

Isolation, Characterization and Antiproliferative Activity of Secondary Metabolites from *Tanacetum alyssifolium* (Bornm.) Grierson

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Abstract: Secondary metabolites of *Tanacetum alyssifolium* (Bornm.) Grierson (Asteraceae) were investigated for the first time. Thirteen compounds including axillarin (1), Luteolin-7-*O*- β -glucoside (8) and rutin (13) as flavonoids, fraxetin (7), isofraxidin (9), isofraxidin-7-*O*-glucoside (11) and fraxidin (12) as coumarins, tatrudin A (2), altissin (3), tamirin (4) and tanachin (5) as sesquiterpene lactones and 2,4-dihydroxy-6-methoxy acetophenone (6) and picein (10) as acetophenone derivatives were isolated from the methanol extract of the species. The structures of all isolated compounds were elucidated by 1D and 2D-NMR techniques and by comparing with the literature data. In addition, in vitro cytotoxic activity of all isolated compounds was evaluated against breast cancer MCF-7 cell line by XTT assay. Sesquiterpene lactones; tatrudin A (2), tamirin (4) and tanachin (5) were found to be the most cytotoxic molecules against MCF-7 cell line.

Keywords: *Tanacetum alyssifolium*; sesquiterpene lactones; cytotoxicity; MCF-7 cell line. © 2020 ACG Publications. All rights reserved.

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1. Introduction

Members of *Tanacetum* (Asteraceae), which display several kinds of biological activities, have been used in folk medicine for many centuries due to their excellent medicinal value. The large number of sesquiterpenoids and sesquiterpene lactones were isolated from *Tanacetum* species previously having large spectrum biological activities. This class of secondary metabolites might be responsible for these effects. *Tanacetum* species also contain other secondary metabolites such as coumarins, flavonoids, and steroids [1]. Plant secondary metabolites may have important biological activities such as reducing agents, free radical scavengers, or antimicrobial activities [2, 3]. Such structures are vital in the defense systems of humans, as they have anti-aging, anti-inflammatory, antioxidant and anti-proliferative activities [4, 5]. *Tanacetum* species have long been used in many parts of the world for the treatment of certain diseases by traditional methods. Although it is most commonly known that *Tanacetum vulgare* L. has an anthelmintic effect and *Tanacetum parthenium* L. has an antipyretic effect, the effective use of other species due to their antiseptic, antibiotic, antioxidant and anti-inflammatory potentials has led them to be preferred in traditional medicine, and these species have been used as folk remedies in Anatolia for many years [6].

Tanacetum alyssifolium is locally called “İliç papatyası” in Turkish [7], and it is distributed in fragmented, small groups in gypsum-bearing areas only in the İliç district of Erzincan in Anatolia. It was first collected from Erzincan in 1989 and introduced to science as a new species. The species was re-collected in 2004. The populations of the species were re-analyzed and the IUCN category was proposed as EN [B2ab (i, ii, iii); C2a (i)] in 2015 [8]. It is a perennial plant whose blooming period is June and July.

Although the essential oil content of *Tanacetum alyssifolium* was investigated, limited studies were found in the literature regarding the isolation of its secondary metabolites. The present study is the first phytochemical study to attempt the determination of secondary metabolites of *Tanacetum alyssifolium* which is endemic to Turkey.

In the present study, secondary metabolites of *Tanacetum alyssifolium* were isolated and characterized by different NMR methods and their antiproliferative activities against the MCF-7 cancer cell line were investigated.

2. Materials and Methods

2.1. General

The column chromatography (CC) was performed on silica gel, sephadex LH-20. MPLC system was used for C18 packing material using solvent mixtures described in the experimental sections. Fractions were monitored by TLC silica gel 60 F₂₅₄ after visualizing under UV (254 and 366 nm) and with ethanol-sulfuric acid reagent on silica gel followed by heating at 100 °C.

The 1D and 2D NMR spectra were recorded using Bruker AVANCE III 400, and Agilent 600 MHz spectrometers. Chemical shifts were reported in δ values in appropriate deuterated solvents. HETCOR, COSY, HMBC, and NOESY experiments were performed to determine of both C-C and C-H interactions to elucidate the structures of the isolated molecules.

2.2. Biological Activity

2.2.1. Cell Culture and Reagents

The breast adenocarcinoma cell line MCF-7 was obtained from the ATCC (American Type Culture Collection, LGC Promochem, UK). The cancer cells were cultured in EMEM (Eagle's Minimum Essential Medium), supplemented with 1% L- Glutamine, 1% Na-pyruvate, 1% penicillin-streptomycin (Pen Strep) solution and 10% fetal bovine serum. Cell cultures were humidified at 37°C with 5% carbon dioxide in an incubator. All studies were performed in a Class II Safety Cabinet.

