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Promising Compounds From *Murraya exotica* for Cancer Metastasis Chemoprevention

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Abstract

Most of the present anticancer drugs are highly cytotoxic and focus mainly on killing tumor cells rather than slowing the progress of cancer metastasis. Evidence has been reported that bridges the mechanisms of inflammation and tumor invasion. Therefore, we evaluated the potency in cancer metastasis chemoprevention of compounds and a coumarin extracted from *Murraya exotica*, which is known for its anti-inflammation bioactivity. By carrying out experiments in vitro, we found the root extracts more efficient than the leaf extracts in restraining cell migration of MDA-MB-231 cells, while leaf extracts presented slightly stronger inhibition of tumor cell adhesion at low concentrations. In addition, compared to root extracts, a novel coumarin identified previously from root extracts showed equal inhibition on cancer cell adhesion and less inhibition on cell migration. All extracts used in this study presented low cytotoxicity in vitro. Through comparison of the contents of leaf and root extracts from *M exotica*, several compounds are considered promising against cancer metastasis. This study evaluates the worth of further development of *M exotica* to find its effect on cancer metastasis chemoprevention.

Keywords

Murraya exotica, coumarin, cancer metastasis chemoprevention, cell migration, cell adhesion

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Introduction

Malignant tumors are becoming the most deadly common disease in the world. Due to lack of valid therapeutic strategies, countless lives have been lost in the past 5 decades. Even in the early stages of cancer, although the primary tumor can be excised by resection, it is nearly inevitable that malignant tumor cells will spread into adjacent tissues or metastasize to distal organs through the vessel system.

As reported, cell adhesion and migration might play significant roles in the process of cancer metastasis,[1] as well as in inflammation[2] and pregnancy.[3] Narod et al demonstrated that oral contraceptive use may reduce the risk of ovarian cancer.[4] The $\alpha_5\beta_1$ integrin was found to contribute to stimulating cellular migration,[5] while this integrin is upregulated in the invading trophoblast[6] and is present in metastatic melanoma cells.[7] Rhim et al[8] treated mice with the anti-inflammation drug dexamethasone, causing a radical suppression of cancer dissemination. The inflammatory protein S100A8 activates NF-κB signaling in macrophages and endothelial cells, resulting in acceleration of lung metastasis formation.[9] All those mechanisms common to nidation, inflammation, and cancer metastasis may have potential connections that could bridge across treatment strategies. Therefore, we provide a new perspective to seek agents for cancer metastasis chemoprevention from materials known for their anti-inflammation and antifertility potency.

Murraya exotica is known as an ornamental and hedge plant[10] for its pleasant smell and beauty as well as a source of herbal therapy for treating stomachalgia, toothache, and body pains from injury or trauma.[11] In southern part of China, cough, fever, and some infectious wounds,[12,13] such as furuncle and carbuncle, can also be treated by *M exotica*.

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Likewise, according to the National Traditional Chinese Medicines Compilation, *M exotica* contributes to antifertility and anti-inflammation uses in local hospitals in Fujian Province. The leaf of *M exotica* is reportedly rich in coumarins,[14-17] which exert antioxidant,[18,19] anticoagulant,[20] antimycobacterial, antitumor,[21-23] antifungal,[24] antiviral, and anti-inflammatory activities.[12,13] The root of *M exotica* is considered rich in coumarins and alkaloids such as paniculidines.[25] Our previous work showed the extract from the root of *M exotica* is more efficient against migration of colon cancer cells than the extracts from other parts of the plant.[26] In the same article, a new coumarin from the root part of *M exotica* was isolated and identified, yet the pharmaceutical activity remains untouched. Therefore, this study was designed to identify the location of potential valuable materials against cancer metastasis from *M exotica* via comparison among extracts from leaf, root, and the novel coumarin.

Lung metastasis is one of the most common outcomes of breast cancer according to medical publications. Given the simulation of the in vivo microenvironment where intravasation or extravasation most frequently occurs, all in vitro experiments were based on the use of MDA-MB-231 cells and human pulmonary microvascular endothelial cells (HPMECs).

Materials and Methods

Materials

MDA-MB-231 cells were purchased from ATCC (Manassas, VA) and cultured in Leibovitz's L-15 Medium (L-15, Gibco, Carlsbad, CA), including 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) as supplement at 37°C with 100% air. HPMECs were purchased from FuDan IBS Cell Center (Shanghai, China) and cultured in endothelial cell medium (ECM, Sciencell, San Diego, CA) at 37°C with 5% CO_2. ECM consists of basal medium, 5% of FBS (Cat. No. 0025, Sciencell), 5% of endothelial cell growth supplement (ECGS, Cat. No. 1052, Sciencell), and 5% penicillin/streptomycin solution (P/S, Cat. No. 0503, Sciencell).

