## SUPPLEMENTARY MATERIAL

# Chemical Composition and Biological Activities of the Essential Oil from *Pulicaria undulata* (L.) C. A. Mey. Growing Wild in Egypt

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## Abstract

*Pulicaria undulata* is used as antiinflammatory plant in Saudi Arabia and Egypt. We used gas chromatography-mass spectrometry (GC-MS) for analysis of essential oil of this plant growing wild in Egypt and 64 compounds were identified. The oil was rich in oxygenated monoterpenes (64.0%) and aromatic derivatives (18.8%). The major components were carvacrol (46.5%), xanthoxylin (18.1%) and carvotanacetone (8.7%). The oil of the Egyptian plant showed significant differences from the oil results reported on this species derived from different accessions. Antioxidant activity was performed by FRAP, DPPH and ABTS assays, and the oil demonstrated a powerful antioxidant properties. Furthermore, cytotoxic activity was assessed using MTT assay against three cell lines (A375, T98G, HCT116) and the oil showed moderate results with $IC_{50}$ of 18.53, 40.64 and 22.23 μg/ml; respectively. Anti-acetylcholinesterase activity was examined using Ellman method and the oil showed noteworthy values ($IC_{50}$= 139.2 ± 2 μg/ml).

**Key words:** *Pulicaria undulata*, Asteraceae, Essential oil, GC–MS, Biological activities**.**

## Experimental Section

### *Plant materials*

The aerial parts of *Pulicaria undulata* (L.) C. A.Mey. were collected in Sinai (Egypt), in July 2015. The plant was botanically identified and confirmed by Prof. Azza El-Hadidy (Professor of Plant Taxonomy, Herbarium of the Department of Botany, Faculty of Science, Cairo University) and Prof. Samih El-Dahmy (Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University) using available literature (Boulos 2002). We deposited a voucher specimen in the Herbarium of Pharmacognosy Department (Faculty of Pharmacy, Zagazig University, Egypt) under the accession code ZAGPU 55.

### *Isolation of essential oil*

80 g of air dried aerial parts were packed in a 2 L flask filled with 800 ml of deionized water and subjected to hydrodistillation using a Clevenger apparatus for 3 hrs. Once separated from the aqueous layer, the oil was dehydrated using anhydrous sodium sulphate and stored in sealed vials at 4 °C before chemical investigation and biological assays. The percentage of the essential oil yield was 0.17%.

### *Chemicals*

For retention index determination, we purchased the alkane standard mixture from Sigma-Aldrich (Milano, Italy). We purchased the analytical grade *n*-hexane from Carlo Erba (Milan, Italy). $Na_2SO_4$ was of analytical grade from J.T. Baker (Deventer, Holland). In case of cytotoxicity study, we purchased MTT dye (3-(4,5- Dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide) from Sigma, St. Louis, MO. We purchased 1,1-diphenyl-2- picrylhydrazyl radical (DPPH), butylated hyroxytoluene (BHT), Trolox and other chemicals used in the antioxidant experiments from Sigma-Aldrich-Fluka (Milan, Italy).

### *GC–MS analysis*

We performed the GC–MS analyses of the essential oil by an Agilent 6890N integrated with a 5973N mass spectrometer. We used HP-5 MS capillary column (5phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness; J and W Scientific, Folsom, CA) as stationary phase. The flow rate of helium (carrier gas) was 1 ml/ min. We used gradient temperature program started

from 60 °C (held 5 min) to 220 °C with an increase of 4 °C/min, then up to 280 °C at 11°C/min (held 15 min). Fragmentation was done using electron impact mode with an electron energy of 70 eV and mass spectra were generated; the acquisition operated in full scan in the range 29 - 400 m/z. We diluted essential oils in n-hexane (1:100) and 2 µl were injected and analyzed three times. A run of n-alkanes mixture C8 - C30 was done using the same operating conditions to calculate the temperature-programmed retention indices (RIs) using the formula of Van den Dool and Kratz (1963). We analyzed data by the NIST Mass Spectral Search Program, MSD ChemStation software, and NIST Tandem Mass Spectral Library v. 2.3. For peak assignment, we compared mass spectrum (MS), retention time and RI of chromatographed peaks with peaks of available standards (see Table 1). In addition, the correspondence of RI and MS with those present in a homemade library, NIST 17, FFNSC2, ADAMS was used as a reliable criterion to assign peaks. The semi-quantitative values for separated components were obtained by peak area integration with no use of response factors.

