ความเป็นพิษต่อเซลล์มะเร็งผิวหนังชนิดแพร่กระจายของรากมะหาด

Cytotoxic activity against B16F10 metastatic melanoma cells of *Artocarpus lakoocha* root

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บทคัดย่อ

มะหาดเป็นพืชสมุนไพรไทยที่แพร่หลายด้านสุขภาพ จัดเป็นเครื่องสำอางสมุนไพรสำหรับบำรุงผิวและทำให้ผิวขาว ผลทดสอบฤทธิ์เบื้องต้นต่อการต้านเซลล์มะเร็งผิวหนังชนิดแพร่กระจาย B16F10 พบว่าสารสกัดหยาบส่วนเปลือกรากมีฤทธิ์ดีกว่าสารสกัดหยาบส่วนแก่นราก 8 เท่า เมื่อนำสารสกัดหยาบชั้นเอทิลอะซิเตทที่มีฤทธิ์ดีกว่ามาศึกษาองค์ประกอบทางเคมี และวิเคราะห์ข้อมูลทางสเปกโทรสโกปี พบสารหลัก 2 ชนิด คือ artolakoochol (**1**) และ oxyresveratrol (**2**) เมื่อนำสาร **1** และ **2** ไปทดสอบฤทธิ์ยับยั้งเซลล์มะเร็งผิวหนังชนิด B16F10 melanoma พบว่าสาร **1** และ **2** มีค่า IC_{50} 5.96±0.068 และ 41.48±5.14 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ รายงานนี้เป็นการรายงานครั้งแรกของฤทธิ์ยับยั้งเซลล์มะเร็งผิวหนังชนิดแพร่กระจาย B16F10 melanoma ของสารสกัดจากรากและสารหลักที่พบในส่วนราก เป็นการเพิ่มมูลค่าให้กับเครื่องสำอางสมุนไพรของมะหาดอีกด้วย

ABSTRACT

Artocarpus lakoocha is a popular Thai medicinal plant for health benefit, in particular the herbal cosmetic ability for nourishing and whitening skin. Preliminary *in vitro* cytotoxic screening against the B16F10 metastatic melanoma cells showed that the root bark extract of this plant species was 8-fold more potent than that of the heart root. Further phytochemical investigation of the more active EtOAc soluble fraction led to isolate and identify the two major types of compounds, artolakoochol (**1**) and oxyresveratrol (**2**), based on their spectroscopic data analyses. The isolates **1** and **2** exhibited inhibitory property on B16F10 cells with respective IC_{50} values of 5.96±0.068 and 41.48±5.14 μg/mL. This is the first report on inhibitory effect against B16F10 metastatic melanoma cells of the root extracts and their major constituents provides additional valuable information towards the herbal property of *A. lakoocha*.

คำสำคัญ: มะหาด อาร์โทลากูชอล ออกซีเรสเวอราทรอล ความเป็นพิษต่อเซลล์ เซลล์มะเร็งผิวหนังชนิดแพร่กระจาย B16F10

Keywords: *Artocarpus lakoocha*, Artolakoochol, Oxyresveratrol, Cytotoxic activity, B16F10 metastatic melanoma cells

INTRODUCTION

Cancer, the second leading cause of death after cardiovascular disease, are predicted by the World Health Organization (WHO) that about 23.6 million cases worldwide in 2030 and 68% of which to be occurring in developing countries (Eid et al., 2015). Melanoma cells, one of three most common skin cancers, develop in melanocytes (pigment cells). Although melanoma cells are almost the last order of cancer, in contrast, the rates of patients with skin cancer could further increase every year. In 2015, a total of 12,960 patients in Australia were reported and the amount is expected to enhance to 17,570 by 2020 (Santhanam et al., 2016). UV radiation is the main risk factor for this cancer. Currently, many processes for the treatment of skin cancer have been used such as surgical removal, radiation therapy, chemotherapy, or cryosurgery. Even, chemotherapy can still be effective treating for various cancers, including lung and melanoma cancers but its side effects are unwanted. Consequently, the traditional medicines from plants are considered as a rich source of potential anti-skin cancer agents.

