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Flavonoids of *Cynometra cauliflora*, their Plausible Biosynthetic Pathway and SAR Study against DPPH Radicals

Nik Fatini Nik Azmin[1], Norizan Ahmat[2]*, Aisyah Salihah Kamarozaman[2], Noor Safina Sulaiman[2], Juriyati Jalil[3] and Mulyadi Tanjung[4]

[1]Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, 40450 Shah Alam, Selangor, Malaysia
[2]Centre of Foundation Studies, Universiti Teknologi MARA Cawangan Selangor, Kampus Dengkil, 43800 Dengkil, Selangor, Malaysia
[3]Centre for Drug and Herbal Development, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
[4]Natural Product Chemistry Research Group, Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga 60115 Surabaya, Indonesia
*Corresponding author (e-mail: noriz118@uitm.edu.my)

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. This study was undertaken to isolate the flavonoids from *Cynometra cauliflora*, and to evaluate their antioxidant properties, together with their structure-activity relationship (SAR) study. The chemical constituents were isolated and purified by combination of chromatographic methods which are; vacuum liquid chromatography (VLC), radial chromatography (RC) and preparative thin layer chromatography (pTLC). The compounds were elucidated using Nuclear Magnetic Resonance (NMR), Ultraviolet-Visible (UV-Vis), Infrared (IR) and Mass spectrometry (MS), as well as comparison with the previous literature. Antioxidant activity of the compounds was determined by 1,1-diphenyl-2-picrylhadrazyl (DPPH) assay. A total of 8 flavonoids (**1-8**), which consist of two flavanones and six flavones were successfully purified from the acetone extract of the twigs part of *C. cauliflora*. The biosynthesis pathway of the isolated flavonoids was reviewed. This plant reported the occurrence of (*R*)-isomer type of flavonoids. 3', 4', 7-trihydroxyflavone (**6**) displayed the highest antioxidant activity with IC_{50} value of 27.5 ± 1.91 uM, verifying that flavonoids having an unsaturated C2-C3 bond in conjugation with a C4-oxo function as potent antioxidants. This study should prompt further studies of the antioxidant properties of 3', 4', 7-trihydroxyflavone, which may be used for the development of natural and safe antioxidant compounds.

Keywords: *Cynometra*; flavonoids; biosynthesis; antioxidant; Structure Activity Relationship

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Flavonoids are among the most ubiquitous phenolic compounds found in nature having a benzo-γ-pyrone structure. The flavonoids structures are characterized by flavan nucleus, which consists of 15 carbon atoms derived from a C6-C3-C6 skeleton. These are group of structurally related compounds with a chromane-type skeleton having phenyl substituent in C2-C3 position [1]. These metabolites account for much of the red, blue, and purple pigmentation found in plants and increasingly for their association with the health benefits of wine, chocolate, and generally with diets rich in fruits and vegetables [2]. Flavonoids have diverse physiological and pharmacological activities such as estrogenic, anti-tumor, antimicrobial, antiallergic, and antiinflammatory effects [3]. They are well-known antioxidants and metal ion chelators [4]. Flavonoids are generated from phenylalanine through the phenylpropanoid pathway, while phenylalanine is synthesized via the shikimate pathway [5]. These pathway, depending on the polyketide synthase (PKS) activity, which are, in this case, chalcone synthase (CHS), will allow the generated tetraketide intermediate to undergo a C-acylation that produces chalcone, which followed by subsequent modifications, lead to the various structure of flavonoids.

Antioxidants protect cells from the harmful impacts of free radicals, which are unsteady molecules produced by radiation, pollution, and normal bodily processes. These unstable molecules can initiate oxidative stress, leading to aging, cellular harm, and the development of long-term diseases. Antioxidants protect DNA, proteins, and lipids from damage by neutralizing free radicals through the donation of electrons [6]. They play a vital role in upholding cellular health, defending against oxidative stress, and lowering the chances of developing different chronic conditions [7]. The antioxidant properties of flavonoids are responsible for the majority of their positive health effects. Flavonoids' protective benefits in living organisms are attributed to their ability to pass electrons to free radicals, bind to metal catalysts, stimulate antioxidant enzymes, decrease alpha-tocopherol radicals, and block oxidases [8-11].