***Cytotoxic activity***

*Cell culture*

The human malignant melanoma (A375), human glioblastoma multiforme (T98G), and human colon carcinoma (HCT116) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM) and RPMI 1640 medium; respectively. We supplemented all cells with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10 % heat inactivated foetal bovine serum (HI-FBS). We cultured cells in a humidified atmosphere at 37 °C and 5% $CO_2$.

*MTT bioassay*

Cytotoxicity of the essential oil was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as reported previously by (Quassinti et al. 2013). Cytotoxic potency was indicated as $IC_{50}$ (the concentration of oil needed to inhibit cell growth by 50%) and GraphPad Prism 4 software (GraphPad Software, S. Diego, CA, USA) was used to determine the $IC_{50}$ values.

***Antioxidant Activity***

The antioxidant activity of the essential oil of *P. undulata* was evaluated by $DPPH^{\cdot}$, $ABTS^{+}$ and

FRAP assays. For the DPPH· assay, we used microplate assay mentioned by (Srinivasan et al. 2007). To determine the total radical scavenging capacity with $ABTS^+$ assay, the method previously described by (Re et al. 1999) was adopted. Serial dilution of essential oils (1 - 0.016 mg/ml) were used for determination of $IC_{50}$ in both assays. The ferric reducing antioxidant power (FRAP) assay was performed as described by (Firuzi et al. 2005). In all three assays, we compared the radicals scavenging ability of essential oil to Trolox as a positive control and the results were expressed in μM Trolox equivalents (TE)/g of essential oil (tocopherol-equivalent antioxidant capacity, TEAC).

***Acetyl-cholinesterase inhibition assay***

The anti-AChE activity of the *P. undulata* essential oil was tested using a modified microplate version of the Ellmann assay (Ellman et al. 1961). For every set of experiments, we used freshly prepared assay solution, consists of 25 μL of acetylthiocholine iodide (15 mM in phosphate buffer pH 7), 125 μL of dithionitrobenzoic acid (3 mM in phosphate buffer pH 8) and 75 μl of phosphate buffer (50 mM, pH 8) plus various concentrations of essential oils (8 - 0.06 mg/ml). 25 μl of buffer solution was used as a control. We used the AChE inhibitor galantamine hydrobromide (2 mg/ml) dissolved in methanol as a positive control and the calibration curve was generated by testing AChE inhibition of different concentrations of galantamine. Reactions were started by adding 25 μl of acetylcholinesterase (AChE from electric eel, 3 U/ml in phosphate buffer pH 8) into the reaction mixture. We monitored the reaction for 3 min at $\lambda = 412$ nm using an Omega plate reader (BMG-Labtech). The inhibitory activity, was expressed as galantamine equivalent activity and as the percentage of AChE inhibition.

***Statistical Analysis***

All cytotoxic, antioxidant and acetyl-cholinesterase inhibition tests and analyses were performed in triplicate and the average was considered. We used one-way analysis of variance (ANOVA; $p < 0.05$) to process the results. Duncan's multiple-range tests were applied to determine the significant differences between means. The SPSS 13.0 software was used to perform all statistical evaluations.

