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Achillea santolina: Growth Dependent Variation in Essential Oil Composition and Some *in–vitro* Bioactivity Studies

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Authors' contributions

This work was carried out in collaboration between all authors. Authors HIAJ, MHAZ and FUA designed the study, confirmed the taxonomic identity of the plant material, supervised the laboratory work, analyzed the data and contributed to critical reading of the manuscript. Authors IFA and MB contributed by plant collection. Authors DFK, IFA contributed to preparation of the essential oil samples. Authors FUA and IFA contributed to chromatographic analysis. Author HMH contributed to the antiplatelet activity assay. Author AMS contributed to the anti-proliferative activity assay. Authors MAAQ and JYAH contributed to the critical reading of the manuscript. All authors have read the final manuscript and approved the submission.

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ABSTRACT

Achillea santolina is used in Jordanian herbal traditional medicine for the treatment of diabetes, intestinal disorders, inflammations and for wound healing.

Aims: The present study aimed at investigating the variation of the hydro-distilled oil composition extracted from fresh and air dried flowering parts of *A. santolina* collected at different growth stages. In addition, the antiplatelet and antiproliferative activities of the essential oil obtained from the air dried flowers were also investigated.

Study Design: The essential oils were extracted by hydro-distillation and their chemical composition was determined using GC/MS technique. The essential oil of the air dried flowers was evaluated for its inhibition of platelet aggregation caused by ADP and collagen. This oil was also assayed for its antiproliferative activity against MCF-7, MDA-MB-231 and PC-3 cancer cell lines.

Results: Oxygenated monoterpenoids were the main constituents in all plant samples. In the fresh plant, eucalyptol was the main constituent at the pre-flowering (18.05%), flowering (20.51%) and post flowering (14.70%), while in the dry plant samples, it dominated the pre-flowering (16.41%) and flowering (17.82%) stages with camphor (17.80%) at the post-flowering stage. The oil of the air dried flowering parts exhibited a dose dependent inhibition of platelet aggregation caused by ADP and collagen, with the highest inhibition at concentration of 60 µg/ml (93.75±3.13; 91.67±4.17., respectively). No antiproliferative activity was detected at concentration1mg/ml against the tested cell lines MCF-7, MDA-MB-231 and PC-3.

Conclusion: During the different growth stages, the essential oils of fresh and air dried flowering parts of *A. santolina* was dominated by oxygenated monoterpenoids. The hydro-distilled oil obtained from air dried flowers has shown a dose dependent antiplatelet activity on both ADP and collagen. This oil was inactive against MCF-7, MDA-MB-231 and PC-3 cancer cell lines at the stock concentrations of 1 mg/ml.

Keywords: Achillea santolina; essential oil; GC/MS; oxygenated monoterpenes; antiplatelet activity; antiproliferative activity.

1. INTRODUCTION

The Asteraceae family (previously known as *Compositae*) is the richest and most common vascular plant family in the world, comprising 1600–1700 genera [1] and about 24000–30000 species [2] distributed wildly around the globe except for Antarctica. The genus *Achillea* L. is a member of this family that is wildly distributed in the world and has been well know and recognized since ancient times for its medicinal uses. There are about 130 flowering and perennial species of the *Achillea* genus that occur in Europe and temperate areas of Asia and North America. Generally, these plants are known for their hairy aromatic leaves and for having flat clusters of small flowers on the top of the stem [3].

Achillea genus (referred to as yarrow) acquired its name in reference to Achilles, the Trojan War hero who used these plants to treat soldiers wounds. These plants are generally famous for their characteristic aromatic odor. The essential oil obtained from hydro-distillation of the leaves and flowers is known to be a source of medicinal preparations used in traditional medicine. In Turkish folk medicine, *Achillea* species are recognized as an important herbal remedy used against gastro-intestinal complaints (stomachache, abdominal pain, flatulence, diarrhea and hemorrhoids), inflammatory disorders (rheumatic pain, abscess maturation, and eye inflammations), for wound healing, as emmenagogue, as diuretic, against jaundice and for many other complaints [4-10]. *A. millefolium* L., recorded as an official plant in the European Pharmacopoeia, is used as a herbal bile remedy [11] and is known to possess anti-inflammatory, antiseptic, antispasmodic, choleretic, antibacterial and astringent effects. Moreover, it is prescribed as herbal tea, as sitz bath, or pressed juice [12].

