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RESEARCH ARTICLE

SYNTHESIS, ANTICANCER AND ANTIOXIDANT ACTIVITIES OF 6,8-DIBROMOFLAVONES

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ABSTRACT

A series of 6,8-dibromoflavones were synthesized by the Mannich base condensation reaction of arylaldehyde with a new compound 1,3-bis(3,5-dibromo-4-alkoxy-2-hydroxyphenyl)-but-3-en-1-one obtained during the reaction of bromine with 4-alkoxy-5-bromo-2-hydroxyacetophenone. All the synthesized 6,8-dibromoflavones were screened for antioxidant and anticancer activities. Among the eight compounds, 6b possess highest antioxidant activity and compound 6b, 6c, 6d possesses anticancer activity.

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INTRODUCTION

Flavones occupy a special place in the realm of natural and synthetic organic chemistry owing to their useful biological activities such as anxiolytic, anticancer, analgesic, and antimicr obial. During the past few years, various methods have been re ported for the synthesis of flavones (Theja *et al*., 2011). They are also active as anti-oxidants although the *in vivo* anti-oxidant activity is very limited due to weak absorption (around 5%) in the small intestine, together with rapid metabolizing and excretion. Because of their broad range of significant biological activities, this family of molecules has been extensively investigated and more than 4000 chemically unique flavonoids have been isolated from plants. They have varied bioactivities and applications in cosmetics, pharmaceuticals, food, flavoring and Agrochemicals (Bennardi *et al.,* 2008). In consequence, these compounds are very interesting targets to organic chemists, and several strategies for their synthesis have been developed. There are a number of methods available for preparing dibromoflavones. Medina *et al*., 1997 reported that some brominated flavones, particularly 6-bromoflavone and 6-bromo-3'-nitroflavone, showed activities higher than that of diazepam. Some 8-bromoflavone derivatives, namely 8-bromo-5,7,4'-trimethoxyflavone might be useful synthetic precursors for the synthesis of biologically active natural products such as vitexin (Frick *et al.,*1989) and aciculatin (Carte *et al.,* 1991). Brominated flavones are first reported by Wheeler and Hutchins from the chalcone by bromination using molecular bromine followed by cyclisation under basic conditions. Latter on, Chen and his group reported the synthesis of brominated flavones in a sequence that used SeO_2 oxidation. Most of the current syntheses for new flavonoids are based on the pioneer work developed by Robinson (Allan *et al.,* 1924) or exerted by the Baker – Venkataraman (Mahal *et al.,* 1933) synthesis *via* chalcones (Iinuma *et al.,* 1984) and synthesis via an intramolecular Wittig reaction involving a number of steps giving too low yields or from encountering considerable challenges due to irreproducible workout. Therefore, the development of new methods for efficient synthesis of flavones is strongly desirable. In continuation of our ongoing research for the development of simple and efficient methods for the synthesis of various heterocyclic compounds herein, we have presented a novel, mild and efficient method for synthesis of 6,8-dibromoflavones from 1,3-bis(3,5-dibromo-4-alkoxy-2-hydroxyphenyl)-but-3-en-1-one using Mannich base condensation reaction with arylaldehyde (Scheme 1). The

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compound 1,3-bis(3,5-dibromo-4-alkoxy-2-hydroxyphenyl)-but-3-en-1-one is the unexpected compound obtained during the reaction of bromine with 4-alkoxy-5-bromo-2-hydroxyacetophenone. The following mechanism has been proposed for supporting the formation of compound **(5)** and product **(6)** (Scheme 2). Synthesized compounds were evaluated for cytotoxicity using HeLa cells. Antioxidant activities of the compounds were determined using DPPH assay (Table 1). The cytotoxicity was assessed by the MTT assay (Table 2). In addition, treatment with HeLa cells also resulted in nuclear DNA fragmentation, as seen in agarose gel electrophoresis (Figure 1). This is a hallmark of cells undergoing apoptosis. Confirmation of apoptosis was performed by staining the cells with Annexin V. Annexin V-positive cells were defined as apoptotic cells (Figure 2).

MATERIALS AND METHODS

Melting points were determined in open capillaries and are uncorrected. The IR spectra were recorded on an 8400S SHIMADZU spectrometer and the UV spectra on a SHIMADZU UV-1700 UV – Vis spectrophotometer. The ^{1}H NMR and ^{13}C NMR spectra were obtained Bruker 400 MHz spectrometer in $CDCl_3$ (Chemical shifts in δ, ppm relative to TMS as an internal standard). Elemental analyses were done on Elementar Vario EL III.

Synthesis of 1-(5-bromo-4-methoxy/ethoxy-2-hydroxyphenyl)ethanone (4a-b)

2-Hydroxy-4-methoxy/ethoxy acetophenone (0.01 mmol) was dissolved in 10 ml of glacial acetic acid in a 100 ml conical flask and 0.5 ml of molecular bromine was added to this solution from a dropping funnel by shaking vigorously. After the completion of the reaction, the reaction mixture was treated with ice cold water, when 1-(5-bromo-4-ethoxy/methoxy-2-hydroxyphenyl)ethanone separated as a solid. It was crystallized from ethanol as colorless needles.

Synthesis of 1,3-bis(3,5-dibromo-4-methoxy/ethoxy-2-hydroxyphenyl)but-3-en-1-one (5a-b)

1-(5-bromo-4-methoxy/ethoxy-2-hydroxyphenyl)ethanone (0.01 mmol) was dissolved in 100 ml glacial acetic acid in a 100 ml conical flask and 0.5 ml of molecular bromine was added to this solution from a dropping funnel by shaking vigorously during the period of 30 minutes. After the completion of the reaction, the reaction mixture was treated with ice cold water. The viscous mass obtained was extracted with diethyl ether (2 × 25 ml), washed with water and dried over anhydrous sodium sulphate (2 g). Evaporation of ether gave 1,3-bis(3,5-dibromo-4-ethoxy/methoxy-2-hydroxy phenyl)but-3-en-1-one as a semi-solid. It was crystallized from ethanol as pale yellow crystals.

