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ARTICLE

Biological evaluation of the diterpenes from *Croton macrostachyus*

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Abstract To clarify whether the seeds of *Croton macrostachyus* can induce apoptosis, its methanolic extract was first subjected to a clonogenic survival assay, which measures long-term cytotoxic effects. Since it showed cytotoxicity on HCT116 human colon cancer cell lines, further separation was performed, and two single diterpene compounds were obtained. One of them was identified to be a novel compound, methyl 2-(furan-3-yl)-6a,10b-dimethyl-4-oxo-2,4,4a,5,6,6a,10a,10b-octahydro-1*H*-benzo[*f*]isochromene-7-carboxylate, based on the interpretation of the nuclear magnetic resonance spectroscopic and mass spectrometric data. Its treatment of HCT116 cells with 50 μg/mL triggered the cleavage of both initiator caspase-9 and effector caspase-7. Moreover, the cleavage of poly (ADP-ribose) polymerase, a substrate of caspase-7, increased after 24 h of treatment. These results demonstrate that this compound exerts antitumor activity by triggering caspase-mediated apoptotic cell death in HCT116 human colon cancer cells.

Keywords *Croton macrostachyus* · Seed · Crotomacrine · Clonogenicity · Apoptosis

Introduction

The genus *Croton* belongs to the Euphorbiaceae family, which is distributed throughout the tropical and subtropical areas. It has traditionally been used to treat human diseases including flu, endocrinopathy, convulsion, and it has reported to exhibit antivirus, antibacterial, antiparasitic, anticancer, and anti-inflammatory activities [1–8]. Some *Croton* species are indigenous to the eastern part of central Africa, as well as Asia and South America. Among them, *Croton macrostachyus* is known as bisana in Amharic, broad-leaved croton in English, musogasoga in Luganda, tambuks in Shona, and islami in Tigrigna [9].

The roots of *C. macrostachyus* have been shown to exhibit antimalarial activity against a protozoan parasite, *Plasmodium berghei* [10]. Moreover, its leaves have anticancer, anti-tuberculosis, and antibacterial activities [11–13]. Further, the roots and leaves of *C. macrostachyus* have been reported to exert several biological effects; however, the seeds of *C. macrostachyus* are rarely studied. We evaluated whether the methanolic extract of the seeds of *C. macrostachyus* can induce apoptosis because the ability to induce apoptosis is an important characteristic of chemotherapeutic agents [14]. In the present study, two single compounds were separated from the seeds of *C. macrostachyus*. One of them was identified as crotomacrine, which was previously reported as one of the

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components of *C. macrostachyus* [15]. Another component was determined to be a crotomacrine derivative, which is a novel compound. Here, we report the identification and biological evaluation of the components isolated from *C. macrostachyus*.

Materials and Methods

Croton macrostachyus was collected from the Oromia regional state, Ethiopia, in December 2012, and a voucher herbarium specimen was deposited at Konkuk University, Korea. Its dried seeds (1178 g) were extracted three times with methanol for 3 days. The methanolic extract (135.33 g, 11.48%) was subjected to sequential separation with solvents of different polarity, from *n*-hexane to water. Five fractions were obtained: *n*-hexane (11.48%), chloroform (21.49%), ethyl acetate (0.89%), *n*-butanol (8.32%), and aqueous fraction (28.19%). Whole fractions were freeze-dried and stored at −20 °C for further experiments. Preparative high-performance liquid chromatography (prep-HPLC) was performed to obtain the single compounds. All chemical reagents for separation and purification were purchased from local chemical companies in Korea. Prep-HPLC was conducted using an Agilent 1260 Infinity HPLC system (Agilent Technologies, USA) fitted with a RP-C18 column (Luna C-18 (II), 5 μm, 10.0 × 250 mm; Phenomenex, USA). In addition, analytical HPLC was performed using an Agilent 1100 series HPLC system fitted with a RP-C18 column (Gemini, 5 μm, 4.6 × 250 mm; Phenomenex) at room temperature. A UV/VIS detector (Agilent Technologies) was used at 260 nm. The mobile phase was 65% aqueous acetonitrile containing 0.1% trifluoroacetic acid, and the flow rate was 3.0 mL/min. All nuclear magnetic resonance (NMR) spectroscopic data were collected using a Bruker Avance 400 MHz (9.4T) spectrometer (Bruker, Karlsruhe, Germany). Samples for NMR experiments were dissolved in deuterated dimethylsulfoxide (DMSO-d6) and then transferred to a 2.5-mm NMR tube. The NMR experiments including 1D and 2D NMR were performed as described previously [16]. High-resolution electron impact ionization mass spectrometry (MS) was performed by using a JMS700 spectrometer (JEOL, Tokyo, Japan) at the Korea Basic Science Institute at Daegu, Republic of Korea [17]. Fast atomic bombardment mass spectrometry was performed using a JEOL DX 303 spectrometer (JEOL, Tokyo, Japan) [18].

