



Sterols, triglycerides and essential fatty acid constituents of *Brassica oleracea* varieties, *Brassica juncea* and *Raphanus sativus*

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ABSTRACT

The dichloromethane extracts of the leaves of *Brassica oleracea* var *capitata* f. *rubra* L (red cabbage) and *Brassica oleracea* L (green/white cabbage) and the stem of *Brassica oleracea* L var. *italic* (broccoli) afforded β -sitosterol (**1**) and unsaturated triglycerides (**2**). The red cabbage also afforded stigmasterol (**3**), while the green/white cabbage and broccoli stem also yielded the essential fatty acid, linoleic acid (**4**). *Brassica juncea* (mustard) leaves and *Raphanus sativus* (radish) roots afforded **1**, and the essential fatty acids **4** and α -linolenic acid (**6**). Mustard leaves also yielded trilinolenin (**5**), lutein (**7**) and β -carotene (**8**), while radish roots also afforded **2**. These compounds were reported to exhibit anticancer properties.

Keywords: *Brassica oleracea* var *capitata* f. *rubra* L, red cabbage, *Brassica oleracea* L., green/white cabbage, *Brassica oleracea* L var. *italic*, broccoli, *Brassica juncea*, mustard, *Raphanus sativus*, radish, β -sitosterol, triglycerides, linoleic acid, trilinolenin, linolenic acid, stigmasterol, lutein, β -carotene