The plants material was collected from Zhangzhou, Fujian Province, China, by Zhou Jiang and identified to be *Murraya exotica* by Prof Lee Jia. The voucher specimen was deposited at the Laboratory of Cancer Metastasis Alert and Prevention Center, Fuzhou University. Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). The filtration membrane (0.22 µm) was purchased from Xinjinghua Co (Shanghai, China).

Extraction and Elucidation

The root and leaf part of *M exotica* was pulverized and dried. Then same weight (15 g) of the powdered parts were refluxed with 80% ethanol (HCl, pH 3) and the solution filtered. The solution was rotary evaporated to remove a large part of the ethanol and subsequently re-filled with water and filtered again. Then, the filtrates were added with 1 mol/L NH_3H_2O to adjust the pH value to 9. Chloroform and methylene chloride were used to, respectively, extract root extract and leaf extract. The organic solvent was removed by rotary evaporation to acquire the extract in dried powder form.

Liquid chromatography-mass spectrometry (LC-MS) analysis was performed to analyze the ingredients of the root extract with an Acquity UPLC system (Waters, Milford, MA) in the positive electrospray mode. An Acquity UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 µm) was purchased from Waters Xevo TQD (Waters). The extract was separated with isocratic elution using H_2O-acetonitrile (4:1, v/v) with 0.1% formic acid as the mobile phase. The flow rate was 200 µL/min. Detection: PDA 200 to 600 nm. The ion scan was in full scan mode across the 50 to 2000 *m*/*z* range. All scan events were acquired with a 200-ms maximum ionization time. The ion source–dependent parameters were optimized as follows: electrospray voltage, 4.0 kV; capillary temperature, 350°C; sheath gas flow rate, 35 units; auxiliary gas flow rate, 5 units; tube lens, 60 V.

A coumarin of *M exotica*, 7-methoxy-8-(5-(prop-1-en-2-yloxy)penta-1,3-dien-1-yl)-coumarin (CM1), was selected through the LC-MS analysis and then isolated, using an high-performance liquid chromatography (HPLC) system, from the ethyl acetate extract of the root of *M exotica*. The semipreparative HPLC procedure was carried out using Agela Venusil XBP C18 (10 mm × 250 mm, 5 µm) column. The sample concentration was 5 µg/mL for root extracts. The elution program was as follows: isocratic elution with mixture of acetonitrile and water (1:4), flow rate 1.25 mL/min, detection wavelength 350 nm. The corresponding HPLC peak was collected and followed by rotary evaporation and lyophilization. The final product was obtained as a white amorphous powder.

To verify the newly isolated component as CM1, we used a reverse-phase HPLC system to compare CM1 with the root extract. The reverse-phase HPLC system consisted of a model set of dual pumps, a model SIL-20AT autosampler, a model CTO-10A thermostat, and a model SPD-20AV UV detector (Shimadzu, Tokyo, Japan). The extract was separated on a GraceSmart RP C18 analytical column (150 mm × 2.1 mm, 5 µm) at 200 µL/min. Unless otherwise specified, the injection volume was 20 µL and the detection wavelength was set at 350 nm, and the column temperature was set at 35°C. ^1H nuclear magnetic resonance (NMR) spectrum of the CM1 obtained from the HPLC system was recorded on Varian Unity+ 500 at 500 MHz, with *d*6-DMSO (dimethyl sulfoxide) as solvent and tetramethyl silane as the internal standard.

For subsequent experiments, all plant extracts were dissolved in DMSO to reach a homogenous solution.

Bioactivity Tests of Plant Extracts

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to test the cytotoxicity of CM1, leaf extract, and root extract on breast cancer cell line MDA-MB-231 cells. MDA-MB-231 cells were plated on a 96-well plate with about 5×10^3 cells in each well for overnight incubation. Then the plant extract was added to each well to reach a final concentration of 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL in the 200 μL medium for a 24-hour treatment. Wells filled with full L-15 medium and 0.1% DMSO were set as the blank control group. Each group had 5 replications for each concentration. At the indicated time point, the supernatant was replaced with the MTT solution, consisting of 10% MTT mother solution (50 mg/mL) and 90% L-15 medium without phenol red. Then, the MTT solution was replaced for 100 μL DMSO in each well after 4-hour treatment. The plate was then gently shaken for 15 minutes and the OD value at 572 nm was determined with a microplate autoreader (L311sx, Bio-Tek Instruments, Inc, Winooski, VT). Percent viability of cells exposed to treatments was calculated as follows:

$$\text{Survival rate (\%)} = (A - A_0)/A_0 \times 100\%$$

where A is the OD value of the extract (coumarin) group and A_0 is the OD value of the blank control group.