General procedure for the synthesis of flavones (6a-h)

An ethanolic solution of 1,3-bis(3,5-dibromo-4-ethoxy/methoxy-2-hydroxyphenyl)but-3-en-1-one (0.005 mmol), arylaldehyde (0.005 mmol) and ammonium acetate (0.005 mmol) were taken in a 50 ml beaker. The mixture was heated in a water bath for 15 minutes. After the completion of the reaction, the reaction mixture was treated with ice cold water. The solid obtained was filtered and crystallized from ethanol as pale yellow crystals. It was negative towards a neutral ferric chloride solution.

1-(5-bromo-2-hydroxy-4-methoxyphenyl)ethanone (4a): Colorless needles, yield 70%, m.p 166-170 °C; IR (γ_{max}, cm^{-1}): 3431, 3078, 2922, 1627, 1485, 1440, 1174, 1047, 746, 663. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.56 (3H, s, $COCH_3$), 3.92 (3H, s, OCH_3), 6.45 (1H, s, H-3), 7.85 (1H, s, H-6), 12.67 (1H, s, OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.26 ($COCH_3$), 56.58 (OCH_3), 100.75 (C-3), 101.09 (C-5), 114.65 (C-1), 134.72 (C-6), 161.84 (C-4), 164.37 (C-2), 201.97 (C=O); Anal. Calcd for $C_9H_9BrO_3$ (243.97): C, 44.11; H, 3.70%. Found: C, 44.28; H, 3.81%.

1-(3,5-dibromo-4-ethoxy-2-hydroxyphenyl)ethanone (4b): Colourless needles, yield 75%, m.p 158-160 °C; IR (γ_{max}, cm^{-1}): 3431, 3078, 2987, 1633, 1597, 1473, 1199, 1045, 746, 646. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (3H, t, J = 7 Hz, CH_3), 2.55 (3H, s, $COCH_3$), 4.10 (2H, q, J = 6.96 Hz, OCH_2), 7.84 (1H, s, H-6), 13.02 (1H, s, OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.50 (CH_3), 26.62 ($COCH_3$), 70.01 (OCH_2), 107.09 (C-3), 108.62 (C-5), 117.70 (C-1), 133.45 (C-6), 159.89 (C-4), 160.34 (C-2), 202.53 (C=O); Anal. Calcd for $C_{10}H_{11}BrO_3$ (257.99): C, 46.36; H, 4.28%. Found: C, 46.86; H, 4.28%.

1,3-bis(3,5-dibromo-2-hydroxy-4-methoxyphenyl)but-3-en-1-one (5a): Yellow crystals, yield 75%, m.p 91-93 °C; IR (γ_{max}, cm^{-1}): 3431, 3078, 2987, 1633, 1597, 1473, 1199, 1045, 746, 646. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.97 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 4.39 (2H, s, CH_2), 4.75 (2H, s, $=CH_2$), 7.26 (1H, s, C-1'-OH), 7.84 (1H, s, H-6), 7.95 (1H, s, H-6'), 12.51 (1H, s, C-1-OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.16 (CH_2), 60.13 (OCH_3), 106.29 (C-3, 3'), 107.81 (C-5, 5'), 114.17 (C-1'), 116.84 ($=CH_2$), 118.30 (C-1), 132.15 (C-6'), 132.47 (C-6), 159.34 (C-9), 60.29 (C-2'), 160.68 (C-4'), 194.37 (C-2), 195.37 (C-4), 201.53 (C=O); Anal. Calcd for $C_{18}H_{14}Br_4O_5$ (629.75): C, 34.32; H, 2.24%. Found: C, 34.82; H, 2.42%.

1,3-bis(3,5-dibromo-4-ethoxy-2-hydroxyphenyl)-but-3-en-1-one (5b): Yellow crystals, yield 70%, m.p 88-90 °C; IR (γ_{max}, cm^{-1}): 3431, 3078, 2987, 1633, 1597, 1473, 1199, 1045, 746, 646. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (6H, t, J = 6.98 Hz, 2 × CH_3), 4.05 (4H, q, J = 6.96 Hz, 2 × OCH_2), 4.28 (2H, s, CH_2), 4.74 (2H, s, $=CH_2$), 7.22 (1H, s, C-1'-OH), 7.68 (1H, s, H-6), 7.78 (1H, s, H-6'), 12.57 (1H, s, C-1-OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.25 (CH_3), 28.16 (CH_2), 65.23 (OCH_2), 106.13 (C-3, 3'), 107.70 (C-5, 5'), 114.02 (C-1'), 116.84 ($=CH_2$), 118.00 (C-1), 132.05 (C-6'), 132.14 (C-6) 59.04 (C-9), 160.09 (C-2'), 160.38 (C-4'), 194.07 (C-2), 195.07 (C-4), 201.94 (C=O); Anal. Calcd for $C_{20}H_{18}Br_4O_5$ (657.78): C, 36.51; H, 2.76%. Found: C, 36.91; H, 2.88%.

6,8-dibromo-7-methoxyflavone (6a): Yellow crystals, yield 85%, m.p 149-152 °C; UV (λ_{max}, nm): 334, 267. IR (γ_{max}, cm^{-1}): 3047, 2943, 1701, 1647, 1547, 1591, 1170, 1045, 729, 690. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.0 (3H, s, OCH_3), 6.9 (1H, s, H-3), 7.42-7.51 (3H, m, H-3', 4', 5'), 7.90-7.97 (3H, m, H-5,

2’, 6’). ^{13}C NMR (75 MHz, $CDCl_3$): δ 61.18 (OCH_3), 102.14 (C-8), 113.21 (C-3), 114.75 (C-6), 119.64 (C-10), 127.20 (C-4’), 128.95 (C-2’, 6’), 130.43 (C-1’), 131.45 (C-5), 131.80 (C-3’, 5’), 146.69 (C-9), 160.66 (C-2), 163.45 (C-7), 181.72 (C=O); Anal. Calcd for $C_{16}H_{10}Br_2O_3$ (409.90): C, 46.86; H, 2.46%. Found: C, 47.02; H, 3.27%.