To confirm whether the components obtained from the seeds of *C. macrostachyus* exhibit anticancer activities, they were subjected to a clonogenic long-term survival assay. HCT116 human colon cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS; Hyclone, USA) at 37 °C in 5% CO_2 atmosphere. HCT116 cells were seeded (5×10^3 cells/well) onto 24-well tissue culture plates (BD Falcon, Flintshire, UK) in DMEM supplemented with 10% FBS. The cells were treated with different concentrations of the fractions (0–100 μg/mL) for 6 days. Thereafter, the cells were fixed with 6% glutaraldehyde and stained with 0.1% crystal violet. The clonogenic survival density was measured using densitometry (Multi-Gauge, Fujifilm, Japan) [19]. All experiments were performed in triplicate. Statistical analysis was carried out using the Student's *t* test with the GraphPad Prism version 7.0 software (GraphPad Software Inc., La Jolla, CA) [20].

Cell vitality was determined by measuring the level of cellular free thiols using VB-48TM, a cell-permeable thiol-reacting probe that stains viable cells in an intensity-dependent manner [21]. HCT116 cells were treated with 50 μg/mL fraction II for 24 h. The cell suspension was mixed with 10 μg/mL PI and 5 μM VB-48TM, and cellular fluorescence was immediately quantified using a NucleoCounter NC-3000 image cytometer (Chemometec Inc., Allerod, Denmark), according to the manufacturer's instructions.

Immunoblot analysis was performed as described previously [19]. Briefly, HCT116 cells were treated with different concentrations of fraction II for 24 or 48 h, and was then harvested and lysed in a buffer consisting of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.2), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride. For immunoblot analysis, the protein extracts (20 μg each) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The blots were incubated with antibodies against cleaved caspase-9, cleaved caspase-7, and poly (ADP-ribose) polymerase (PARP) (all from Cell Signaling Technology, Danver, MA, USA). Signals were analysed using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA) [19].

Results and Discussion

There are many methods for measuring anticancer activities. One of them is by evaluating the cytotoxicity against cancer cell lines using a clonogenic assay, which measures the long-term cytotoxicity of anticancer agents [19]. To identify the components in *C. macrostachyus* that exert anticancer effects, their activity on the clonogenicity of HCT116 human colon cancer cell lines was evaluated. Among the five fractions obtained by sequential separation

based on polarity, the chloroform fraction showed the best cytotoxicity. Further separation was performed using prep-HPLC [Supplementary Data Figure S1]. Two fractions I and II collected at retention times of 14.0 and 17.8 min, respectively, were determined to be single compounds based on the interpretation of the data measured by a photodiode array detector [Supplementary Data Figures S2A and S2B]. To confirm whether they exert cytotoxicity, a clonogenic assay was carried out. As shown in Fig. 1, both fractions I and II showed cytotoxicity. Based on their survival densities measured using densitometry (Multi-Gauge, Fujifilm, Japan), the half-maximal cell growth inhibitory concentration (GI_{50}) values were calculated, which were 23.5 and 53.0 μg/ml, respectively.

