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Original Article

CYTOTOXIC AND ANTIOXIDANT ACTIVITIES OF SECONDARY METABOLITES FROM *PULICARIA UNDULATA*

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ABSTRACT

Objective: To evaluate the *in vitro* cytotoxicity, antioxidant activities and structure-activity relationship of secondary metabolites isolated from *Pulicaria undulata*.

Methods: The methylene chloride-methanol (1:1) extract of the air-dried aerial parts of *Pulicaria undulata* was fractionated and separated to obtain the isolated compounds by different chromatographic techniques. Structures of the isolated compounds were determined on the basis of the extensive spectroscopic analysis, including 1D and 2D NMR and compared with the literature data. The crude extract and the isolated compounds were evaluated for *in vitro* antioxidant activity using the 2,2 diphenyl dipicryl hydrazine (DPPH) method and cytotoxic assay using human breast cancer (MCF-7) and hepatoma (Hep G2) cell line.

Results: Nine secondary metabolites were isolated from *Pulicaria undulata* in this study. Of which two terpenoidal compounds; 8-epi-ivalbin and 11β, 13-dihydro-4H-xanthalongin 4-*O*-β-D-glucopyranoside firstly isolated from the genus *pulicaria* and three flavonoids; eupatolitin, 6-methoxykaempferol, and patulitrin firstly isolated from *P. undulata*. 6-methoxykaempferol (IC_{50} 2.3 µg/ml) showed the most potent antioxidant activity. The highest cytotoxic effect against MCF-7 and Hep G2 cells was obtained with eupatolitin (IC_{50} 27.6 and 23.5 µg/ml) respectively. The structure-activity relationship was also examined and the findings presented here showed that 3, 5, 7, 4' and 3, 5, 4', 5'-hydroxy flavonoids were potent antioxidant and has cytotoxic activity.

Conclusion: *Pulicaria undulata* is a promising medicinal plant, and our study tends to support the therapeutic value of this plant as antioxidant drug and in the treatment of cancer.

Keywords: *Pulicaria undulata,* Sesquiterpenes, Ditepenoids, Flavonoids, Cytotoxicity, Antioxidant

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INTRODUCTION

Medicinal plants are a rich source of secondary metabolites with interesting biological activities. Therefore, these secondary metabolites have an important source with a variety of structural arrangements and properties [1-3]. The genus *Pulicaria*, belonging to the tribe Inuleae of the Asteraceae family, consists of ca. 100 species with a distribution from Europe to North Africa and Asia, particularly around the Mediterranean [4]. Phytochemical analysis of certain *Pulicaria* species led to the isolation of monoterpene, sesquiterpenes, diterpenes, triterpenes, phenolics, flavonoids and steroids [5]. Various biological activities have been reported for some species of *Pulicaria*, such as cytotoxic activity of *Pulicaria crispa* and *Pulicaria orientalis* [6-7], antibacterial activity of *Pulicaria undulata* and *Pulicaria dysenterica* [8, 9], antimicrobial activity of *Pulicaria odor* L. [10], antispasmodic activity of *Pulicaria glutinosa* [11] and antihistaminic effect of *Pulicaria dysenterica* [12]. *Pulicaria undulata* (L.) KOSTEL [syn. *P. crispa* FORSSK. BENTH. *et* HOOK. f.; *Francoeuria crispa* (FORSSK.) CASS.] is an annual herb or sometimes a perennial subshrub, producing small bright yellow flower. It is commonly used traditionally to treat inflammation, insect repellent, and even as an herbal tea [13]. As a part of our continuing search for natural antioxidant and cytotoxic agents, an attempt has been made to isolate and elucidate the structures of secondary metabolites from the *Pulicaria undulata* aerial part.

The antioxidant activities of crude extract (CH_2Cl_2/MeOH) (1:1) and the isolated compounds were evaluated by measuring their ability to scavenge the radical DPPH. Furthermore, the effects of *P. undulata* crude extract and the isolated metabolites on inhibition of cell proliferation in human breast cancer cells (MCF-7) and hepatoma cells (Hep G2) were also examined. In addition, the other objective of this study is to investigate the structure-activity relationship of identified terpenoids and flavonoids.

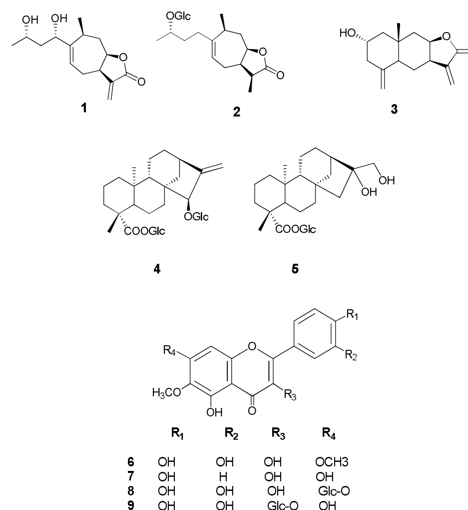


Fig. 1: Structures of the isolated compounds from *P. undulata* in the present study