Artocarpus lakoocha (Moraceae family), known in Thai as Mahaad, is indigenous in the regions of South and South-East Asia, including India, Sri Lanka, Laos, Malaysia, Myanmar, Thailand and Vietnam (Charoenlarp et al. 1981). Its heartwood has been used as anthelmintic agent to treat tapeworm infection in Thai folk medicine. In earlier phytochemical reports, flavonoids, stilbenoids and 2-arylbenzofurans were found as predominant constituents (Jagtap and Bapat, 2010). Oxyresveratrol (or 2,4,3′,5′-tetrahydroxystilbene), the most abundant stilbenoid purified from the *A. lakoocha* heartwood, exhibited potent anti-tyrosinase activity which involved in catalyzing of rate limiting steps in biosynthesizing human melanin pigment. It showed higher anti-tyrosinase activity than two tyrosinase inhibitors (kojic acid and licorice extract) commonly used in whitening agents (Tengamnuay et al., 2006). Oxyresveratrol has been claimed as a potent ingredient containing in *A. lakoocha* extract lotion and applied for effective skin whitener in cosmetic product. Recently, this compound obtained from *A. lakoocha* heartwood can decrease cell viabilities lower than about 80% and 60% at 15 and 17.5 μg/mL, respectively, on B16 melanoma cells by MTT assay (Rodboon et al., 2015). 2-Arylbenzofuran found in Brazilian red propolis showed inhibitory effect on B16BL6 melanoma cells (Li et al., 2008). The root bark of *A. lakoocha* also found to be a rich source of 2-arylbenzofurans from previous literatures (Hakim, 2010). This observation motivated us to look for constituents in *A. lakoocha* which may regard the cytotoxicity against B16F10 metastatic melanoma cells.

As part of our ongoing research on bioactive substances from Thai medicinal plants (Nontakham et al., 2014; Paseeta et al., 2011), *in vitro* comparative cytotoxic activity screening against B16F10 metastatic melanoma cells of *A. lakoocha* root bark and heart root extracts reviewed that the former was more potent. Reported herein are the isolation and structural elucidation of two representative-types of compounds **1** and **2** and their cytotoxic activity against melanoma cells (B16F10).

EXPERIMENTAL

General: The NMR spectra were recorded on a Bruker Avance 300 FT-NMR and are reported in ppm relative to the reference solvent of the sample in which they were run; coupling constants are reported in hertz (Hz). The chemical shifts were referenced to the residual solvent peaks (δ_H 7.24 and δ_C 77.00 for $CDCl_3$). ESI-MS Mass spectra were obtained from Finnigan LCQ. All column chromate-graphies (CC) were carried out on Merck silica gel 60 (particle size of 230-400 mesh). All organic solvents were distilled prior to use. Fractions were monitored by TLC, performed on precoated silica gel GF254 TLC plates. Spots on TLC were visualized under UV light (254 and 365 nm) and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

Plant Materials: The root bark and heart root of *A. lakoocha* were collected from Krabi in 2015 and a voucher specimen (UN003) is deposited at the Natural Product Unit, Department of Chemistry, Faculty of Science, Srinakharinwirot University, Thailand.

Extraction and isolation: The air-dried heart root of *A. lakoocha* (100 g), was milled and successively extracted with EtOAc (3 x 400 mL) and then with MeOH (3 x 400 mL) at 50 $^{\circ}$C, respectively. The EtOAc (2.1 g) and MeOH (5.7 g) extracts were obtained. The air-dried root bark of *A. lakoocha* (1.1 kg), was milled and successively extracted with EtOAc (3 x 5 L) and then with MeOH (3 x 5 L) at 50 ^{o}C, respectively. The EtOAc (34.8 g) and MeOH (69.6 g) extracts were furnished after removal of the solvent. The EtOAc extract obtained from the root bark was carried out on Quick Column Chromatography (QCC) and eluted with a gradient of hexane–EtOAc (80:20 to 0:100) and EtOAc–MeOH (80:20 to 60:40). The fractions were combined according to TLC profiles into 16 fractions (A-P). From TLC profiles, fraction E gave a major spot. Thus, purification of the fraction E (1.3 g) was carried out twice on silica gel columns with isocratic elution of CH_2Cl_2–MeOH (99:1) to furnish the major constituent **1** (131 mg, 0.003%). Fraction L (2.1 g) was further rechromatographed on a silica gel column with gradient system of CH_2Cl_2-MeOH (96:4 to 90:10) followed by a silica gel column with isocratic eluent of CH_2Cl_2-MeOH (94:6) to give compound **2** (23 mg, 0.0006%).