2.2. 2. Cell Viability Assay

To perform the cell viability assay, the MCF-7 cells (100 μ L) were seeded in 96-well plates at a concentration of 5×10^3 cells/well and incubated in a CO₂ incubator for 24 h. Next, the cells were treated with the isolated compounds (**1-13**) for 24 h. Each compound was dissolved in DMSO (dimethyl sulfoxide) and diluted with complete medium between the range of 2 to 500 μ M. Complete medium containing 0.5% DMSO was used as negative control group. After the incubation of the cancer cells with the tested compounds, the absorbance of each well was measured colorimetrically at 450 nm by Epoch Microplate Reader (BioTek, USA). Antiproliferative activity results were expressed as IC₅₀ (μ M) value, which is defined as the concentration of the tested complex that reduces the cellular viability by 50%. Each value represents the standard deviation of three independent experiments (\pm S.D). Doxorubicin that is commonly used to treat cancer was evaluated as a reference drug.

2.3. Statistical Analysis

Statistical analyses were performed for evaluation of cytotoxicity assay results by one-way ANOVA with unpaired t-test using the statistical program GraphPad Prism 6 (GraphPad, La Jolla, CA, Software 7.0). All results were expressed as means with their standard deviation (SD). The minimum level of significance was taken as $p < 0.05$.

2.4. Plant Material

T. alyssifolium was collected from Yahşiler Village, Kemah, Erzincan at flowering stage in June 2014. A voucher specimen was authenticated by Prof. Dr. Ali Kandemir and deposited at Gazi University Herbarium (AK10111).

2.5. Extraction and Isolation

Well-grounded aerial parts of *T. alyssifolium* (1.5 kg) were extracted with hexane (5 x 4 L), and methanol (5 x 4 L), successively, then solvents were evaporated. The methanol extract was suspended in water at 60 °C then cooled and non-soluble parts were removed by filtration. The solution was extracted with ethyl acetate and butanol, respectively. The final water solution was lyophilized and kept at 4 °C. A portion of the EtOAc extract (50 g) was applied to SiO₂ column chromatography (CC) then eluted with DCM-EtOAc-MeOH. A gradient of increasing polarity was employed, starting at 10% dichloromethane and finishing with methanol using 400 mL of each mixture. A total of 196 fractions were collected and similar fractions were combined to afford 13 subfractions. The subfraction 4 (10 g) was applied to SiO₂ CC eluting with DCM-MeOH mixture by increasing polarity by 0.5% for every 200 mL. After checking TLC, 7 subfractions were obtained. The subfraction 5 was purified over SiO₂ to give **1** (200 mg). 10 g of EtOAc extract was fractionated over MPLC equipped with a C18 packed glass column using the mixtures of MeOH:Water as mobile phase with 10 mL/min flow rate: 0:100, 10:90, 20:80, 30:70, 40:60 and finally 50:50. The fractions eluted with 20:80 mixture were combined and evaporated to dryness. The EtOAc soluble parts of this fraction were applied to SiO₂ CC and eluted isocratically by 60:40 hexane-EtOAc mixture. The flow rate was adjusted to about 0.1 mL/min. A total of 82 fractions were collected and similar fractions were combined to give 6 subfractions. The subfractions from 3 to 6 were purified using prep-HPLC over C18 prep-scale column (50:50 MeOH:Water, 235 nm, 8 mL/min) to give **2** (12 mg), **3** (17 mg), **4** (9 mg) and **5** (34 mg), respectively.

The BuOH extract was evaporated to dryness (40 g) and then applied to SiO₂ column eluting with increasing polarity of hexane-DCM-EtOAc-MeOH to afford 13 (A1 to A13) main fractions according to TLC basis. A2 was applied to Sephadex LH-20 using MeOH:CHCl₃ (60:40) as mobile phase. 20 fractions in total were collected and the subfractions between 15-17 were eluted over C18 using the following mixtures of MeOH:H₂O: 1:9, 2:8, 3:7, 4:6 and 5:5. Altogether 28 fractions were collected. After TLC development, fractions 12-19 were combined and purified over sephadex LH-20 to give **6** (18 mg). The main fraction A5 was fractionated over SiO₂ using hexane-EtOAc-MeOH system with increasing polarity and 42 fractions were collected. Fractions 7-14 were combined according to TLC profile and purified

over sephadex LH-20 to give **7** (24 mg). Fractions of A7-A9 were combined (8 g) then applied on sephadex LH-20 using MeOH:CHCl₃ (65:35) as mobile phase to give **8** (84 mg) and **9** (25 mg). A11 was eluted over sephadex LH-20 as above to afford **10** (8 mg) from the 56th fraction of 70 subfractions. A13 was fractionated over sephadex LH-20 using MeOH:CHCl₃ (60:40) and 24 fractions were collected. Two main subfractions were obtained according to TLC. The subfraction 1 purified over sephadex LH-20 to give **11** (18 mg). The subfraction 2 was applied to SiO₂ CC eluting with 6:4 (EtOAc:Hexane). The fractions 16-24 gave **12** (34 mg).

