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Anti-proliferative Effects of a Coumarin Benjaminin on Four Human Cancer Cell Lines

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ABSTRACT

Cancer is a major health issue around the globe. Chemotherapy and radiation therapy are no longer the standard treatments for patients as these remedies lead to many adverse effects. Thus, the development of alternative effective drugs from lead compounds, especially natural products is necessary. This study aimed to isolate cytotoxic phytochemical constituents from a plant *Calophyllum inophyllum*, which has been used as traditional medicine since ancient time. The root of *C. inophyllum* was extracted and subjected for phytochemical constituent isolation. A coumarin, benjaminin (**1**) was isolated successfully from the chloroform extract and its structural elucidation was performed by spectroscopy analyses of MS, IR and NMR. The anti-proliferative effect of **1** on four cancer cell lines, leukemia (K562), stomach (SNU-1), liver (Hep-G2) and lung (NCI-H23) cancers were evaluated by using colorimetric MTT assay. Benjaminin (**1**) exhibited cytotoxic effects towards all cancer cells and showed the strongest inhibition towards SNU-1 cell proliferation with an IC_{50} value of 70.42 µM. The outcome of this study revealed that **1** is a potential cytotoxic lead compound that could be further developed into anti-cancer drug. Thus, the study on the structure-activity relationship of **1** is highly recommended in due course to improve its anti-proliferative effects on cancer cells.

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INTRODUCTION

Cancer is a growing health problem worldwide and characterized by uncontrollably cell growth. It is a result of mutations that inhibit oncogene and tumor suppressor gene function, which lead to uncontrollable cell growth in our body. The cancer cells do not experience programmatic death, apoptosis, resulting in a mass of abnormal cells. Standard treatments of cancer are surgery, chemotherapy, radiation, hormonal therapy, immune therapy and gene therapy. The treatment of cancer depends on the types and stages of cancer, as well as the physical status of patients. As such, the patients often receive a combination of therapies. The search for novel selective anti-cancer agents has become a priority in pharmaceutical industries. Thus, the discovery of lead compounds for anti-cancer drugs is in urgent need. Natural products are a reliable source of pharmacologically active phytochemical constituents that are potential to be the lead compounds. It has led to our study on the isolation of bioactive constituent from the natural resources, particularly *Calophyllum inophyllum*.

Calophyllum species have been used as folk medicine for rheumatism, haemorrhoids and ulcers. Previous studies revealed that it has potential inflammatory (Tsai et al., 2012), antimicrobial (Alkhamaiseh et al., 2012; Khan et al, 2002), anti-*Helicobacter pylori* (Souza et al., 2009) and cytotoxic (Alkhamaiseh et al., 2011; Mah et al., 2013) properties. These properties are deduced to be contributed by the bioactive secondary metabolites present in their plant extracts, especially coumarins (Ee et al., 2011; McKee et al., 1996). Coumarins isolated from *Calophyllum* species are well-known for numerous pharmacological effects such as anti-HIV (Spino et al., 1998), anti-bacterial (Verotta et al., 2004) and cytotoxicity (Guilet et al., 2001a; Win et al., 2008). In this study, we reported for the first time the isolation of a coumarin, Benjaminin (**1**) from the roots of *C. inophyllum*. The chemical structure of **1** was determined by the analyses of spectroscopic data, including mass spectrometry (MS), infrared (IR) and nuclear magnetic resonance (NMR). The coumarin was further evaluated for its anti-proliferative effects against four cancer cells, which are K562 (chronic myelogenous leukemia), SNU-1 (gastric carcinoma), Hep-G2 (hepatocellular carcinoma) and NCI-H23 (adenocarcinoma) cell lines

Table 1. The ^{1}H- and ^{13}C-NMR data for Benjaminin (1)