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Studies of Structure-Activity Relationships (SAR) are crucial in medicinal chemistry and drug discovery. Comprehending the correlation between a molecule's structural features and its biological effects is essential in SAR. The in vitro antioxidant activity of flavonoids and their metabolites is determined by how the functional groups are positioned around the nuclear structure [12]. Research on structure-activity relationships (SAR) has provided insights into how flavonoids are processed in the body, including their absorption and metabolism, leading to a clearer grasp of their antioxidant and prooxidant effects [13]. However, the variety of structures in flavonoids, their numerous action mechanisms, and the different experimental approaches to measuring antioxidant activity make it difficult to establish a universal system of structure-activity relationships [12]. Although there are some conflicting pieces of evidence, numerous SAR have been firmly established *in vitro*. The structure-activity relationships (SAR) of naturally existing flavonoids provide initial understanding of how these changes in metabolism influence different antioxidant mechanisms [12]. *Cynometra cauliflora* L. from Fabaceae family is indigenous to eastern Peninsular Malaysia and is widely distributed in South East Asia, Ceylon and western and southern Peninsular of India [14]. Usually, this plant growth in wet tropical lowlands, but may also grow well in climates with a more distinct dry season and resistant to wind [15]. Their native name is 'Buah Katak Puru' or 'Nam-nam. This plant is a typical underutilized fruit tree that has the medicinal values in folk traditional medicine and cultivated as an ornamental plant in the village [16, 17]. Decoction of the leaves is traditionally used for treating diabetes and hyperlipidemia. The mature fruit can be eaten raw or cooked with sugar to make sweet compote, or fried in batter. The fruits have also been reported to have useful medicinal properties and used as folk medicine. The fruits have been used to cure loss of appetite, while the seed oil is used for curing skin diseases. The fruit has a large seed that was reported to have low antioxidant capacity and moderately high in total phenolic content [18]. Limited works have been conducted on *C. cauliflora* regarding its chemical composition. In view of this, the present study aimed to identify the constituents, together with their proposed bio-synthesis pathway, from the twigs of *C. cauliflora* and to evaluate their antioxidant properties. In addition, their Structure Activity Relationship (SAR) was also discussed.

EXPERIMENTAL

Plant Materials

The plant part used in this study is the twig. *C. cauliflora* was collected by author in 2012 at Kota Bharu, Kelantan and was identified by Mr. Sani Miran, a botanist from Universiti Kebangsaan Malaysia (UKM), Bangi, without voucher specimen being deposited. The dried powdered twigs were macerated in acetone for more than 24 hours, filtered and concentrated under reduced pressure. This step was repeated for five times to get as much crude extract.

Chemicals

The chromatographic separation and purification were conducted using the following adsorbents: Silica gel G60 (230 - 400 mesh, Merck catalog number: 1.09385) was used for Column Chromatography (CC), Silica Gel 60 PF254 Gypsum (Merck catalog number: 1.07749) for Radial Chromatography (RC), TLC plates, Kiesel-gel 60 F254 0.25 mm Silica Gel 60 PF254 (Merck catalog number: 1.00747) was used for Vacuum Liquid Chromatography (VLC) and Silica Gel 60 (0.2-0.5 mm) (Merck catalog number: 1.07733) was used for samples preparation. Thin Layer Chromatography (TLC) Silica Gel 60 PF254 (aluminium sheets) (Merck catalog number: 1.05554) was used for TLC analysis. The industrial (distilled before use) and analytical grade reagent of the common solvents of n-hexane (Hex), dichloromethane (DCM), chloroform ($CHCl_3$), ethyl acetate (EtOAc), acetone and methanol (MeOH) were used for extraction, fractionation, purification and isolation of the compounds.

Instrumentation

The ^{1}H-NMR and ^{13}C-NMR were recorded in acetone-d_6 on Bruker 300 Ultrashield NMR spectrometer measured at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Chemical shifts were reported in ppm (δ) and the coupling constants (J) were given in Hz. The mass spectra were measured on Agilent Technologies LC-MS. Melting point was measured by Fisher Johns 'micro melting point apparatus'. Optical rotation was determined using Perkin-Elmer 341 polarimeters in MeOH. The compound's profile on the TLC plates were observed under UV light at wavelength of 254 and 365 nm. DPPH experiments were analyzed on Gen-5 Biotek Synergy HT Microplate reader.