The water extract firstly fractionated with MPLC over C18 packed column using water:MeOH mixture as mobile phase with 20 mL/min flow rate with increasing MeOH starting from 0:100 to 100:0. Totally 70 fractions were collected and combined according to TLC view to give 6 subfractions. The subfraction 4 was chromatographed over C18 CC with 65:35 water: MeOH system, isocratically to give 36 fractions which was combined in three groups. The second group was purified over sephadex LH-20 column using MeOH as mobile phase to give **13** (38 mg) and **9** (24 mg).

3. Results and Discussion

The methanol extract of the aerial parts of *T. alyssifolium* afforded two acetophenone derivatives: 2,4-dihydroxy-6-methoxy acetophenone (**6**) [9] and picein (**10**) [10], three flavonoids; axillarin (**1**) [11], Luteolin-7-*O*- β -glucoside (**8**) [12] and rutin (**13**) [13], four sesquiterpene lactones tatrudin A (**2**) [14], altissin (**3**) [15], tamirin (**4**) and tanachin (**5**) [16], four coumarin derivatives; fraxetin (**7**) [17], isofraxidin (**9**) [18], isofraxidin-7-*O*-glucoside (**11**) [19] and fraxidin (**12**) [20]. The occurrence of acetophenone derivatives (6 and 10) in *Tanacetum* species was firstly reported in this study. Flavone derivatives were common for *Tanacetum* species [21-23].

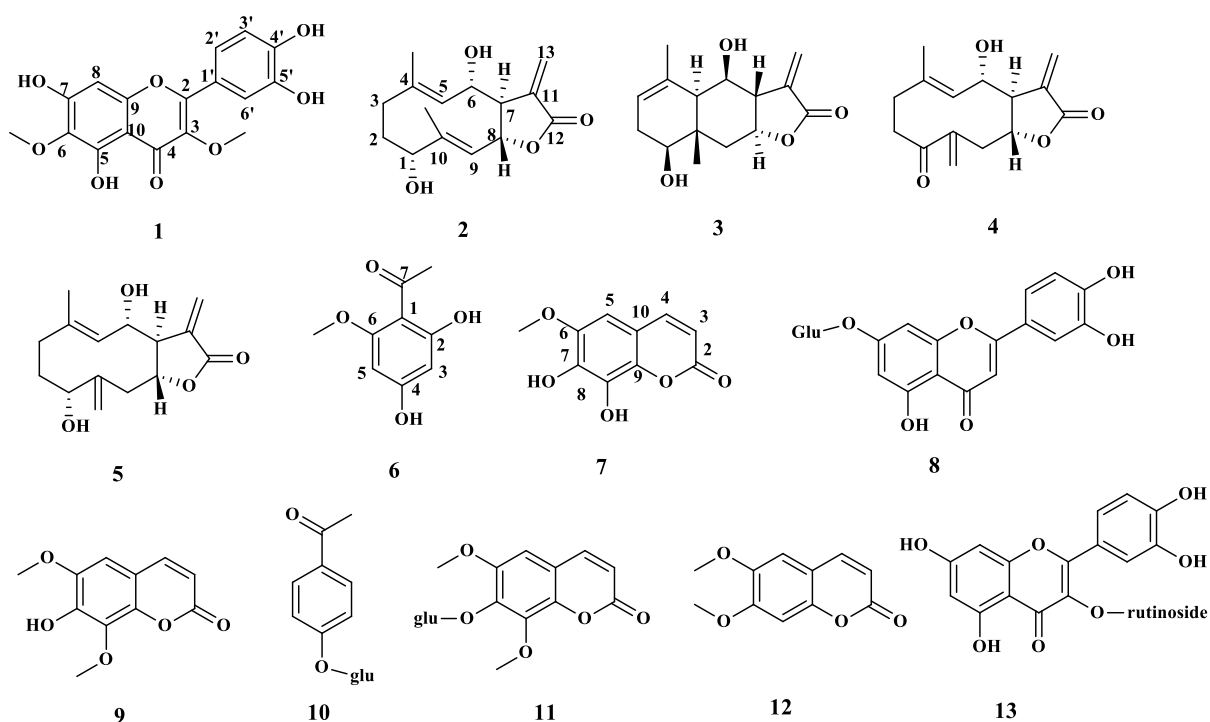


Figure 1. Chemical structures of isolated molecules (**1-13**)