A. santolina L., is a biennial to perennial herb, 10-30 cm long with many weak bending stems and aromatic smell. The leaves are narrow, linear, pinnatisect. Heads are small, yellow, 5-8 cm in diameter, arranged in small, flat inflorescence. The plant is known to grow wildly in the waste ground and cultivated marginal lands across Jordan including Irbid, Ajloun, Jarash, Madaba, Mafraq and the capital Amman. Flowering occurs during the spring season

extending from March to May [13]. It has been reported that infusions prepared from the aerial parts of *A. santolina* are used for the treatment of diabetes, intestinal colics, anaemia, dysentery and wound healing. It is also said that the infusion has tonic, vermifugal and carminative potential. The essential oil of *A. santolina* is known to possess insecticidal, nematicidal, antibacterial, antispermatogenic, antifungal, molluscicidal and cercaricidal properties [14]. Also in local Jordanian traditional medicine, other *Achillea* species are used as herbal remedies for the treatment of similar diseases. Young flowering branches of *A. santolina* are used as antidiabetic [15-17]. People generally use the fresh plant during the spring season and preserve the air dried plant material for use in other seasons as herbal remedy.

Literature survey revealed that researchers determined the composition of the volatile oil of *A. santolina* from air dried plant samples collected during flowering stage. With the best of our knowledge, despite the fresh plant is widely used in herbal medicine, the oil obtained from fresh aerial parts of *A. santolina* has never been investigated for its chemical composition. Thus, the current study was designed to compare the chemical composition of the hydro-distilled oil of the fresh and air dried *A. santolina* at different growth stages. Moreover, prior the present study, neither the antiplatelet activity, nor the antiproliferative efficacy against MCF-7, MDA-MB-231 and PC-3 cell lines were evaluated for the hydro-distilled oil.

2. MATERIALS AND METHODS

2.1 Plant Material

Aerial flowering parts of *A. santolina* collected at the different flowering stages (Pre-, Flowering and Post-flowering were collected in the spring season from an area near the University of Jordan (Amman, 31°57'N and 35°56'E, Jordan) during the period extending from April to May (2011). The plant was identified by one of the authors (Dr Hala I. Al-Jaber). A voucher specimen (No BAU/03/0511) has been deposited in the Department of Physics and Basic Sciences, Faculty of Engineering Technology, Al-Balqa Applied University, Amman, Jordan. The flowering aerial parts were air dried in the shade, at room temperature, until constant weight was achieved, and were subsequently assayed for essential oil extraction and identification.

2.2 Extraction of Essential Oil

Ground fresh (400 g each) and air dried (150 – 200 g, equivalent of 400 g fresh) plant material were subjected to hydro-distillation using a Clevenger type apparatus for 3 h. The extraction procedure was repeated twice for both types of samples. The oils obtained were pooled separately, dried over anhydrous sodium sulfate (Na_2SO_4) and then stored in amber glass vials (at 4℃) until analysis.

2.3 GC-MS and GC-FID Analysis

The essential oils were analyzed using a Varian Chrompack CP-3800 GC/MS/MS-200 (Saturn, Netherlands) system. The compounds were separated on DB-5 (5% diphenyl, 95% dimethyl polysiloxane) GC capillary column (30 m × 0.25 mm i.d., 0.25 µm film thicknesses). The ionization voltage of the MS was 70 eV and the ionization source temperature was 180℃. The column temperature was held constant at 60℃ for 1 min, the temperature was then increased from 60℃ to 246℃ at a rate of 3℃/min after which it was held constant at 246℃ for 3 minutes. The flow rate of the carrier gas (Helium) was 0.9 ml/min. Using an automatic injector in the split mode, about 1 µL aliquot of each essential oil was injected into the GC. The constituents of the essential oils were identified using the built-in search engines (including NIST Co and Wiley Co, USA), by comparing their calculated retention indices relative to (C_8-C_{20}) *n*-alkanes with literature values measured with columns of identical polarity [18] and/or co-injection of pure authentic compounds. Authentic samples including the pinene isomers (α- and β), limonene, linalool were purchased form Fluka (Buchs, Switzerland). Eugenol and sabinene hydrate were purchased form Sigma-Aldrich (Buchs, Switzerland). *n*-Hexane (both GC- and analytical reagent grades) and Na_2SO_4 (anhydrous) were purchased from Scharlau (Barcelona, Spain) and UCB (Bruxelles, Belgium), respectively.