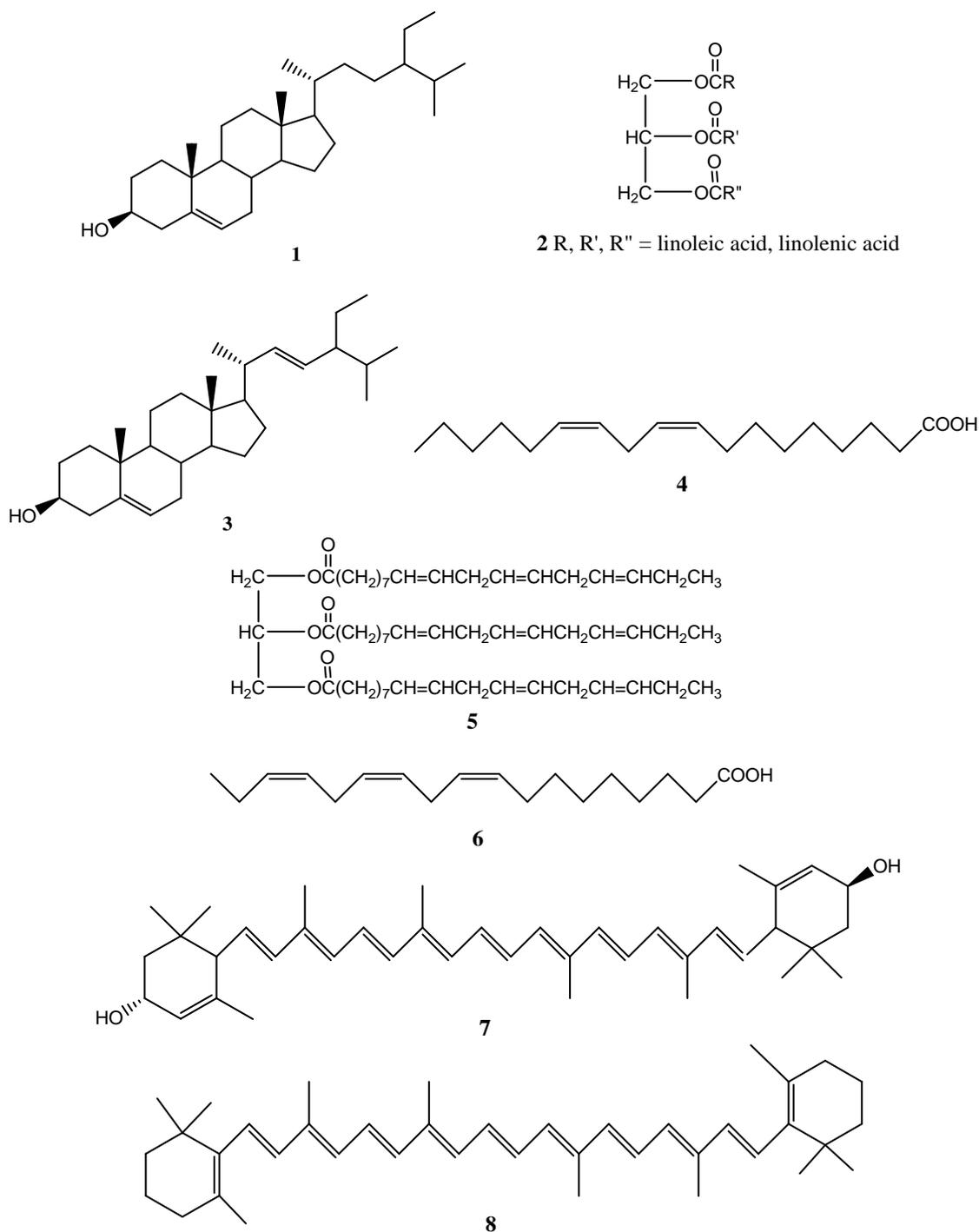
INTRODUCTION

The vegetables of the Brassicaceae family include *Brassica oleracea* var *capitata* f. *rubra* L (red cabbage), *Brassica oleracea* L. (green/white cabbage), *Brassica oleracea* L var. *italic* (broccoli), *Brassica juncea* (mustard) and *Raphanus sativus* (radish) which have been shown to exhibit cancer preventing effects due to their high content of glucosinolates which hydrolyze to form products such as bioactive isothiocyanates [1,2].

Phytochemical screening on the leaf extracts of *B. oleracea* L. var. *capitata* revealed the presence of alkaloids, tannins, saponins, phenols, glycosides, steroids, terpenoids and flavonoids. The ethanolic extract with the highest phenolic compounds exhibited the greatest anti-oxidant activity [3]. The leaf extract of *B. oleracea* L. var. *capitata* possesses significant hepatoprotective activity [4]. Chemical components analysis of *B. oleracea* L. var. *capitata* has shown that it is rich in phenolic compounds [5], carotenoids [6] and glucosinolates [7].

Methanolic extracts of *B. oleracea* var *capitata* f. *rubra* L were shown to exhibit synergistic anticancer effects against HeLa and HepG2 cells [8]. An earlier study reported that the major constituents of red cabbage are isothiocyanates (glucosinolate), vitamins A, B, C and anthocyanins [9]. In a recent study, it was reported that

polyphenolic compounds (phenyl propanoids, flavonols and anthocyanins) are the major antioxidants in red cabbage [10].



Chemical constituents of *Brassica oleracea* varieties, *Brassica juncea* and *Raphanus sativus*: β -sitosterol (1), triglyceride (2), stigmasterol (3), linoleic acid (4), trilinolenin (5), α -linolenic acid (6), lutein (7) and β -carotene (8)

β -Sitosterol is a major sterol of *Brassica oleracea* L. var. *botrytis italica* Plenck [11]. The vegetable contains glucosinolates, flavonoids, vitamins and mineral nutrients [12] as well as antioxidants such as β -carotene, α -tocopherol, indoles and isothiocyanates [13]. 3,3'-Indolylmethane and indole-3-carbinol, hydrolysis by-products of

broccoli, exhibited multiple chemo-preventive effects through the inhibition of androgen and estrogen related mechanisms and signaling pathways mediating the cell cycle.[14]. The isothiocyanates commonly found in broccoli, sulforaphane and erucin, inhibited the expression of LasR, a transcriptional activator, through the disruption of quorum sensing in *Pseudomonas aeruginosa*[15]. Mammospheres or stem/progenitor cells from breast adenocarcinoma immortalized cell lines, MCF7 and SUM159, were treated with sulforaphane. Anti-proliferation of these cells was induced due to the decreased presence of aldehyde dehydrogenase enzyme activity.[16]

The major constituents of the essential oils in the seeds of *Brassica juncea* (L.) Coss., grown in Hebei, Shaanxi and Shandong Province were allyl isothiocyanate (61.3%), diallyl trisulfide (9.7%) and 3-butenyl isothiocyanate (5.9%) [17]. Another study reported that *B. juncea* seed oil afforded β -sitosterol, campesterol and brassicasterol [18]. The chemical constituents of *B. juncea* include glucosinolates, ascorbate, folate and sterols (brassicasterol, sitosterol and brassinosteroids) [19].