EXPERIMENTAL PROCEDURE

Extraction and Isolation

The ground, air-dried powder of the twigs of *C. cauliflora* (3.1 kg) was macerated with acetone (10 L) and allowed to stand for 24 hours at room temperature. The acetone extract was then filtered and evaporated under reduced pressure using rotary evaporator at 40°C. This process was repeated for five times to finally gave a dark brown residue (149.3 g). For the removal of tannins, the crude extract was dissolved in methanol and then diethyl ether was added to precipitate the tannin constituents. Decantation and evaporation at 40°C of the ether-soluble fraction yielded crude extract with less tannin (60 g) which was further fractionated with VLC and eluted with the mixtures of Hex:EtOAc (6:4 until 0:10), followed by EtOAc:MeOH (9:1). Ten major fractions were obtained (CC1-CC10).

Fraction CC2 (209.4 mg) was subjected to radial chromatography using solvent system of Hex: EtOAc (10:0 until 5:5) to give naringenin (**1**) (6.3 mg). Fraction CC4 (1.54 g) was refractionated with VLC (Hex:EtoAc 8:2, 7:3, 6:4, 5:5, 4:6, 3:7) which yielded seven subfractions (CC41-CC47). Subfraction CC42 was identified as apigenin (**8**) (0.5 mg). Subfraction CC46 (104 mg) was further processed with radial chromatography (eluent, $CHCl_3$: Acetone [9:1 until 7:3]) and produced 4', 7-dihydroxyflavone (**7**) (4 mg). Fraction CC5 (360 mg) was subjected to radial chromatography with solvent system Hex: EtoAc (9:1 until 6:4) and gave seven subfractions (CC51-CC57). Subfraction CC57 (200 mg) once again was purified by radial chromatography using $CHCl_3$: Acetone (9:1 until 7:3) as the eluent, to give pure compound eriodictyol (**2**) (8.1 mg). Fraction CC10 (6.76 g) was subjected to VLC with solvent system Hex: EtoAc of increasing polarity (3:7 until EtoAc: MeOH 8:2) to give nine subfractions (CC10-1-CC10-9). Using RC, subfraction CC10-3 (150 mg) was purified with solvent system of $CHCl_3$: Acetone (9:1 until 7:3) and luteolin (**3**) (5.8 mg) was obtained. Subfraction CC10-4 (38 mg) gave out 3', 4',7-trihdroxyflavone (**6**) (3.3 mg) with pTLC technique using $CHCl_3$: Acetone (7:3) as the eluent, while CC10-9 (75 mg) yielded luteolin-3', 5-dimethyl ether (**4**) (1.5 mg) and acacetin (**5**) (2.0 mg) by purification using RC with $CHCl_3$: Acetone (9:1 until 5:5) as solvent system.

DPPH Free Radical Scavenging Assay

The antioxidant assay was measured based on the scavenging activity of the stable free radicals, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) based on literature with slight modification [19] and was performed in a 96-well microplate. The stock solution (200µg/ml) and substock solution (concentration ranging from 5-100 g/ml) for each sample were prepared with methanol and was introduced into the respective 96 wells. The reaction mixtures in the 96-well plates consisted of sample (195 µl) and DPPH solution [(5 µL, 0.2 mM) dissolved in methanol. The plate was shaken to ensure thorough mixing of the mixture. The mixtures were then incubated for 30 min in the dark at room temperature. After incubation, the absorbance was read at wavelength of 517 nm against a blank. All determinations were performed in triplicates. The scavenging rates towards DPPH radical were calculated according to equation below;

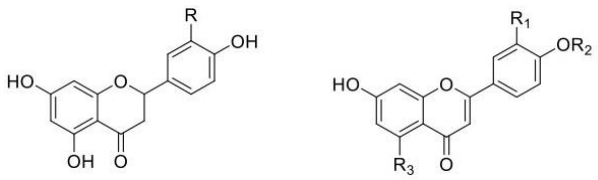
Percentage Inhibition (%) =
[(Absorbance of control - Absorbance of sample)/ Absorbance of control] × 100

IC_{50} values were calculated to determine the 50% inhibition of DPPH, using prism. Trolox was used as standard.