Artolakoochol (**1**) (131 mg): Yellow sticky oil; ^{1}H–NMR ($CDCl_3$, 300 MHz): δ_H 7.38 (1H, d, *J* = 8.3 Hz, H-4), 6.95 (1H, d, *J* =1.9 Hz, H-7), 6.75 (1H, dd, *J* = 8.3 Hz, 1.9 Hz, H-5), 6.73 (1H, s, H-3), 6.70 (1H, s, H-6'), 6.66 (1H, d, *J* = 10.0 Hz, H-1'''), 5.57 (1H, d, *J* = 10.0 Hz, H-2'''), 5.16 (1H, br t, *J* = 6.4 Hz, H-2''), 5.09 (1H, br t, *J* = 7.0 Hz, H-7'''), 3.44 (2H, d, *J* = 6.4 Hz, H-1''), 2.10 (2H, m, H-6'''), 1.75 (2H, m, H-5'''), 1.71 (3H, s, H-4''), 1.67 (3H, s, H-5''), 1.64 (3H, s, H-10'''), 1.56 (3H, s, H-9'''), 1.36 (3H, s, H-4'''); ^{13}C–NMR ($CDCl_3$, 75 MHz): δ_C 155.3 (C-7a), 154.4 (C-2), 153.5 (C-6), 152.3 (C-3'), 149.1 (C-5'), 131.7 (C-8'''), 131.1 (C-3''), 130.3 (C-1'), 128.6 (C-2'''), 124.1 (C-7'''), 123.6 (C-2''), 122.9 (C-3a), 121.1 (C-4), 120.1 (C-2'), 116.9 (C-1'''), 111.8 (C-5), 109.5 (C-4'), 106.9 (C-6'), 105.5 (C-3), 98.1 (C-7), 78.4 (C-3'''), 41.3 (C-5'''), 26.2 (C-4'''), 25.7 (C-1'',5''), 25.6 (C-10'''), 22.9 (C-6'''), 18.1 (C-4''), 17.6 (C-9''').

Oxyresveratrol (**2**) (23 mg): Pale yellow crystal, ^{1}H–NMR ($CDCl_3$ + DMSO-d_6, 300 MHz): δ_H 7.31 (1H, d, *J* = 8.4 Hz, H-6), 7.29 (1H, d, *J* = 16.3 Hz, H-7), 6.78 (1H, d, *J* = 16.3 Hz, H-8), 6.50 (1H, br d, *J* = 1.6 Hz, H-2', 6'), 6.42 (1H, d, *J* = 2.0 Hz, H-3), 6.35 (1H, dd, *J* = 8.4, 2.0 Hz, H-5), 6.24 (1H, br s, H-4'); ^{13}C–NMR ($CDCl_3$, 75 MHz): δ_C 157.9 (C-3',5'), 157.5 (C-4), 155.6 (C-2), 140.1 (C-1'), 126.7 (C-6), 125.0 (C-8), 123.4 (C-7), 116.0 (C-1), 107.2 (C-5), 104.5 (C-2',6'), 102.8 (C-3), 101.4 (C-4').

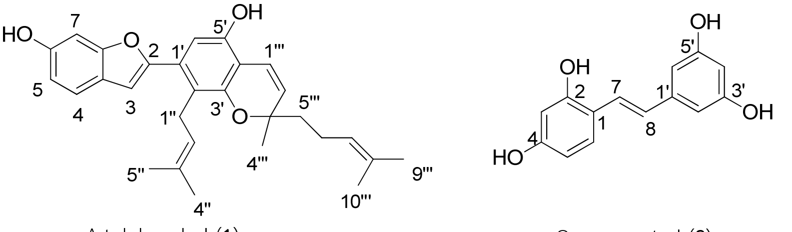

Figure 1 Structures of compounds 1 and 2

Cytotoxicity Assay

Cytotoxicity assay was assessed using the MTT colorimetric assay, as described previously (Siripong et al., 2006). Briefly, log-phase of B16F10 metastatic melanoma cells (ATCC®CRL 6475, USA: $3x10^3$ cells/mL) suspended in 100 μL of DMEM medium containing 10% fetal bovine serum, 1% antibiotic, were seeded onto a 96-well culture plate (Costar, Cambridge, MA, USA). After 24 h pre-incubation, the cells were exposed with serial concentrations of the tested compounds; (0.01-100 μM) for the indicated times (24, 48 and 72 h). Doxorubicin (0.01-30 μM) and 0.1% DMSO in medium were used as positive and negative controls. At the end of each incubation period, 20 μL MTT solution (5 mg/mL in PBS) was added to each well and further incubated at 37°C for 3 h. After centrifugation at 1,400 rpm for 5 min at 4°C, the medium was aspirated and the formazan product in each well was solubilized with 100 μL DMSO. The absorption at 550 nm wavelength was recorded on a Microplate reader (Benchmark 550, Bio-Rad, USA). Each concentration of drug was performed in six wells for three independent experiments. The IC_{50} value was calculated by plotting of the percentage of cell viability versus drug concentrations.

RESULTS AND DISCUSSION

In the search for bioactive compounds from Thai natural resources, by using the MTT assay, the *in vitro* inhibitory potency testing on B16F10 melanoma of extracts obtained from the root bark and the heart root of *A. lakoocha* were carried out and their IC_{50} values were shown in Table 1. The most potent root bark EtOAc extract (IC_{50} 27.65±3.59 μg/mL), which was approximately 6.5 times higher than that of the MeOH fraction (IC_{50} 178.39±6.08 μg/mL) and approximately 8 times higher than that of the heart root (IC_{50} 227.74±6.92 μg/mL), was then subjected to chromatographic purification to yield two major phenolic compounds of different type, an 2-arylbenzofurans (0.003%, based on dry plant material) and a stilbene (0.0006%, based on dry plant material). By using NMR spectral data analysis and by comparison with literature values (Sritularak et al., 2010, Djapić et al., 2003), both compounds were characterized as artolakoochol (**1**) and oxyresveratrol (**2**).