A scratch assay was used to test the migration of MDA-MB-231 cells under the treatment of CM1, leaf extract, and root extract. MDA-MB-231 cells were seeded on a 12-well plate and cultured to a confluent cell monolayer. Two or 3 parallel scratches per well were made by tips of a 2.5 μL pipette. Supernatant was then replaced, followed by a 48-hour incubation, with L-15 basic medium containing 1% FBS and plant extract with concentrations of 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL. The well filled with L-15 basic medium with 1% FBS and 0.1% DMSO was set as the control group. To evaluate the migration of breast cancer cells, the area of the wounds were measured at indicated time points by Adobe Photoshop CS6. Percent migration of cells exposed to plant extract was calculated as follows:

$$\text{Wound healing } (\%) = B / B_0 \times 100\%$$

where B is the decrement value of the wound area of the coumarins group and B_0 is the decrement value of the wound area of the blank control group.

Static adhesion test was carried out to evaluate the cell-cell adhesion between HPMECs and MDA-MB-231 cells under the treatment of CM1, leaf extract, and root extract. HPMECs were first plated on the bottom of wells to reach a confluent cell monolayer. MDA-MB-231 cells were digested with trypsin to reach a homogenous cell suspension, followed by 20-minute treatment with rhodamine B. After being washed by phosphate-buffered saline (PBS) 3 times, MDA-MB-231 cell suspension was added to wells to ensure the ratio between cell counts of MDA-MB-231 and HPMEC was 1 to 2. All 3 types of plant extract were applied to the wells with concentrations of 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL. The control group was supplemented with the plant extract solvent (DMSO) at a final concentration less than 0.1%. Each group had at least 3 replicates. After 1-hour incubation with plant extract, cells were rinsed by PBS 3 times and then sent for photography under a 10× lens of an inverted fluorescence microscope. Adhesion density was evaluated by measuring the area of fluorescent dots in the photograph via Adobe Photoshop CS6.

$$\text{Adhesion rate (\%)} = C/C_0 \times 100\%$$

where C is the area of the fluorescent dots of the experimental group and C_0 is the area of the fluorescent dots of the control group.

All data were analyzed using SPSS software and expressed as the mean ± SD. Statistical comparisons between different groups were performed using 2-way ANOVA *t* test.

Results

Composition of the Extractions

Leaf and root extract compounds of *M exotica* were acquired according to our previous protocols.[26] UPLC-TQ-MS and UPLC-PDA methods were developed to rapidly identify the compounds (see Figure 1). The mass spectral signals of leaf and root extracts of *M exotica* are listed in Table 1. In the chromatogram of leaf extract, 7 characteristic peaks were identified separately at 3.05 minutes, 3.57 minutes, 4.57 minutes, 6.83 minutes, 7.87 minutes, 8.98 minutes, 11.34 minutes. In the chromatogram of the root extract, 5 characteristic peaks were identified separately at 1.89 minutes, 3.05 minutes, 3.57 minutes, 9.08 minutes, and 11.42 minutes. According to our previous work,[26] the peak at 1.89 minutes was named CM1 and isolated from the root extract through an Agela Venusil XBP C18 (10 mm × 250 mm, 5 μm) column. Furthermore, HPLC method was performed to verify the component extracted from the root part of *M exotica*. And the isolated product (CM1, retention time at 5.0 minutes) showed satisfying purity (Figure 2). Then, the content of CM1 in the extraction of root was determined to be 16% by HPLC method.