MATERIALS AND METHODS

General experimental procedure

^{1}H NMR (600 MHz, $CDCl_3$ and CD_3OD), ^{13}C NMR (150 MHz, $CDCl_3$ and CD_3OD) and the 2D spectra (^{1}H-^{1}H COSY, HMQC, and HMBC) were recorded on the JEOLEAC 600 MHz spectrometer, with tetramethylsilane (TMS) as an internal standard. The IR spectrum

(KBr) was taken on a HORIBA FT-720 spectrometer. Optical rotation was determined with the help of an HORIBA SEPA-300 spectro-polarimeter. Electron impact mass spectrometry (El-MS) analyses were recorded on a JEOLSX102A mass spectrometer. Column chromatography was carried out on silica gel 60 (Merck; 230-400 mesh) and sephadex LH-20 (Pharmacia Co. Tokyo, Japan). TLC was performed on silica gel 60 F_{254} plated (0.25 mm, Merck Co.), Column chromatography (CC) was carried out on kieslgel 60 (Merck; 230-400 mesh) and spots were detected under UV light and colored by spraying with 10% H_2SO_4 solution followed by heating.

Plant material

P. undulata plant was collected in March 2013, from North Sinai, Egypt. A voucher specimen (no. PU-51-03-13) was deposited in the Herbarium of Saint Katherine protectorate, Egypt. The collection was taking place under the permission of Saint Katherine protectorate for scientific purposes.

Extraction and Isolation

Aerial parts (1.5 kg) of *P. undulata* were powdered and extracted with CH_2Cl_2-MeOH (1:1) at room temperature. The extract was concentrated *in vacuo* to obtain a residue of 112 g. The residue was fractionated by silica gel CC (6×120 cm) eluted with n-hexane (2500 ml) followed by a gradient of n-hexane-$CHCl_3$ up to 100 % CH_2Cl_2 and CH_2Cl_2-MeOH up to 15% MeOH (2000 ml of each solvent mixture) with increasing degree of polarity. The n-hexane–chloroform (1:2) fraction (250 mg) was pre-fractionated by CC using sephadex LH-20 (2×40 cm) and eluted with n-hexane–methylene chloride-methanol 7: 4: 0.5 (3000 ml). Fractions were obtained and combined into two main portions: A (90 mg) and B (100 mg). Sub-fraction A was repurified by reversed phase HPLC using MeOH/H_2O (65–30 % 2500 ml) to afford compound 1 (10 mg), 2 (12 mg), 3 (15 mg), 4 (10 mg) and 5 (15 mg). Sub-fraction B repurified by reversed phase HPLC using MeOH/H_2O (65–40 % 1500 ml) to afford compound 6 (30 mg), 7 (16 mg), 8 (20 mg) and 9 (22 mg).

8-epi-Ivalbin (1)

Colourless crystals; IR (KBr, ν_{max}, cm^{-1}): 1760, 1660, 1630; ^1H NMR (600 MHz, $CDCl_3$) δ: 4.23 (1H, dd, *J* = 5.5, 8.5 Hz, H-2), 1.69 (IH, m, H-3α), 1.55 (1H, m, H-3β), 4.05 (1H, m, H-4), 5.79 (1H, dd, *J* = 6.2, 8.9 Hz, H-5), 2.43 (1H, m, H-6 α), 2.30 (1H, m, H-6 β), 3.31 (1H, m, H-7), 4.62 (1H, ddd, *J* = 2.7, 9.6, 12.3 Hz, H-8), 2.05 (1H, m, H-9 α), 1.81 (1H, m, H-9 β), 2.60 (1H, m, H-10), 6.26 (1H, d, *J* = 2.7 Hz, H-13α), 5.52 (1H, d, *J* = 2.7 Hz, H-13β), 1.17 (3H, d, *J* = 6.2 Hz, H-14), 1.22 (3H, d, *J* = 6.2 Hz, H-15); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 148.5 (C-1), 77.3 (C-2), 44.4 (C-3), 69.2 (C-4), 122.2 (C-5), 26.1 (C-6), 41.6 (C-7), 79.1 (C-8), 37.0 (C-9), 33.3 (C-10), 138.8 (C-11), 170.1 (C-12), 121.9 (C-13), 24.5 (C-14), 22.0 (C-15).