A Hewlett-Packard HP-8590 gas chromatograph equipped with a split-splitless injector (split ratio 1:50), a flame ionization detector (FID) and an optima-5 (5% diphenyl, 95% dimethyl polysiloxane) fused silica capillary column (30 m ×0.25 mm, 0.25 film thickness) was used for quantitative analysis. The temperature of the injector was fixed 250℃, while the detector was maintained at 300℃. The column temperature was programed from 60℃ to 250℃ at a rate of

10℃/min, the temperature was then held constant at 250℃ for 5 min.

2.4 Platelet Aggregation Assay

ADP and collagen were the products of Chrono-Log Corp. The antiplatelet activity of the essential oil of *A. santolina* was determined in human whole blood *in vitro.* Blood samples were obtained from healthy non-smoker volunteers based on the criteria that they had not taken any medications, including aspirin, within the last two weeks. Vacutainer containing 3.8% sodium citrate (9:1 v/v) were used for blood withdrawal. The blood samples were diluted with normal saline (1:1 dilution ratio). The essential oil was dissolved in dimethyl sulfoxide (DMSO) to obtain concentration of 2 µg/µl.

Essential oil (5, 10, 20, 25 and 30 µl) was added to a cuvette containing the diluted whole blood. The resulted mixture was incubated at 37℃ for 4 min prior to the addition of ADP (10 µM) or collagen (2 µg/ml). The total volume of the mixture was 1 ml. The final concentrations of the essential oil in the prepared mixtures were (10, 20, 40, 50, and 60) µg/ml. The platelet aggregation was measured by the whole blood Chrono-log 700 lumi-aggregometer using an electrical impedance method. The mean platelet aggregation in whole blood was measured as a change in impedance over 6 min after the addition of the inducers by comparison with that of a control group impedance. The final concentration of DMSO in the whole blood was 0.5% to eliminate the effect of the solvent on the aggregation [19]. Aspirin was used as a positive control.

2.5 Antiproliferative Activity Assay

Human breast adenocarcinoma cell lines (MCF-7 ATCC No: HTB 22 and MDA-MB-231 ATCC No: HTB-26), and human prostate adenocarcinoma cell line (PC-3, ATCC No: CRL-1435) were used for the antiproliferative activity assay.

The tissue culture media used were supplemented with 10% heated foetal bovine serum, 1% of 2 mM l-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. Doxorubicin (Ebewe Pharma GMBH Nfg. KG, Austria) was used as a positive control. For adherent cells (PLF), 1 $\times 10^4$ cells were seeded in each well. Colorimetric Cell Titer 96 non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) was used to detect cells proliferation in each well according to the manufacturer's instructions. Briefly, 15 µl of dye solution were added on each well. The cells were incubated at 37℃ and 5% CO_2 for 4 h. Then, 100 µl of the solubilisation solution were added to solubilize formazan precipitate. The absorbance was recorded at 570 nm using a colorimetric plate reader (Sunrise-Basic TECAN, Austria). The percentage of cell survival was calculated as [Mean (OD test−OD blank)/Mean (OD control−OD blank)] × 100%. The IC_{50} (the concentration at which 50% of cells died in comparison to control) was calculated using the 'GraphPad prism 6' software.

3. RESULTS AND DISCUSSION

3.1 Chemical Compositions of the Hydro-Distilled Oils

The GC/MS analysis of the essential oils obtained from the fresh and air dried flowering parts of *A. santolina* collected at different flowering stages resulted in the identification of a total of 88 compounds (Table 1). These oils were generally dominated by oxygenated monoterpenes that accounted always for more than 70.0% of the total oil content. In total, 56, 65 and 44 constituents were identified at the pre-flowering, flowering and post-flowering stages of fresh *A. santolina*. These constituents amounted to 99.36%, 99.54% and 99.69% of the total oil content, respectively. In the fresh pre-flowering stage oil, oxygenated monoterpenes amounted for 74.24% of the total oil content. Eucalyptol (18.05%), camphor (9.51%) and borneol (7.81%) were the main constituents of the oil. Additionally, monoterpene hydrocarbons (11.69%), sesquiterpne hydrocarbons (4.89%), oxygenated sesquiterpenes (25.40%), aliphatic hydrocarbons and their derivatives (2.38%) as well as aromatics (0.76%) were detected.