CM1 was then elucidated by ^{1}H NMR and MS assay and data were acquired as follows: ^{1}H NMR (500 MHz, d6-DMSO): δ 1.81 (s, 3H); δ 2.55 (s,1H); δ 4.59 (dd, 1H); δ 4.64 (d, 1H); δ 4.73 (d, 1H); δ 4.86 (s, 1H); δ 4.95 (s, 1H); δ 5.11 (m, 2H). MS (*m*/*z*): $[M + H]^+$ calculated for CM1, 299.2; elimination of isopropenyl residue, 259.1; continuous

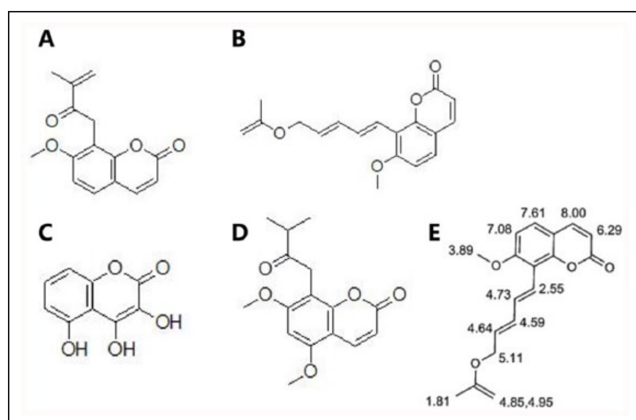

Figure 1. Structure of identified compounds. (A) Structure of murrayone (7-methoxy-8-(3-methyl-2-oxobut-3-enyl)chromen-2-one) (retention time at 7.87 minutes in leaf extracts). (B) Structure of 7-methoxy-8-(2′-methyl-2′-formylpropyl)-coumarin (retention time at 4.57 minutes in leaf extracts). (C) Structure of trihydroxy coumarin (retention time at 8.98 minutes in leaf extracts). (D) Structure of 5,7-dimethoxy-8-(3-methyl-2-keto-butyl) coumarin (retention time at 9.08 minutes in root extracts). (E) The elucidated chemical structure 7-methoxy-8-(5-(prop-1-en-2-yloxy)penta-1,3-dien-1-yl)-coumarin (CM1, retention time at 1.89 minutes in root extracts), labeling with the assignment of H atoms.

elimination of CH_2OH residue at *m*/*z* 231.1. The structure of coumarin was identified as 7-methoxy-8-(5-(prop-1-en-2-yloxy)penta-1,3-dien-1-yl)-coumarin (Figure 1E).[26] Structures of the distinguished compounds in leaf or root extracts were elucidated in our previous work and are illustrated in the subsequent figure (Figure 1A-D).

Evaluation on Cancer Metastasis In Vitro

MTT assay was performed to evaluate the cytotoxicity of extracts of *M exotica* on breast cancer cell line MDA-MB-231. Compared to the control group, during 24-hour incubation, only with 100 μg/mL root extract the survival rate of cells showed a statistically significant decline to 65%. In addition, the survival rate of cells under the treatment of 100 μg/mL leaf extract and 100 μg/mL CM1 presented significant differences compared to the root group (Figure 3A). The IC_{50} value of the CM1 group, leaf group, and root group are all larger than 100 μg/mL.

Migration of MDA-MB-231 exposed to leaf extract, root extract, and CM1 was also tested via scratch assay (Figure 3B and D). The area of wounds was used to evaluate the migration capacity of cells. According to the results, except for the leaf group and CM1 group at 1 μg/mL, all experimental groups showed significant differences compared to the control group at all concentrations. In the leaf group, the wound healing rate is 76%, 66%, and 58%, respectively, at 10 μg/mL, 50 μg/mL, and 100 μg/mL. In the CM1 group, the wound healing rate is 88%, 63%, and 50%, respectively, at 10 μg/mL, 50 μg/mL, and 100 μg/mL. In the root group, the wound healing rate is 68%, 61%, 47%, and 33%, respectively, at 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL. In addition, wound healing rate of leaf group and CM1 group showed no significant difference between them while both presented significant difference compared to the root group at all concentrations. All wound healing value of control group was set as 100%.

The static adhesion test was carried out to evaluate the cell-cell adhesion capacity between MDA-MB-231s and HPMECs under the treatment of plant extracts (Figure 3C and E). The concentrations of leaf extract, root extract, and CM1 were all set to 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL. All data of the experimental groups presented significant differences compared to the control group. According to the results, the adhesion rate of the root group at 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL declined, respectively, to 83%, 70%, 53%, and 35%. The adhesion rate of the leaf group at 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL declined, respectively, to 64%, 52%, 46%, and 44%. The adhesion rate of the CM1 group at 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL declined, respectively, to 67%, 58%, 50%, and 42%. In addition, there is no significant difference in comparison of adhesion rate at 50 μg/mL and 100 μg/mL among the 3 experimental groups. However, there is a significant difference in the adhesion rate at 1 μg/mL and 10 μg/mL between the leaf group and the root group.

