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Chemical constituents of *Cardamine flexuosa*

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ABSTRACT

*Chemical investigation of the whole plant of Cardamine flexuosa led to the isolation of β-sitosterol (**1**), stigmasterol (**2**), lutein (**3**), unsaturated triglycerides (**4**) and the essential fatty acids, linoleic acid (**5**) and linolenic acid (**6**). The structures of **1**-**6** were identified by comparison of their ^{1}H NMR data with those reported in the literature.*

Keywords: *Cardamine flexuosa*, Brassicaceae, β-sitosterol, stigmasterol, lutein, linolenic acid, linoleic acid, triglycerides

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INTRODUCTION

Cardamine flexuosa, commonly known as wavy bittercress, is an herbaceous annual, biennial, or short-lived perennial plant [1]. The leaves and root of *C. flexuosa* may be eaten raw or cooked and could be used as flavouring in salads [2]. The plant belongs to the family Brassicaceae which were shown to exhibit anticancer properties due to their high content of glucosinolates which hydrolyze to form bioactive products such as isothiocyanates [3, 4]. An earlier study reported that *C. flexuosa* contained benzylglucosynolate [5]. Furthermore, 5-methylthiopentyl isothiocyanate and 1-methylpropylisothiocyanate were detected in the GC analysis which indicated the presence of 1-methylpropylglucosynolate in the eight-week-old *C. flexuosa* [6]. The presence of methyl isothiocyanate in the aerial part of *C. flexuosa* corresponding to the parent methylglucosynolate has been reported [7]. Another study identified by GC, 1-methylpropyl, 1-methylethyl, benzyl, and 2-phenylethyl isothiocyanates which indicated the presence of the corresponding glucosynolates in the seeds of *C. flexuosa* [8]. The methanol extract of *C. flexuosa* strongly inhibited the growth of *Microcystis aeruginosa*. An algicidal compound, falcarindiol was isolated from the methanol extract which showed a dose-dependent response to blue-green alga, *M. aeruginosa* and green alga, *Chlorella vulgaris garis* with IC_{50} values of 0.19 and 3.13 μg mL^{-1}, respectively. It also inhibited the growth of duckweed (*Lemna pausicostata*) with an IC_{50} value of 6.17 μg mL^{-1}. It showed a strong potential for preventing the blooming of blue-green algae such as *Microcystis* spp. in pond or for the control of weeds that are harmful to water-logged rice [9]. Moreover, falcarindiol is a known antitumor compound that induces endoplasmic reticulum stress, promoting the death of cancer cells [10]. A review on the chemical constituents and biological activities of the genus *Cardamine* has been provided [5].

This study was conducted as part of our research on the chemical constituents of the family Brassicaceae. We earlier reported the isolation of β-sitosterol and unsaturated triglycerides from the leaves of *Brassica oleracea var capitata f. rubra* L, *B. oleracea* L, and the stem of *B. oleracea* L *var. italic*. The red cabbage also afforded stigmasterol, while the green cabbage and broccoli stem also yielded linoleic acid. *B. juncea* leaves and *Raphanus sativus* roots afforded β-sitosterol, linoleic acid and α-linolenic acid. Mustard leaves also yielded trilinolenin, lutein and β-carotene, while radish roots also afforded unsaturated triglycerides [11]. These compounds were reported to exhibit anticancer properties.

We report herein the isolation and identification of β-sitosterol (**1**), stigmasterol (**2**), lutein (**3**), unsaturated triglycerides (**4**) and the essential fatty acids, linoleic acid (**5**) and linolenic acid (**6**) (Fig. 1) from the whole plant of *C. flexuosa*.

