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Research Article

Anticancer constituents from *Canarium patentinervium* Miq.

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Abstract

Antitumor activities of seven compounds isolated from the ethanol leaf and chloroform bark extracts of *Canarium patentinervium* Miq. including scopoletin (1), scoparone (2), (+)-catechin (3), hyperin (4), cynaroside (5), lioxin (6) and vomifoliol (7) were evaluated *in vitro* against the triple negative human breast cancer cell line MDA-468 using the MTT method. Scopoletin was further tested against three other human cancer lines namely HT29 and HCT 116 colorectal and MCF-7 breast due to good activity and higher yield. Scopoletin exhibited potent and significant anticancer activity against HT-29 and MDA-468 cell lines yielding GI_{50} values of 0.17 µg/ml and 0.09 µg/ml

respectively. In comparison, positive control doxorubicin gave GI_{50} values of 0.69 µg/ml and 0.04 µg/ml respectively against HT-29 and MDA-468 cells.

Keywords: *Canarium patentinervium*, coumarin, anticancer, scopoletin, HT-29, MDA-468.

Introduction

The research for anticancer agents from plant sources started in the 1950's, and plant products have proven to be an important source of anticancer drugs (Cragg et al. 1997). This directly results from the biological and chemical diversity of nature, which allows for the discovery of completely new chemical classes of compounds. At present more than 450 different compounds have been isolated from bioactive plants that have shown in vitro and/or in vivo antitumor activity. Virtually every major class of natural chemical compound is represented in the list of active constituents (Farnsworth & Kaas 1981). Of the 121 medications being prescribed for use in cancer treatment, 90 are sourced from plants. It was also determined that approximately 74% of these discoveries were as a result of an investigation into the claims made by folklore tradition (Shishodia & Aggarwal 2004). In our ongoing study on *Canarium patentinervium* Miq (R Mogana et al. 2011a; Mogana & Wiart 2013; R. Mogana et al. 2011b)., the present study was undertaken to screen the isolated compounds from this plant in various human cancer cell lines. This is the first anticancer study that has been reported on the isolated compounds of this species to date.

Materials and methods

Collection and identification of plant materials: The leaves and barks of *Canarium patentinervium* Miq. were collected from one individual tree from Bukit Putih, Selangor, Malaysia ($3^0$5'24'' N $101^0$46'0''E). The plant was identified by Mr. Kamaruddin (Forest Research Institute of Malaysia). A herbarium sample (PID 251210-12) has been deposited in the Forest Research Institute of Malaysia.

Preparation of extracts: The leaves were air dried and grinded into small particles using an industrial grinder. Dried and grinded plant materials (2.8 kg) were soaked in hexane with the ratio of 1 : 3 parts of sample to solvent for 2h in a 60°C water bath then filtered and concentrated with a rotary evaporator (Buchi, R-200 Switzerland). This was repeated 3 times. Thereafter the leaves and barks were left to air dry completely for 3 days before repeating the whole process with chloroform and then ethanol, respectively. The yield for the hexane (LH), chloroform (LC), and ethanol (LE) extract of leaves were 1.25%, 1.11%, and 6.45%, respectively. The yield for the hexane (BH), chloroform (BC) and ethanol (BE) extract of barks were 1.04 %, 0.40 % and 2.61 %. The extracts were kept at – 20 °C till further use.

Isolation and identification: The ethanol extract of the leaves (75.0 g) was partitioned with petroleum ether, chloroform, and water to yield the respective solvent extracts. The chloroform