11β, 13-dihydro-4H-xanthalongin 4-O-β-D-glucopyranoside (2)

Amorphous white powder; ^1H NMR (600 MHz, CD_3OD) δ: 1.49 (1H, m, H-1α), 1.44 (IH, m, H-2β), 1.72 (1H, m, H-3α), 1.67 (1H, m, H-3β), 3.78 (1H, m, H-4), 5.55 (1H, br d, *J* = 10.5 Hz, H-5), 2.13 (1H, dd, *J* = 12.0, 12.3 Hz, H-6α), 2.03 (1H, m, H-6β), 2.10 (1H, m, H-7), 4.58 (1H, ddd, *J* = 2.7, 9.6, 12.3 Hz, H-8), 2.20 (1H, dd, *J* = 2.1, 15.0 Hz, H-9α), 1.60 (1H, ddd, *J* = 2.7, 4.1, 12.3 Hz, H-9β), 2.51 (1H, m, H-10), 1.63 (1H, m, H-11), 6.26 (IH, d, *J* = 2.7 Hz, H-13α), 5.52 (IH, d, *J* = 2.7 Hz, H-13β), 1.12 (3H, d, J = 6.2, H-14), 1.22 (3H, d, *J* = 6.2 Hz, H-15), 4.29 (1H, d, *J* = 7.8 Hz, H-1'), 3.13 (1H, t, *J* = 8.8 Hz, H-2'), 3.32 (1H, m, H-3'), 3.25 (1H, m, H-4'), 3.24(1H, m, H-5'), 3.81 (1H, m, H-6'α), 3.63 (1H, m, H-6'β); ^{13}C NMR (150 MHz, CD_3OD) δ: 147.3 (C-1), 35.0 (C-2), 36.2 (C-3), 76.1 (C-4), 122.2 (C-5), 23.8 (C-6), 48.1 (C-7), 82.2 (C-8), 36.8 (C-9), 33.9 (C-10), 40.0 (C-11), 181.2 (C-12), 17.1 (C-13), 9.0 (C-14), 20.9 (C-15), 102.9 (C-1'), 74.0 (C-2'), 76.8 (C-3'), 70.3 (C-4'), 76.5 (C-5'), 61.5 (C-6').

2α-Hydroxy-5αH-eudesma-4(15), 11(13)-dien-12,8β-olide (Ivalin) (3)

Amorphous white powder; IR (KBr, ν_{max}, cm^{-1}): 3440, 2924, 1754, 1647, 1457, 1347, 1264, 1137; LC-MS *m/z* 271.1294 [M+Na]$^+$(calcd. For $C_{15}H_{20}O_3Na$: 271.1310), 219.2698 [2M+Na]$^+$(cald. For $C_{30}H_{40}O_6Na$: 519. 2722); ^1H NMR (600 MHz, $CDCl_3$) δ: 2.65 (1H, m, H-1α), 1.98 (1H, m, H-1β), 3.82 (IH, ddd, *J* = 4.8, 11.2, 15.6 Hz, H-2), 1.88 (1H, m, H-3α), 1.17 (1H, m, H-3β), 1.83 (1H, d, *J* = 12.8 Hz, H-5), 1.75 (1H, m, H-6α), 1.33 (1H, m, H-6β), 2.97 (1H, m, H-7), 4.49 (1H, dd, *J* = 4.8, 4.4 Hz, H-8), 2.23 (1H, dd, *J* = 1.3, 14.0 Hz, H-9α), 1.52 (1H, dd, *J* = 15.5, 14.0 Hz, H-9β), 6.12 (1H, br. s, H-13α), 5.59 (1H, br. s, H-13β), 0.82 (1H, s, H-14), 4.87 (1H, br. s, H-15α), 4.54 (1H, br. s, H-15β); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 41.1 (C-1), 67.1 (C-2), 46.3 (C-3), 146.0 (C-4), 45.6 (C-5), 27.3 (C-6), 40.5 (C-7), 76.7 (C-8), 51.0 (C-9), 34.0 (C-10), 141.9 (C-11), 170.6 (C-12), 120.5 (C-13), 18.8 (C-14), 109.4 (C-15)

Crispioside A (4)