Although rutin and axillarin content of some *Tanacetum* species were previously reported [24, 25], the occurrence of luteolin-7-*O*- β -glucoside was not reported in any *Tanacetum* species up to date. *Tanacetum* species are known to be rich in sesquiterpene lactones [26-29]. Although tatrudin A, tamirin and tanachin were isolated from *T. vulgare* previously [30], altissin was not reported before in *Tanacetum* species. Coumarin derivatives such as scopoletin from *T. cadmeum* [31], isofraxidin from *T. chiliophyllum* [32] and *T. parthenium* [33] were reported previously. Chemical structures of all isolated molecules from *T. alyssifolium* are given in Figure 1.

The antiproliferative activities of the compounds (**1-13**) against the MCF-7 cell line were evaluated by XTT assay, which is based on the extracellular reduction of XTT salt by Nicotinamide Adenine Dinucleotide (NADH) in mitochondria. The cancer cells were treated with each compound for 24 h at varying concentrations (2, 10, 25, 50, 100, and 500 μM). The results showed that the acetophenone derivatives (**6** and **10**), flavonoid derivatives (**1**, **8**, and **13**) and coumarin derivatives (**7**, **9**, **11**, and **12**) displayed no activity up to 500 μM against MCF-7 cell line, while the sesquiterpene lactone derivatives (**2**, **3**, **4** and **5**) exhibited very high cytotoxic activities. Figure S28 (see supporting information file) shows the dose dependent cytotoxic activity of the sesquiterpene lactones (**2**, **3**, **4**, and **5**). The compounds **2**, **4** and **5** showed dose-dependent cytotoxic activities between the ranges of 2-100 μM . The IC_{50} values of the compounds are given in the Table 1. The sesquiterpene lactone **4** exhibited the highest cytotoxic activity with the smallest IC_{50} value (40.8 μM). The IC_{50} values of the other two sesquiterpene lactones **2** and **5** were found as 57.1 μM and 65.8 μM , respectively. Considering the IC_{50} value of doxorubicin (2,92 μM), the compounds (**2**, **4**, and **5**) showed remarkable cytotoxic activity against the MCF-7 cell line. On the other hand, acetophenone derivatives (**6** and **10**), coumarin derivatives (**7**, **9**, **11**, and **12**), and flavonoid derivatives (**1**, **8**, and **13**) did not show cytotoxic activity up to 500 μM .

The molecular structures, location of the functional groups, and the relative configurations of sesquiterpene lactones significantly changes the cytotoxic effect and IC_{50} value of the compounds [34, 35]. Robinson *et al.* tested six sesquiterpene lactones from *Saussurea lappa* for their anticancer activity on Colo-205, A-431, A-549, and MCF-7 cell lines. The IC_{50} values of the sesquiterpene lactones against MCF-7 cell lines were measured between the range of 35.1 to 103.4 $\mu\text{g/mL}$. However, the same compounds were found not to show cytotoxic activity against A-549 cell line, indicating the susceptibility of the different cancer cell lines to the same lactone compound [36]. Therefore, our results were in accordance with the studies in the literature.

Table 1. *In vitro* IC_{50} values of the compounds (**1-13**) and doxorubicin against MCF-7 cell line

Compounds	IC_{50} (μM) ^x
1	NA
2	57.1 \pm 0.92 ^a
3	NT
4	40.8 \pm 1.13 ^b
5	65.8 \pm 1.35 ^c
6	NA
7	NA
8	NA
9	NA
10	NA
11	NA
12	NA
13	NA
*Doxorubicin	2,92 \pm 0.85 ^d

The cytotoxic activity of the three sesquiterpene lactones in Table 1 were in the decreasing order of **4**>**2**>**5**. If we consider their structures, the only difference between **4** and **5** is that the group at C1 position changes from a carbonyl in **4** to a hydroxyl in **5**. This indicates that the mechanism of cytotoxicity is different in these two compounds. The presence of the carbonyl group instead of the hydroxyl at C1 may cause more induction of cytotoxic activity in the cell line. On the other hand, considering the structures of **2**, **4**, and **5**, instead of the methyl group at C10 in **2**, there is an exocyclic methylene group at the same position in **4** and **5**. This is also consistent with the common notion that α -methylene- γ -lactone moiety of sesquiterpene lactones is responsible for their diverse biological activities including cytotoxicity because of its interaction with biological nucleophiles, such as thiol groups (SH) of target proteins [37].

As a result, the primary cytotoxicity results of the sesquiterpene lactones, especially compounds **2**, **4**, and **5** may be a good starting point for further investigations of cytotoxic activity on other cancer cell lines, as well as mechanisms of activity and structure activity relationships.

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Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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