During the flowering stage, the oil obtained from fresh plants was rich in oxygenated monoterpenes. The identified 30 different compounds accounted for 78.56% of the total content, with eucalyptol (20.51%) as the major component. Monoterpene hydrocarbons content decreased slightly upon flowering (9.23%) and the oil also contained slightly lower amounts of sesquiterpene hydrocarbons (3.37%). The content of oxygenated sesquiterpenes increased slightly upon flowering (6.27%).

During the post-flowering fresh stage, oxygenated monoterpenes accounted for 82.89% of the total oil content. The main constituents

were eucalyptol (14.70%), *trans*-verbenol (12.25%), artemisia ketone (10.02%) and camphor (7.66%). During this stage, monoterpene hydrocarbons content increased slightly as compared to the flowering stage (9.59%, 9.23%., respectively). Among the nine monoterpene hydrocarbons detected, α-pinene (3.79%) and camphene (1.31%) had the major contribution to this fraction. Aromatic volatile compounds, aliphatic hydrocarbons and their derivatives were detected in very low concentration in the studied oils of the fresh plant (Table 1).

GC/MS analysis of the essential oils obtained from the air-dried flowering parts of *A. santolina* collected at the consecutive flowering stages resulted in the identification and quantification of a total of 53 (pre-flowering), 43 (flowering) and 45 (post-flowering) constituents. These constituents amounted to 99.43%, 99.74% and 98.43% of the total oil content, respectively. All these oils were dominated by oxygenated monoterpenes which accounted for 73.11% (dry pre-flower), 82.86% (dry flower) and 70.64% (dry post-flower) of the total oils contents.

During the air-dried pre-flowering stage, eucalyptol remained the main constituent (16.41%) of the oil. Other oxygenated monoterpenes detected in appreciable concentrations included *trans*-piperitol (9.34%), *cis*-chrysanthenyl acetate (7.79%), terpinene-4-ol (7.69%) and α-terpineol (2.55%). Monoterpene hydrocarbons (12.84%) were represented by γ- and α-terpinene isomers (3.64%, 1.99%., respectively). Sesquiterpene hydrocarbons (4.89%) and their oxygenated derivatives (5.96%) were detected at similar levels as compared to the fresh pre-flowering stage (4.89%, 5.40%., respectively) and were represented by β-caryophyllene (1.85%) and caryophyllene oxide (1.95%), respectively.

The oil of the air dried *A. santolina* at the flowering stage yielded eucalyptol (17.82%), artemisia ketone (12.59%) and camphor (11.12%) as the main constituents. Monoterpene hydrocarbons content changed slightly as compared to the fresh flowering stage (9.74%, 9.23%) with α-pinene (4.26%) and camphene (1.25%) as the main constituents of this fraction. Sesquiterpenoid content (hydrocarbons and their oxygenated derivatives) decreased as compared to the fresh flowering stage (5.63% of the total oil content).

The oil of *A. santolina* collected at the post-flowering stage exhibited the greatest variation in its composition. While camphor had the major contribution to this fraction (17.80%), the content of eucalyptol reached a minimum level amounting only to 3.08% of the total oil composition. Monoterpene hydrocarbons concentration increased as compared to all other stages (fresh and air dried) amounting to 18.67% of the total oil content with α-pinene (9.29%), camphene (1.63%) and limonene (1.51%) being detected as the major constituents of this fraction. Sesquiterpenoids (hydrocarbons and oxygenated derivatives) accounted only for 5.79% of the total oil content.

Table 1. The composition of the fresh and air dried essential oils of *A. santolina* growing wild in Jordan collected at different flowering stages

No	lit RI	RI	Compound	Concentration (%)					
				Pre fresh	Pre dry	Fresh flower	Dry flower	Post fresh	Post dry
1	845	848	Isopropylbutanoate	-	-	-	-	-	0.50
2	881	872	sec-amyl acetate	0.17	-	-	-	-	0.77
3	909	902	santolina triene	-	-	-	0.84	0.78	4.81
4	927	925	tricyclene	0.27	-	-	-	-	-
5	930	926	α-thujene	-	0.37	0.21	-	-	-
6	939	935	α-pinene	1.10	1.24	0.63	4.26	3.79	9.29
7	954	952	camphene	1.98	0.43	1.25	1.25	1.31	1.63
8	975	974	sabinene	2.16	1.71	2.85	0.87	1.05	0.45
9	979	980	β-pinene	0.61	0.43	0.55	0.35	0.37	0.26
10	991	990	myrcene	0.24	0.21	0.18	-	-	-
11	991	992	1,8-dehydrocineol	-	-	-	0.32	0.37	-
12	999	995	yomogi alcohol	-	-	1.22	1.44	1.72	-
13	1000	1003	n-decane	0.13	-	0.25	-	-	0.13

