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Antioxidant Principles of *Tanacetum vulgare* L. Aerial Parts

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The methanolic extract of aerial parts of *Tanacetum vulgare* L. (Asteraceae) and its fractions were investigated for antioxidant activity. The crude extract displayed DPPH radical scavenging effects with an EC_{50} value of 37 ± 1.2 µg/mL (n=3). Activity-guided fractionations of the crude extract resulted in the isolation of three antioxidant compounds; 3,5-*O*-dicaffeoylquinic acid (3,5-DCQA), axillarin and luteolin. 3,5-DCQA was the major constituent with antioxidant activity (IC_{50} = 9.7 µM) comparable with that of the standard quercetin (IC_{50} = 8.8 µM). Though the isolated compounds were previously known for their antioxidant effects, this is the first report on the identification of 3,5-DCQA from *Tanacetum vulgare*. The displayed potent antioxidant activity of the crude extract and isolated active principles is in support of the traditional medicinal uses of the plant for disease conditions such as wound healing, rheumatic arthritis and other inflammatory conditions.

Keywords: *Tanacetum vulgare,* Asteraceae, 3,5-*O*-dicaffeoylquinic acid, axillarin, luteolin, antioxidant.

Recent reports from various laboratories have shown that excessive production of reactive oxygen species can lead to irreversible oxidative damage to biological molecules [1a]. Several studies further established the link between oxidative macromolecular damage and pathological conditions including atherosclerosis, rheumatoid arthritis, diabetes, cancer, ischemia-reperfusion tissue damage and neurodegenerative diseases [1a,1b]. Owing to the therapeutic potential of antioxidant compounds, there has been a growing interest in the identification of reactive oxygen species modulators from natural sources. In this connection, our recent systematic phytochemical and *in vitro* antioxidant activity studies have resulted in the characterization of several promising lead natural products [1a,1c-1g].

T. vulgare L. (Asteraceae), commonly known as tansy, is native to temperate Europe and Asia, where it grows along roadsides, hedgerows and waste places [2a,2b]. The medicinal values of *T. vulgare* preparations include uses for tonic, rheumatic, skin eruption and diuretic conditions [2c-2e]. Other reported medicinal uses include anthelmintic, antihypertensive, stimulant, emmenagogue, carminative, antiseptic, antihypertensive and as an antispasmodic agent [2a,2f]. *T. vulgare* is also known to be used as food flavouring agent [2f]. The scientific basis for the reported traditional uses of *T. vulgare* remains to be established though some studies so far revealed anti-inflammatory, diuretic properties and wound-healing effects [3a-3e]. Since various recent studies established the link between antioxidant activity and *in vivo* anti-inflammatory and/or wound healing effects [3f and references there in], the present study was designed to assess the antioxidant potential of *T. vulgare* and its constituents.

The concentration-dependent radical scavenging activity of the crude extract and fractions is shown in Figure 1. Our data revealed that the crude ethanolic extract displayed potent DPPH scavenging activity with an IC_{50} value of 37 ± 1.2 µg/mL (n=3). Fractionation of the crude extract with solvents of increasing polarity gave the following yield: petroleum ether (14.4%, w/w *vs* the crude extract), chloroform (4.3%), ethyl acetate (10.3%), *n*-butanol (27.3%) and water (34.3%). As shown in Figure 1,

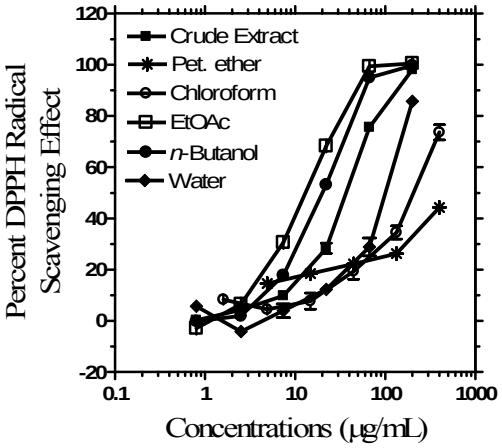

Figure 1: DPPH radical scavenging activity of the crude extract of *T. vulgare* and its fractions (n=3).

the order of potency in radical scavenging activity of the fractions was as follow: ethyl acetate > *n*-butanol > water > chloroform > petroleum ether. It appears from these results (Figure 1) that the ethyl acetate fraction was of interest for further fractionation study while the petroleum ether, chloroform and water fractions displayed activity lower than the crude extract.