fraction (4.6 g) was subjected to silica gel chromatography (4 cm × 90 cm, 0.063–0.200 mesh) using chloroform/methanol as gradient elution (the ratio from 100:0 to 8:100) and preparative TLC (2mm thickness) with solvent system chloroform/methanol (ratio of 100:15) yielding compound **1** (45 mg) and band three yielding compound **2** (9 mg) (Figure S1). The water fraction (19.0 g) was further purified by Sephadex LH-20 with mobile phase ethanol yielding 16 fractions which was then recombined to four fractions. Fraction 2 was loaded on Sephadex LH-20 with mobile phase methanol yielding 3 fractions, whereby fraction 2 was compound **3** (11 mg). Fraction 3 of the water fraction was loaded on Sephadex LH-20 with mobile phase methanol yielding compound **4** (4.9 mg) and compound **5** (4.2mg). Crude chloroform extract (6.9 g) was dissolved in dichloromethane: methanol (2:1) and subjected to PTLC with mobile phase ethyl acetate: methanol (10:1) yielding 3 bands. Band 1 and 2 was run on semi-preparative HPLC with mobile phase ethyl acetate: acetonitrile (6:1) which yielded compound **6** (12 mg) at retention time (Rt) = 14.1 min and compound **7** (3.2mg) at Rt = 15.8. The structure of compounds **1**-**7** was elucidated by NMR and MS spectroscopy techniques. Nuclear magnetic resonance spectroscopy was performed on a Varian 500 MHz NMR spectrometer. Catechin exists in nature as (+) and (-) enantiomers. The optical rotation of the compound in methanol was tested using Schmidt + Haensch Polartronic H532 Polarimeter. The experimental rotation was an average of +69.25 (n=4) concluding that this compound is (+)-catechin. Previous published data on (+)-catechin reports an optical rotation of +56.60 (Sharma et al. 2010). Structures are reported in Fig 1: scopoletin (**1**), scoparone (**2**), (+)-catechin (**3**), hyperin (**4**), cynaroside (**5**), vomifoliol (**6**) and lioxin (**7**).

Scopoletin (**1**) (Shaw et al. 2003; Prachayasittikul et al. 2009; Chang et al. 2012): Pale yellow powder; ^{1}H-NMR (400MHz, $CDCl_3$) δ; 3.98 (6-OCH_3, s, 3H), 6.30 (H-3, d, J = 9.5 Hz, 1H), 6.87 (H-5, s, 1H), 6.95 (H-8, s, 1H), 7.63 (H-4, d, J = 9.5 Hz, 1H); ^{13}C-NMR (125MHz, $CDCl_3$) δ; 56.4 (6-OCH3), 103.2 (C-5), 107.4 (C-8), 111.6 (C-3), 113.5 (C-10), 143.3 (C-4), 144.0 (C-6), 149.7 (C-9), 150.2 (C-7), 161.6 (C-2); EI-MS: m/z (relative intensity): 192 (M^+, 100), 177 (70), 164 (28) 149 (59).

Scoparone (**2**) (November & March 2009; Rumzhum et al. 2012) Pale yellow powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ; 3.98 (7-OCH_3, s, 3H), 3.95 (6-OMe,H-3, s, 3H), 6.32 (H-3,d, J = 9.5 Hz, 1H), 6.88 (H-8, s, 1H), 6.87 (H-5, s, 1H), 7.64 (H-4, d, J = 9.6 Hz, 1H); 13C-NMR (125 MHz, CD3Cl) δ; 56.38 (6-OCH3), 56.40 (7-OCH3), 100.05 (C-5), 107.98 (C-10), 111.45 (C-3), 113.59 (C-9), 143.28 (C- 8), 146.37 (C-7), 150.06 (C-4), 152.87 (C-6), 161.41 (C-2); ESI-MS: *m/z* (relative intensity): 206 (M^+, 100), 191 (39.7), 178 (17.9), 163 (28.9),149 (6.7), 135 (17.2), 107 (12.4), 79 (11.3).

(+)-catechin (**3**) (Meulenbeld et al. 1999; Lin et al. 2009): Slightly pale yellow needles; ^{1}H-NMR (500 MHz, CD_3OD) δ; 2.52 (H-4a, dd, J = 16.0, 8.5 Hz, 1H), 2.87 (H-4b, dd, J = 16.0, 1.5 Hz, 1H), 3.98 (H-3, m, 1H), 4.01 (H-2, d, J = 7.8 Hz, 1H), 4.58 (7-OH, d, 1H), 5.87 (H-8, d, J = 2.0 Hz, 1H), 5.94 (H-6, d, J = 2.0 Hz, 1H), 6.74 (H-6', dd, J = 8.1, 1.8 Hz, 1H), 6.78 (H-5', d, J = 8.1 Hz, 1H), 6.85 (H-2', d, J = 1.8 Hz, 1H); ^{13}C-NMR (125 MHz, CD_3OD) δ; 27.12 (C-4), 67.41 (C-3), 81.46 (C-2), 94.08 (C-8), 94.86 (C-6), 99.40 (C-10), 113.84 (C-2'), 114.66 (C-5'), 121.0 (C-6'), 130.82 (C-1'), 144.83 (C-3'), 144.85 (C-4'), 155.52 (C-9), 156.19 (C-5), 156.45 (C-7); EI-MS: m/z (relative intensity): 290 (M^+, 100), 291 (16), 292 (1.2).