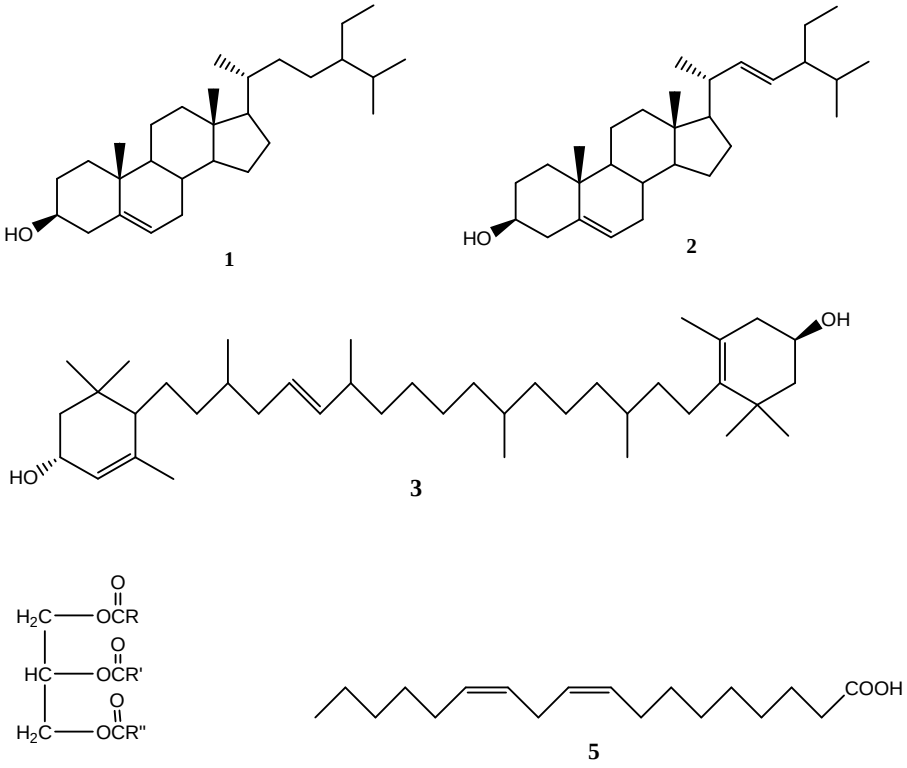

Fig. 1. Chemical constituents of *C. flexuosa*: β-sitosterol (1), stigmasterol (2), lutein (3), unsaturated triglycerides (4), linoleic acid (5) and linolenic acid (6)

MATERIALS AND METHODS

General Experimental Procedure

NMR spectra were recorded on a Varian VNMRS spectrometer in $CDCl_3$ at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR spectra. Column chromatography was performed with silica gel 60 (70-230 mesh). Thin layer chromatography was performed with plastic backed plates coated with silica gel F_{254} and the plates were visualized by spraying with vanillin/H_2SO_4 solution followed by warming.

Sample Collection
Samples of *Cardamine flexuosa* were collected from a private farm in Sitio Usiwan, Barangay Palola, Lucban, Quezon in March 2013. Within this private farm is a patch of secondary forest at 620 m asl, which interestingly harbors a number of endemic species. Voucher specimens were collected and identified by one of the authors (EHM) with collection # and deposited at De La Salle University-Manila.

General Isolation Procedure
A glass column 20 inches in height and 2.0 inches internal diameter was packed with silica gel. The crude extract from the leaves were fractionated by silica gel chromatography using increasing proportions of acetone in dichloromethane (10% increment) as eluents. One hundred milliliter fractions were collected. All fractions were monitored by thin layer chromatography. Fractions with spots of the same R_f values were combined and rechromatographed in appropriate solvent systems until TLC pure isolates were obtained. A glass column 12 inches in height and 0.5 inch internal diameter was used for the rechromatography. Five milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

Isolation
The air-dried whole plant of *C. flexuosa* was ground in a blender, soaked in CH_2Cl_2 for 3 days and then filtered. The filtrate was concentrated under vacuum to afford a crude extract which was chromatographed using increasing proportions of acetone in CH_2Cl_2 at 10% increment. The 30% acetone in CH_2Cl_2 fraction was rechromatographed (2 ×) using 5% EtOAc in petroleum ether to afford **4** (6 mg). The 40% acetone in CH_2Cl_2 fraction was rechromatographed (4 ×) using CH_3CN:Et_2O:CH_2Cl_2 (0.5:0.5:9, v/v) to afford a mixture of **1** and **2** (5 mg) after washing with petroleum ether. The 40% to 50% acetone in CH_2Cl_2 fractions were combined and rechromatographed (3 ×) using acetonitrile:Et_2O:CH_2Cl_2 (0.5:0.5:9, v/v) to afford a mixture of **5** and **6** (8 mg). The 60% acetone in CH_2Cl_2 fraction was rechromatographed (5 ×) using CH_3CN:Et_2O:CH_2Cl_2 (1:1:8, v/v) to afford **3** (3 mg) after washing with petroleum ether, followed by Et_2O.