RESULTS AND DISCUSSION

The extraction and isolation of the acetone extract of *C. cauliflora* Linn twigs by a combination of a repetitive radial and preparative thin layer (pTLC) chromatographic techniques have successfully yielded eight pure flavonoid compounds which are; naringenin (**1**) [20], eriodictyol (**2**) [21], luteolin (**3**) [22], luteolin-3', 5-dimethyl ether (**4**) [23], acacetin (**5**) [24], 3',4', 7-trihydroxyflavone (**6**) [25], 4',7-dihydroxyflavone (**7**) [26], and apigenin (**8**) [27]. The structures of these compounds were determined and confirmed on the basis of analysis of ^1H and ^{13}C NMR, UV and IR spectral evidences and comparison with the published data.

Naringenin (**1**). Yellow amorphous powder (6.3 mg), m.p 249-251°C, HRESITOFMS (positive mode) m/z: $[M+H]^+$ 273.0775 (Calc. for $C_{15}H_{12}O_5$). UV (MeOH) λ_{max} nm: 219, 296, 342 and 403. IR (KBr) ν_{max} cm^{-1}: 3384, 2917, 2824, 1631, 1603, 1519, 1495, 1460, 1313, 1251, 1179, 1157, 1082, 1064, 971, 888, 831. ^1H NMR (300 MHz, acetone-d6): δ_H12.17 (1H, s, OH), 7.38 (2H, d, *J*=8.4 Hz, H-2', 6'), 6.88 (2H, d, *J*=8.7 Hz, H-3',5'), 5.95 (2H, s, H-6,8), 5.44 (1H, dd, *J*=3.0, 12.6 Hz, H-2), 3.17 (1H, dd, *J*=17.1, 12.6 Hz, H-3a) and 2.70 (1H, dd, J=3.0, 17.1 Hz, H-3b). ^{13}C NMR (75 MHz, acetone-d6): 198.0 (C-4), 168.1 (C-5,7), 165.2 (C-9), 159.4 (C-4'), 131.5 (C-1'), 129.8 (C-2',6'), 116.9 (C-3',5'), 103.9 (C-10), 97.5 (C-6), 96.6 (C-8), 80.7 (C-2) and 44.3 (C-3a, 3b).



1;R=H
2;R=OH

3;R_1=R_3=OH,R_2=H
4;R1=R3=OCH3,R2=H
5;R1=R2=H,R3=OCH3
6;R1=OH,R2=R3=H
7;R1=R2=R3=H
8;R1=R2=H,R3=OH

Figure 1. Structure of compounds isolated from *C. cauliflora.*

Eriodictyol (**2**). Yellow amorphous powder (8.1 mg), m.p 268-270°C, HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 287.0578 (Calc. for $C_{15}H_{12}O_6$). UV (MeOH) λ_{max} nm: 216, 295 and 342. IR (KBr) ν_{max} cm^{-1}: 3368, 2917, 1635, 1604, 1451, 1347, 1308, 1259, 1161, 1085, 1067, 858, 825. ^{1}H NMR (300 MHz, acetone-d6): δ_H12.17 (1H, s, OH), 7.03 (1H, s, H-2'), 6.87 (2H, s, H-5',6'), 5.95 (2H, d, *J*=2.7 Hz, H-6,8), 5.38 (1H, dd, *J*=3.0, 12.6 Hz, H-2), 3.13 (1H, dd, *J*=12.6, 17.1 Hz, H-3a) and 2.70 (1H, dd, *J*=3.0, 17.1 Hz, H-3b). ^{13}C NMR (75 MHz, acetone-d6): 196.3 (C-4), 166.5 (C-5,7), 163.5 (C-9), 145.5 (C-4'), 145.1 (C-3'), 130.7 (C-1'), 118.4 (C-6'), 115.1 (C-5'), 113.8 (C-2'), 102.3 (C-10), 95.9 (C-6), 94.9 (C-8), 79.1 (C-2), 42.7 (C-3a, 3b).

Luteolin (**3**). Yellow amorphous powder (5.8 mg), m.p 328-330°C, HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 287.0520 (Calc. for $C_{15}H_{10}O_6$). UV (MeOH) λ_{max} nm: 205, 255, 270 and 371. IR (KBr) ν_{max} cm^{-1}: 3390, 2927, 2851, 2357, 1610, 1506, 1451, 1366, 1256, 1167, 1116, 1028, 809. ^{1}H NMR (300 MHz, acetone-d6): δ_H13.0 (1H, s, OH), 7.51 (1H, d, *J*=2.1 Hz, H-2'), 7.46 (1H, dd, *J*=2.1, 8.1 Hz, H-6'), 7.00 (1H, 6, *J*=8.4 Hz, H-5'), 6.59 (1H, s, H-3), 654 (1H, d, *J*=2.1 Hz, H-8) and 6.26 (1H, d, *J*=2.1 Hz, H-6). ^{13}C NMR (75 MHz, acetone-d6): 183.9 (C-4), 166.6 (C-7), 165.9 (C-2), 164.4 (C-5), 160.2 (C-9), 151.1 (C-4'), 147.2 (C-3'), 124.6 (C-1'), 121.0 (C-6'), 117.5 (C-5'), 115.0 (C-2'), 105.9 (C-10), 105.2 (C-3), 100.6 (C-6), 95.7 (C-8).