Colourless crystals; ^1H NMR (600 MHz, CD_3OD) δ: 1.94 (1H, m, H-1α), 1.28 (IH, m, H-1β), 1.38 (1H, ddd, *J* = 17.1, 4.4, 2.2 Hz, H-2α), 1.34 (1H, ddd, *J* = 17.1, 15.1, 5.1 Hz, H-2β), 2.13 (1H, m, H-3α), 1.04 (1H, m, H-3β), 1.53 (1H, m, H-5), 1.80 (1H, dd, *J* = 6.8, 14.2 Hz, H-6α), 1.78 (1H, dt, *J* = 3.2, 14.2 Hz, H-6β), 1.40 (1H, m, H-7α), 0.98 (1H, m, H-7β), 1.18 (1H, m, H-9), 1.43 (1H, m, H-11α), 1.40 (1H, m, H-11β), 1.41 (1H, m, H-12α), 0.99 (1H, m, H-12β), 2.52 (1H, m, H-13), 1.86 (1H, m, H-14α), 0.90 (1H, brs, H-14β), 3.81 (1H, dd, *J* = 2.0, 3.0 Hz, H-15), 5.26 (1H, d, *J* = 3.0 Hz, H-17α), 4.90 (1H, d, *J* = 3.0, H-17β), 1.20 (3H, s, H-19), 0.94 (3H, s, H-20), 5.38 (1H, d, *J* = 8.0, H-1'), 3.25 (1H, dd, *J* = 8.0, 9.5 Hz, H-2'), 5.27 (1H, dd, *J* = 9.5, 9.5 Hz, H-3'), 3.35 (1H, dd, *J* = 9.5, 9.5 Hz, H-4'), 3.37 (1H, m, H-5'), 3.67 (1H, dd, *J* = 2.5, 12.5 Hz, H-6'α), 3.65 (1H, dd, *J* = 5.0, 12.5, H-6'β), 4.41(1H, d, *J* = 8.0 Hz, H-1''), 3.28 (1H, dd, J= 8.0, 9.5 Hz, H-2''), 3.34 (1H, dd, *J* = 9.5, 9.5 Hz, H-3''), 3.31 (1H, dd, *J* = 9.5, 9.5 Hz, H-4''), 3.21 (1H, m, H-5''), 3.89 (1H, dd, *J* = 5.0, 12.5 Hz, H-6''α), 3.88 (1H, dd, J = 5.0, 12.5 H-6''β); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 37.9 (C-1), 19.0 (C-2), 37.8 (C-3), 46.2 (C-4), 46.0 (C-5), 21.3 (C-6), 36.2 (C-7), 43.9 (C-8), 56.3 (C-9), 39.2 (C-10), 17.7 (C-11), 33.4 (C-12), 40.4 (C-13), 40.5 (C-14), 90.4 (C-15), 151.3 (C-16), 105.1 (C-17), 175.1 (C-18), 27.8 (C-19), 15.4 (C-20), 104.6 (C-1'), 72.7 (C-2'), 76.2 (C-3'), 70.2 (C-4'), 72.7 (C-5'), 61.1 (C-6'), 94.2 (C-1''), 74.1 (C-2'), 76.2 (C-3''), 79.7 (C-4''), 77.0 (C-5''), 77.3 (C-6'').

Crispioside B (5)

Colourless crystals; ^1H NMR (600 MHz, CD_3OD) δ: 1.81 (1H, m, H-1α), 0,78 (IH, m, H-1β), 1.59 (1H, m, H-2α), 1.57 (1H, m, H-2β), 2.14 (1H, m, H-3α), 1.95 (1H, m, H-3β), 1.08 (1H, m, H-5), 1.98 (1H, dd, *J* = 6.8, 14.2 Hz, H-6α), 1.81 (1H, dt, *J* = 3.2, 14.2 Hz, H-6β), 1.93 (1H, m, H-7α), 1.57 (1H, m, H-7β), 1.06 (1H, m, H-9), 1.92 (1H, m, H-11α), 1.90 (1H, m, H-11β), 1.57 (1H, m, H-12α), 1.48 (1H, m, H-12β), 1.94 (1H, m, H-13), 1.26 (1H, m, H-14α), 0.88 (1H, m, H-14α), 3.33 (1H, m, H-15α), 3.32 (1H, m, H-15β), 3.81 (1H, d, *J* = 11.0 Hz, H-17α), 3.56 (1H, d, *J* = 11.0 Hz, H-17β), 1.18 (3H, s, H-19), 0.95 (3H, s, H-20), 5.38 (1H, d, *J* = 8.0 Hz, H-1'), 3.30 (1H, dd, J= 8.0, 9.5 Hz, H-2'), 3.39 (1H, dd, *J* = 9.5, 9.5 Hz, H-3'), 3.25 (1H, dd, *J* = 9.5, 9.5 Hz, H-4'), 3.26 (1H, m, H-5'), 3.29 (1H, m, H-6'α), 3.28(1H, m, H-6'β); ^{13}C NMR (150 MHz, CD_3OD) δ: 40.5 (C-1), 18.8 (C-2), 37.7 (C-3), 44.5 (C-4), 56.0 (C-5), 21.9 (C-6), 36.7 (C-7), 43.7 (C-8), 57.2 (C-9), 39.6 (C-10), 18.3 (C-11), 25.9 (C-12), 44.9 (C-13), 42.0 (C-14), 52.4 (C-15), 81.6 (C-16), 56.5 (C-17), 176.9 (C-18), 27.7 (C-19), 15.0 (C-20), 94.3 (C-1'), 72.7 (C-2'), 77.4 (C-3'), 69.8 (C-4'), 72.7 (C-5'), 61.1 (C-6').

Quercetagetin 6, 7-dimethyl ether (Eupatolitin) (6)

Amorphous white powder; ^1H NMR (600 MHz, CD_3OD) δ: 6.71 (1H, s, H-8), 6.62 (IH, d, *J* = 2.0 Hz, H-2'), 6.88 (1H, d, *J* = 8.5 Hz, H-5'), 7.52 (1H, dd, J= 2.0, 8.5 Hz, H-6'), 3.76 (3H, s, OCH_3), 3.94 (3H, s, OCH_3); ^{13}C NMR (150 MHz, CD_3OD) δ: 154.5 (C-2), 138.1 (C-3), 178.9 (C-4), 148.6 (C-5), 105.5 (C-6), 149.8 (C-7), 90.2 (C-8), 157.0 (C-9), 115.1 (C-10), 121.7 (C-1'), 121.0 (C-2'), 145.1 (C-3'), 145.3 (C-4'), 115.2 (C-5'), 129.5 (C-6'), 55.1 (OCH_3), 59.1 (OCH_3).