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**Table S1.** Chemical composition of the essential oil of *Pulicaria undulata* growing wild in Egypt; RSDs % were ranged from 0.37 to 8.81

| N. | Component [a] | RI calc.[b] | RI lit.[c] | Content [%][d] | ID [e] |
|---|---|---|---|---|---|
| 1 | 1-Octen-3-ol | 975 | 974 | Tr [f] | All components identified by MS & RI |
| 2 | *p*-Cymene | 1022 | 1020 | 0.2 | |
| 3 | 1,8-Cineole | 1028 | 1026 | Tr | |
| 4 | Santolina alcohol | 1035 | 1034 | 0.2 | |
| 5 | *γ*-Terpinene | 1055 | 1054 | Tr | |
| 6 | *cis*-Linalool oxide | 1070 | 1067 | Tr | |
| 7 | Terpinolene | 1095 | 1086 | Tr | |
| 8 | Linalool | 1100 | 1095 | 1.8 | |
| 9 | *cis*-Thujone | 1104 | 1101 | 0.2 | |
| 10 | *trans*-Thujone | 1113 | 1112 | 0.1 | |
| 11 | Isophorone | 1118 | 1124 | 0.2 | |
| 12 | Chrysanthenone | 1121 | 1124 | 0.6 | |
| 13 | *trans*-Pinocarveol | 1133 | 1135 | 0.1 | |
| 14 | Camphor | 1138 | 1141 | 0.6 | |
| 15 | Borneol | 1160 | 1165 | 0.1 | |

| | | | | |
|---|---|---|---|---|
| 16 | Terpinen-4-ol | 1172 | 1174 | 0.5 |
| 17 | *p*-Cymen-8-ol | 1183 | 1179 | Tr |
| 18 | *α*-Terpineol | 1187 | 1186 | 0.3 |
| 19 | Myrtenal | 1191 | 1195 | Tr |
| 20 | Coahuilensol, methyl ether | 1213 | 1219 | 0.2 |
| 21 | Carvomenthol | 1216 | 1225 | 0.1 |
| 22 | Nerol | 1228 | 1227 | Tr |
| 23 | Carvotanacetone | 1243 | 1244 | 8.7 |
| 24 | Thymoquinone | 1247 | 1248 | 2.0 |
| 25 | Piperitone | 1254 | 1249 | 0.1 |
| 26 | *cis*-Chrysanthenyl acetate | 1259 | 1261 | 0.1 |
| 27 | *(E)*-Cinnamaldehyde | 1267 | 1267 | 0.1 |
| 28 | Isopiperitenone | 1273 | 1270 | 0.2 |
| 29 | Thymol | 1294 | 1289 | 0.9 |
| 30 | Carvacrol | 1306 | 1298 | 46.5 |
| 31 | Piperitenone | 1336 | 1340 | 0.1 |
| 32 | *α*-Terpinyl acetate | 1347 | 1346 | 0.2 |
| 33 | Eugenol | 1355 | 1356 | 0.1 |
| 34 | *α*-Copaene | 1369 | 1374 | 0.1 |
| 35 | Modheph-2-ene | 1369 | 1382 | 0.1 |
| 36 | *β*-Bourbonene | 1376 | 1387 | Tr |
| 37 | *(E)-β*-Damascenone | 1380 | 1383 | Tr |
| 38 | *β*-Elemene | 1386 | 1389 | Tr |
| 39 | *(E)*-Jasmone | 1390 | 1390 | 0.1 |
| 40 | *(Z)*-Jasmone | 1395 | 1392 | 0.7 |
| 41 | Methyl eugenol | 1406 | 1403 | Tr |
| 42 | *(E)*-Caryophyllene | 1409 | 1417 | 2.1 |
| 43 | 2,5-Dimethoxy-p-cymene | 1424 | 1424 | 0.1 |
| 44 | *α*-Humulene | 1443 | 1452 | 0.3 |
| 45 | allo-Aromadendrene | 1451 | 1458 | 0.7 |
| 46 | *(E)-β*-Farnesene | 1457 | 1454 | 0.7 |
| 47 | *β*-Chamigrene | 1468 | 1476 | 0.1 |
| 48 | Germacrene D | 1472 | 1484 | 0.4 |
| 49 | *β*-Selinene | 1476 | 1489 | Tr |
| 50 | *ar*-Curcumene | 1479 | 1479 | 0.1 |
| 51 | Bicyclogermacrene | 1486 | 1500 | 1.0 |
| 52 | *α*-Muurolene | 1494 | 1500 | 0.1 |
| 53 | (*E*)-Methyl isoeugenol | 1497 | 1491 | 0.5 |
| 54 | *trans-β*-Guaiene | 1500 | 1502 | 0.1 |
| 55 | *γ*-Cadinene | 1506 | 1513 | 0.2 |
| 56 | *δ*-Cadinene | 1517 | 1522 | 0.6 |
| 57 | Caryophyllene oxide | 1572 | 1583 | 1.2 |
| 58 | Viridiflorol | 1592 | 1592 | 0.1 |
| 59 | Benzophenone | 1617 | 1626 | Tr |
| 60 | *epi-α*-Cadinol | 1633 | 1638 | 0.8 |
| 61 | *β*-Eudesmol | 1639 | 1649 | 0.6 |

