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-1-picrylhydrazine (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, Diterpenoids, Flavonoids, Cytotoxicity, Antioxidant

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$_2$Cl$_2$/MeOH) (1:1) and the isolated compounds were measured by using 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

1H NMR (600 MHz, CDCl$_3$ and CD$_3$OD), 13C NMR (150 MHz, CDCl$_3$ and CD$_3$OD) and the 2D spectra (1H-1H COSY, HMOC, and HMBC) were recorded on the JEOL LEAC 600 MHz spectrometer, with tetramethylsilane (TMS) as an internal standard. The IR spectrum
13β), 1.12 (3H, d, J = 6.2, H-14), 1.22 (3H, d, J = 6.2, H-15); 1.52 (1H, δ: 148.5 (C-1), 77.3 (C-2), 154.5 (C-2'), 138.1 (C-3), 178.9 (C-4), 152.3 (C-5), 131.0 (C-6), 157.1 (C-7), 93.4 (C-8), 115.1 (C-9), 157.0 (C-10), 114.0 (C-11), 150.0 (C-12), 146.0 (C-13), 45.6 (C-15), 27.3 (C-16), 40.5 (C-17), 7.67 (C-18), 5.10 (C-19), 34.0 (C-20), 141.9 (C-11), 170.6 (C-12), 120.5 (C-13), 18.8 (C-14), 10.94 (C-15).

Crispoides A (4)

Crispoides B (5)

Crispoides C (6)

Quercetagin 6, 7-dimethyl ether (Eupatalon) (6)

6-Methoxykaempferol (7)

Patialon 7-O-β-d-glycopyranoside (Patalurin) (8)
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-glucopyranoside (9) (91.3%) > patulitrin (8) (90.8%), and the crude extract (CH<sub>3</sub>C<sub>6</sub>OH) (76.2%) > ivalbin (1) (61.5%), with the IC<sub>50</sub> values of 2.3, 27, 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.

RESULTS AND DISCUSSION

Identification of purified compounds

The extract CH<sub>3</sub>C<sub>6</sub>OH (1:1) of *P. undulata* aerial parts were subjected to silica gel GC 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: queretetargin 6, 7-dimethyl ether (eupatolitin) (6) [21], 6-methoxykaempferol (7) [22], patuletin 7-O-β-D-gluco-
possess potential anticancer activity. The cytotoxic activity of the crude extract and the isolated compounds of \textit{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, and 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\textsubscript{50} 27 \textmu g/ml), followed by compound 2, 7, 3, 4, 1 and 8 with respective IC\textsubscript{50} of 35.9, 37.3, 39.6, 47.9, 62.1 and 87.1 \textmu g/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\textsubscript{50} 23.5 \textmu g/ml), followed by compound 3, 2, 7, 4, 8, 1, 5 and 9 with respective IC\textsubscript{50} of 31.6, 39.5, 40.2, 49.6, 72.0, 80.1, 82.3 and 85.0 \textmu g/ml. In addition, the crude extract (CH\textsubscript{2}Cl\textsubscript{2}/MeOH) showed a good cytotoxic activity against both MCF-7 cells and Hep G2 cells with IC\textsubscript{50} 41.6 and 40.7 \textmu g/ml respectively.

**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 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\textsubscript{50} 2.3 \textmu g/ml) showed the most potent DPPH scavenger in this study, not only possesses the 2, 3-double bond in conjunction 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\textsubscript{50} 2.7 \textmu g/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 aroxy radicals, possibly through hydrogen bonding, and which participates in the electron dislocation [30, 31]. Also, it possesses the 2,3-double bond in conjunction 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 (Methoxylolation) 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\textsubscript{50} 6.7 \textmu g/ml) and compound 9 (IC\textsubscript{50} 6.0 \textmu g/ml) possesses the 2,3-double bond in conjunction 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\textsubscript{50} 93.4 \textmu g/ml), showing the lowest antioxidant potency, the presence of two free hydroxyl groups, one CH\textsubscript{2} and one CH\textsubscript{3} 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\textsubscript{50} 27.6 and 23.5 \textmu g/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\textsubscript{50} 37.3 and 40.2 \textmu g/ml) respectively and came in the second rank after compound 6. Comparing compound 6 and 7, it is concluded that hydroxylaton 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 \mu g/ml) compared with compound 6 (IC_{50} 27.6 and 23.5 \mu g/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 \mu g/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 \mu g/ml) and compound 3 (IC_{50} 39.6 and 31.6 \mu g/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 [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 [39, 40].

Also, the presence of both 3-free hydroxyl group and the 7-methoxy group is essential for flavonoids cytotoxicity.

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 DPHI and the viability assay, respectively. The findings presented here showed that 3, 5, 7, 4’, 3, 5, 4’, 5’-hydroxy flavonoids were a potent antioxidant and cytotoxic activity. It can be suggested 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_2 and CH_3 groups in sesquiterpenes skeletons gives bacterial molecules a polar character that enhances the cytotoxicity. 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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