Carbon Number	Chemical Shift, δ (ppm)	
	^{1}H-NMR	^{13}C-NMR
1	-	-
2	-	179.3
3	2.74 (2H, *d*)	39.2
4	3.51 (1H, *m*)	32.7
4a		115.2
5	-	165.0
6	-	115.3
7	-	160.2
8	-	109.1
8a		158.6
1′	1.70 (2H, *m*)	33.9
2′	1.11 (2H, *m*)	27.8
3′	1.24 (2H, *m*)	32.0
4′	1.22 (2H, *m*)	22.6
5′	0.82 (3H, *t*)	14.2
1″	3.25 (2H, *m*)	22.7
2″	5.19 (1H, *t*)	122.9
3″	-	131.8
4″	1.67 (3H, *s*)	25.8
5″	1.74 (3H, *s*)	18.0
1‴	-	200.4
2‴	2.53 (1H, *m*)	46.2
3‴	4.13 (1H, *m*)	78.8
4‴	1.49 (3H, *d*, J = 6.87 Hz)	19.7
5‴	1.19 (3H, *d*, J = 6.87 Hz)	10.3
7-OH	12.28 (1H, *s*)	-
5-OCH_3	3.72 (3H, *s*)	62.9

by using MTT assay. This study also represents the first scientific report of the anti-proliferative effect of **1** on cancer cells.

METHODS

General

The infrared spectrum was obtained from FT-IR spectrometer (Perkin-Elmer 100 Series) by using the universal attenuated total reflection (UATR) technique. The mass spectrum was recorded by GC-MS spectrophotometer (Shimadzu GC-MS model QP2010 Plus) using electron ionization (EI) method. The 1D- and 2D-NMR spectra were collected by using NMR spectrophotometer (JEOL FT-NMR 400 MHz). The melting point was recorded by using a melting point device (Electrothermal IA9100).

Plant Material

The roots of *C. inophyllum* was collected in 2012 from Universiti Putra Malaysia, Serdang, Malaysia. The specimen of the plant was identified by Associate Professor Dr. Rusea Go, a botanist from the Department of Biology, Universiti Putra Malaysia and was kept in

the Herbarium, Department of Biology, UPM with a voucher specimen number of RG5016.

Extraction and Isolation

The roots of *C. inophyllum* (1 kg) was air-dried and ground for the extraction process by using maceration method where the solvents were used to soak the plant materials. The powdery plant materials were extracted sequentially by using hexane and chloroform. The solvents were filtered and replaced with fresh hexane after being macerated for three days. The extraction process was repeated for three times. The filtered extracts were dried under vacuum by using rotary evaporator to obtain the dried crude extracts.

The chloroform extract was subjected to purification by using column chromatography with a series of solvent systems. Approximately 20 g of the crude extract was loaded into an opened glass column chromatography with pre-packed silica gel and eluted with hexane, chloroform and methanol in a stepwise gradient system. A total of 43 fractions were obtained and fractions pooling were performed. Fractions 5-11 were pooled and further purified using the same chromatographic technique to give another 13 fractions. Subsequently, fractions 4-8 gave a yellowish oily gum and was further purified to afford yellowish crystals (22 mg). The structural elucidation of this compound was achieved using NMR, GC-MS and FT-IR spectroscopic methods.

Spectral Data

Benjaminin (**1**). Yellow crystals. m.p. 245-246 ºC. EI-MS: *m/z* 432 with molecular formula $C_{25}H_{36}O_6$. ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1.

MTT Assay

The antiproliferative assays were examined by colorimetric MTT assay as described by Mosmann et al (1983). In this study, four cancer cell lines, K562, SNU-1, Hep-G2 and NCI-H23 cells from ATCC were used. Cisplatin was used as a standard drug in this assay. All the cancer cells were cultured in the RPMI medium supplemented with 10% fetal bovine serum. The concentration of the K562 cells used was 2.0 x 10^5 cells/mL while SNU-1, Hep-G2 and NCI-H23 cells were 1.0 x 10^5 cells/mL. Serial dilution of **1** was performed to obtain five different concentrations. For suspension cells (K562 and SNU-1 cells), the concentrations prepared were 200.00, 100.00, 50.00, 25.00, 12.50, 6.25 µg/mL. On the other hand, 100.00, 50.00, 25.00, 12.50, 6.25 and 3.13 µg/mL were the concentrations prepared for anchorage-dependent cells (Hep-G2 and NCI-H23 cells). The assay was conducted in triplicate for both the compound and standard drug.