6-Methoxykaempferol (7)

Yellow amorphous powder; ^1H NMR (600 MHz, CD_3OD) δ: 6.45 (1H, s, H-8), 6.85 (IH, d, *J* = 8.0 Hz, H-2',6'), 8.05 (1H, d, *J* = 8.0 Hz, H-3',5'), 3.85 (3H, s, OCH_3);^{13}C NMR (150 MHz, CD_3OD) δ: 147.0 (C-2), 135.6 (C-3), 176.3 (C-4), 152.3 (C-5), 131.0 (C-6), 157.1 (C-7), 93.4 (C-8), 151.7 (C-9), 104.0 (C-10), 122.4 (C-1'), 129.4 (C-2',6'), 115.0 (C-3',5'), 159.3 (C-4'), 59.6 (OCH_3).

Patuletin 7-*O*-β-D-glucopyranoside (Patulitrin) (8)

Yellow amorphous powder; ^1H NMR (600 MHz, CD_3OD) δ: 3.28–3.86 (6H, m, H-2''-6''), 5.07 (1H, d, *J* = 8.0 Hz, H-1''), 6.86 (1H, s, H-8), 7.74

(IH, d, *J* = 2.0 Hz, H-2'), 6.85 (1H, d, *J* = 8.5 Hz, H-5'), 6.65 (1H, dd, *J* = 2.0, 8.5 Hz, H-6'), 3.86 (3H, s, OCH_3); ^{13}C NMR (150 MHz, CD_3OD) δ: 147.7 (C-2), 132.0 (C-3), 176.3 (C-4), 156.3 (C-5), 100.7 (C-6), 162.0 (C-7), 95.0 (C-8), 151.8 (C-9), 114.8 (C-10), 122.6 (C-1'), 114.9 (C-2'), 144.9 (C-3'), 147.7 (C-4'), 120.6 (C-5'), 132.0 (C-6'), 105.3 (C-1''), 73.4 (C-2''), 77.2 (C-3''), 69.8 (C-4''), 77.4 (C-5''), 61.2 (C-6''), 60.2 (OCH_3).

Patuletin 3-*O*-β-D-glucopyranoside (9)

Yellow amorphous powder; ^{1}H NMR (600 MHz, CD_3OD) δ: 3.20–3.67 (6H, m, H-2''-6''), 5.23 (1H, d, *J* = 8.0 Hz, H-1''), 6.48 (1H, s, H-8), 7.68 (IH, d, *J* = 2.0 Hz, H-2'), 6.84 (1H, d, *J* = 8.5 Hz, H-5'), 7.55 (1H, dd, *J* = 2.0, 8.5 Hz, H-6'), 3.84 (3H, s, OCH_3); ^{13}C NMR (150 MHz, CD_3OD) δ: 157.5 (C-2), 133.9 (C-3), 178.4 (C-4), 144.6 (C-5), 94.3 (C-6), 157.8 (C-7), 93.6 (C-8), 152.3 (C-9), 104.8 (C-10), 121.7 (C-1'), 114.7 (C-2'), 131.3 (C-3'), 148.5 (C-4'), 116.2 (C-5'), 121.9 (C-6'), 101.9 (C-1''), 74.4 (C-2''), 77.0 (C-3''), 69.9 (C-4''), 78.1 (C-5''), 61.2 (C-6''), 59.7 (OCH_3).

Antioxidant assay

The antioxidant activity was determined by the DPPH free radical scavenging assay in triplicate and average values were considered [14]. Freshly prepared (0.004 % w/v) methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10 °C in the dark. A methanol solution of the test samples was prepared. A 40 µl aliquot of the methanol solution was added to 3 ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Miton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

$$PI = [\{(AC-AT)/AC\} \times 100].$$

Where; AC = Absorbance of the control at t = 0 min and AT = Absorbance of the sample+DPPH at t = 16 min.

Cytotoxic assay

The human breast adenocarcinoma cell line (MCF-7), and the human hepatocarcinoma cell line (Hep G2) cell line, which was purchased from the American Type Culture Collection (ATCC) USA, were used to evaluate the cytotoxic effect of the crude extract and the compounds. A control of untreated cells was made in the absence of the testing sample. A positive control containing doxorubicin drug was also tested as a reference drug for comparison. Six wells were used for each concentration of the tested samples. The number of the surviving cells was determined by staining the cells with crystal violet followed by cell lysing 33% glacial acetic acid and read the absorbance at 590 nm using ELISA reader (SunRise, TECAN, inc, USA) after well mixing [15,16]. The absorbance values from untreated cells were considered as 100 % proliferation. The number of viable cells was determined using ELISA reader as previously mentioned before and the percentage of viability was calculated as $[1-(ODt/ODc)] \times 100$ %. Where ODt is the mean optical density of untreated cells. The 50 % inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots.