6,8-dibromo-7-methoxy-2-(4-nitrophenyl)-4H-chromen-4-one (6b): Yellow crystals, yield 75%, m.p 200-203 ^oC; UV (λ_{max}, nm): 337, 267. IR (γ_{max}, cm^{-1}): 3071, 2943, 2840, 1709, 1651, 1599, 1174, 1044, 847, 831. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.0 (3H, s, OCH_3), 6.9 (1H, s, H-3), 8.0 (1H, s, H-5), 8.11 (2H, d, J = 8.8 Hz, H-2’, 6’), 8.36 (2H, d, J = 8.8 Hz, H-3’, 5’). ^{13}C NMR (75 MHz, $CDCl_3$): δ 62.33 (OCH_3), 100.71 (C-8), 106.72 (C-3), 112.12 (C-6), 114.97 (C-9), 125.09 (C-1’), 128.62 (C-2’, 6’), 133.10 (C-4’), 138.77 (C-3’, 5’), 148.94 (C-10), 165.09 (C-7), 162.14 (C-2), 177.25 (C=O); Anal. Calcd for $C_{16}H_9Br_2NO_5$ (454.88): C, 42.23; H, 1.99; N, 3.08%. Found: C, 42.39; H, 2.81; N, 3.08%.

6,8-dibromo-2-(4-chlorophenyl)-7-methoxy-4H-chromen-4-one (6c): Colourless crystals, yield 80%, m.p 141-145 ^oC; UV (λ_{max}, nm): 339, 269. IR (γ_{max}, cm^{-1}): 3073, 2952, 2854, 1712, 1652, 1585, 1172, 1043, 890, 823. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.04 (3H, s, OCH_3), 6.90 (1H, s, H-3), 7.48 (2H, d, J = 8.8 Hz, H-2’, 6’), 7.90 (2H, d, J = 8.8 Hz, H-3’, 5’), 7.99 (3H, s, H-5). ^{13}C NMR (75 MHz, $CDCl_3$): δ 62.33 (OCH_3), 103.45 (C-3), 114.35 (C-8), 120.61 (C-6), 128.26 (C-10), 128.36 (C-1’), 130.26 (C-2’, 6’), 131.04 (C-4’), 133.79 (C-3’, 5’), 133.88 (C-5), 137.47 (C-9), 137.56 (C-7), 147.89 (C-2), 182.72 (C=O); Anal. Calcd for $C_{16}H_9Br_2ClO_3$ (443.86): C, 43.23; H, 2.04%. Found: C, 43.39; H, 2.85%.

6,8-dibromo-2-(4-bromophenyl)-7-methoxy-4H-chromen-4-one (6d): Yellow crystals, yield 80%, m.p 198-200 ^oC; UV (λ_{max}, nm): 340, 289. IR (γ_{max}, cm^{-1}): 3072, 2983, 2943, 1710, 1653, 1596, 1173, 1043, 894, 812. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.95 (3H, s, OCH_3), 6.79 (1H, s, H-3), 7.55 (2H, d, J = 8.4 Hz, H-2’, 6’), 7.73 (2H, d, J = 8.4 Hz, H-3’, 5’), 7.89 (1H, s, H-5). ^{13}C NMR (75 MHz, $CDCl_3$): δ 60.80 (OCH_3), 104.43 (C-3), 108.65 (C-8), 113.16 (C-6), 120.26 (C-10), 122.63 (C-4’), 128.62 (C-2’, 6’), 129.34 (C-1’), 131.57 (C-3’, 5’), 134.18 (C-5), 153.91 (C-9), 163.36 (C-7), 163.61 (C-2), 177.52 (C=O); Anal. Calcd for $C_{16}H_9Br_3O_3$ (505.95): C, 37.90; H, 5.37%. Found: C, 39.46; H, 1.86%.

N-(4-(6,8-dibromo-7-methoxy-4-oxo-4H-chromen-2-yl)phenyl)acetamide (6e): Yellow crystals, yield 80%, m.p 252-254 ^oC; UV (λ_{max}, nm): 352, 273. IR (γ_{max}, cm^{-1}): 3068, 2943, 2872, 1706, 1668, 1664, 1595, 1419, 1217, 1169, 1138, 896, 833. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.03 (3H, s, OCH_3), 25 (3H, s, $COCH_3$), 6.93 (1H, s, H-3), 7.33 (1H, s, NH), 7.67 (2H, d, J = 6.6 Hz, H-2’, 6’), 7.95 (2H, d, J = 6.6 Hz, H-3’). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.49 ($COCH_3$), 61.55 (OCH_3), 114.44 (C-8), 119.39 (C-6), 120.12 (C-10), 126.10 (C-1’), 127.29 (C-2’, 6’), 133.04 (C-3’, 5’), 139.40 (C-4’), 141.50 (C-5), 144.40 (C-9), 169.26 (C-2), 181.00 (C=O); Anal. Calcd for $C_{18}H_{13}Br_2NO_4$ (466.92): C, 46.28; H, 2.81; N, 3.00%. Found: C, 46.86; H, 2.99; N, 3.00%.

6,8-dibromo-7-ethoxyflavone (6f): Yellow crystals, yield 75%, m.p 125-127 ^oC; UV (λ_{max}, nm): 335, 267. IR (γ_{max}, cm^{-1}): 3063, 2980, 1705, 1647, 1593, 1415, 1384, 1269, 1172, 1132, 887, 813. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, t, J = 6.8 Hz, CH_3), 4.15 (2H, q, J = 7.2 Hz, OCH_2), 6.85 (1H, s, H-3), 7.32-7.44 (3H, m, H-3’, 4’, 5’), 7.84-7.89 (3H, m, H-2’, 6’, 5). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.56 (CH_3), 70.59 (OCH_2), 102.77 (C-3), 113.70 (C-8), 114.85 (C-6), 119.64 (C-10), 127.34 (C-1’), 129.11 (C-2’, 6’), 130.54 (C-4’), 130.70 (C-5), 131.96 (C-3’, 5’), 146.96 (C-9), 160.35 (C-7), 163.75 (C-2), 182.02 (C=O); Anal. Calcd for $C_{17}H_{12}Br_2O_3$ (423.91): C, 48.15; H, 2.85%. Found: C, 48.31; H, 3.66%.