Luteolin 3', 5-dimethyl ether (**4**). White amorphous powder (1.5 mg), m.p 273-276°C. HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 315.0866 (Calc. for $C_{17}H_{14}O_6$). UV (MeOH) λ_{max} nm: 200, 236, 266, and 354. IR (KBr) ν_{max} cm^{-1}: 3401, 2934, 2373, 1603, 1355, 1289, 1267, 1209, 1179, 1130, 1056, 1032, 834. ^{1}H NMR (300 MHz, methanol-d4): δ_H7.49 (1H, d, *J*=2.1 Hz, H-6'), 7.46 (1H, dd, *J*=2.1, 3.9 Hz, H-2'), 6.92 (1H, d, *J*=8.4 Hz, H-5'), 6.58 (1H, d, *J*=2.4 Hz, H-8), 6.57 (1H, s, H-3), 6.43 (1H, d, *J*=2.1 Hz, H-6), 3.96 (3H, s, OCH3-3') and 3.90 (3H, s, OCH3-5) ^{13}C NMR (75 MHz, methanol-d4): 178.9 (C-4), 163.6 (C-2), 162.3 (C-7), 161.0 (C-9), 159.9 (C-5), 150.2 (C-3'), 148.1 (C-4'), 122.3 (C-1'), 119.9 (C-6'), 115.3 (C-5'), 111.3 (C-10), 108.9 (C-2'), 105.4 (C-3), 96.1 (C-6), 95.0 (C-8), 55.2 (OCH3-3'), 55.0 (OCH3-5).

Acacetin (**5**). White amorphous powder (2.0 mg), m.p 258-260°C, HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 285.0916 (Calc. for $C_{16}H_{12}O_5$). UV (MeOH) λ_{max} nm: 206, 265 and 344. IR (KBr) ν_{max} cm^{-1}: 3400, 2917, 2368, 1635, 1609, 1382, 1354, 1248, 1207, 1177, 1111, 831. ^{1}H NMR (300 MHz, methanol-d4): δ_H7.81 (2H, d, *J*=8.7 Hz, H-2',6'), 6.91 (2H, d, *J*=8.7 Hz, H-3',5'), 6.57 (1H, d, *J*=2.1 Hz, H-8), 6.54 (1H, s, H-3), 6.43 (1H, d, *J*=2.1 Hz, H-6) and 3.90 (3H, s, OCH3). ^{13}C NMR (75 MHz, methanol-d4): 180.3 (C-4), 165.1 (C-2), 163.8 (C-7), 161.9 (C-4'), 162.3 (C-5), 160.6 (C-9), 129.1 (C-2',6'), 123.3 (C-1'), 117.0 (C-3',5'), 108.1 (C-10), 106.6 (C-3), 97.6 (C-6), 96.5 (C-8), 56.5 (OCH3).

3', 4', 7-trihydroxyflavone (**6**). Yellow amorphous powder (3.3 mg), m.p 314-316°C, HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 271.0601 (Calc. for $C_{15}H_{10}O_5$). UV (MeOH) λ_{max} nm: 203, 232, 318 and 358. IR (KBr) ν_{max} cm^{-1}: 3538, 3117, 2362, 1627, 1602, 1569, 1544, 1511, 1458, 1385, 1342, 1322, 1229, 1193, 1174, 1149, 1124, 1048, 982, 943, 860, 806. ^{1}H NMR (300 MHz, methanol-d4): δ_H7.96 (1H, d, *J*=8.7 Hz, H-5), 7.42 (1H, d, *J*=2.4 Hz, H-2'), 7.40 (1H, dd, *J*=2.1, 8.4 Hz, H-6'), 6.96 (1H, d, *J*=1.8 Hz, H-8), 6.91 (1H, dd, *J*=2.4, 8.7 Hz, H-6), 6.90 (1H, d, *J*=3.0 Hz, H-5') and 6.63 (1H, s, H-3). ^{13}C NMR (75 MHz, methanol-d4): 180.3 (C-4), 166.1 (C-2), 164.9 (C-7), 159.7 (C-9), 150.8 (C-4'), 147.1 (C-3'), 127.8 (C-5), 124.0 (C-1'), 120.2 (C-6'), 117.2 (C-10), 116.8 (C-5'), 116.3 (C-6), 114.2 (C-2'), 105.2 (C-3), 103.5 (C-8).