No	lit RI	RI	Compound	Concentration (%)					
				Pre fresh	Pre dry	Fresh flower	Dry flower	Post fresh	Post dry
14	1003	1014	α-phellandrene	0.55	0.46	0.33	-	-	0.29
15	1007	1013	pentylpropanoate	0.36	0.46	0.56	-	-	0.72
16	1017	1018	α-terpinene	1.10	1.99	0.73	0.55	0.77	0.43
17	1025	1026	p-cymene	0.40	0.69	0.43	0.48	0.75	0.45
18	1029	1030	limonene	1.66	1.70	1.28	0.81	0.82	1.51
19	1031	1034	eucalyptol	18.05	16.41	20.51	17.82	14.70	3.08
20	1062	1057	artemisia ketone	-	-	3.17	12.59	10.02	0.35
21	1060	1064	γ-terpinene	1.60	3.64	0.97	0.56	0.70	-
22	1070	1073	*cis*-sabinene hydrate	4.68	3.76	3.53	1.56	1.73	0.58
23	1084	1081	artemisia alcohol	-	0.43	2.09	6.48	5.97	0.31
24	1089	1088	terpinolene	0.42	0.66	0.25	0.25	-	-
25	1100	1100	isoamyl 2-methyl butanoate	0.77	-	-	0.36	0.43	-
26	1097	1102	linalool	1.61	6.22	3.36	-	-	2.56
27	1098	1105	*trans*-sabinene hydrate (*trans* IPP vs OH)	3.91	3.80	3.32	2.02	2.66	-
28	1103	1108	isoamyl isovalerate	0.51	0.82	-	0.67	0.60	-
29	1102	1112	α-thujone	-	-	7.21	0.30	0.29	3.58
30	1114	1121	β-thujone	-	-	0.94	-	-	0.60
31	1123	1125	*trans*-p-mentha-2,8-dien-1-ol	-	-	0.26	0.94	1.08	-
32	1128	1126	chrysanthenone	-	-	-	-	-	0.69
33	1122	1127	*cis*-*p*-menth-2-en-1-ol	1.60	1.68	-	-	0.86	1.05
34	1134	1128	1-terpineol	-	-	0.59	0.59	-	-
35	1126	1129	α-campholenal	-	-	-	0.58	0.58	0.61
36	1138	1140	*cis*-*p*-mentha-2,8-dien-1-ol	-	-	0.11	-	0.30	-
37	1139	1145	*trans*-pinocarveol	-	-	0.14	4.76	6.06	2.01
38	1141	1145	*trans*-*p*-menth-2-en-1-ol	0.88	0.96	0.43	-	-	-
39	1145	1150	*trans*-verbenol	-	0.72	0.45	10.02	12.25	-
40	1146	1152	camphor	9.51	0.93	4.56	11.12	7.66	17.80
41	1159	1157	β-pinene oxide	1.62	1.99	2.78	0.88	0.91	1.23
42	1173	1163	artemisyl acetate	-	0.25	0.78	-	-	-
43	1164	1166	*cis*-chrysanthenol	1.65	1.91	-	-	-	16.16
44	1165	1166	pinocarvone	-	-	0.42	3.33	3.16	-
45	1170	1171	umbellulone	0.43	0.23	0.27	-	-	0.18
46	1166	1176	δ-terpineol	-	-	0.60	1.69	2.15	-
47	1169	1177	borneol	7.81	1.95	9.36	-	-	0.97
48	1177	1184	terpinene-4-ol	4.91	7.69	4.46	2.75	3.89	-
49	1189	1191	*trans*-isocarveol	-	-	0.08	0.39	0.64	-
50	1189	1198	α-terpineol	2.88	2.55	2.85	1.18	1.60	2.30
51	1205	1212	verbenone	-	-	-	1.66	2.50	-
52	1208	1213	*trans*-piperitol	4.38	9.34	0.86	-	-	5.48
53	1216	1219	fragranol	0.72	0.89	-	-	0.29	-
54	1217	1224	*trans*-carveol	-	1.24	-	0.44	0.62	0.76
55	1237	1232	isogeraniol	1.15	0.59	2.69	-	0.48	0.32
56	1243	1249	carvone	-	0.45	-	-	-	-
57	1265	1259	*cis*-chrysanthenyl acetate	1.72	7.79	0.67	-	-	9.06
58	1286	1289	isobornyl acetate	0.19	0.46	0.21	-	-	-
59	1290	1292	thymol	0.36	0.35	0.38	-	-	-
60	1342	1335	*trans*-carvyl acetate	0.51	0.87	-	-	-	0.96
61	1338	1338	δ-elemene	-	-	-	-	-	0.58
62	1344	1344	verbanol acetate	5.72	-	0.13	-	0.40	-
63	1359	1359	eugenol	-	-	-	-	-	0.35
64	1349	1360	α-terpinyl acetate--	0.31	-	0.51	-	-	-
65	1419	1419	β-caryophyllene	1.98	1.85	1.95	0.52	0.36	0.23
66	1455	1456	α-humulene	0.15	0.38	0.14	-	-	-