Discussion

This study preliminarily explored the potency of CM1 on cancer metastasis chemoprevention. We found that CM1 had strong inhibition of cell migration and adhesion exhibited in in vitro experiments. Based on our previous publication, this study further implies strong potential of antimetastatic efficacy of the root extract and leaf extract from *M exotica* with low cytotoxicity. Based on the bioactivity experiments and previous elucidation of the root and leaf extract compounds, more explorations shall be made using 5,7-dimethoxy-8-(3-methyl-2-keto-butyl) coumarin and other identified compounds.

MTT assay demonstrated similar and low cytotoxicity of CM1, root extract, and leaf extract. Only with 100 μg/mL treatment did the cell survival rate show a significant difference between CM1 and the other 2 extract mixtures. Another study found that leaf extract of *M exotica* decreased chondrocyte apoptosis in a dose-dependent manner.[27] Although the plant extracts used in this study did not protect MDA-MB-231 cells from apoptosis, they did not cause much damage to the breast cancer cells. Studies have been conducted to demonstrate efficient cytotoxicity against tumor cells by coumarins from *M exotica.* However, structure-activity relationship

Table 1. The Mass Signals and Assignment of the Root Extract of *Murraya exotica*.

t_R Root (Minutes)	t_R Leaf (Minutes)	*m*/*z*	Structure
1.89		231.1, 259.1, 299.2, 553.4, 575.4	Undetermined
3.05	3.05	163.1, 185.1	Hydroxy coumarin
3.57	3.57	231.1, 259.1, 299.2, 553.4, 575.4	Undetermined
	4.57	261.1, 301.2, 557.4, 579.5	7-Methoxy-8-(2′-methyl-2′-formylpropyl)-coumarin
	6.83	231.1, 299.1, 553.4, 575.4	Unreported
	7.87	259.1, 281.1	Murrayone
	8.98	163.1, 195.1, 217.1	Trihydroxy coumarin
9.08		163.1, 217.1, 291.2, 331.3	5,7-Dimethoxy-8-(3-methyl-2-keto-butyl) coumarin
11.42	11.34	231.1, 259.1, 327.6	Murpanicin

Abbreviation: t_R, retention time.

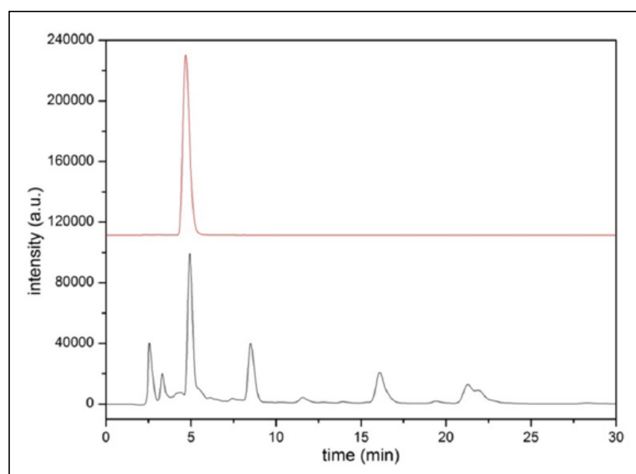

Figure 2. HPLC result of CM1. CM1 at retention time of 5 minutes is purified by a semipreparative HPLC procedure. Purification is further confirmed by UV detection.

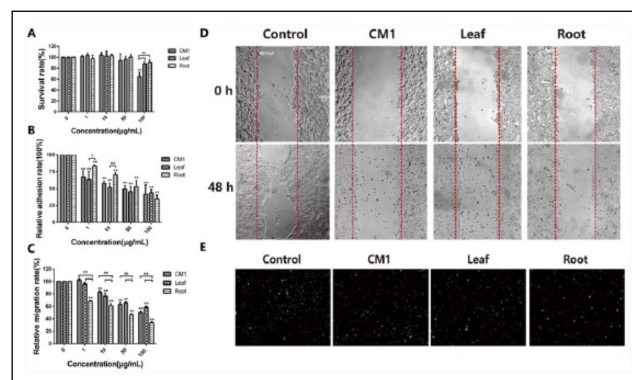

Figure 3. Bioactivity of CM1, root extracts, and leaf extracts. (A) MTT test results of MDA-MB-231 cells under the treatment of CM1, leaf extract, and root extract. (B) Static adhesion test between MDA-MB-231 cells and HPMEC cells under the treatment of CM1, leaf extract, and root extract. (C) Wound healing assay results of MDA-MB-231 cells under the treatment of CM1, leaf extract, and root extract. (D) Sample photographs of CM1 group, leaf group, and root group at 50 μg/mL in comparison with control group in the scratch assay. (E) Sample photographs of CM1 group, leaf group, and root group at 50 μg/mL in comparison with control group in the adhesion assay. Single asterisk stands for significant difference with $P < .05$. Double asterisks stand for significant difference with $P < .01$.

studies indicated that 2 adjacent phenolic hydroxyl groups at the C-6 and C-7 positions in the coumarin skeleton were necessary for the antiproliferative and antioxidant effect,[28] while none of the compounds in Table 1 showed similar structure.