Since a higher amount of fraction II (30 mg) was obtained compared to that of fraction I (20 mg), its structure was elucidated first. The NMR spectroscopic data of fraction II were collected. Twenty-one carbon signals were observed in the ^{13}C NMR spectrum [Supplementary Data Figure S3]. Two ^{13}C peaks at 166.7 and 171.6 ppm showed the existence of two carboxyl groups. The carbon peak at 166.7 ppm showed long-range coupling to the proton peak at 6.70 ppm in the heteronuclear multiple bonded connectivities (HMBC) spectrum [Supplementary Data Figure S4]. This proton was correlated with two protons at 6.22 and 6.31 ppm in the correlated spectra (COSY) [Supplementary Data Figure S5]. One of them, the ^{1}H peak at 6.22 ppm showed two long-range couplings with the ^{13}C peaks at 139.5 and 52.8 ppm in the HMBC spectrum. The latter was attached directly to the ^{1}H peak at 2.25 ppm in the heteronuclear multiple quantum coherence (HMQC) spectrum [Supplementary Data Figure S6]. This proton was long-range coupled to the ^{13}C peak at 139.5 ppm, which showed long-range coupling with the ^{1}H peak at 6.22 ppm as mentioned above. Among the three protons that correlated with each other in COSY spectra, the ^{1}H peak at 6.70 ppm was long-range coupled to the ^{13}C peak at 37.3 ppm. As a result, a partial structure with a six-membered ring was determined, which contains a carboxyl group as mentioned above. Four protons belonging to this partial structure were neighboured based on the coupling

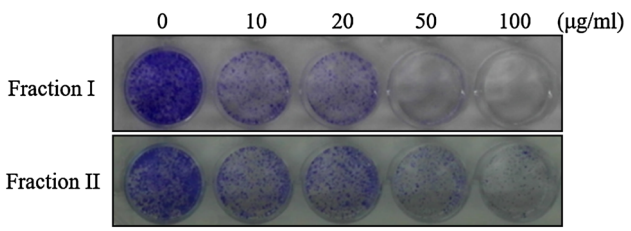

Fig. 1 Effect of the fractions on the clonogenicity of HCT116 colon cancer cells. HCT116 cells (5×10^3) were cultured in the absence or presence of the fractions. After 6 days of treatment, colonies were stained with 0.1% crystal violet

constants determined by the ^{1}H NMR spectrum [Supplementary Data Figure S7]. The ^{1}H peak at 2.25 ppm showed long-range coupling with a methyl carbon at 14.8 ppm, which was attached directly to the ^{1}H peak at 1.10 ppm in the HMQC spectra. Since this proton was long-range coupled to the carbon at 37.3 ppm, this methyl group should be attached to one of the carbons of the six-membered ring. The carbon peak at 166.7 ppm showed another long-range coupling with a methyl proton at 3.65 ppm, indicating that this methyl group was attached to the carboxyl group. Two protons at 1.35 and 2.42 ppm were attached to the methylene carbon at 33.1 ppm, which were long-range coupled to the methyl carbon at 14.8 ppm. Therefore, this carbon should be attached to the six-membered ring. Since the carbon signal at 35.9 ppm showed long-range couplings with the protons at 6.31 and 2.25 ppm, it was also attached to the six-membered ring. A methyl carbon at 15.3 ppm was long-range coupled to the proton at 2.25 ppm, and its proton peak at 1.03 ppm was long-range coupled to the carbon at 52.8 ppm. As a result, this methyl group was found adjacent to the carbon at 35.9 ppm. Two protons at 1.75 and 2.35 ppm attached to the methylene carbon at 41.5 ppm showed long-range couplings with the methyl group at 35.9 ppm and the methine carbon at 52.8 ppm; thus, this methylene group should be attached to the carbon at 35.9 ppm. Since these protons correlated with the proton at 5.58 ppm attached to the carbon at 71.3 ppm, they were found to be adjacent to each other. As a result, the methine carbon at 71.3 ppm should be attached to the methylene carbon at 41.5 ppm. Since the methylene protons at 1.56 and 1.90 ppm attached directly to the carbon at 18.1 ppm showed long-range couplings with two carbons at 37.3 and 35.9 ppm, this methylene group was attached to another methylene group at 33.1 ppm. The methine proton peak at 2.49 ppm attached directly to the carbon at 49.1 ppm correlated with the protons at 1.35, 1.56, 1.90, and 2.42 ppm, which belong to two methylene groups in COSY spectrum; thus, it was determined to be attached to the methylene group at 18.1 ppm. As a result, another six-membered ring was determined as a partial structure. The proton peak at 2.49 ppm was long-range coupled to the carbonyl carbon at 171.6 ppm. Five carbons adjacent to each other were found at 171.6, 49.1, 35.9, 41.5, and 71.3 ppm. Based on the existence of a carbonyl group and the chemical shift of 71.3 ppm, a third six-membered ring formed by five carbons was determined. The carbon peak at 125.7 ppm was long-range coupled to the carbon at 41.5 ppm; thus, it was found to be adjacent to the carbon at 71.3 ppm. Since the two carbons at 140.3 and 109.2 ppm showed long-range couplings with the proton at 5.58 ppm, they should be attached to the carbon at 125.7 ppm. The proton peak at 7.66 ppm showed long-range coupling to the carbon at