No	lit RI	RI	Compound	Concentration (%)					
				Pre fresh	Pre dry	Fresh flower	Dry flower	Post fresh	Post dry
67	1460	1462	alloarmandrene	-	0.52	-	-	-	-
68	1463	1468	dehydroaromandrane	-	0.85	-	-	-	-
69	1485	1484	germacrene D	1.11	0.39	0.57	0.80	0.65	-
70	1495	1495	γ-amorphene	-	-	0.14	0.24	-	-
71	1500	1500	bicyclogermacrene	1.03	0.90	0.49	0.38	-	-
72	1516	1516	sesquicineole	0.62	-	0.08	-	-	-
73	1533	1528	*Z*-nerolidol	0.16	-	-	-	-	-
74	1575	1561	α-cedrene epoxide	-	0.48	-	-	-	-
75	1564	1573	geranyl butanoate	0.21	-	0.25	-	-	-
76	1572	1574	*n*-tridecanol	-	0.31	-	-	-	-
77	1578	1579	spathulenol	0.69	1.36	1.13	0.67	0.80	0.24
78	1583	1585	caryophyllene oxide	0.65	1.95	1.16	0.99	1.10	1.20
79	1632	1637	γ-eudesmol	0.38	0.51	0.58	-	0.51	1.01
80	1641	1639	caryophylla-4(14),8(15)-dien-5α-ol	0.24	-	0.33	0.33	-	-
81	1641	1643	caryophylla-4(14),8(15)-dien-5β-ol	0.73	0.61	0.97	1.12	1.55	-
82	1640	1646	τ-cadinol	0.49	-	0.50	-	-	0.69
83	1660	1654	neo-intermedeol	0.46	0.38	0.41	0.58	0.46	1.17
84	1651	1660	β-eudesmol	0.52	0.67	0.51	-	-	0.67
85	1668	1677	14-hydroxy-9-epi-E-caryophyllene	-	-	0.24	-	-	-
86	1686	1690	α–bisabolol	1.08	-	0.15	-	-	-
87	1743	1751	khausimol	-	-	0.29	-	-	-
88	1834	1849	cyclopentadecanolide	0.23	-	0.24	-	-	0.41
Monoterpene hydrocarbons				11.69	12.85	9.23	9.74	9.59	18.67
Oxygenated monoterpenes				74.24	73.11	78.56	82.86	82.86	70.64
Sesquiterpene hydrocarbons				4.89	4.89	3.37	1.94	1.01	0.81
Oxygenated sesquiterpenes				5.40	5.96	6.27	3.69	4.42	4.98
Aliphatic hydrocarbons & their derivatives				2.38	1.59	1.30	1.03	1.03	2.53
Aromatics				0.76	1.04	0.81	0.48	0.75	0.80
Total identified (%)				99.36	99.43	99.54	99.74	99.69	98.43