Raphanus sativus (radish) sprouts and mature radish taproot contained glucosinolates, isothiocyanates, phenolics and anthocyanins [20]. The major fatty acids in seed lipids of radish were erucic, oleic, linoleic, and linolenic acids, while the major fatty acids in radish family lipids were linolenic acid (52–55%), erucic acid (30–33%), and palmitic acid (20–22%)[21].

This study was conducted on the dichloromethane extracts of the *Brassica* vegetables red cabbage (*Brassica oleracea var capitata f. rubra* L), green/white cabbage (*Brassica oleracea* L.), broccoli (*Brassica oleracea* L var. *italica*), mustard (*Brassica juncea*) and radish (*Raphanus sativus*). Results of this study identified the relatively non-polar constituents which may contribute to the anticancer properties of these vegetables. We report herein the sterols, triglycerides and essential fatty acid constituents of local collections of *Brassica oleracea* varieties, *Brassica juncea* and *Raphanus sativus*. Carotenoids were also isolated as the major constituents of *Brassica juncea*. These compounds have been reported to exhibit anticancer properties.

EXPERIMENTAL SECTION

General Experimental Procedures

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 F₂₅₄ and the plates were visualized by spraying with vanillin/ H_2SO_4 solution followed by warming.

Plant Materials

Five (5) kg each of red and green cabbage and broccoli were purchased from *Arranque* market in September 2011. The samples were authenticated as *Brassica oleracea var capitata f. rubra* L (red cabbage), *Brassica oleracea* L (green/white cabbage) and *Brassica oleracea* L var. *italica* (broccoli) at the Bureau of Plant industry, Quirino Avenue, Manila, Philippines. One (1) kg of mustard was collected from a private farm in Sitio Usiwan, Barangay Palola, Lucban, Quezon in March 2013. It was identified as *Brassica juncea* at the Bureau of Plant industry, Quirino Avenue, Manila, Philippines. One (1) kg of radish roots was bought from *Arranque* market in March 2013. It was identified as *Raphanus sativus* at the Botany Division, Philippine National Museum.

Extraction and Isolation

The air-dried leaves of *Brassica oleracea var capitata f. rubra* L (red cabbage) (480 g), *Brassica oleracea* L (green/white cabbage) (515.5 g) and the freeze-dried stems of *Brassica oleracea* L var. *italica* (broccoli) (87.5 g) were separately ground in a blender, soaked in CH_2Cl_2 for three days, and then filtered. The filtrates were concentrated under vacuum to afford the crude extracts: red cabbage (6.2 g), green/white cabbage (3.4 g) and broccoli (0.6 g). The freeze-dried mustard leaves (379.4 g) and radish roots (56.5 g) were ground in a blender, soaked in CH_2Cl_2 for three days, and then filtered to afford crude extracts: mustard leaves (9.8 g) and radish roots (170.5 mg). The crude extracts were separately fractionated by silica gel chromatography using increasing proportions of acetone in CH_2Cl_2 (10% increment) as eluents. A glass column 18 inches in height and 1.0 inch internal diameter was used for the fractionation of the crude extracts. Five milliliter fractions were collected. 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. Two milliliter fractions were collected. Final purifications were conducted using Pasteur pipettes as columns. One milliliter fractions were collected.

The 20% to 40% acetone in CH₂Cl₂ fractions from the chromatography of the crude red cabbage extract were combined and rechromatographed in 15% EtOAc in petroleum ether. The less polar fractions were rechromatographed (4 ×) in 5% EtOAc in petroleum ether to afford a mixture of **2** (25 mg). The more polar fractions were rechromatographed (2 ×) in 15% EtOAc in petroleum ether to afford a mixture of **1** and **3** (12 mg) in about 7:1 ratio after washing with petroleum ether.

The CH₂Cl₂ to 40% acetone in CH₂Cl₂ fractions from the chromatography of the crude green/white cabbage extract were combined and rechromatographed in 15% EtOAc in petroleum ether. The less polar fractions were rechromatographed (3 ×) in 5% ethyl acetate in petroleum ether to afford **2** (12 mg). The more polar fractions were rechromatographed in 15% ethyl acetate in petroleum ether. The less polar fractions were rechromatographed (2 ×) in 15% ethyl acetate in petroleum ether to afford **1** (12 mg) after washing with petroleum ether. The more polar fractions were rechromatographed in 15% ethyl acetate in petroleum ether to afford **4** (18 mg).