125.7 ppm. Three protons at 6.63, 7.66, and 7.75 ppm correlated with each other; thus, the corresponding carbons at 109.2, 143.9, and 140.3 ppm were neighbored. In addition, the carbon at 125.7 ppm was adjacent to these carbons. As a result, four carbons were determined to be contained in a furan based on their chemical shifts. The final structure obtained from the NMR data can be determined as shown in Fig. 2A.

The important connectivities obtained from the HMBC and COSY interpretation are shown in Supplementary Data Figure S8. To confirm the structure determined by NMR spectroscopy, the molecular mass and formula were determined using high-resolution electron impact ionisation mass spectrometry (HR/MS) [Supplementary Data Figure S9], which were 356.1620 and $C_{21}H_{24}O_5$, respectively. Since the calculated mass was 356.1624, the structure was correct. The name of the fraction II was methyl 2-(furan-3-yl)-6a,10b-dimethyl-4-oxo-2,4,4a,5,6,6a,10a, 10b-octahydro-1*H*-benzo[*f*]isochromene-7-carboxylate. The complete assignments of the ^{1}H and ^{13}C NMR data are listed in Supplementary Data Table S1.

Similarly, the NMR data of fraction I were obtained. Its ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC data are shown in Supplementary Data Figures S10-S14. The assignments of the ^{1}H and ^{13}C NMR data obtained from the interpretation of the 1D and 2D NMR data are listed in Supplementary Data Table S2. Comparing this data with the data of fraction II, all NMR data except those regarding three positions were similar to each other. Unlike fraction II, fraction I contains a hydroxyl group at the C-5 position and a double bond between C-4a and C-5 [Fig. 2B]. As a result, the molecular mass of fraction I was expected to be 370.1416, and the mass determined by fast atomic bombardment mass spectrometry (FAB/MS) was 371.0 (M + H) [Supplementary Data Figure S15]. Its molecular formula was $C_{21}H_{22}O_6$. As a result, the name of fraction I was methyl 2-(furan-3-yl)-5-hydroxy-6a,10b-dimethyl-4-oxo-2,4,6,6a,10a,10b-hexahydro-1*H*-benzo[*f*]isochromene-7-carboxylate. This result was consistent with previous findings [22]. While fraction I has been reported as crotomacrine, fraction II has not been discovered. Therefore, of the two compounds isolated from *C. macrostachyus*, one is a known compound and the other is a novel compound. There are many properties to predict the bioavailability. The logP values calculated using the ChemDraw program of fraction I and II were 2.83 and 1.12, respectively. Their calculated molar refractivities were 97.7 and 100.1, respectively. These values satisfy Lipinski's rule which is used to evaluate druglikeness.

Since fraction II was a novel compound, further biological experiments were conducted. To address whether fraction II induces cytotoxicity, cell viability was determined by measuring the intracellular levels of reduced thiols, a key event of early apoptosis [21, 23, 24], by using a thiol-reactive probe VitaBright-48TM (VB-48TM). Since propidium iodide (PI) is not permeant to live cells, it was used as a counterstain to differentiate non-viable cells. As shown in Fig. 3, the majority of the control cells (~96%) exhibited a high VB-48 fluorescence intensity, indicating a healthy status. However, in the presence of fraction II, the percentage of VB-48-stained cells gradually decreased in a time-dependent manner (from 96 to 87% at 24 h and to 47% at 48 h), accompanied by increases in the PI-stained subpopulations. These data suggest that fraction II exerted cytotoxicity in HCT116 cells. Caspases are cysteine proteases that play a pivotal role in mediating apoptosis. To determine whether fraction II activates caspases, HCT116 cells were treated with fraction II for various durations. As