6,8-dibromo-7-ethoxy-2-(4-nitrophenyl)-4H-chromen-4-one (6g): Yellow crystals, yield 70%, m.p 210-219 ^oC; UV (λ_{max}, nm): 328, 267. IR (γ_{max}, cm^{-1}): 2993, 2814, 1710, 1597, 1417, 1388, 1271, 1170, 1134, 895, 854. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (3H, t, J = 6.8 Hz, CH_3), 4.19 (2H, q, J = 7.2 Hz, OCH_2), 6.84 (1H, s, H-3), 7.90 (1H, s, H-5), 8.01 (2H, d, J = 8.4 Hz, H-2’, 6’), 8.26 (2H, d, J = 8.8 Hz, H-3’, 5’)., ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.69 (CH_3), 70.93 (OCH_2), 102.99 (C-3), 111.20 (C-8), 114.53 (C-6), 119.15 (C-10), 124.31 (C-2’, 6’), 127.77 (C-1’), 132.29 (C-3’, 5’), 138.04 (C-4’), 148.16 (C-5), 148.45 (C-9), 161.13 (C-7), 164.13 (C-2), 181.90 (C=O); Anal. Calcd for $C_{17}H_{11}Br_2NO_5$ (468.90): C, 43.53; H, 2.36; N, 2.99%. Found: C, 48.69; H, 2.88; N, 3.30%.

6,8-dibromo-2-(4-chlorophenyl)-7-ethoxy-4H-chromen-4-one (6h): Colourless crystals, yield 75%, m.p 177-176 ^oC; UV (λ_{max}, nm): 339, 269. IR (γ_{max}, cm^{-1}): 3070, 2982, 2810, 1710, 1626, 1593, 1417, 1384, 1276, 1170, 1134, 895, 819. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (3H, t, J = 6.9 Hz, CH_3), 4.17 (2H, q, J = 7.0 Hz, OCH_2), 6.79 (1H, s, H-3), 7.34 (2H, d, J = 8.4 Hz, H-2’, 6’), 7.77 (2H, d, J = 8.4 Hz, H-3’, 5’), 7.78 (1H, s, H-5). ^{13}C NMR (75 MHz, $CDCl_3$): δ 15.56 (CH_3), 70.65 (OCH_2), 102.76 (C-3), 113.32 (C-8), 113.89 (C-6), 119.51 (C-10), 127.40 (C-1’), 129.45 (C-2’, 6’), 130.20 (C-4’), 132.98 (C-3’, 5’), 136.62 (C-5), 147.05 (C-9), 160.49 (C-7), 163.60 (C-2), 181.84 (C=O); Anal. Calcd for $C_{17}H_{11}Br_2ClO_3$ (457.87): C, 44.53; H, 2.42%. Found: C, 44.70; H, 3.03%.

Bioassays

DPPH free radical scavenging activity

The free radical scavenging activity of 6,8-dibromoflavone and its derivatives were measured using DPPH according to Blois method. Exactly 0.1 mmol/L solution of DPPH in ethanol was prepared and 2 ml of this solution was added to 2 ml of the test solutions at different levels (5–25 μM). After 30 minutes, absorbance was measured at 517 nm using UV-vis. Spectrophotometer. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage of inhibition,

$$\% \text{ inhibition} = [(A_{control} - A_{sample})/A_{control} \text{ x}100]$$

The EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plotting between %

inhibition and concentrations. All tests were performed in triplicate. Ascorbic acid was used as the reference compound.

Cytotoxicity Assay

The HeLa cell line was maintained with IMDM (Ischove's Modified Dulbecco's Medium) medium supplemented with Fetal Bovine Serum (FBS) 10%, penicillin 100 IU/ml, and streptomycin 100 mg/ml. The HeLa cells were incubated at 37°C in 5% atmospheric CO_2 and used for *in vitro* experiments.

MTT Assay / Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay was carried out to examine the cytotoxic effect of 6,8-dibromoflavones **(6a-h)** at varying concentrations in accordance to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). This assay is based on the MTT reduction by the mitochondrial dehydrogenase enzyme present in the viable cells resulting in the blue formazan product. Briefly, 1×10^5 cells of HeLa were plated onto 96-well microtitre plates and then treated with various concentrations of 6,8-dibromoflavones **(6a-h).** The treated HeLa cells were incubated at 37 °C in a humidified chamber for 24 hours. After incubation, 10 ml of MTT (5 mg/ml in PBS) was mixed with the contents of each well and then the plate was further incubated for four hours at 37 °C. About 100 µL of dissolving buffer was added to the resulting formazan and its absorbance was measured using a multiwell spectrophotometer (Biorad, Model 680, Japan). All the experiments were carried out thrice and the CC_{50} values were then determined.

Caspase 3 Assay / Apoptotic Assay

The cells were lysed with the lysis buffer provided in the caspase 3 assay kit (Sigma, St. Louis, MO) and kept on ice for 15-20 minutes. The assay is based on the hydrolysis of the peptide substrate, Ac-DEVD-pNA, by caspase 3, resulting in the release of Ac-DEVD and *p*-nitroaniline (pNA) which absorbs light significantly at 450 nm. Briefly, for 1.0 ml of the reaction mixture, 10 ml of the cell lysate from treated samples was added along with 980 ml of assay buffer, followed by the addition of 10 ml of 20 mm caspase 3 colorimetric substrates (Ac-DEVD pNA). The cell lysates of the drug (8 compounds) treated HeLa cells were then incubated at 37°C with the caspase 3 substrate for two hours and the absorbance was read at 450 nm in a double-beam UV - visible spectrophotometer (Shimadzu, Japan). The assay was also performed with non-induced cells and in the presence of caspase 3 inhibitor for a comparative analysis.