RESULTS AND DISCUSSION

Identification of purified compounds

The extract CH_2Cl_2-MeOH (1:1) of *P. undulata* aerial parts were subjected to silica gel CC and reversed phase HPLC resulted in the isolation of nine secondary metabolites (1-9) (fig. 1). Of which three sesquiterpenes (1-3) were identified as 8-epi-ivalbin (1) [17], 11β,13-dihydro-4H-xanthalongin 4-*O*-β-D-glucopyranoside (2) [18], ivalin (3) [19], two diterpenes (4-5) were identified as crispioside A (4) and crispioside B (5) [20], in addition to four flavonoids (6-9) were identified as; quercetagetin 6, 7-dimethyl ether (eupatolitin) (6) [21], 6-methoxykaempferol (7) [22], patuletin 7-*O*-β-D-glucopyranoside (patulitrin) (8) [23] and patuletin 3-O-β-D-glucopyranoside (9) [24]. Compounds (6), (7) and (8) firstly isolated from *P. undulata* and also previously isolated from other species of *Pulicaria* [21-23]. As far as could be ascertained, this is the first report of compounds (1) and (2) from the genus *Pulicaria*.

Determination of antioxidant activity

Although various assays were reported to estimate the free radical scavenging activity [25], one common method is DPPH. The change in absorbance produced by reducing DPPH was used to evaluate the ability of testing compounds as antioxidant activity. Based on the principle, the antioxidant of the tested samples can be expressed as its ability in scavenging the DPPH radicals. The dose-response curves for the tested samples showed that for each sample six concentrations (µg/ml) were tested (fig. 2). It was found that the crude extract and the isolated compounds of *P. undulata* showed variable degrees of free radical scavenging property that increased in a dose-dependent manner. The inhibition percentage of DPPH radical formation ranged from 18.9 % to 96.3 % at the highest tested dose (128 µg/ml) and from 3.8 % to 23.1 % at the lowest tested dose (1 µg/ml) (fig. 2). The obtained results showed that the DPPH scavenging percentage of the different tested samples at the same concentration (128 µg/ml) is as follows: 6-methoxykaempferol (7) (96.3%)>eupatolitin (6) (94.7%)>patuletin 3-O-β-D-gluco-pyranoside (9) (91.3 %)>patulitrin (8) (90.8%)>the crude extract (CH_2Cl_2/MeOH) (76.2%)>ivalbin (1) (61.5%), with the IC_{50} values of 2.3, 2.7, 6.0, 6.7, 43.9 and 93.4 µg/ml respectively.

However, the other compounds 11β,13-dihydro-4H-xanthalongin 4-O-β-D-glucopyranoside (2), ivalin (3), crispioside A (4) and crispioside B (5) showed poor antioxidant activity, the values of DPPH scavenging percentage are 18.9, 23.7, 27.3 and 38.9 % respectively.

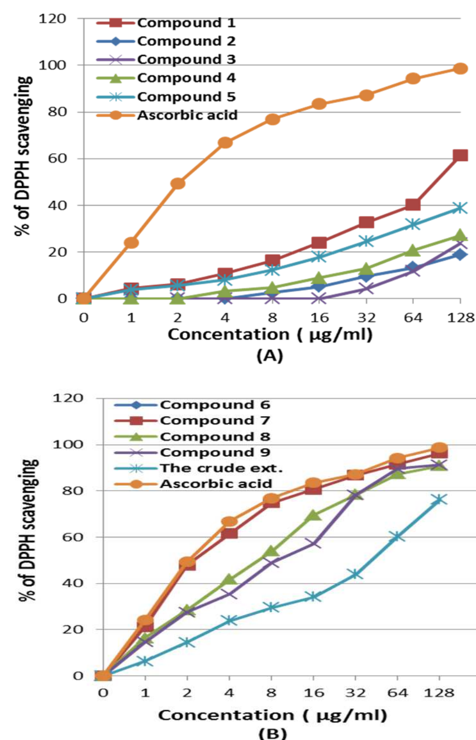


Fig. 2: A) Antioxidant activity of terpenoids (1-5), B) Flavonoids (6-9) and the crude extract compared with standard Ascorbic acid

Data are means±standard deviation of triplicate experiments

Determination of cytotoxic activity

Cancer is a public health problem all over the world [26]. A Large number of plants and their isolated constituents has been shown to