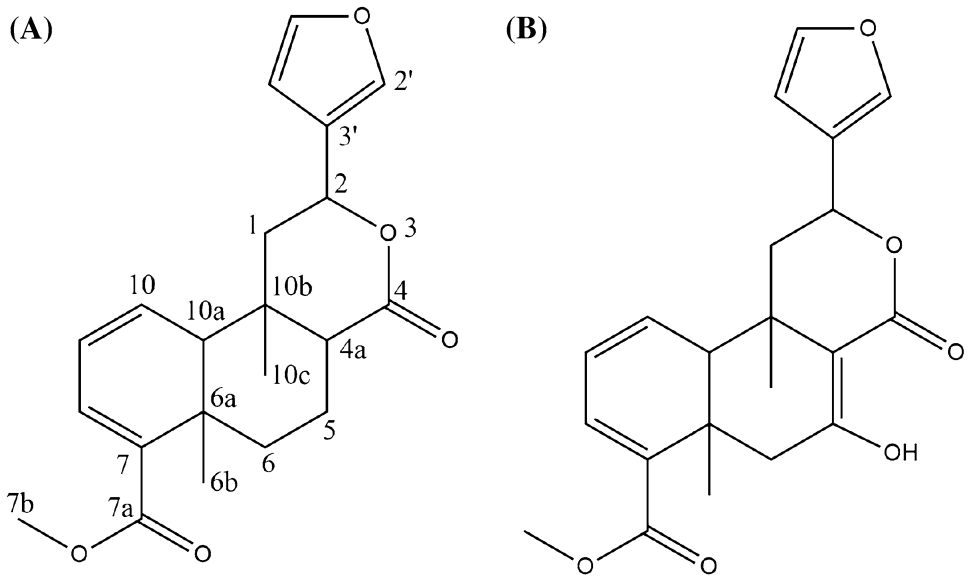

Fig. 2 The structures and numbering of (**A**) fraction II and (**B**) fraction I

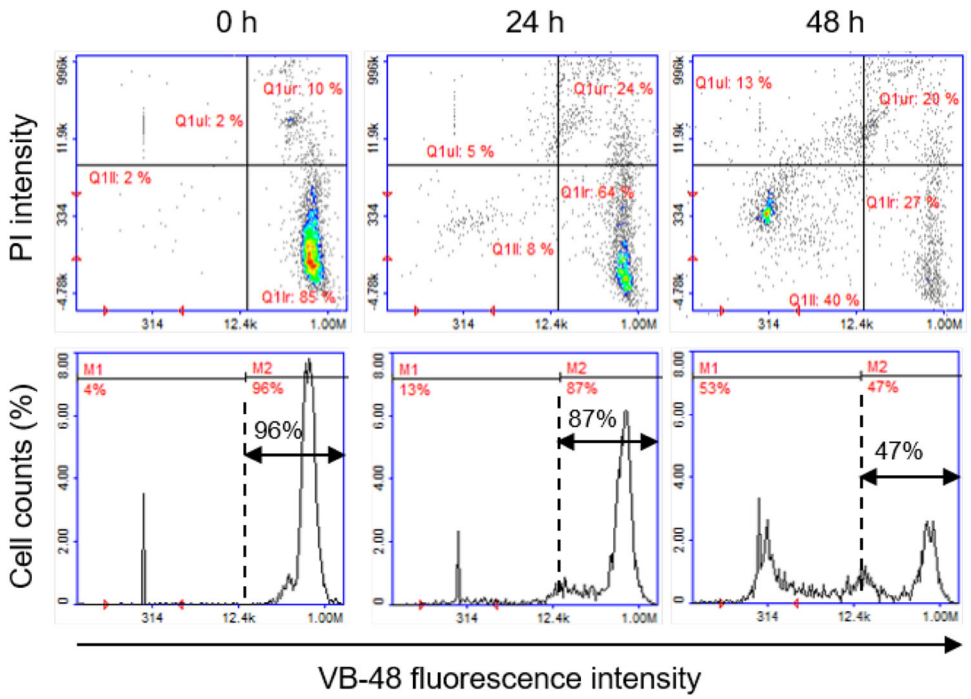

Fig. 3 Effect of fraction II on the depletion of the thiol groups. HCT116 cells were treated with 50 μg/mL fraction II for various durations (0, 24, and 48 h). Cells were stained with VB-48 and PI, and the fluorescence intensities were analysed using a NucleoCounter NC-3000. Scatter plots (*top panels*) show VB-48 versus PI intensity. Histograms (*bottom panels*) show the cell population (%) versus VB-48 intensity