DNA Fragmentation Assay

The cells (1×10^6) treated with the 6,8-dibromoflavones **(6a-h)** were lysed in 250 µL cell lysis buffer containing 50 mM Tris HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid, 0.1 M NaCl, and 0.5% sodium dodecyl sulfate. The lysate was incubated with 0.5 mg/ml RNase A at 37 °C for one hour, and then with 0.2 mg/ml proteinase K at 50 °C overnight. Phenol extraction of this mixture was carried out, and DNA in the aqueous phase was precipitated by 25 µL (1/10 volume) of 7.5 mM ammonium acetate and 250 µL (1/1 volume) isopropanol. DNA electrophoresis was performed in 1% agarose gel containing 1 µg/ml ethidium bromide at 70 V and the DNA fragments were visualized by exposing the gel to ultraviolet light, followed by photography using Alpha Imager, the Gel documentation instrument.

Statistical Analysis

Data were analyzed using the software SigmaPlot for Windows (Version 11.0). Values were expressed as mean ± standard deviation of the mean values of three independent experiments followed by student t-test. Statistical significance was acceptable to a level of $p < 0.05$.

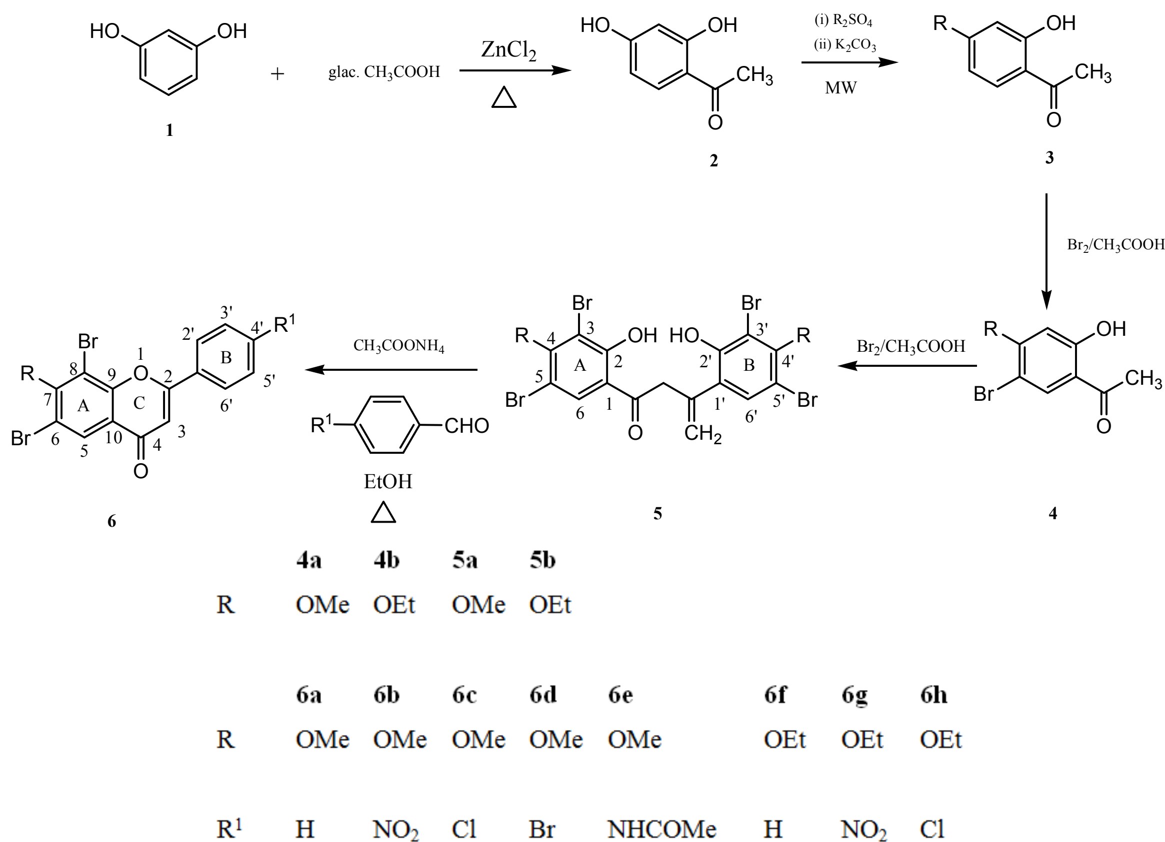
RESULTS AND DISCUSSION

All the compounds were characterized by elemental analysis, UV, FT-IR, ^{1}H NMR and ^{13}C NMR spectra. The UV spectra of all compounds showed two maxima, one around at 267 – 273 nm and the other at 328 – 352 nm. Flavones and flavonols exhibit two major absorption bands in the ultraviolet / visible region, Band I in the 320 – 385 nm range representing the B ring absorption, and Band II in the 250 – 285 nm range representing A ring absorption (Carte *et al.,* 1991). The infrared spectra of the flavones **6a-h** showed carbonyl absorption in the region 1626 – 1668 cm^{-1}, aromatic C–H stretching band in the region 3063-3073 cm^{-1}, and aliphatic stretching band in the region 2814 – 2983 cm^{-1}. In the ^{1}H NMR spectra of the flavones, apart from the expected aromatic protons in the region 7.32 – 8.36 δ, a one proton singlet observed in the region 6.79 – 6.93 δ was assigned to C-3 proton. This singlet indicated the formation of products **6a-h**. The formation of products was further confirmed by ^{13}C NMR spectra.

DPPH radical scavenging activity

Free radicals and active oxygen species have been related to cardiovascular and inflammatory diseases, and even with a role in cancer and ageing. Efforts to counteract the damage caused by these species are gaining acceptance as a basis for novel therapeutic approaches and the field of preventive medicine is experiencing an upsurge of interest in medically useful antioxidants (Torres de Pinedo *et al.,* 2007). Flavonoids have attracted the interest of researchers because they showed promise of being powerful antioxidants which can protect the human body from free radicals (Mellou *et al.,* 2005; Zhao *et al.,* 2003). Many flavonoids such as quercetin, luteolin and cathechins, are better antioxidants than the nutrient antioxidants such as vitamin C, vitamin E and β-carotene (Rice-Evans *et al.,* 1997). The function of an antioxidant is to intercept and react with free radicals at a rate faster than the substrate. Since free radicals are able to attack at a variety of targets, including lipids, fats and proteins, it is believed that they may damage organisms, leading to disease, poisoning and including aging (Wright *et al.,* 2001). The scavenging effect of the synthesized compounds on the DPPH radical was evaluated according to the Blois method (Blois, 1958).