Hyperin (**4**) (Lee et al. 2007; Liu 2008): Pale yellow powder: ^{1}H-NMR (500 MHz, CD_3OD), δ; 3.45 (H-5", t, *J* = 9.0 Hz, 1H), 3.56 (H6b", dd, *J* =11.0, 3.0 Hz, 1H), 3.60 (H-3", t, *J* =9.0 Hz, 1H), 3.67 (H-6a", dd, *J* =11.0, 5.8 Hz, 1H), 3.84 (H-2", t, *J* =9.0 Hz 1H), 3.87 (H-4", t, *J* =9.0 Hz, 1H), 6.23 (H-6, d, J = 2.0 Hz, 1H), 6.43 (H-8, d, *J* = 2.0 Hz, 1H), 6.88 (H-5', d, *J* = 8.5 Hz, 1H), 7.86 (H-2', d, *J* = 2.0 Hz, 1H); ^{13}C-NMR (125 MHz, CD3OD), δ; 60.53 (C-6"), 70.2 (C-4"), 74.0 (C-2"), 75.80 (C-3"), 77.88 (C-5"), 93.34 (C-8), 98.55 (C-6), 103.99 (C-1"), 104.17 (C-10), 114.67 (C-2'), 116.36 (C-5'), 121.51 (C-1', C-6'), 134.36 (C-3), 144.43 (C-3'), 148.56 (C-4'), 157.08 (C-9), 157.37 (C-2), 161.63 (C-5), 164.90 (C-7).

Cynaroside (**5**) (Chiruvella et al. 2007; Rzywacz et al. 2009): Light yellow powder: ^{1}H-NMR (500 MHz, CD_3OD, δ) 7.86 (H-2", d, 1H, J = 2.6 Hz), 7.60 (H-6', dd, 1H, J = 2.6, 8.6 Hz,), 6.88 (H-5', d, 1H, J = 8.6 Hz), 6.43 (H-8, d, 1H, J = 2.1 Hz), 6.23 (H-6, d, 1H, J = 2.1 Hz), 5.19 (H-1", d, 1H, *J* = 8.2 Hz), 3.30 (H-4", t, 1H, J = 9.0 Hz), 3.20 (H-2", t, 1H, *J* = 9.0 Hz), 3.67 (H-6"a, dd, 1H, J = 11.0, 3.0 Hz), 3.56 (H-6"b, dd, 1H, J = 11.0, 5.8 Hz), 3.60 (H-3", t, 1H, J = 9.0 Hz) and 3.45 (H-5", t, J = 9.0 Hz, 1H); ^{13}C-NMR (125 MHz, CD3OD),δ: 178.1 (C-4), 164.90 (C-7), 161.63 (C-5), 157.37 (C-2), 157.08 (C-9), 148.56 (C-4'), 144.43 (C-3'), 134.36 (C-3), 121.51 (C-1', C-6'), 116.36 (C-5'), 114.67 (C-2'), 104.17 (C-10), 103.99 (C-1"), 98.55 (C-6), 93.34 (C-8), 77.88 (C-5"), 75.80 (C-3"), 73.77 (C-2"), 69.62 (C-4"), 60.53 (C-6").

