbons	-	-	6.17
Oxygenated compounds				
	Oxygenated monoterpenes	13.21	42.30	23.52
	Oxygenated sesquiterpenes	17.55	7.93	7.91
	Phenols and phenolic ethers	8.06	5.64	3.95
	Non-terpenoid compounds	2.97	4.40	-

Results and Discussion

The method of preparation of the essential oil affected not only the yield of the oil [0.8%, 0.6%, and 1.5% (v/w) of essential oil was obtained from HD, DE, and SE samples, respectively], but also the colour (yellow, light yellow, and dark yellow, respectively) and specific gravity of the samples (0.72033, 0.83056, and 0.8950 of HD, DE, and SE samples, respectively). The influence of the method of preparation on the qualitative and quantitative composition of the different oil samples was noticeable (Table I). The total number of identified compounds was 29 constituting 98.48% of the HD sample, 39 compounds constituting 99.7% of the DE sample, and 35 compounds constituting 76.48% of the SE sample. A variability in the type and amount of the compounds detected in the analysed samples was obvious; for example, HD essential oil was dominated by *β*-myrcene (18.81%), sabinene (18.49%), *trans-iso*-elemicin (12.90%), and terpinen-4-ol (8.09%), while the major components in the DE sample were terpinen-4-ol (24.65%), sabinene (7.38%), *γ*-terpinene (7.27%), and *β*-myrcene (5.53%). The major components in the SE sample were terpinen-4-ol (15.40%), dill apiol (7.90%), and *allo*-ocimene (4*E*,6*Z*) (6.00%).

It is also interesting to note that the different methods used for preparation had an influence on the percentage of chemical classes. In fact, significant differences were evident especially in the content of monoterpene hydrocarbons and oxygenated monoterpenes. The highest percentage of oxygenated monoterpenes was found in the DE sample (42.30%). Aromatic hydrocarbons were only detected in the HD sample, which may be artifacts due to heating.

The essential oils prepared by HD, DE, and SE were found to be safe up to the maximum dose of 8.9, 9.1, and 7.5 g/kg, respectively.

Natural products have served to provide a basis for many pharmaceutical agents in current cancer therapy (Pietras and Weinberg, 2005). The use of chemotherapeutic drugs involves the risk of life threatening host toxicity. The search, therefore, moves towards the development of drugs which selectively act on tumour cells. So, cytotoxic activity of the three prepared samples was tested on three human cancer cell lines: liver cancer cell line (HEPG2), colon cancer cell line (HCT116), and breast cancer cell line (MCF7) (Table II). The three samples showed cytotoxic activity, the DE sample being the most potent as it showed the lowest IC_{50} values (1.67, 1.34, and 3.38 μg/ml) against the liver, colon, and breast cancer cell lines, respectively. The major components of the DE sample (terpinen-4-ol, sabinene, *γ*-terpinene, and *β*-myrcene) were isolated using the preparative TLC technique and subsequently tested for cytotoxic activity against the three human cancer cell lines. Terpinen-4-ol, *γ*-terpinene, and *β*-myrcene were the most potent on the basis of their IC_{50} values. Although their effects on the liver (HEPG2) and colon (HCT116) cancer cell lines were weaker than those of the whole oil sample; their effects on the breast cancer cell line (MCF7) was more potent than that of the whole oil sample on the basis of the IC_{50} value.

Table II. *In vitro* cytotoxicity of the essential oils of *P. tortuosus* (Desf.) Benth and Hook.

Sample	IC_{50} [μg/ml]		
	HEPG2	HCT116	MCF7
Sample HD	2.31	5.37	9.93
Sample DE	1.67	1.34	3.38
Sample SE	4.33	8.93	3.58
Terpinen-4-ol	4.29	4.28	2.78
Sabinene	19.6	17.6	11.4
γ-Terpinene	2.67	3.43	2.67
β-Myrcene	2.51	3.28	2.82
Doxurubicin	0.67	0.69	0.7

HD, sample prepared by hydrodistillation; DE, sample prepared by hydrodistillation-solvent extraction; SE, sample prepared by conventional solvent extraction

The cytotoxic activity of *Pituranthos* essential oil may be due to the presence of phenols, aldehydes, and alcohols (Bakkali *et al*., 2008). In addition, the cytotoxic activity of the oil could be also attributed to the presence of sesquiterpenes (Sylvestre *et al*., 2005). The high activity of the oil prepared by the DE method could be attributed to the presence of a high percentage of terpinen-4-ol (24.65%), the most active compound of tea tree oil, which inhibits human melanoma cells *in vitro* through caspase-dependent apoptosis (Calcabrini *et al*., 2004). Furthermore, minor compounds detected in the sample prepared by the DE method may potentiate, in a synergistic way, the cytotoxic action of compounds known to be cytotoxic. *β*-Myrcene (5.53% in the DE sample) displayed antimutagenic effects against oxidative mutagenesis in the oxyR deficient *E. coli* WP2 IC202 strain (Mitic-Culafic *et al*., 2009). *α*-Terpinene and *α*-terpineol (3.83% and 1.99%

in the DE sample) modulate hepatic mono-oxygenase activity, such as CYP1A1 and CYP2B1, thus interacting with promutagen or procarcinogen xenobiotic biotransformation (Bakkali *et al*., 2008). Carvacrol (0.72% in the DE sample) has an important *in vitro* cytotoxic activity against tumour cells resistant to chemotherapy, as well as a significant antitumour effect in mice (Ait M'Barek *et al*., 2007). Linalool (2.00% in the DE sample) has cytotoxic activity against murine B16 melanoma and human HL-60 leukemia cells (Usta *et al*., 2009). In addition, many terpenes are reported to be cytotoxic, *viz.* thymol, citronellol, and *trans-iso*-elemicin (2.28%, 1.47%, and 5.03% in the DE sample) (Hasheminejad and Caldml, 1994; Bakkali *et al*., 2008). In general, the cytotoxic activity of *Pituranthos* essential oil may be attributed to the synergistic effect of all constituents.

The results of the present study indicate the potent cytotoxic activity of the essential oil prepared by DE from the aerial parts of *P. tortuosus* and its major components terpinen-4-ol, γ-terpinene, sabinene, and β-myrcene against liver (HEPG2), colon (HCT116), and breast cancer (MCF7) cells. Although, the actual mechanism by which they exhibit antitumour activity is not known, it may be due to their interference with cell development. This will be the object of future research. The results of this investigation may improve our understanding of the use of this plant in an alternative cytotoxic therapy.

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