Briefly, 100 µL of the cancer cells were seeded into the 96 well plate and followed by 100 µL of compound. For the suspension cells, the compound was added immediately after the addition of cells while the compound was added after 24 hours of cell seeding for anchorage-dependent cells. The plate was then incubated for 72 hours in an incubator with 5% CO_2 and temperature of 37 °C. The addition of 20 µL of MTT solution into each well was carried out and the plate was incubated for another 3 hours. Afterwards, 150 µL of supernatant of each well were discarded and replaced with the same amount of DMSO to dissolve the purple formazan. The absorbance of each well was read at 550 nm after to obtain the percentage of cell viability. The IC_{50} values of the compound for each cancer cell were obtained from the graph of percentage of cell viability *vs.* concentration.

Statistical Analysis

All the data were expressed as mean ± standard deviation (SD). The statistical analysis was conducted by using Graph Pad Prism with *t*-test or one-way ANOVA. The level of significance used was $p < 0.05$.

RESULTS AND DISCUSSION

Benjaminin (**1**) was isolated successfully from the chloroform extract of the root of *C. inophyllum*. It is a coumarin with the chemical structure presented in Figure 1. The compound appears as yellow crystals with melting point of 245-246 °C. The mass spectrum of the compound demonstrated a molecular ion peak $[M]^+$ at *m/z* 432 which is consistent with the molecular formula of $C_{25}H_{36}O_6$. The FT-IR spectrum exhibited absorptions of hydroxyl group (OH) at 3296 cm^{-1}, sp^2 and sp^3 C-H stretch at 2970 and 2925 cm^{-1}, conjugated carbonyl (C=O) at 1705 cm^{-1} and aromatic group (C=C aromatic) at 1626 and 1470 cm^{-1}.

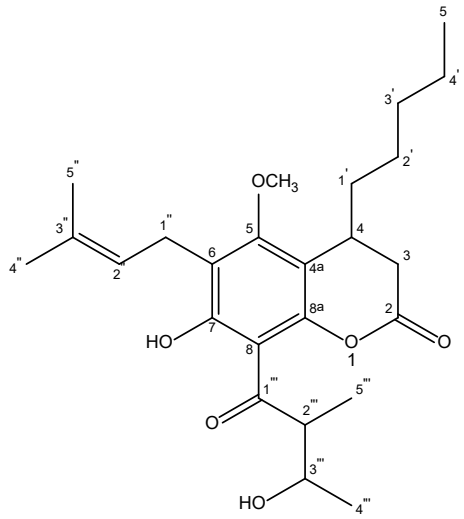

Figure 1. Chemical structure of Benjaminin (1)

The ^{1}H- and ^{13}C-NMR data of **1** were presented in Table 1. The spectrum of ^{1}H NMR implied the presence of a chelated hydroxyl group with a chemical shift found at δ 12.28, which correlates to δ 109.1 (C-8) and 115.3 (C-6) and δ 160.2 (C-7) through ^{2}J and ^{3}J correlations in HMBC spectrum, indicating its attachment to C-7 of the coumarin. Moreover, the chemical shifts of δ 3.25 (H-1″), 5.19 (H-2″), 1.67 (H-4″) and 1.74 (H-5″) demonstrated the presence of a prenyl moiety. The proton signal at δ 3.25 (H-1'') shows coupling with δ 22.7 (C-1'') in HMQC spectrum and long-term correlations with δ 115.3 (C-6), δ 160.2 (C-7) and δ 165.0 (C-5) in HMBC spectrum. It confirms that the prenyl moiety is attached to the coumarin at position C-6. Another substituent group, *n*-pentyl that is attached at C-4 were observed by a series of upfield chemical shifts, including δ 1.70 (H-1′), 1.11 (H-2′), 1.24 (H-3′), 1.22 (H-4′) and 0.82 (H-5′), in addition to HMBC correlations of H-4 and H-3 with δ 33.9 (C-1') and δ 27.8 (C-2'), respectively. Besides that, the chemical shift of δ 3.72 belongs to the methoxyl group at C-5. Consequently, the side chain of 3-hydroxy-2-methylbutanoyl is attached to the coumarin at position C-8. For the ^{13}C NMR spectrum, the number of peaks obtained are in accordance with the total number of carbons present in the structure of **1**. Both the ^{1}H and ^{13}C NMR spectra are consistent with the previously reported data (Sahimi et al., 2015). Thus, Benjaminin (**1**) is confirmed to be isolated for the first time from the root of *C. inophyllum*.