7, 4'-dihydroxyflavone (**7**). Yellow amorphous powder (4.0 mg), m.p 318-320°C, HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 255.0635 (Calc. for $C_{15}H_{10}O_4$). UV (MeOH) λ_{max} nm: 223, 319 and 344. IR (KBr) ν_{max} cm^{-1}: 3400, 3230, 1626, 1604, 1506, 1454, 1384, 1270, 1236, 1220, 1180, 826. ^{1}H NMR (300 MHz, methanol-d4): δ_H7.96 (1H, d, *J*=8.7 Hz, H-5), 7.87 (2H, d, *J*=9.0 Hz, H-2',6'), 6.98 (1H, d, *J*=2.1 Hz, H-8), 6.93 (2H, d, *J*=8.7 Hz, H-3',5'), 6.92 (1H, dd, *J*=2.1, 8.7 Hz, H-6), and 6.69 (1H, s, H-3). ^{13}C NMR (75 MHz, methanol-d4): 179.7 (C-4), 165.8 (C-2), 164.5 (C-7), 162.4 (C-4'), 159.4 (C-9), 129.4 (C-2',6'), 127.8 (C-5), 123.3 (C-1'), 118.0 (C-10), 117.1 (C-6,3',5'), 105.2 (C-3), 103.5 (C-8).

Apigenin (**8**). Yellow amorphous powder (0.5 mg), m.p 348-350°C, HRESITOFMS (positive mode) *m/z*: $[M+H]^+$ 270 (Calc. for $C_{15}H_{10}O_5$). ^{1}H NMR (300 MHz, acetone-d6): δ_H13.01 (1H, s, OH), 7.93 (2H, d, *J*=8.7 Hz, H-2',6'), 7.01 (2H, d, *J*=9.0 Hz, H-3',5'), 6.63 (1H, s, H-3), 6.54 (1H, d, *J*=2.1 Hz, H-8) and 6.25 (1H, d, *J*=2.1 Hz, H-6).

Basically, flavonoids are synthesized by the phenylpropanoid metabolic pathway in which the amino acid phenylalanine is used to produce 4-coumaroylCoA which combined with three molecules of malonyl-CoA, to yield the true backbone of flavonoids, a group of compound called chalcone which consist of two phenolic groups connected by an open three carbon bridge, in the presence of chalcone synthase (CHS). A flavonoid-class containing three rings, the flavanones can be derived from the chalcone structure. Based on these flavanones, all the other flavonoid-classes are generated including isoflavones, flavanols, anthocyanidins, flavonols and flavones [28].

As three molecules of malonyl-CoA and one pcoumaryl-CoA condensed in a reaction catalyzed by

CHS, naringenin chalcone is created. Later, normally, a stereospecific ring closure isomerization step of naringenin chalcone catalyzed by chalcone isomerase (CHI), converts the chalcone into the three-ringed structure of (*S*)-enantiomer flavanone called (2*S*)-naringenin. This is because enzyme-catalyzed reactions are expected to be stereospecific. However, this plant reported the occurrence of (2*R*)-naringenin (**1**). This mean that the enzyme in *C. cauliflora* involved in the biopathway is stereoselective, which favor the formation of (*R*)-isomer over (*S*)-isomer flavonoids. Naringenin (**1**) then undergone oxidative hydroxylation at C-3' of ring B with the presence of flavonoid-3'-hydroxylase (F3'H) which resulted in the formation of eriodictyol (**2**). Based on flavanone structure, all other flavonoid-classes are generated. In the presence of enzyme flavone synthase (FNS), **1** is converted into flavone apigenin (**8**). Luteolin (**3**) can also be formed from **2** using the same enzyme. On the other hand, **8** can also be converted into luteolin (**3**) with F3'H enzyme. The 4'-*O*-methylation of **8** resulted in the formation of acacetin (**5**). Meanwhile 3' and 5-*O*-methylation on **3** yield luteolin-3', 5-dimethyl ether (**4**). The *O*-methylation process takes place in the presence of flavonoid-*O*-methyltransferase enzyme. The pathway of 4', 7-dihydroxyflavone (**7**) can be suggested from the dehydroxylation process of **8** at C-5 while, the same process on **3** produced 3', 4', 7-trihydroxyflavone (**6**) [29]. However, both 5-deoxyflavone compounds could also be produced at the beginning of the pathway as naringenin chalcone undergone the removal of hydroxyl group at C-2' to form isoliquiritigenin intermediate which then cyclized into 4', 7-dihyroxyflavanone (liquiritigenin) with CHI. The flavanone then produces **7** in the presence of FNS. Another 5-deoxyflavone, compound **6** can be formed directly from **7** with F3'H as the catalyst. Although isoliquiritigenin and liquiritigenin are not isolated from this species, the occurrence of these two intermediates within the Fabaceae family made this pathway possible to take place [30].