Vomifoliol (**6**) (Lu & Foo 1997; S. Hammami , H. Ben Jannet , A. Bergaoui , L. Ciavatta 2004): White solid; ^{1}H-NMR (CD_3OD, 500 MHz) δ; 1.03 (H-11, s, 3H), 1.06 (H-12, s, 3H), 1.26 (H-10, d, 3H), 1.94 (H-13, s, 3H), 2.18 (3a, d, 1H), 2.54 (3b, d, 1H), 4.34 (H-9, m, 1H), 5.81 (H-5, m, 1H), 5.82 (H-8, m, 1H), 5.90 (H-5, m, 1H); ^{13}C-NMR (125 MHz, CD_3OD) δ; 18.15 (C-13), 22.05 (C-12), 22.41 (C-10), 23.06 (C-11), 41.03 (C-2), 67.34 (C-9), 78.34 (C-1), 125.69 (C-5), 128.71 (C-7), 135.49 (C-8), 166.10 (C-6), 199.85 (C-4). EI-MS: m/z (relative intensity): 224.14 (M^+, 100), 225.14 (14.1), 226.15 (1.6).

Lioxin (**7**) (Hansen et al. 2009; Bogdan et al. 2009): Slightly pale yellow needles; 1H-NMR (500 MHz, CD_3OD) δ; 3.80 (10-OCH3, s, 3H), 6.77 (OH, s, 1H), 7.30 (H-5, d, 1H), 7.30, 7.31 (H-2, H-6, m, 2H), 9.57 (H-7, s, 1H); 13C-NMR (125 MHz, CD3OD) δ; 54.8 (10-OCH3), 110.07 (C-2), 115.51 (C-5), 125.64 (C-), 127.72 (C-1), 141.96 (C-), 147.08 (C-4), 191.60 (C-7); ESI-MS: m/z (relative intensity): 151 (M+, 100), 137 (6), 123 (13)

Cell lines and culture media: The cell lines used in the current study were derived from human carcinoma as shown in Table S1. Three human carcinoma cell lines were procured from the Centre for Biomolecular Sciences, School of Pharmacy, University of Nottingham UK Campus. Cell lines include breast carcinoma cell line MDA-468 (estrogen receptor-negative, ER–), breast carcinoma cell line MCF-7 (estrogen receptor-positive, ER+), colorectal carcinoma cell line HCT-116 and colorectal adenocarcinoma cell line HT-29.

Each cell line was routinely maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma- Aldrich, USA) supplemented with 2 mM of L-glutamine (Sigma-Aldrich, USA) and 10% (v/v) of fetal bovine serum (FBS) (Sigma-Aldrich, USA) at 37°C in a humidified 5% (v/v) of CO_2 incubator (Binder, Germany), and subcultured twice weekly to maintain continuous logarithmic

growth.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: The anticancer effects of crude extracts were investigated against human breast and colorectal carcinoma cell lines using MTT assay. Each cell line was seeded in 96- well microtiter plates (Jet Biofil, China) at a density of 5000 cells/well, and allowed to adhere for 24 h before compounds were introduced. All samples were tested against human derived cell lines MDA 468 [(ER-) breast carcinoma]. Samples were prepared as 1 mg/ml stock solutions, dissolved in DMSO, and stored at 4 ^{o}C, protected from light for a maximum period of 4 weeks. Serial dilutions were prepared in medium immediately prior to each assay. At the time of sample addition and following 72 hr exposure, MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) 400 µg/mL (Sigma- Aldrich, USA) was added to each well. Incubation at 37 °C for 4 hr allowed reduction of MTT by viable cells to an insoluble formazan product. Well contents were aspirated and formazan solubilized by addition of DMSO:glycine buffer (pH 10.5) (4:1). Absorbance was read on an Anthos Labtec System plate reader at 550 nm as a measure of cell viability, thus cell growth or extract toxicity was determined. Due to good activity exhibited, scopoletin was also tested against HT-29, MCF-7 and HCT 116 cell lines. Doxorubicin was used as positive control.

Statistical analysis: Concentration–response curves were calculated using Microsoft Excel and data were reported as mean and SD values obtained from three independent experiments performed in triplicates, minimum of three determinations. Graph was plotted with SD and 95% confidence interval.