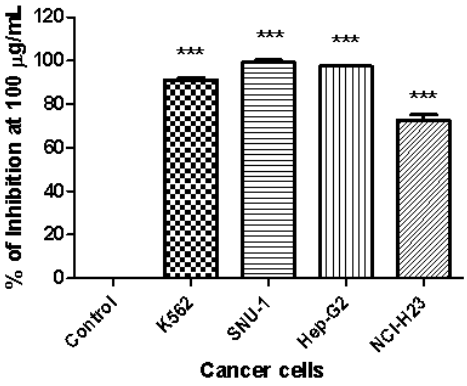

Figure 2. Anti-proliferative effects of Benjaminin (1) at the concentration of 100 µg/mL on K562, SNU-1, Hep-G2 and NCI-H23 cells. The values are expressed as mean ± SD from three separate experiments. *** p < 0.05 by t-tests

Benjaminin (**1**) was evaluated for its anti-proliferative activities against four cancer cell lines by MTT method and reported for the first time in this study. The cell lines used were human erythroleukemia (K562), human gastric cancer (SNU-1), human liver hepatocellular carcinoma (Hep-G2) and human lung cancer (NCI-H23) cell. The anti-proliferative effects of these cancer cells proliferative by **1** at the concentration of 100 µg/mL are presented in Figure 2. This compound showed potential inhibitory effects against all the cancer cell lines with the strongest effect observed for SNU-1 cell line (99.36 ± 0.93 %). It is followed by Hep-G2 and K562 cell lines with 97.42 ± 0.50 % and 91.19 ± 0.82 %, respectively. Lastly, the anti-proliferative effect against NCI-H23 cell line is the weakest with an inhibition percentage of 72.72 ± 2.42 %.

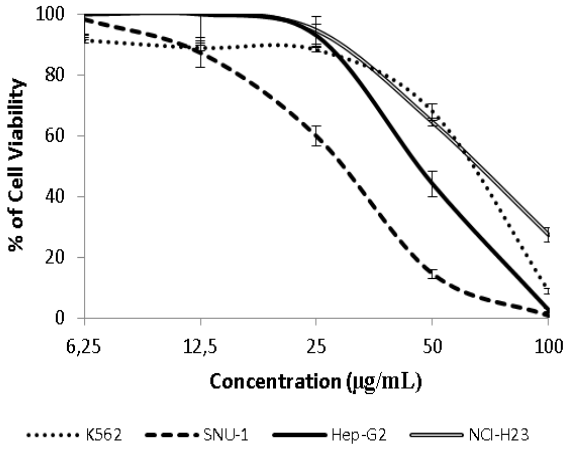

Figure 3. Cytotoxicity of Benjaminin (1) towards K562, SNU-1, Hep-G2 and NCI-H23 cells at different concentrations. Each data point represents the mean ± SD of three independent experiments.