Further activity-directed fractionation of the ethyl acetate fraction using sephadex column chromatography resulted in the isolation of three major antioxidant compounds: 3,5-DCQA, luteolin and axillarin (Figure 2). The identification of these compounds was based on analysis of the ^{1}H and ^{13}C NMR data together with UV, IR and MS studies. Assignments of ^{1}H and ^{13}C data, as well as confirmation of structural assignments, were further supported by means of 2D NMR experiments (COSY, HSQC, HMBC and NOESY). The data obtained for these compounds in general were in good agreement with those published previously for 3,5-DCQA [4a], luteolin [4b,4c] and axillarin [3a]. The comparative antioxidant activity of the active principles and the standard antioxidant compound, quercetin, was evaluated using the DPPH radical scavenging study (see Table 1). While 3,5-DCQA and quercetin displayed the highest radical scavenging activity, axillarin and luteolin showed moderate and weak activity respectively (Table 1). These results were in agreement with previous antioxidant activity studies where DPPH radical scavenging activity was observed for 3,5-DCQA [5a-5c], luteolin [5d], quercetin [5e] and axillarin [5f].

It is worth noting that 3,5-DCQA (Figure 2) is the major constituent of the ethyl acetate fraction which

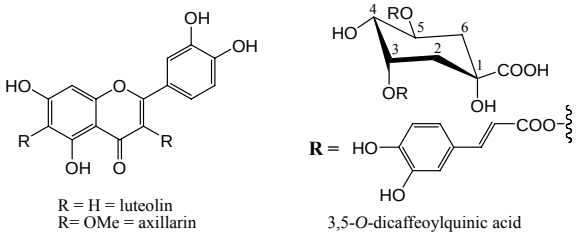

Figure 2: Structures of antioxidant principles from the ethyl acetate fraction of *T. vulgare* extract.

was isolated with the highest yield (21.1% of the crude extract). Though 3,5-DCQA and various caffeoyl esters of quinic acids, including 4,5-DCQA, 3,4-DCQA and 1,5-DCQA have been isolated from many medicinal plants [4a,5c,6a], this is the first report of 3,5-DCQA found in *T. vulgare*. Dicaffeoylquinic acids have been shown to display various biological properties including antioxidant, neuroprotective, hepatoprotective, analgesic and anti-inflammatory properties [6b,6c]. Potent antiviral properties including anti-HIV of 3,5-DCQA has also been reported [4b,6d]. The claimed medicinal uses of the plant could thus be in part attributed to the major biologically active constituent, 3,5-DCQA.

Flavonoids are common plant metabolites with diverse biological properties such as antioxidant, anti-inflammatory, antimicrobial and chemo-prevention [6e]. Several flavonoid aglycones including axillarin and luteolin were previously isolated from the aerial parts of *T. vulgare* and a related species, *T. parthenium* [3a].

Table 1: IC_{50} values obtained for the isolated compounds and the standard reference compound, quercetin.

Compounds	IC_{50} ± SEM, µM[a]
3,5-DCQA	9.70 ± 0.43
Axillarin	23.10 ± 2.89
Luteolin	111.90 ± 7.87
Quercetin	8.80 ± 0.15

[a]Data are mean and SEM values from three separate determinations.

Previous phytochemical studies on *T. vulgare* were also extended to essential oil analysis where thujone, eucalyptol, camphor and myrtenol were identified as major constituents [7a,7b]. Monoterpenes and sesquiterpene lactones which are common to the genus have been shown to occur in *T. vulgare* [2c,7c-7e]. All of these identified constituents may play a role in the medicinal effect of *T. vulgare* preparations but our activity guided-isolation procedure resulted in the identification of 3,5-DCQA, luteolin, quercetin and axillarin as the major antioxidant principles.