β-Sitosterol (**1**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.50 (m, H-3), 2.28, 2.21 (H_2-4), 5.33 (dd, *J* = 3.0, 1.8 Hz, H-6), 0.66 (s, H_3-18), 0.99 (s, H_3-19), 0.90 (d, *J* = 6.6 Hz, H_3-21), 0.79 (d, *J* = 7.2 Hz, H_3-26), 0.82 (d, *J* = 6.6 Hz, H_3-27), 0.85 (t, *J* = 7.2 Hz, H_3-29).

Stigmasterol (**2**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 3.50 (m, H-3), 5.33 (dd, *J* = 3.0, 1.8 Hz, H-6), 0.68 (s, CH_3-18), 0.99 (s, CH_3-19), 1.01 (d, *J* = 6.6 Hz, CH_3-21), 5.13 (dd, *J* = 9.0, 15.0 Hz, H-22), 5.00 (dd, *J* = 9.0, 15.0 Hz, H-23), 0.84 (d, *J* = 6.6 Hz, CH_3-26), 0.83 (d, *J* = 6.6 Hz, CH_3-27), 0.80 (t, *J* = 6.6 Hz, CH_3-29).

Lutein (**3**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.05 (s, 2 ring A CH_3), 0.83 (s, ring B CH_3), 0.98 (s, ring B CH_3), 1.60 (allylic CH_3), 1.72 (allylic CH_3), 1.89 (allylic CH_3), 1.952 (allylic CH_3), 1.945 (2 allylic CH_3), 1.45, 1.75 (CH_2), 1.35, 1.85 (CH_2), 2.35, 2.00 (allylic CH_2), 2.38 (allylic CH), 4.23 (br s, CHOH), 4.00 (m, CHOH), 5.52 (br s, =CH), 5.41 (dd, *J* = 9.0, 15.0 Hz, =CH), 6.10 (=CH), 6.22 (=CH), 6.32 (=CH), 6.36 (=CH), 6.60 (=CH).

Triglyceride (**4**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.27 (dd. *J* = 4.8, 12.0 Hz), 4.12 (dd, *J* = 6.0, 12.0 Hz, glyceryl CH_2O), 5.32 (glyceryl CHO), 2.30 (t, *J* = 7.2 Hz, α-CH_2), 5.34 (olefinic H), 2.03 (allylic, CH_2), 1.22-1.35 (CH_2), 2.79 (t, *J* = 6.6 Hz, double allylic CH_2), 2.75 (t, *J* = 6.6 Hz, double allylic CH_2), 0.96 (t, *J* = 7.2 Hz, CH_3), 0.87 (t, *J* = 6.6 Hz, CH_3), and 0.85 (t, *J* = 6.6 Hz, CH_3).

Linoleic Acid (**5**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.34 (m, olefinic H), 2.75 (t, *J* = 6.6 Hz, double allylic CH_2), 2.30 (t, *J* = 7.2 Hz, α CH_2), 2.03 (allylic CH_2), 1.60 (β CH_2), 1.23-1.36 (CH_2), 0.86 (t, *J* = 6.6 Hz, CH_3).

Linolenic Acid (**6**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.34 (m, olefinic H), 2.79 (t, *J* = 7.2 Hz, double allylic CH_2), 2.30 (t, *J* = 7.2 Hz, α CH_2), 2.03 (allylic CH_2), 1.60 (β CH_2), 1.23-1.36 (CH_2), 0.96 (t, *J* = 7.2 Hz, CH_3).

RESULTS AND DISCUSSION

Silica gel chromatography of the dichloromethane extract of the air-dried leaves of the whole plant of *C. flexuosa* afforded a mixture of β-sitosterol (**1**) [12] and stigmasterol (**2**) [13] in a 2:1 ratio, lutein (**3**) [14], unsaturated triglycerides (**4**) [15] and a mixture of the essential fatty acids, linoleic acid (**5**) [16] and linolenic acid (**6**) [17] in a 1:1 ratio. The ratio of the mixture of **1** and **2** was deduced from the integrations of the ^{1}H NMR resonances for the