Another study has claimed that ethanol extract of *M exotica* inhibits IL-1β, which can induce VCAM-1 expression in cancer cells to enhance adhesion ability.[29] It might be consistent with our study since the adhesion test demonstrated a restraining effect on the breast cancer cell adhesion rate by CM1, leaf extract, and root extract of *M exotica*. Furthermore, at a low concentration (1 μg/mL, 10 μg/mL) range, the adhesion rate was decreased to a slightly lower level by the leaf extract than by the root extract, with a significant difference. Since murrayone, trihydroxy coumarin, and 7-methoxy-8-(2′-methyl-2′-formylpropyl)-coumarin were identified as the components of leaf extract differing from the root extract, their efficiency on blockage of cell adhesion requires more attention. Liu and colleagues found murrayone could significantly inhibit the platelet aggregation induced by ADP, which may be through regulating on integrin $\alpha_{IIb}\beta_3$ modulated the interaction and adhesion between carcinoma cells and platelets.[30] Our adhesion test result is intended to demonstrate effects on adhesion between tumor cells and endothelial cells. Although integrin $\alpha_{IIb}\beta_3$ is negative on the surface of endothelial cells, it was discovered on the plasma membrane of tumor cell lines.[31]

In the scratch assay, root extract exerts significantly more inhibition on cell migration than CM1 and leaf extract at all

concentration points. According to Table 1, the main compounds that the root extract has distinctive from leaf extract are CM1 and 5,7-dimethoxy-8-(3-methyl-2-keto-butyl) coumarin. Besides CM1, the latter has no relevant information regarding to its biological activity in any publications hitherto. However, like murrayone, they both have a dimethoxy at C7 of the skeleton as well as a keto and a methyl at the C2 and C3 positions of the substituent group, respectively. Regarding cell migration, integrins were also seen as pseudopodia in the membrane flow model.[32] And murrayone was reported to inhibit integrin $\alpha_{IIb}\beta_3$ involved in platelet aggregation. The degree of P-selectin was markedly reduced in tissue sections from rats receiving the treatment of a coumarin derivative, which has an ethoxycarbonyl-methoxy subsituent group at C7 of the coumarin skeleton.[33] Therefore, our study may imply restraining effects on integrins relevant to cancer cell migration by 5,7-dimethoxy-8-(3-methyl-2-keto-butyl) coumarin, which shares a similar region of structure with murrayone.

There are only a few relevant bioactivity studies about *M exotica* or similarly structured coumarins mentioned in this article. Even though this study implied promising compounds on cancer metastasis chemoprevention, further mechanism studies should be carried out to validate those hypotheses based on bioactivity experiments. In addition, isolation of certain compounds ought to be performed to determine the real potency via comparison with the general mixture. Given the efficiency of blocking tumor cell adhesion exhibited by CM1 in this study, proteome study needs to be carried out to search for promising targets of CM1. Furthermore, most studies about *M exotica* focused on its aerial part and most studies about coumarins pay attention on their cytotoxicity against tumor cells. Yet none of them tend to explore the potency against cancer metastasis based on the antifertility and anti-inflammation effect of *M exotica*. Here we demonstrated that in comparison with leaf extracts, root extracts of *M exotica* may be more efficient in cancer metastasis chemoprevention rather than killing cancer cells.

Conclusion

In summary, according to the results, root extract exerts the most promising effect on cancer metastasis chemoprevention. In the root extract mixture, 7-methoxy-8-(5-(prop-1-en-2-yloxy)-penta-1,3-dien-1-yl)-coumarin and 5,7-dimethoxy-8-(3-methyl-2-keto-butyl) coumarin (CM1), respectively, presented strong and distinguishing inhibition on tumor cell adhesion and migration. This finding may allow insight into the efficiency against cancer metastasis of the compounds in the root part of *Murraya exotica*.

Authors' Note

Authors Su-Dan He and Xing-Tian Yang contributed equally to this work.

Declaration of Conflicting Interests

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