Experimental

Materials and chemicals used: Silica gel (Kiesel gel, 70-230 Mesh, 60), lipophilic Sephadex LH-20 (25-100 bead size), 2,2-di-phenyl-1-picrylhydrazyl (DPPH) and quercetin were products of Sigma-Aldrich Chemical Company (Dorset, UK). All solvents were of analytical grade and obtained from Fisher Scientific-UK (Loughborough, UK). *T. vulgare* from authenticated source was grown for two years at the Hadlow Horticultural Gardens (Hadlow Agricultural College, Hadlow, UK). The established plant during the flowering stage was harvested in July 2006 and appropriate voucher specimen was deposited at the School of Science (University of Greenwich) collections.

General methods: ^{1}H NMR, ^{13}C NMR and 2D-NMR (COSY, NOESY, HSQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument. The VG BIO-Q mass spectrometer equipped with an ESI z-spray ion source was used. Samples dissolved in methanol were directly injected into the mass spectrometer operating in a negative ion mode at a flow rate of 50 μL/mL. Either Sephadex LH-20 or Silica Gel 60 was used as the adsorbent for open column chromatography. Silica gel 60 F_{254} plates (Sigma) were used for thin-layer chromatography (TLC). A solvent mixture of chloroform:methanol (1:1) was routinely used for open column Sephadex LH-20 chromatography.

Extraction and initial fractionations: Dried powdered aerial parts of *T. vulgare* (750 g) were extracted twice by soaking the plant material into methanol (2. 5 L) for two weeks. Evaporation of the extraction solvent under reduced pressure followed by freeze-drying resulted in 55.3 g (7.37%) of the extract residue. A portion of the crude extract (30 g) was suspended in 400 mL of water and then fractionated using solvents of increasing polarity: 500 mL each of petroleum ether (yield: 4.3g), chloroform (yield: 1.2 g), ethyl acetate (yield: 3.1 g), *n*-butanol (yield: 8.2 g) and water (yield: 10.3 g). Following the radical scavenging activity test, the ethyl acetate fraction which showed the highest activity was taken for further fractionations.

A portion of the ethyl acetate fraction (2.2 g) was applied onto a silica gel column (18 cm x 2.5 cm) and elution done with chloroform, ethyl acetate and methanol mixtures of increasing polarity. After analysis of fractions by TLC, a total of eight major fractions were obtained: Fraction A (40.1 mg, 0-25% EtOAc), B (59.3 mg, 25%-30% EtOAc), C (285.5 mg, 30-35% EtOAc), D (131.9 mg, 35%-45% EtOAc), E (72.2 mg, 50-75% EtOAc), F (353.0 mg, 80-90% EtOAc) and G (826.6 mg, 100% EtOAc) were elution with EtOAc mixtures in chloroform; H (409.8 mg) was collected by elution with 10-40% methanol in EtOAc.

Isolation of antioxidant compounds: Fraction C (280 mg) which showed a good antioxidant activity was applied onto a sephadex column (34 cm x 2.0 cm) as the mobile phase leading to seven major fractions; I (90 mL), II (165 mL,), III (90 mL), IV (15 mL), V (165 mL), VI (90 mL), VII (120 mL). The most active radical scavenging fractions, VI and VII, were taken for further separations. Fraction VI which contained predominantly axillarin was purified by silica gel column (13 cm x 1 cm) chromatography with 3%-10% methanol in chloroform as a solvent to yield 75 mg of the pure compound. Similarly fraction VII was purified using a silica gel column (12 cm x 1 cm) and elution done with 10%-30% chloroform in MeOH to yield pure luteolin (80 mg).

Fraction F (325 mg) was applied onto a sephadex column (34 cm x 2.5 cm) leading to three fractions: A (176 mL), B (150 mL) and C (100 mL). Fraction B yielded pure 3,5-DCQA (50 mg) while similar treatment of fraction G (826 mg) resulted in the further isolation of 410 mg of pure 3,5-DCQA.

DPPH radical scavenging assay: The DPPH radical scavenging assay was carried out by using our previously described method [1d]. Briefly, the test samples in three-fold dilutions were prepared and mixed with 0.1 mM solution of DPPH. After 20 minutes of incubation at room temperature, the DPPH colour bleaching by antioxidants was assessed.

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