Results and Discussion

In our previous study the ethanol extract of leaves and chloroform extract of barks abrogated the survival of human cancer lines (R. Mogana et al. 2011). A chromatographic separation of the ethanol extract of leaves yielded scopoletin (1), scoparone (2), (+)-catechin (3), hyperin (4) and cynaroside (5) while the chloroform extract of barks yielded lioxin (6) and vomifoliol (7) (Figure 1). The anticancer activity of the compounds that were tested is shown in Table 1. Isolated compound with the highest yield scopoletin was tested against four cell lines, two human breast cancer cell lines, MCF-7 (ER+) and MDA 468 (ER-), and two colon cancer cell line, HCT 116 and HT-29. Scopoletin exhibited potent growth inhibition of MDA 468 (GI_{50} 0.09±0.25 µg/ml) (Figure and vomifoliol and HT-29 (GI_{50} 0.17±0.05 µg/ml) (Figure 3) with significantly lower GI_{50} values compared to positive control doxorubicin (GI_{50} 0.69±0.06 µg/ml) for the HT-29 cell line. MCF-7 and HCT 116 cells were less sensitive to scopoletin with GI_{50} values >2 µg/ml. Scoparone, (+)-catechin, hyperin, cynaroside, vomifoliol and lioxin were tested against the MDA 468 cell line only and all revealed GI_{50} values >2 µg/ml. Please refer graphical abstract Figure 4.