^{1}H–NMR spectrum of **1** ($CDCl_3$) showed a set of ABX aromatic protons at δ_H 7.38 (d, *J* = 8.3 Hz, H-4), 6.95 (d, *J* =1.9 Hz, H-7), 6.75 (dd, *J* = 8.3 Hz, 1.9 Hz, H-5), together with two singlet signals at δ_H 6.73 (H-3) and 6.70 (H-6'), which corresponding for the presence of 3',5',6-trioxygenated 2-arylbenzo-furan moiety. Two doublet signals at δ_H 6.66 (H-1''') and 5.57 (H-2''') with a coupling constant value of 10.0 Hz were assignable to two *cis*-coupled olefinic protons of a pyrano ring moiety. The rest of signals appeared as the typical signals of isoprene and modified geranyl substituents. The ^{13}C NMR spectrum provided 29 carbons, including 14 carbons of 2-arylbenzofuran skeleton and 15 carbons of the substituents, one prenyl and one modified geranyl. Furthermore, the ^{13}C-NMR showed the presence of a separated carbon signal at δ_C 78.4 (C-3'''), which was characteristic of quaternary carbon located on the 2,2-dimethylpyran ring. Comparison of ^{1}H and ^{13}C-NMR data between compound **1** and reported artolakoochol, it was shown that they were very similar (Sritularak et al., 2010). Compound **1** was therefore identified as artolakoochol.

^{1}H–NMR data of compound **2** ($CDCl_3$ + DMSO–d_6) displayed two sets of aromatic systems: a ABX aromatic system at δ_H 7.31 (d, *J* = 8.4 Hz, H-6), 6.42 (d, *J* = 2.0 Hz, H-3) and 6.35 (dd, *J* = 8.4, 2.0 Hz, H-5), and AB_2 aromatic system at δ_H 6.50 (br d, *J* = 1.6 Hz, H-2',6') and 6.24 (br s, H-4')'. Two doublet signals at δ_H 7.29 (H-7) and 6.78 (H-8) with a coupling constant value of 16.3 Hz were characteristic of *trans* conjugated double bond. The ^{13}C NMR and DEPT spectra exhibited 14 carbons due to two methylenes, two methines, six aromatic and four oxygenated quaternary carbons. These data implied for the presence of stilbene skeleton. In addition, the signals at δ_C 157.9 (C-3',5'), 157.5 (C-4) and 155.6 (C-2) were further confirmed that the compound had four oxygenated quaternary carbons. By comparison of the spectroscopic data (^{1}H- and ^{13}C-NMR) with those reported in literature (Djapić et al., 2003), compound **2** was thus identified as oxyresveratrol.

The isolated constituents were consequently evaluated for cytotoxic potency against the B16F10 metastatic melanoma cells. From the results, artolakoochol (**1**) displayed an interesting IC_{50} value of 5.96 μg/mL (Table 1). Under the same evaluation, oxyresveratrol (**2**) showed weaker activity with IC_{50} 41.48 μg/mL, which was almost 7-fold less potent than that of **1**. Oxyresveratrol of *A.*

lakoocha heartwood has been reported to decrease cell viabilities of B16 melanoma cells (Rodboon et al., 2015). To the best of our knowledge, this is the first report for the naturally isolated 2-arylbenzofurans from plant and of the *A. lakoocha* root extracts with inhibitory property on B16F10 cells.

Table 1 Cytotoxic activity on B16F10 metastatic melanoma cells of *A. lakoocha* extracts and compounds **1** and **2**

Plant part/ Compounds	IC_{50} (μg/mL)	
	EtOAc ext.	**MeOH**
Root bark	27.65±3.59	178.39±6.08
Heart root	227.74±6.92	219.58±9.96
Artolakoochol (**1**)	5.96±0.068	
Oxyresveratrol (**2**)	41.48±5.14	
Doxorubicin (Positive control)	0.025±0.002	

CONCLUSION

This study has shown for the first time the cytotoxic ability against B16F10 metastatic melanoma cells of *A. lakoocha* root extracts and their major type of compounds, artolakoochol (**1**) and oxyresveratrol (**2**), in which the former compound was about 7 times more active than that of the latter. Our finding provided value-added information towards *A. lakoocha*. Thus, *A. lakoocha* root extract might be a promising candidate for new anti-skin cancer agent development.

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