Different monoterpenoid skeletons were detected during the different growth stages of *A. santolina*. These included the pinane (α & β-pinenes), the thujane (sabinene) and the *p*-menthane (limonene, γ-terpinene, α-terpineol and eucalyptol) skeletons. The *p*-menthane skeleton, represented mainly by eucalyptol, was the main abundant skeleton of monoterpenoids detected in all tested oil samples. Alpha terpenyl cation is known to be responsible for the production of many monoterpenes detected in the current investigation including camphene, α– & β–pinenes, sabinene, borneol, terpinolene, γ-terpinene, limonene and α-terpineol [20]. Alpha-terpineol, served as the precursor for the formation of eucalyptol which was the most prominent oxygenated monoterpenoid detected. It was noticed that the content of its precursors (limonene and α-terpineol) has declined upon development. The concentration of borneol, sabinene and its hydrated derivatives reached a maximum level upon flowering. The concentration of borneol was unfavorably altered during the post-flowering stage upon oxidation to camphor.

A thorough literature investigation revealed that the composition of the essential oil obtained from aerial air dried parts of *A. santolina* has been the subject of investigation in different parts of the world. However, the chemical composition of the oil obtained from hydro-distilled fresh plant has been investigated only in Egypt [21]. In this study, the plant was collected from wild populations located near Alexandria Province and the hydro-distilled oils obtained from different organs were screened for their chemical composition. Interestingly, fragranyl acetate (flowers 51.70%, leaves 47.14% and stems 45.10%) and fragranol (11.84%, leaves 13.22%, stems 18.69%) were detected as the main constituents. Nenaah [22] investigated the chemical composition of aerial air dried Egyptian *A. santolina* collected from different locations from Sinai desert. Again, fragranyl acetate and

fragranol were the main contributors for this oil (27.3%., 8.2%) but detected at much lower concentrations as compared to the findings of El-Shazly et al. [21]. Moreover, the oil of *A. santolina* collected from different locations of Allamain desert and Sinai Peninsula, contained thujone in appreciable concentrations (8.4% of total oil content) while 1,8-cineol (eucalyptol 7.1%) and camphor (6.7%) were detected at lower concentrations as compared to the findings of the current investigation (17.82%, 11.12%., respectively) [22]. Fragranyl acetate and fragranol were completely absent in the current study. In Iran [23], the essential oil of *A. santolina* collected from Southern Iranian region contained different types of terpenoids, of which, camphor was the main component detected at higher concentration as compared to the current investigations (26.7%, 11.12%., respectively). Eucalyptol, on the other hand, was detected in much lower concentrations as compared to our results (8.26%, 17.82%., respectively). In a recent investigation [24], the essential oil obtained by hydro-distillation of the different organs of *A. santolina* collected from north-eastern Iran was investigated. The different oils were dominated by fragranyl acetate (28.4% flower, 34.0% leaves and 37.0% stems), fragranol (flower 8.1%, leaves 9.1%, stems 7.8%) that were both not detected in the study by Ahmadi and his co-workers [23].

Earlier, Bader et al. [11] investigated the chemical composition of the air dried flowering parts of *A. santolina* from Jordan. Interesting qualitative and quantitative differences were noticed as compared to the findings of the current investigation. While eucalyptol was detected at similar levels (17.6%, 17.82%., respectively), camphor was reported at much higher concentrations (17.5%, 11.12%., respectively) compared to the findings of the present study. Interestingly, artemisia ketone, the second major oxygenated monoterpene detected in the current investigation was not detected in the essential oil of the mentioned study of the year 2003 using the plant from Jordan. Table 2 lists the major differences in the essential oil composition of *A. santolina* investigated from different locations.

Generally, investigations of the composition of the essential oils of aromatic plants are performed on the air dried plant materials submitted to hydro-distillation. Drying, as well as exposure to heat may affect the actual composition of the oils in the fresh plant. Additionally, in comparison of the composition of the volatile oils from different sources, environmental conditions, soil characteristics, time of harvest and climatic factors which may all affect the composition of the oil should be taken in consideration [17,25].

3.2 Antiplatelet Activity

Antiplatelet activity of the essential oil obtained from the air dried flowering parts of *A. santolina* from Jordan was determined in human whole blood *in vitro* (Table 3), Aspirin was used as a positive control. As expected, ADP and collagen showed 100% platelet aggregation that was inhibited dose-dependently upon addition of the essential oil of *A. santolina*.