Scheme 1. Synthesis of 6,8-dibromoflavones

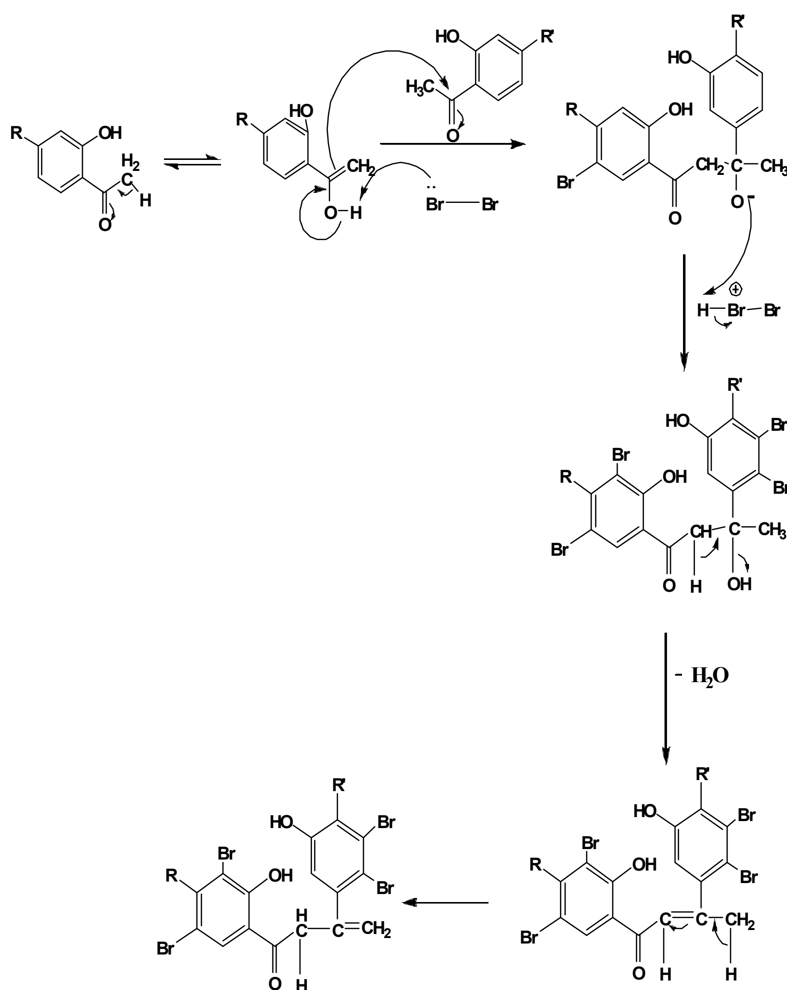


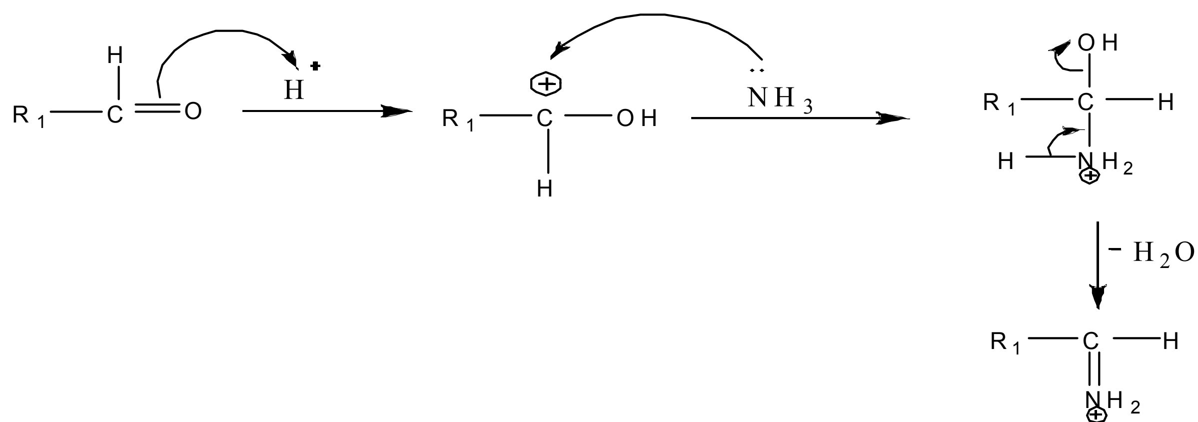
	4a	4b	5a	5b
R	OMe	OEt	OMe	OEt

	6a	6b	6c	6d	6e	6f	6g	6h
R	OMe	OMe	OMe	OMe	OMe	OEt	OEt	OEt
R[1]	H	NO_2	Cl	Br	NHCOMe	H	NO_2	Cl

Scheme 2. Mechanism of the synthesis of 6,8-dibromoflavones (6a-h).

The following mechanism was proposed for the reactions given in Scheme 1.




H
R1 — C = O
H+
R1 — C — OH
NH3
OH
R1 — C — H
H — NH2
- H2O
R1 — C — H
NH2

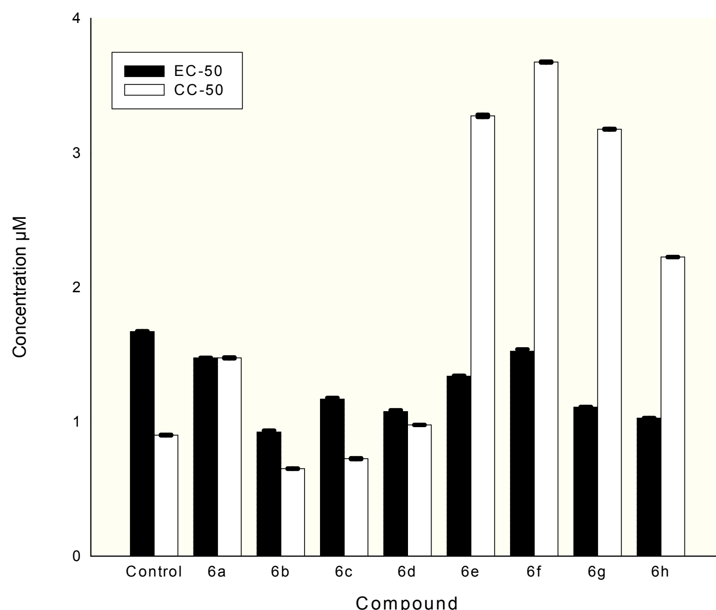


Fig.1. Comparison between EC_{50} and CC_{50} values. Each bar represents the mean _ SD for three Separate experiments. Statistical analysis was performed by ANOVA ($p < 0.05$)