The cytotoxic effects of **1** towards cancer cells are shown in a concentration-dependent manner (See Figure 3). Thus, the IC_{50} values of cytotoxicity of **1** against all the human cancer cell lines were determined from the graph of percentage of cell proliferative inhibition against the concentration and summarized in Table 2. Cisplatin, which was used as a standard drug showed cytotoxic activity with the IC_{50} values ranging from 4 to 10 µM. The lowest IC_{50} value of cytotoxicity for **1** was observed for the stomach cancer cells, SNU-1 which is 70.42 ± 3.56 µM, indicating the strongest inhibition towards SNU-1 cell proliferation. The IC_{50} values of **1** obtained for the liver cancer cells, Hep-G2 cells is 109.65 ± 2.36 µM, which is higher than SNU-1 cells. The anti-proliferative activities of K562 and NCI-H23 cell lines were found to be weaker with the IC_{50} values of 150.72 ± 3.21 and 160.42 ± 6.46 µM, respectively. These data suggest that **1** could be a potential cytotoxic lead compound to be further developed as anticancer drug. The potential cytotoxic properties of coumarins present in *Calophyllum* species were supported by previous studies.

Six coumarin derivatives were previously isolated successfully from *C. dispar* by Guilet et al. (2001a) and these coumarins were claimed to have cytotoxicity against KB cells. The coumarins, Isodispar B, disparpropylinol B and disparinol B showed significant

Table 2. The IC_{50} values of cytotoxicity of Benjaminin (1) towards K562, SNU-1, Hep-G2 and NCI-H23 cells

Cancer Cell Line	IC_{50} (μM)	
	Benjaminin (1)	Cisplatin
K562	150.72 ± 3.21	4.08 ± 0.09
SNU-1	70.42 ± 3.56	9.64 ± 0.59
Hep-G2	109.65 ± 2.36	8.67 ± 1.22
NCI-H23	160.42 ± 6.46	5.32 ± 1.69

The values are expressed as mean ± SD in triplicate.

effects by inhibiting 50% of the cellular growth at the concentration ranging from 4 to 8 μg/mL. In the same year, the authors reported the isolation of another eleven furanocoumarins from the same plant together with their anti-proliferative effects against the same cell line, KB cells. Mammea A/BA cyclo F, mammea A/AA cyclo F, mammea A/AB cyclo F and mammea A/AC cyclo F are the coumarins that exhibited significant antiproliferative effects against KB cells with the ED_{50} values of less than 10 μg/mL (Guilet et al, 2001b). Furthermore, Reyes-Chilpa et al. (2004) reported that these mammea-typed coumarins, which were isolated from *C. brasiliense*, were cytotoxic against three human tumor cell lines, K562, U251, and PC3 cells. Mammea A/BA possessed the strongest activities among the coumarins with the IC_{50} values of less than 0.59 μM for all cell lines (Reyes-Chilpa et al., 2004).

In terms of structure-activity relationship, the cytotoxic effect of **1** is deduced to be contributed by the prenyl moiety attached to position C-6 of the coumarin. This statement is supported by a previous study that examined the inhibitory effects of EBV-EA activation in Raji cells on ten 4-phenylcoumarins isolated from *C. inophyllum*. The authors suggested that Calocoumarin-A, which has a prenyl moiety at C-6, exhibited the most potent inhibitory activity (Itoigawa et al., 2001). This is further confirmed by another study on thirty-two 7-methoxycoumarins isolated from other plants, *Murraya* and *Citrus* species. This investigation study indicated that the coumarin with a prenyl moiety at C-6 showed significant inhibitory effects, as seen in 6,8-di(30-methyl-20-butenyl)-7-methoxycoumarin, which is the most potent compound among the coumarins tested (Ito et al., 1999). As a summary, benjaminin (**1**) is a valuable potential cytotoxic lead compound that can be developed as an anti-cancer drug. Therefore, a future study on the design and synthesis of more potent analogues, particularly for the anti-proliferative effects on stomach cancer cell line, SNU-1 is worthwhile.

CONCLUSIONS

A coumarin, benjaminin (**1**) was successfully isolated from the root of *C. inophyllum*. This compound has anti-proliferative effects on four cancer cell lines, K562, SNU-1, Hep-G2 and NCI-H23 cells with the strongest effect towards SNU-1 cells. This study confirms that coumarins have potent cytotoxic effects as reported previously. However, the level of cytotoxicity might vary due to the presence of different side chains. Therefore, further study on structure-activity relationship on benjaminin derivatives could be performed to improve its cytotoxicity towards cancer cells.

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