The 40% acetone in CH₂Cl₂ fraction from the chromatography of the crude broccoli stems extract was rechromatographed (3 ×) in 15% EtOAc in petroleum ether to afford **4** (9 mg). The 60% acetone in CH₂Cl₂ fraction from the chromatography of the crude broccoli extract was rechromatographed in 15% EtOAc in petroleum ether. The less polar fractions were rechromatographed (3 ×) in 5% EtOAc in petroleum ether to afford **2** (14 mg). The more polar fractions were rechromatographed (2 ×) in 15% EtOAc in petroleum ether to afford **1** (11 mg) after washing with petroleum ether.

The 10% acetone in CH₂Cl₂ fraction from the chromatography of the crude mustard leaves extract was rechromatographed (3 ×) in 2.5% EtOAc in petroleum ether to afford **8** (13 mg) after washing with petroleum ether. The 30% acetone in CH₂Cl₂ fraction from the chromatography of the crude mustard leaves extract was rechromatographed (3 ×) in 7.5% EtOAc in petroleum ether to afford **5** (15 mg). The 40% to 50% acetone in CH₂Cl₂ fractions from the chromatography of the crude mustard extract were combined and rechromatographed in 20% EtOAc in petroleum ether. The less polar fractions were rechromatographed (2 ×) in 20% EtOAc in petroleum ether to afford **1** (8 mg) after washing with petroleum ether. The more polar fractions were rechromatographed (3 ×) in acetonitrile:Et₂O:CH₂Cl₂ (0.5:0.5:9 by volume) to afford a mixture of **4** and **6** (14 mg) in a 1:2 ratio and **7** (19 mg) after washing with petroleum ether, followed by diethyl ether.

The 10% acetone in CH₂Cl₂ fraction from the chromatography of the crude radish roots extract was rechromatographed (3 ×) in 5% EtOAc in petroleum ether to afford **2** (6 mg). The 40% acetone in CH₂Cl₂ fraction from the chromatography of the crude radish roots extract was rechromatographed (2 ×) in 15% EtOAc in petroleum ether to afford **1** (1 mg) after washing with petroleum ether. The 50% acetone in CH₂Cl₂ fraction from the chromatography of the crude radish roots extract was rechromatographed (4 ×) in acetonitrile:Et₂O:CH₂Cl₂ (0.5:0.5:9 by volume) afford a mixture of **4** and **6** (3 mg).

RESULTS AND DISCUSSION

The dichloromethane extracts from the leaves of *Brassica oleracea var capitata f. rubra* L (red cabbage) and *Brassica oleracea* L (green/white cabbage) and the stem of *Brassica oleracea* L var. *itali* (broccoli) afforded **1** and **2**. The red cabbage also yielded **3**, while the green/white cabbage and broccoli stem also yielded **4**. The structures of **1**, **2**, **3** and **4** were identified by comparison of their ¹³C NMR data with those reported in the literature for β-sitosterol [22], unsaturated triglyceride [23], stigmasterol [22] and linoleic acid [24], respectively.

The dichloromethane extracts of the leaves of *Brassica juncea* (mustard) and the roots of *Raphanus sativus* (radish) yielded **1** and the essential fatty acids, **4** and α-linolenic acid (**6**). Mustard leaves also afforded trilinolenin (**5**), lutein (**7**) and β-carotene (**8**), while radish roots also yielded **2**. The structures of **5**, **6**, **7** and **8** were identified by comparison of their ¹³C NMR data with those reported in the literature for trilinolenin [25], α-linolenic acid [24], lutein [26] and β-carotene [22], respectively.

The presence of α-linolenic acid (**6**) in the triglyceride was deduced from the methyl triplet at δ 0.96 (t, *J* = 7.8 Hz), the double allylic methylenes at δ 2.78 and the olefinic protons at δ 5.34 (m) [27]. The presence linoleic acid (**4**)