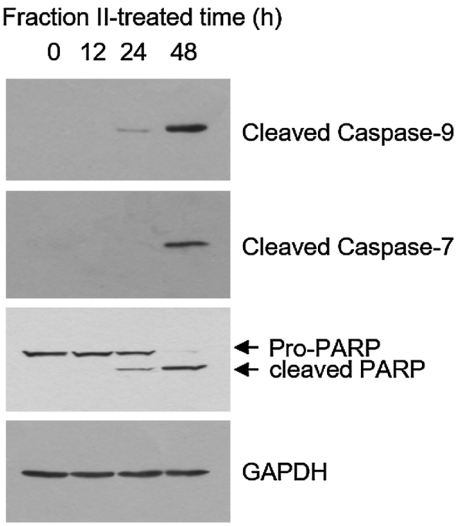

Fig. 4 Effect of fraction II on the activation of the caspase cascade. HCT116 cells were treated with 50 μg/mL fraction II for various durations (0, 24, and 48 h). Whole-cell lysates were prepared and subjected to immunoblotting using antibodies against cleaved caspase-9, cleaved caspase-7, and PARP. GAPDH was used as an internal control to show equal loading of the protein. *Each blot* represents at least three individual experiments

shown in Fig. 4, the treatment of HCT116 cells with 50 μg/mL fraction II triggered the cleavage of both initiator caspase-9 and effector caspase-7. Moreover, the cleavage of poly (ADP-ribose) polymerase (PARP), a substrate of caspase-7 [25], increased after 24 h of treatment. These results demonstrate that fraction II exerts antitumor activity by triggering caspase-mediated apoptotic cell death in HCT116 human colon cancer cells.

Medicinal plants have long been used to treat human diseases, such as *Euphorbiaceae*, which are a large family of flowering plants including herbs and also some shrubs and trees in the tropics [26]. *C. macrostachyus*, which belongs to the *Euphorbiaceae* family, has been used for various conditions: its leaves were used for malaria, bloating, constipation [27], and wound; its bark for tapeworm infection, syphilis, asthma [28], and liver disease/jaundice [29]; and its fruits and decoction of it roots have been used for venereal disease [30]. Moreover, whole plant extract was investigated for antibacterial activity [13] and anti-mycobacterial activity [31]. In particular, its seeds have been used to induce abortion [30] and for tapeworm infection [32]. Several compounds have been isolated from various parts of *C. macrostachyus* including the leaves, roots, barks, fruits, seeds, or whole plants. From the stem barks and twigs, compounds including fatty acids, beta-sitosterol, stigmasterol, lupeol, betulin, and one cyclohexane diepoxide named crotepoxide have been isolated. Other compounds such as clerodane diterpenes namely trachyloban-19-oic acid, trachyloban-18-oic acid, neoclerodan-5,10-en-19,6b;20,12-diolide, 3a,19-dihydroxytrachyloban, 3a,18,19-trihydroxytrachyloban, and the triterpene 3b-acetoxytaraxer-14-en-28-oic acid were separated from its roots. In addition to lupeol, betulin, and crotepoxide, other diterpenes namely crotomacrine, floridolide A, hardwickiic acid, and 12-oxo-hardwickic acid were obtained from the

fruits and stem barks of *C. macrostachyus* [1]. Of the two compounds isolated in the current study, fraction I was identified to be crotomacrine, which has previously been established as a component from the fruits of *C. macrostachyus* [22]; however, fraction II has not been reported yet. Its structure was determined using NMR spectroscopy and mass spectrometry, and it showed antitumor activity by triggering caspase-mediated apoptotic cell death in HCT116 human colon cancer cells.

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