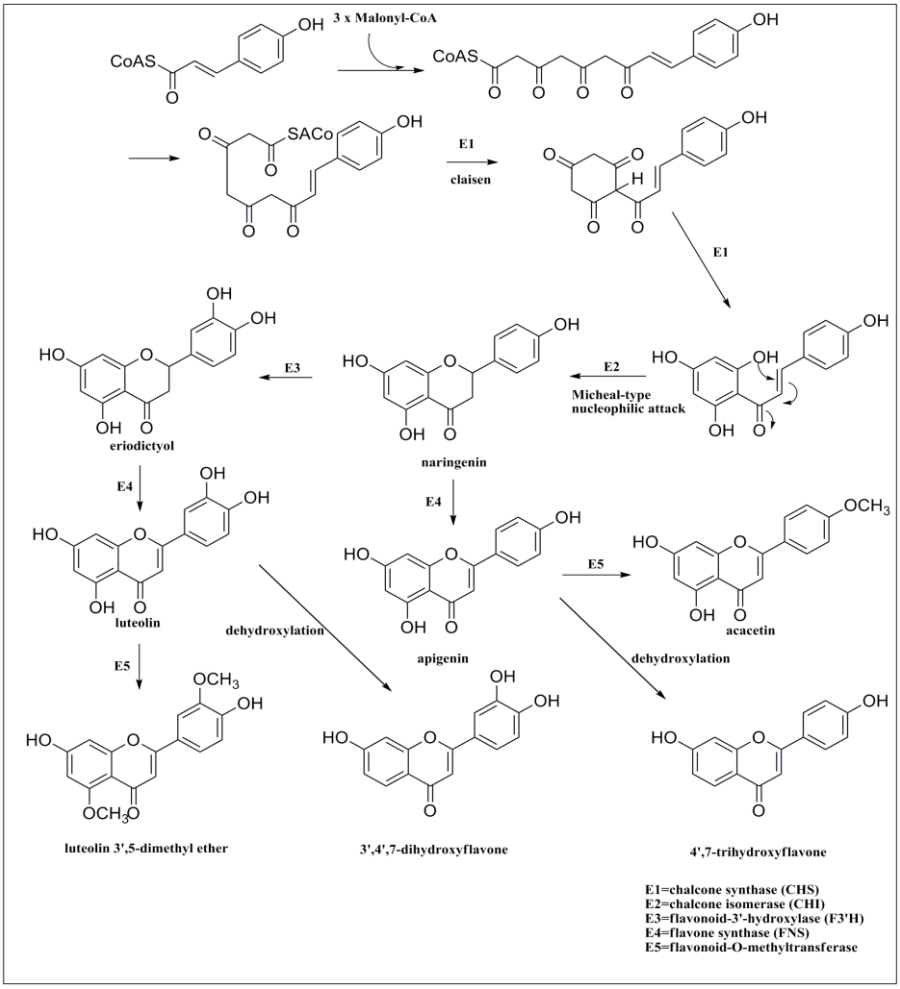


Figure 2. The biosynthesis pathway of flavonoids from *C. cauliflora.*

Table 1. %Inhibition and IC_{50} of compounds against DPPH Free Radical Scavenging Activity.