olefinic protons of **1** at δ 5.33 (dd, *J* = 1.8, 3.0 Hz, H-6) and **2** at δ 5.33 (dd, *J* = 1.8, 3.0 Hz, H-6), 5.13 (dd, *J* = 9.0, 15.0 Hz, H-22) and 5.00 (dd, *J* = 9.0, 15.0 Hz, H-23). The presence of linoleic acid (**5**) in the mixture of fatty acids was deduced from the methyl triplet at δ 0.86 (t, *J* = 6.6 Hz), the double allylic methylene at δ 2.75 and the olefinic protons at δ 5.34 (m) [16], while α-linolenic acid (**6**) was detected from the methyl triplet at δ 0.96 (t, *J* = 7.2 Hz), the double allylic methylenes at δ 2.79 and the olefinic protons at δ 5.34 (m) [17]. Based on integrations of the methyls at δ 0.86 (t, *J* = 6.6 Hz) for linoleic acid and δ 0.96 (t, *J* = 7.2 Hz) for linolenic acid, their ratio in the mixture is about 1:1. The structures of **1**-**6** were identified by comparison of their ^{1}H NMR data with those reported in the literature [12-17].

Although no biological activity tests were conducted on the isolated compounds (**1**-**6**), literature search revealed that these have diverse biological activities as follows.

β-Sitosterol (**1**) was reported to exhibit growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells [18]. It was shown to be effective for the treatment of benign prostatic hyperplasia [19]. It attenuated β-catenin and PCNA expression, as well as quenched radical *in-vitro*, making it a potential anticancer drug for colon carcinogenesis [20]. It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells [21]. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake [22].

Stigmasterol (**2**) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions [23]. It lowers plasma cholesterol levels, inhibits intestinal cholesterol and plant sterol absorption, and suppresses hepatic cholesterol and classic bile acid synthesis in Winstar as well as WKY rats [24]. Other studies reported that stigmasterol showed cytostatic activity against Hep-2 and McCoy cells [25], markedly inhibited tumour promotion in two stage carcinogenesis experiments [26], exhibited antimutagenic [27], topical anti-inflammatory [28], anti-osteoarthritic [29] and antioxidant [30] activities.

Dietary lutein (**3**), especially at 0.002%, inhibited tumor growth by selectively modulating apoptosis, and by inhibiting angiogenesis [31]. Another study reported that the chemopreventive properties of all-*trans* retinoic acid and lutein may be attributed to their differential effects on apoptosis pathways in normal *versus* transformed mammary cells [32]. Moreover, very low amounts of dietary lutein (0.002%) can efficiently decrease mammary tumor development and growth in mice [33]. Another study reported that lutein and zeaxanthine reduces the risk of age related macular degeneration [34].

Triglycerides (**4**) exhibited antimicrobial activity against *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans*, and *T. mentagrophytes* [35]. Another study reported that triglycerides showed a direct relationship between toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation [36].

Linoleic acid (**5**) belongs to the omega-6 fatty acids. It was reported to be a strong anticarcinogen in a number of animal models. It reduces risk of colon and breast cancer [37] and lowers cardiovascular disease risk and inflammations [38].

Linolenic acid (**6**) belongs to omega-3 fatty acid. A previous study reported that α-linolenic acid (ALA) inhibited the human renal cell carcinoma (RCC) cell proliferation [39]. Another study reported that apoptosis of hepatoma cells was induced by the α-linolenic acid enriched diet which correlated with a decrease in arachidonate content in hepatoma cells and decreased cyclooxygenase-2 expression [40]. γ-Linolenic acid (GLA) and α-linolenic acid (ALA) exhibited greater than 90% cytotoxicity between 500 μM and 1 mM against all but two malignant micro-organ cultures tested in 5-10% serum. GLA and ALA killed tumor at concentrations of 2 mM and above in tests using 30-40% serum [41].

CONCLUSION

Chemical investigation of the dichloromethane extract the whole plant of *Cardamine flexuosa* yielded compounds similar to those we previously isolated from *Brassica oleracea var capitata f. rubra* L, *B. oleracea* L *var. italic*, *B. juncea* and *Raphanus sativus* [8] which belong to the Brassicaceae family. All the compounds isolated from *C. flexuosa* are known anticancer compounds. Thus, the anticancer properties of Brassicaceae

vegetables may not only be attributed to the high content of glucosynolates, but also to the presence of sterols, carotenoid, unsaturated triglycerides and essential fatty acids.

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