was deduced from the methyl triplet at δ 0.86 (t, $J = 6.6$ Hz), the double allylic methylene at δ 2.8 and the olefinic protons at δ 5.34 (m) [28]. Based on integrations of the triglyceride methyls at δ 0.96 (t, $J = 7.8$ Hz) and δ 0.86 (t, $J = 6.6$ Hz), the ratio of linolenic acid and linoleic acid in the triglycerides (**2**) of red cabbage and radish is about 1:1. On the other hand, the triglyceride in mustard is trilinolenin (**5**) based on the methyl resonance at δ 0.96 (t, $J = 7.8$ Hz) [27]. No other methyl resonance in the shielded region of the spectrum was detected for the triglyceride in mustard. A mixture of α -linolenic acid (**6**) and linoleic acid (**4**) in a 1:1 ratio was deduced from the integrals of the methyl triplets at δ 0.96 (t, $J = 7.8$ Hz) and δ 0.86 (t, $J = 7.2$ Hz) for the fatty acids in red cabbage and radish. In mustard, a mixture of α -linolenic acid (**6**) and linoleic acid (**4**) in a 2:1 ratio was deduced from the integrals of the methyl triplets at δ 0.96 (t, $J = 7.8$ Hz) and δ 0.86 (t, $J = 7.2$ Hz) for the fatty acids.

Although no biological activity tests were conducted on the isolated compounds, literature search revealed that these have several known bioactivities. It is interesting to note that **1-8** were all reported as anticancer compounds.

β -Sitosterol (**1**) was observed to have growth inhibitory effects on human breast MCF-7 and MDA-MB-231 adenocarcinoma cells [29]. It was shown to be effective for the treatment of benign prostatic hyperplasia [30]. It was also reported to attenuate β -catenin and PCNA expression, as well as quench radical *in-vitro*, making it a potential anticancer drug for colon carcinogenesis [31]. It can inhibit the expression of NPC1L1 in the enterocytes to reduce intestinal cholesterol uptake [32]. It was reported to induce apoptosis mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells [33]. On the other hand, stigmasterol (**3**) shows therapeutic efficacy against Ehrlich ascites carcinoma bearing mice while conferring protection against cancer induced altered physiological conditions [34].

A triglyceride, trilinolein exhibited protective effects against cardiovascular disorders [35]. It also inhibits ischemia-induced ventricular arrhythmias and it exhibits anti-oxidant effect [36]. It was also reported to inhibit the growth of human non-small cell lung carcinoma A549 and induce apoptosis in a dose- and time- dependent manner [37]. Another study reported that triglycerides showed a direct relationship between toxicity and increasing unsaturation, which in turn correlated with increasing susceptibility to oxidation. Trilinolenin (18:3; μ -3) (**5**) was toxic only after prolonged incubation [38].

Linoleic acid (**4**) 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 [39] and lowers cardiovascular disease risk and inflammations [40].

Omega-3 polyunsaturated fatty acids (n-3 PUFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA) (**6**), and their fatty acid ethyl esters, exhibited strong antibacterial activity against various oral pathogens, including *Streptococcus mutans*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*. They also showed anti-inflammatory effects [41]. Peroxisome proliferator-activated receptor- γ (PPAR- γ) and cyclooxygenase-2 (COX-2) inhibition serve as two signaling pathways for the inhibitory effects of α -linolenic acid (ALA) on the human renal cell carcinoma (RCC) cell proliferation [42]. 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 [43]. γ -Linolenic acid (GLA) and α -linolenic acid (ALA) exhibited greater than 90% cytotoxicity between 500 μ M 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 [44].

Dietary lutein (**7**), especially at 0.002%, inhibited tumor growth by selectively modulating apoptosis, and by inhibiting angiogenesis [45]. Another study reported that the chemopreventive properties of all-*trans* retinoic acid (ATRA) and lutein may be attributed to their differential effects on apoptosis pathways in normal *versus* transformed mammary cells [46]. A previous study reported that very low amounts of dietary lutein (0.002%) can efficiently decrease mammary tumor development and growth in mice [47].

β -carotene (**8**) dose-dependently induced apoptosis and cell differentiation in cultured leukemia cells, but not in normal cells [48]. Another study reported that β -carotene could reduce damage caused by radiation therapy and decrease local cancer recurrence [49]. It also inhibited angiogenesis by altering the cytokine profile and the activation and nuclear translocation of transcription factors [50].

CONCLUSION

The dichloromethane extracts of *Brassica vegetables* (red cabbage, green cabbage, broccoli and mustard) and radish afforded similar major constituents: β -sitosterol, unsaturated triglycerides and essential fatty acids (linoleic and linolenic acids) which were reported to exhibit anticancer properties. Thus, the anticancer properties of *Brassica vegetables* and radish are not only attributed to the high content of glucosinolates, but also to the presence of sterols, unsaturated triglycerides and essential fatty acids.

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