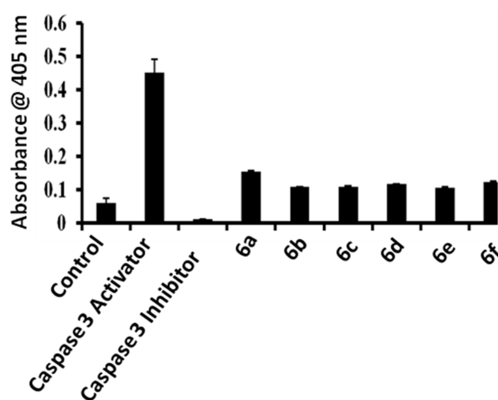


Fig.2. Apoptosis of HeLa cells induced by 6,8-dibromoflavones

Table 1. Radical modulation assay of compounds 6a-h

Tested compound	EC_{50} (μM)
6a	1.474 ± 0.002
6b	0.923 ± 0.011
6c	1.168 ± 0.009
6d	1.075 ± 0.010
6e	1.338 ± 0.004
6f	1.524 ± 0.014
6g	1.108 ± 0.002
6h	1.026 ± 0.002
Ascorbic acid	1.670 ± 0.003

Table 2. MTT Assay of Compound 6a-h

Tested compound	CC_{50} (μM)
6a	1.474 ± 0.004
6b	0.650 ± 0.002
6c	0.724 ± 0.004
6d	0.975 ± 0.001
6e	3.274 ± 0.009
6f	3.674 ± 0.004
6g	3.175 ± 0.003
6h	2.224 ± 0.001
Control	0.9 ± 0.003

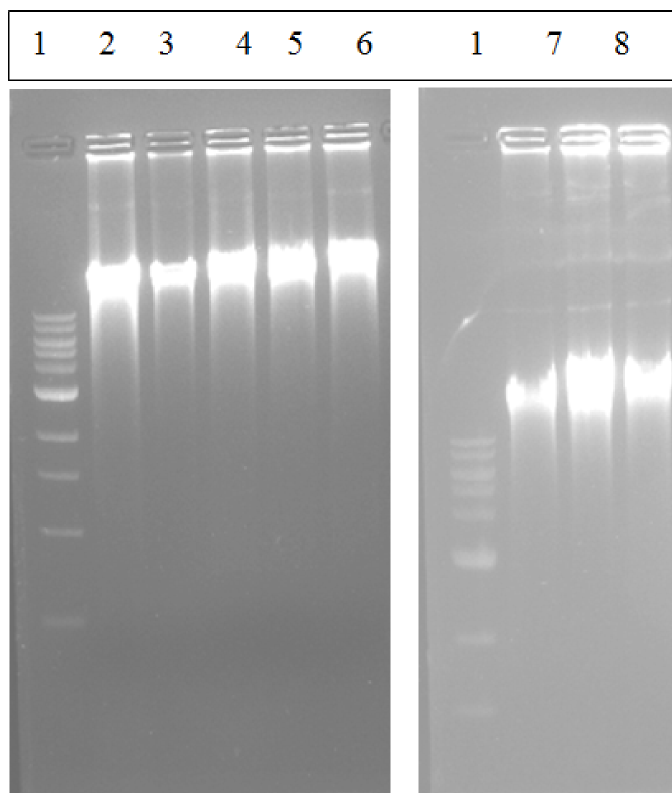


Fig.3. DNA fragmentation assay of 6,8-dibromoflavones

Lane 1 - control
Lane 2 - 6a Lane 6 - 6e
Lane 3 - 6b Lane 7 - 6f
Lane 4 - 6c Lane 8 - 6g
Lane 5 - 6d Lane 9 - 6h

The EC_{50} values of these compounds are presented in Table 1. Among the eight compounds **6a-h**, **6b** possesses highest activity with EC_{50} values of 0.923 µM. The phenolic compounds (e.g., flavones) are considered to play an important role in the prevention of oxidative damage in living systems. Moreover, in capturing free radicals, their antioxidant activity is highly influenced by the presence of oxygenated groups (e.g., hydroxyl and methoxy) on the aromatic rings (Ahmed *et al.,*2005). Other structural features important for antioxidant nature include the presence of 2, 3 unsaturation in conjugation with a 4 – Oxo – function in the C- ring (Williams *et al.,* 2004). The introduction of methoxy group, increases the hydrogen-donating ability and therefore increases the radical scavenging capacity of the antioxidant. This effect may be due to the fact that methoxy groups are electron-donating groups, which help to stabilize the phenoxy radicals (Torres de Pinedo *et al.,* 2007). The flavone derivative **6b** showed the highest radical modulation activity compared to other tested compounds due to the presence of the Nitro group in the *para* position. A recent report with 2,3-diarylchromanones also revealed the same (Kanagalakshmi *et al.,* 2010).