possess potential anticancer activity. The cytotoxic activity of the crude extract and the isolated compounds of *P. undulata* were also assessed against MCF-7 and Hep G2 cells. We have examined the effect of each sample on the proliferation of MCF-7 and Hep G2 cells *in vitro* at six different concentrations (3.125-100 µg/ml) using the viability assay. The results obtained from this study showed that the tested samples inhibited the proliferation of MCF-7 cells (fig. 3) and Hep G2 cells (fig. 4) at various levels, and the cytotoxic activity increased in a dose-dependent manner. The strongest cytotoxic effect was obtained, with compound 6 against MCF-7 cells (IC_{50} 27 µg/ml), followed by compound 2, 7, 3, 4, 1 and 8 with respective IC_{50}s of 35.9, 37.3, 39.6, 47.9, 62.1 and 87.1 µg/ml. The MCF-7 cells were resistant to compounds 5 and 9 that also showed a weak cytotoxicity against Hep G2 cells. On the other hand, compound 6 is also the strongest cytotoxic effect against Hep G2 cells (IC_{50} 23.5 µg/ml), followed by compound 3, 2, 7, 4, 8, 1, 5 and 9 with respective IC_{50}s of 31.6, 39.5, 40.2, 49.6, 72.0, 80.1, 82.3 and 85.0 µg/ml. In addition, the crude extract (CH_2Cl_2/MeOH) showed a good cytotoxic activity against both MCF-7 cells and Hep G2 cells with IC_{50} 41.6 and 40.7 µg/ml respectively.

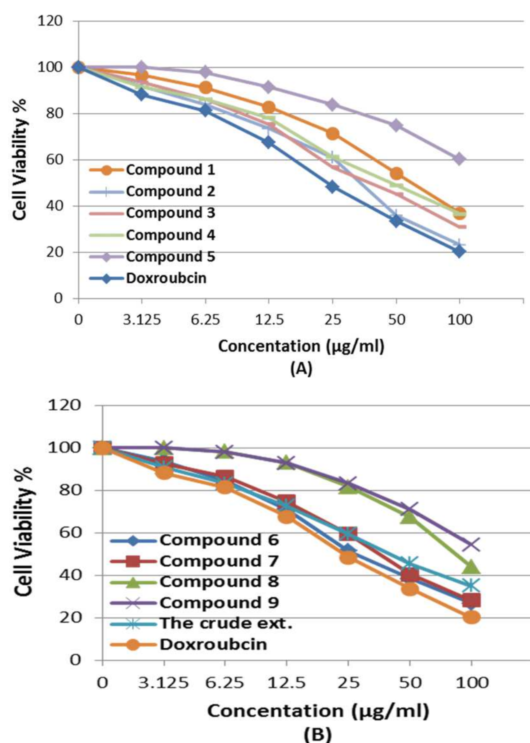
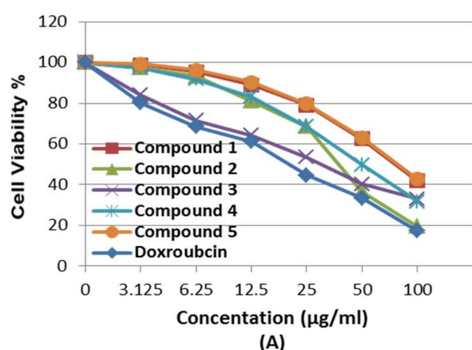


Fig. 3: A) Cytotoxic activity of terpenoids (1-5), B) Flavonoids (6-9) and the crude extract compared to Doxorubicin as positive control on MCF-7 cells *in vitro*

Data are means±standard deviation of triplicate experiments



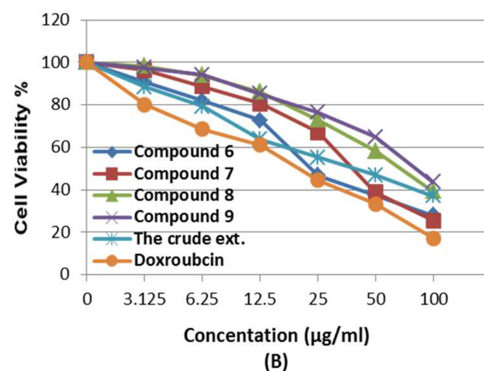


Fig. 4: A) Cytotoxic activity of terpenoids (1-5), B) Flavonoids (6-9) and the crude extract compared to Doxorubicin as positive control on Hep G2 cells *in vitro*

Data are means±standard deviation of triplicate experiments

Structures-activity relationship

Flavonoids are powerful antioxidant against free radicals because they act as ''radical scavengers''. This activity is attributed to their hydrogen-donating ability. Where the phenolic groups of flavonoids serve as a source of a ready available ''H'' atoms, allowing as delocalization over the flavonoids structure of the subsequent radicals produced. In fact, the antioxidant capacity of a flavonoid is linked to its particular chemical structures [27, 28].