| | | | | |
|---|---|---|---|---|
| 62 | $\alpha$-Cadinol | 1646 | 1652 | 0.6 |
| 63 | Xanthoxylin (2,4-dimethylether-phloroacetophenone) | 1667 | 1667 | 18.1 |
| 64 | Hexahydrofarnesyl acetone | 1845 | 1845 | Tr |
| | Total identified [%] | | | 93.9 |
| | MO (monoterpenes oxy.) | | | 64.0 |
| | MH (monoterpenes hydr.) | | | 0.2 |
| | SO (sesquiterpenes oxy.) | | | 3.2 |
| | SH (sesquiterpenes hydr.) | | | 6.5 |
| | Aromatics (Benzene dvs) | | | 18.8 |
| | Others | | | 1.2 |

a Compounds are listed in order of their elution from a HP-5MS column.
b Linear retention index on HP-5MS column, experimentally determined using homologous series of C8-C30 alkanes.
c Linear retention index taken from Adams (2007), FFNSC2 (2012) or NIST 08 (2008).
d Relative content expressed as percentage of the total oil composition. Relative percentage values are means of three determinations ± SD.
e Identification methods: MS, based on comparison with WILEY, ADAMS, FFNSC2, NIST 08 and home-made MS databases; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC2 and NIST 08.
f Tr, % below 0.1%

**Table S2.** *In vitro* cytotoxic activity of *P. undulata* essential oil.

| | **Cell line ($IC_{50}$ µg/ml)[a]** | | |
|---|---|---|---|
| | **A375[b]** | **T98G[c]** | **HCT116[d]** |
| *P. crispa* essential oil | 18.53 | 40.64 | 22.23 |
| 95% C.I.[e] | 16.15 - 21.27 | 36.07 - 45.79 | 20.18 - 24.50 |
| **Positive control** | | | |
| Cisplatin | 0.51 | 2.38 | 2.21 |
| 95% C.I | 0.46 - 0.58 | 2.09 - 2.59 | 1.96 - 2.41 |

[a] $IC_{50}$ = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation).
[b] Human malignant melanoma cell line. [c] Human glioblastoma multiforme cell line. [d] Human colon carcinoma cell line.
[e] Confidence interval.

**Table S3.** Antioxidant activity of essential oil of *P. undulata*.

| | DPPH | | ABTS | | FRAP |
|---|---|---|---|---|---|
| | **TEAC[a] µmol TE/g** | **$IC_{50}$[b] µg/ml** | **TEAC µmol TE/g** | **$IC_{50}$ µg/ml** | **TEAC µmol TE/g** |
| Essential oil | 35.9 ± 0.9 | 422.5 ± 3.2 | 1060.1 ±74.3 | 7.9 ± 0.2 | 236.5 ± 25.5 |
| **Positive control** | | | | | |
| Trolox | | 3.8 ± 0.1 | | 2.1 ± 0.2 | |

[a] TEAC = Trolox equivalent (TE) antioxidant concentration.

[b] $IC_{50}$ = The concentration of compound that affords a 50% reduction in the assay.

**Table S4.** Acetylcholinesterase inhibitory properties of essential oil from *P. undulata*.

| | **$IC_{50}$ µg/ml** | **mg GEIC/gr** |
|---|---|---|
| Essential oil | 139.3 ± 2 | 18.3 ± 1.46 |
| Galantamine | 2.55 ± 0.2 | ……. |

GEIC = Galantamine-equivalent inhibition capacity