MTT Assay / Cell Viability Assay

The cytotoxicity of the flavone derivatives **6a-h** was studying in HeLa cells. The cytotoxicity of the compounds was determined after five days of exposure and its CC_{50} values were calculated. Among the tested compounds, flavones **6b**, **6c** and **6d** exhibited highest cytotoxicity with CC_{50} of 0.650 µM, 0.724 µM and 0.975 µM respectively (Table 2) at very low concentration. Their cytotoxicity seems to be specific for tumor cells since normal human lymphocytes were not susceptible. It is realized, however, that normal and neoplastic cells have different rates of proliferation and it is not surprising that an active drug is ineffective on slow-growing normal cells. The EC_{50} and CC_{50} values were compared using one-way analysis of variance ANOVA (Figure 1). Among the three flavones, **6b** possesses the highest cytotoxicity than **6c** and **6d** due to the presence of the Nitro group in the C-4' position. Generally, in flavones, the presence of methoxy group and Nitro group will enhance the activity and the presence of ethoxy group will decrease the activity. Since the drugs were able to inhibit the viability of the cells, even at very low concentrations, they can be utilized as potential antitumor therapeutic molecules. Thus, the three flavones **6b**, **6c** and **6d** have been selected for further studies.

Caspase 3 Assay / Apoptotic Assay and DNA Fragmentation Assay

The caspase 3 assay was carried in order to examine whether the 6,8-dibromoflavones **(6a-h)** are inducing apoptotic cell death to the HeLa cells especially at the minimal inhibitory concentration. Since the drugs were seemed to be affecting

normal metabolic activities of the cell, the role of caspase-3 enzyme, a key factor in the apoptotic pathway of the cells were assessed. The activity of caspase 3 enzyme was found in the cells treated with drugs at their corresponding CC_{50} values as shown in Figure 2. The enzyme activity was found to be higher in the cells treated with **6a** and moderate for the other drugs. This activation of caspase enzymes when treated with various drugs suggests that they induce cell death only by means of apoptosis and was also supported by the corresponding cell DNA fragmentation Figure 3. When compared with the control, the DNA ladder indicates that the double stranded DNA breaks, thereby further substantiating the potential antitumor activity of the chemical drugs.

Conclusion

High yields, mild reaction conditions, and short reaction times are the notable advantages of this procedure. We believe that this procedure will provide a better scope and a more practical alternative to the existing methods for the synthesis of flavones. We have designed a new method and synthesized a series of new flavones derivatives with potent antioxidant activity against DPPH as well as anticancer activity against HeLa cells in vitro. Among the eight compounds, **6b** possesses highest antioxidant activity and compounds **6b**, **6c** and **6d** exhibited potential anticancer activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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REFERENCES

Ahmed, N., Ali, H. and Van Lier, J. E. 2005. Silica gel supported InBr and InCl 3: new catalysts for the facile and rapid oxidation of 2'-hydroxychalcones and flavanones to their corresponding flavones under solvent free conditions. *Tetrahedron Letters*, 46: 253-256.

Allan, J. and Robinson, R. 1924. CCXC.- An accessible derivative of chromonol. *Journal of the Chemical Society*, 2192-2195.

Bennardi, D. O., Ruiz, D. M., Romanelli, G. P., Baronetti, G. T., Thomas, H. J. and Autino, J. C. 2008. Efficient Microwave Solvent-Free Synthesis of Flavones, Chromones, Coumarins and Dihydrocoumarins. *Letters in Organic Chemistry,* 5: 607-615.

Blois, M. S. 1958. Antioxidant Determinations by the Use of a Stable Free Radical. *Nature,* 181: 1199-1200.

Carte, B. K., Carr, S., DeBrosse, C., Hemling, M. E., MacKenzie, L., Offen, P. and Berry, D. E. 1991. Aciculatin, a novel flavone-C-glycoside with DNA binding activity from chrysopogonaciculatis. *Tetrahedron*, 47: 1815-1822.

Frick, W. and Schmidt, R. R. 1989. Einfache Synthese von C-β-D-Glucopyranosylaromaten.-Synthese des 5,7,4'-Tri-O-methyl-vitexins. *Liebigs Annalen der Chemie*, 565-570.

Iinuma, M., Iwashima, K. and Matsuura, S. 1984. Synthetic Studies on Flavone Derivatives. XIV. Synthesis of 2',4',5',Trioxygenated Flavones. *Chemical and Pharmaceutical Bulletin,* 32: 4935-4941.

Kanagalakshmi, K., Premanathan, M., Priyanka, R., Hemalatha, B. and Vanangamudi, A. 2010. Synthesis, anticancer and antioxidant activities of 7-methoxyiso flavanone and 2,3-diarylchromanones. *European Journal of Medicinal Chemistry,* 45: 2447-2452.

Mahal, H. S. and Venkataraman, K. 1933. A Synthesis of Flavones at Room Temperature. *Current Science* 4: 214.

Medina, J. H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D. and Paladini, A.C. 1997. Overview-Flavonoids: A New Family of Benzodiazepine Receptor Ligands. *Neurochemical Research,* 22: 419-425.

Mellou, F., Lazari, D., Skaltsa, H., Tselepis, A. D., Kolisis, F. N. and Stamatis, H. 2005. Biocatalytic preparation of acylated derivatives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. *Journal of Biotechnology,* 116: 295-304.

Rice-Evans, C., Miller, N. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. *Trends in Plants Science Reviews,* 2: 152-159.

Theja, N. D., Choudary, P. T., Madhusudhana reddy, I., Gupta, A. V. S. S. and Koti reddy, U. 2011. A facile synthesis of Flavone derivatives used as potent anti-inflammatory agents. *International Journal of Pharmacy and Pharmaceutical Sciences,* 3: 51-54.

Torres de Pinedo, A., Penalver, P. and Morales, J. C. 2007. Synthesis and evaluation of new phenolic-based antioxidants: Structure-activity relationship. *Food Chemistry,* 103: 55-61.

Williams, R. J., Spencer, J. P. E. and Rice – Evans, C. 2004. Flavonoids: Antioxidants or Signalling Molecules?. *Free radical Biology and Medicine,* 36: 838-849.

Wright, J. S., Johnson, E. R. and Dilabio, G. A. 2001. Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. *Journal of American Chemical Society,* 123: 1173-83.

Zhao, F. P., Strack, D., Baumert, A., Subramaniam, R., Ngoh, K. G., Tet, F. C., Swee, N. T. and Lian, S. C. 2003. Antioxidant flavonoids from leaves of Polygonum hydropiper L. *Phytochemistry,* 62: 219-228.

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