Sample	DPPH Scavenging	
	% Inhibition	IC_{50} ± SD (uM)
1	21.04	Nd
2	95.27	42.01 ± 5.93
3	94.51	31.40 ± 0.39
4	53.99	323.73 ± 3.63
6	93.73	27.5 ± 1.91
7	22.03	Nd
Trolox	96.47	83.22 ± 0.22

Six of the isolated flavonoids (**1**-**4**, **6**-**7**) were tested for their DPPH radical scavenging activity. The results (Table 1) showed that eriodictyol (**2**) exhibited the highest scavenging activity with percentage inhibition of 95.27%, and the lowest was displayed by naringenin (**1**) with 21.04%. In general, the radical-scavenging activity of flavonoids depends on the molecular structure and the substitution pattern of hydroxyl groups. Previous studies on the structure-activity relationship (SAR) of flavonoids have discussed the importance of the number and location of the phenolic OH groups present for the antiradical efficacy [31,32]. According to kinetic studies of aroxyl radical and decomposition reactions, the antioxidant capacity of flavonoid is linked to its three structural groups which are, the *ortho*-dihydroxy (catechol) structure in the B ring, the C2-C3 double bond in conjugation with a C4-oxo function, and the presence of both C3- and C5-hydroxyl groups [33]. These structural features contributed to the increase of the phenoxyl radical stability, which allowed the rapid donation of H atom thus, increase the radical scavenging activity of the parent flavonoid.

Two flavanones, naringenin (**1**) and eriodictyol (**2**), show a large difference in their activity as eriodictyol exhibited a 5-fold increase in its activity with IC_{50} of 42.01 uM. This was due to the effect of an additional hydroxyl group in *ortho*-position of eriodictyol structure that is the position of 3', 4'-OH (catechol) in the B-ring, which is the salient feature of the most potent scavengers that strongly enhances its activity because it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical [34]. Both flavones luteolin (**3**) and 3', 4', 7-trihydroxyflavone (**6**) displayed significant activities, verifying that flavonoids having an unsaturated C2-C3 bond in conjugation with a C4-oxo function as potent antioxidants [33]. This can be further explained as the conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical [32], hence the presence of both elements potentiated the flavonoids radical scavenging capability.

Although **3** have an extra number of hydroxyl groups at ring A as compare to **6**, higher activity was observed in **6** by a 0.9-fold. The presence of C5-OH alone in **3** does seem to contribute to its activity, however, the absence of such group resulted in a better activity of **6**. This might be due to the chelation of the hydroxyl group by the adjacent carbonyl group which strengthen the O-H bond energy, thus make it difficult to donate the H atom. Meanwhile, 4', 7-dihydroxyflavone (**7**) only displayed 24.8% inhibition due to lack number of hydroxyl groups and the absence of the catechol group, which resulted in a 4.25-fold decreased in its activity as compared to **6**. Then, luteolin-3', 5-dimethyl ether (**4**) showed a higher activity compared to **7,** with 54.3% inhibition at the same concentration. The addition of methoxy group at C-3' which is in *ortho*-position to the OH group seems to give a positive contribution to the activity of **4**.

CONCLUSION

The phytochemical investigation on the twig part of *Cynometra cauliflora* resulted in the isolation of 8 flavonoid compounds, namely; naringenin (**1**), eriodictyol (**2**), luteolin (**3**), luteolin-3',5-dimethyl ether (**4**), acacetin (**5**), 3',4',7-trihydroxyflavone (**6**), 4',7-dihydroxyflavone (**7**) and apigenin (**8**). Flavonoids are products from a cinnamoyl-CoA starter unit with chain extension from three molecules of malonyl-CoA that gives a polyketide. This polyketide, with the presence of different enzymes, can be folded in two different ways. Chalcone synthase resulted in Claisen-like reaction that fold the polyketide into a chalcone. Chalcones then act as a precursor and undergone Micheal-type nucleophilic attack of a phenol group to the unsaturated ketone which gave a flavanone structure (**1, 2**), which then can give rise to the structure of the other flavones (**3**-**8**). Eriodictyol (**2**), luteolin (**3**) and 3',4',7-trihydroxyflavone (**6**), showed significant antioxidant activity comparable to the standard trolox (83.22 uM) with IC_{50} of 42.01, 31.40 and 27.50, respectively. Based on this study, the 3', 4'-catechol moeity in the B-ring in flavonoid structure is the most important factors in order to exert flavonoids antioxidant activity.

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