บทความนี้เป็นการรายงานครั้งแรกของฤทธิ์ยับยั้งเซลล์มะเร็งผิวหนังชนิดแพร่กระจาย B16F10 melanoma ของสารสกัดจากรากและสารหลักที่พบในส่วนรากเป็นการเพิ่มมูลค่าให้กับเครื่องสําอางสมุนไพรของมะหาดอีกด้วย

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 IC₅₀ 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.

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 (δH 7.24 and δC 77.00
for CDCl3). ESI-MS Mass spectra were obtained
from Finnigan LCQ. All column chromatoge-
raphies (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-H2SO4 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 °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 °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$_2$Cl$_2$–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$_2$Cl$_2$–MeOH (96:4 to 90:10) followed by a silica gel column with isocratic eluent of CH$_2$Cl$_2$–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”).

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’).
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; 3×10³ 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 IC₅₀ 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 IC₅₀ values were shown in Table 1. The most potent root bark EtOAc extract (IC₅₀ 27.65±3.59 µg/mL), which was approximately 6.5 times higher than that of the MeOH fraction (IC₅₀ 178.39±6.08 µg/mL) and approximately 8 times higher than that of the heart root (IC₅₀ 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).

\[ ^1H-\text{NMR spectrum of 1 (CDCl}_3 \text{) showed a set of ABX aromatic protons at } \delta_H 7.38 \text{ (d, } J = 8.3 \text{ Hz, H-4), 6.95 \text{ (d, } J = 1.9 \text{ Hz, H-7), 6.75 \text{ (dd, } J = 8.3 \text{ Hz, 1.9 Hz, H-5), together with two singlet signals at } \delta_H 6.73 \text{ (H-3) and 6.70 (H-6), which corresponding for the presence of 3',5',6-trioxygenated 2-arylbenzofuran moiety. Two doublet signals at } \delta_H 6.66 \text{ (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 \text{ 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-\text{NMR showed the presence of a separated carbon signal at } \delta_C 78.4 \text{ (C-3'''), which was characteristic of quaternary carbon located on the 2,2-dimethylpyran ring. Comparison of } ^1H- \text{ and } ^{13}C-\text{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.}

\[ ^1H-\text{NMR data of compound 2 (CDCl}_3 + \text{DMSO-}d_6 \text{) displayed two sets of aromatic systems: a ABX aromatic system at } \delta_H 7.31 \text{ (d, } J = 8.4 \text{ Hz, H-6), 6.42 \text{ (d, } J = 2.0 \text{ Hz, H-3) and 6.35 \text{ (dd, } J = 8.4, 2.0 \text{ Hz, H-5), and AB}_2 \text{ aromatic system at } \delta_H 6.50 \text{ (br d, } J = 1.6 \text{ Hz, H-2',6') and 6.24 \text{ (br s, H-4'). Two doublet signals at } \delta_H 7.29 \text{ (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 \text{ 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 } \delta_C 157.9 \text{ (C-3',5'), 157.5 \text{ (C-4) and 155.6 (C-2) were further confirmed that the compound had four oxygenated quaternary carbons. By comparison of the spectroscopic data (} ^1H- \text{ and } ^{13}C-\text{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\textsubscript{50} value of 5.96 \mu\text{g/mL} (Table 1). Under the same evaluation, oxyresveratrol (2) showed weaker activity with IC\textsubscript{50} 41.48 \mu\text{g/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>
<thead>
<tr>
<th>Plant part/ Compounds</th>
<th>IC₅₀ (µg/mL)</th>
<th>MeOH</th>
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<tr>
<td></td>
<td>EtOAc ext.</td>
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<tr>
<td>Root bark</td>
<td>27.65±3.59</td>
<td>178.39±6.08</td>
</tr>
<tr>
<td>Heart root</td>
<td>227.74±6.92</td>
<td>219.58±9.96</td>
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<tr>
<td>Artolakoochol (1)</td>
<td>5.96±0.068</td>
<td></td>
</tr>
<tr>
<td>Oxyresveratrol (2)</td>
<td>41.48±5.14</td>
<td></td>
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<tr>
<td>Doxorubicin (Positive control)</td>
<td>0.025±0.002</td>
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</table>

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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REFERENCES