Compound 7 (IC_{50} 2.3 µg/ml) showed the most potent DPPH scavenger in this study, not only possesses the 2, 3-double bond in conjugation with 4-oxo function in the C-ring, responsible for the electron dislocation from the B-ring, but also possesses both 3 and 5-hydroxyl groups and another free 7-OH in the A-ring, which are among the essential structural elements of potent radical-scavenging activities of the flavonoids [28, 29]. Compound 6 (IC_{50} 2.7 µg/ml) is also a potent antioxidant and came in the second rank, possesses a 3', 4'-catechol structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding, and which participates in the electron dislocation [30, 31]. Also, it possesses the 2,3-double bond in conjugation with a 4-oxo function and both 3 and 5-hydroxyl groups. On the other hand, the presence of two methoxy groups at 6 and 7 positions (Methoxylation) in compound 6 might play a certain role in reducing its antioxidant activity when compared with compound 7 [27, 32]. Although, both compound 8 (IC_{50} 6.7 µg/ml) and compound 9 (IC_{50} 6.0 µg/ml) possesses the 2,3-double bond in conjugation with a 4-oxo function and 3', 4'-catechol structure, but their antioxidant activity highly reduced compared to compounds 6 and 7. The combination of a glucose moiety of compounds 8 and 9 at 7 and 3 positions respectively, may be playing an important role in reducing their antioxidant activity [28, 29]. On the other hand, the sesquiterpenoidal compound 1 (IC_{50} 93.4 µg/ml), showing the lowest antioxidant potency, the presence of two free hydroxyl groups, one CH_3 and one CH_2 in its structure, seems to confer only satisfies reducing potential. However, the other terpenoids 2, 3, 4 and 5 showed poor antioxidant activity, the DPPH scavenging percentage of them less than 40 %.

The highest cytotoxic effect against MCF-7 and Hep G2 cells was obtained with compound 6 (IC_{50} 27.6 and 23.5 µg/ml) respectively; this activity could be explained by the presence of a C2-C3 double bond and the 3-hydroxyl group of the ring A that important factors for the anti-proliferative activity of flavonoids [33]. Compound 7 is also a potent cytotoxic activity against MCF-7 and Hep G2 cells (IC_{50} 37.3 and 40.2 µg/ml) respectively and came in the second rank after compound 6. Comparing compound 6 and 7, it is concluded that hydroxylation at C-5' and O-methylation at C-7 in compound 6 seem to enhance its cytotoxicity more than compound 7 [34, 35]. In fact, the presence of methoxyl substituent has modulated the cytotoxicity of flavonoids [36-38]. The presence of 7-O glucose in compound 8 instead of the 7-methoxy group in compound 6, highly reduced the

cytotoxicity of compound 8 (IC_{50} 87.1 and 72 µg/ml) compared with compound 6 (IC_{50} 27.6 and 23.5 µg/ml) against MCF-7 and Hep G2 cells respectively. Compound 9 is the lowest cytotoxic activity against MCF-7 and Hep G2 cells, (IC_{50}>100 and 85 µg/ml) respectively, that possesses 3-O glucose and 7-OH instead of 3-OH and 7-methoxy groups in compound 6. We can conclude that the presence of both 3-free hydroxyl group and the 7-methoxy group is essential for flavonoids cytotoxicity.

The terpenoidal compounds 1-5 showed the variable potency of cytotoxic activity, compound 2 (IC_{50} 35.9 and 39.5 µg/ml) and compound 3 (IC_{50} 39.6 and 31.6 µg/ml) showed the most potent cytotoxic against MCF-7 and Hep G2 cells, respectively, compared with the other terpenoids. The cytotoxicity of compound 2 and 3 could be explained by the presence of a number of CH_2 and CH_3 groups in both skeletons that enhanced their polarity and cytotoxicity [39, 40]. Also, the presence of 4-O glucose in compound 2 instead of the free 4-OH group in compound 1, highly enhanced the cytotoxicity of compound 2 (IC_{50} 35.9 and 39.5 µg/ml) more than compound 1(IC_{50} 62.1 and 80.5 µg/ml) against MCF-7 and Hep G2 cells, respectively. On the other hand, compound 4 (IC_{50} 47.9 and 49.6 µg/ml) is more potent in cytotoxicity than compound 5 (IC_{50}>100 and 82.3 µg/ml) against MCF-7 and Hep G2 cells, respectively, compound 4 is highly more polar than compound 5, that may play important role in enhancing its cytotoxicity [39, 40].

To the authors' knowledge, this is the first report concerning the antioxidant and cytotoxic terpenoids and flavonoids from *P. undulata* with the study of their structure-activity relationship.

CONCLUSION

In this study, the antioxidant and cytotoxic potential of flavonoids and terpenoids isolated from *P. undulata* aerial part were evaluated using *in vitro* DPPH and the viability assay, respectively. The findings presented here showed that 3, 5, 7, 4' and 3, 5, 4', 5'-hydroxy flavonoids were a potent antioxidant and cytotoxic activity. It can suggest that the C2-C3 double bond, in conjugation with a 4-oxo function in the C-ring and OH substitution on the A-ring play important roles in the antioxidant and cytotoxic capacity of these flavonoids. Furthermore, the ortho-dihydroxy (catechol) structure in the B-ring and the 7-methoxylation in the A-ring also affect significantly the antioxidant and cytotoxic potential. On the other hand, among the tested terpenoids in this study, the presence of a number of CH_3, CH_2 and sugar moiety in sesqui/diterpenes skeletons gives their molecules a polar character that enhances the cytotoxic activity. The investigation of such structure–activity relationship in this study afforded important information that may participate in the development of the future design for antioxidant and cytotoxic agents

CONFLICT OF INTERESTS

Declared none

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