Essential oil concentrations of 50 and 60 µg/ml gave more than 90% inhibition of platelet aggregation induced by ADP, and concentration of 60 µg/ml gave more than 90% inhibition of platelet aggregation induced by collagen. A thorough literature survey reveals that camphor and eucelaptol were investigated for their *in vitro* antiplatelet potency, both on ADP and collagen, and were found to be inactive [26,27]. However, the observed activity, in this case, could be attributed to the synergistic effect of the different constituetns. The essential oil of *A. biberstienii* has been reported to inhibit platelet aggregation [17]. Interestingly, the hydro-alcoholic extracts of different *Achillea* species including *A. biberestineii* [28], *A. falcata* [29] *and A. fragrantissima* [30] were found to enhance platelet aggregation, in support of the traditional use of *Achillea* species as a haemostatic.

3.3 Antiproliferative Activity

The essential oil of air-dried flowering parts of *A. santolina* growing wild in Jordan were investigated for their antiproliferative activity against MCF-7, MDA-MB-231 283 and PC-3 cancer cell lines and was found to be inactive at the stock concentrations of 1 mg /ml. Many factors could explain the lack of activity of the investigated oil including solubility, low concentration of active compounds (sesquiterpenoids and phenyl propanoids) in all samples. It is worth mentioning that the ethanol extract obtained from air dried aerial parts of *A. santolina* from Jordan had shown interesting antiproliferative activity against MCF-7 cancer cell lines (IC_{50} 24.12 µg/ml) [31].

Table 2. Major Variation in the composition of the fresh and air dried essential oils of *A. santolina* from different locations

Component	Current Results	*Iran	♦Iran			⊥Egypt			⁂Egypt
			Flower	Leaves	Stem	Flower	Leaves	Stem	
□-Pinene	4.26	10.14	1.2	1.4	0.9	0.70	0.35	0.15	0.5
Camphene	1.25	9.09	1.4	1.3	1.2	0.60	0.35	0.15	0.9
□-Thujone	0.3*	-	-	-	-	-	-	-	8.4
Eucalyptol	17.82	8.26	5.0	4.5	3.0	3.0	1.93	0.69	7.1
Camphor	11.12	26.7	4.2	4.1	3.8	3.67	3.03	3.40	6.7
Boreneol	-	4.85	1.5	3.5	4.5	4.50	4.80	3.56	1.7
4-Terpineol	2.75	-	6.4	7.1	6.1	6.60	6.53	5.89	1.1
Fragranol	-	-	8.1	9.1	7.8	11.84	13.22	18.69	8.2
trans-Carveol	-	-	-	-	-	-	-	-	2.4
Fragranyl acetate	-	-	28.4	34.0	37.0	51.70	47.14	45.10	27.3

**Sistan Region, South Iran, 2008 [23]; ♦Air dried plant Khorasan-Razavi Province, North-East Iran, [24]; ⊥Fresh aerial parts of the plant collected from wild populations near Alexandria Province [21]; ⁂collected from different locations of Allamain desert and Sinai Peninsula, [22]*

Table 3. Percentage inhibition of essential oil of air dried *A. santolina* and Aspirin (positive control) on platelet aggregation of human whole blood induced by ADP (10 µM) and collagen (2 µg/mL)

Essential oil µg/mL	ADP % inhibition	Collagen % inhibition	Aspirin µg/mL	ADP	Collagen
10	58.33±4.17	20.83±2.08	20	16.00±0.00	35.00±5.01
20	58.33±4.17	29.17±2.08	40	45.45±0.00	55.00±5.01
40	87.50±6.25	41.67±4.17	80	45.45±0.00	90.00±0.00
50	93.75±3.13	62.50±6.25	120	100.00±0.00	100.00±0.0 0
60	93.75±3.13	91.67±4.17	-	-	-

Values are presented as mean ± SE. Experiments were done in duplicates

4. CONCLUSION

Affected by the different growth stages and the status of plant used (fresh or air dried), the current study revealed interesting qualitative and quantitative variations in essential oil composition of *A. santolina*. The *p*-menthane monoterpenoid skeleton, represented by eucalyptol, had the main contribution to all tested samples. Moreover, while the oil of the air dried flowers exhibited interesting *in vitro* dose dependent antiplatelet potency, both on ADP and collagen, it did not show antiproliferative activity against the tested cancer cell lines at concentration of 1 mg/ml.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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