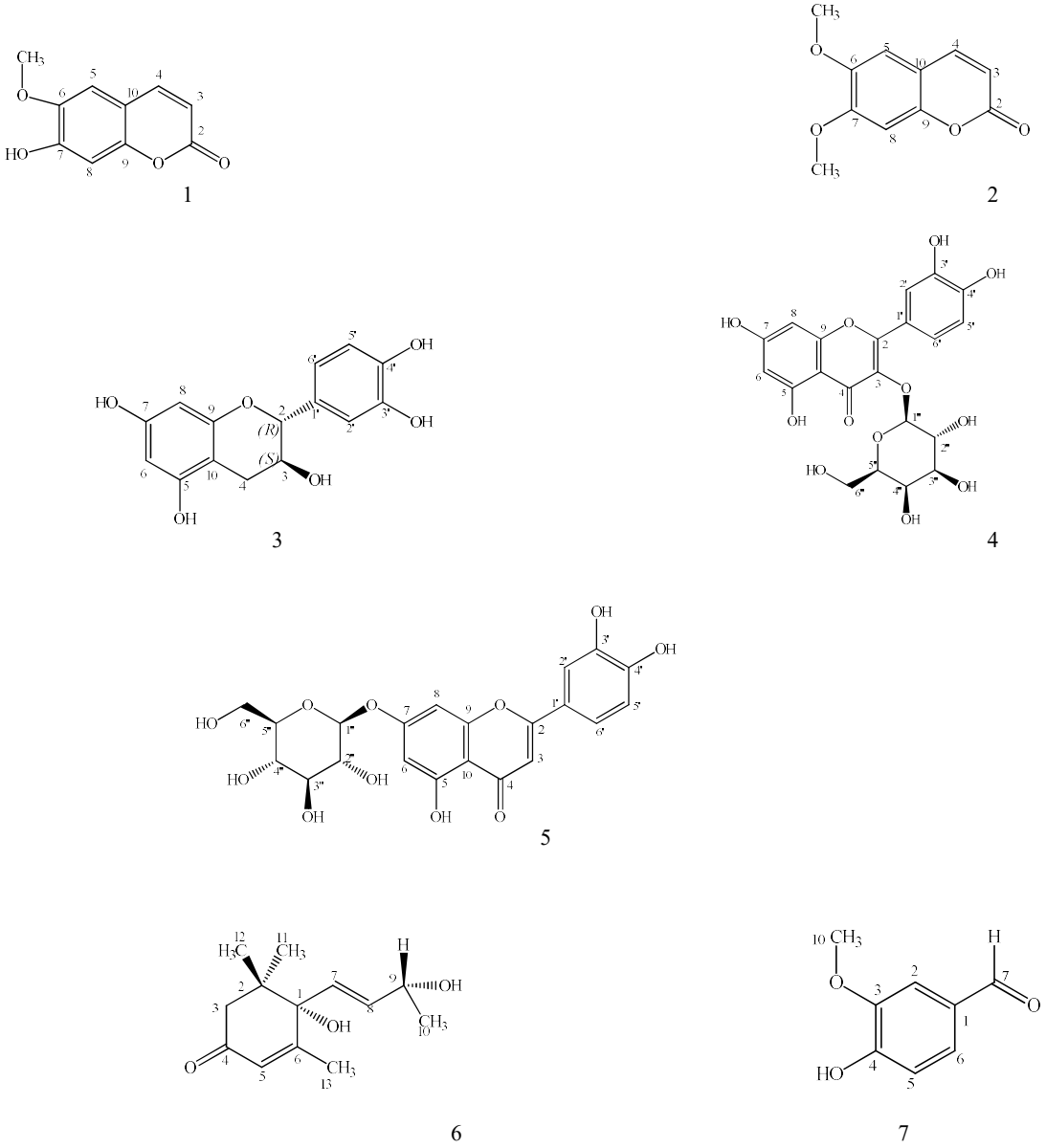

Figure 1. Bioactive compounds isolated from *Canarium patentinervium* Miq.

(scopoletin (1), scoparone (2), (+)-catechin (3), hyperin (4), cynaroside (5), lioxin (6))

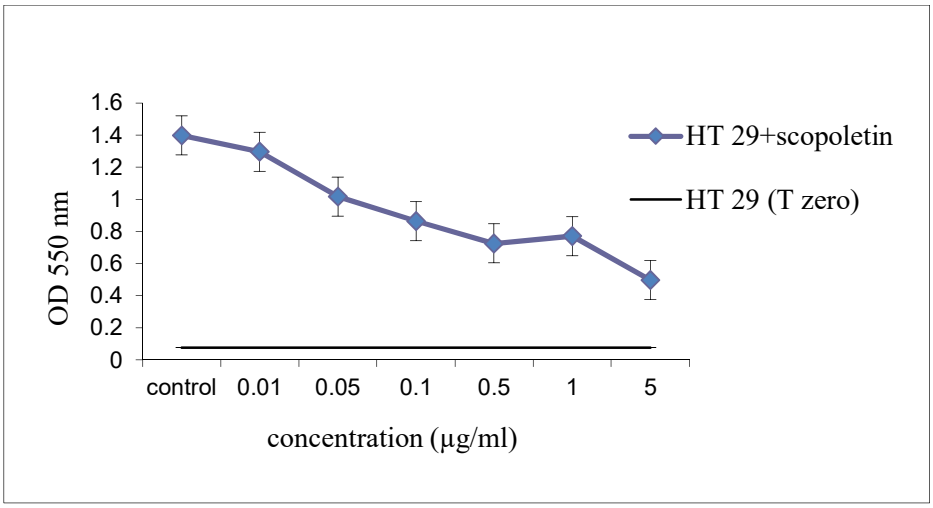

Figure 3: Effect of scopoletin isolated compound from *Canarium patentinervium* Miq. against growth of colon carcinoma cell line HT-29 (GI_{50} = 0.17 µg/ml)

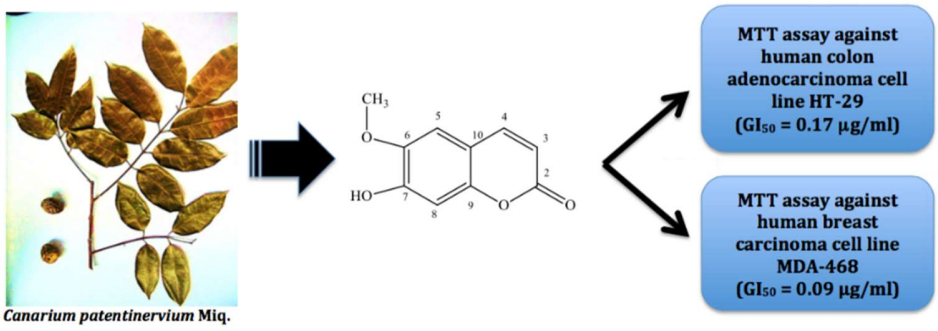

Figure 4: Graphical abstract for the anticancer constituents from *Canarium patentinervium* Miq.

Conclusion

The present study demonstrates that *Canarium patentinervium* Miq. contains scopoletin which has potent anticancer activity *in vitro* against MDA-468 and HT-29 human cancer cell lines. Therefore, the appraisal of scopoletin *in vivo* cancer models and mechanism of growth inhibition is warranted.

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Declaration of conflict of interest

No conflict of interest is associated with this work.

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