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Original Article

An Antioxidative and Cytotoxic Substance Extracted from *Curcuma comosa* Roxb.

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Abstract

The powerful antioxidative and HeLa cytotoxic activity of crude ethanol extract of *Curcuma comosa* Roxb. prompted us to carry out biologically-guided separation of the active constituent. Chromatographic separation of the ethanol extract yielded fractions Zcc(R)-E-1 and Zcc(R)-E-2. The potent fraction Zcc(R)-E-1 was subsequently bioassay-guided separated and the active fraction Zcc(R)-E-15 was obtained. Recrystallization of fraction Zcc(R)-E-15 yielded compound A, exhibited antioxidant power 1064.73 ± 60.96 μM vitamin C equivalence and HeLa cell cytotoxicity with IC50 4.44 ± 0.85 μg/ml. With structure elucidation via spectroscopic techniques, compound A was identified as 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone.

Key words: ***Curcuma comosa*, antioxidant, 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone**

Introduction

Several plants belonging to the genus Curcuma of the family Zingiberaceae, i.e., *Curcuma aromatica* Salisb., *C. comosa* Roxb., *C. longa* Linn., *C. parviflora* Wall., *C. xanthorrhiza* Roxb. and *C. zedoaria* Roxb., have been used as traditional medicine for the treatment of a wide range of diseases in many Asian countries including Thailand. Phytochemical investigations of these plants led to the isolation of several compounds, some of which possess interesting biological activities. *C. comosa* was originally cultivated in Indonesia, where it is known as Temoelawak, and spread to several areas in the Indo-Malaysian territory as well as Thailand. In Thailand, this plant is commonly known as *Waan chak mod luuk*[1], and has been used widely by traditional practitioners for the treatment of postpartum uterine inflammation and also as an aromatic stomachic and cholerctic[2]. In 1994 Jurgen et al.[3] reported the nematicidal activity of diarylheptanoids, and the active principal agents in hexane-soluble fraction of the methanol extract of *C. comosa*. Piyachaturawat et al.[4-8] investigated the biological activities of *C. comosa* crude extracts. It was

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found that the hexane extract was effective in the uterotropic activity by increasing uterine weight and glycogen content in rats, whereas the ethylacetate extract exhibited the hypolipidemic activity in mice. The isolation and initial assessment of the hypolipidemic effect of two new phenolic diarylheptanoids of *C. comosa* was reported in 1997.

Since *C. comosa* expresses assorted activities, the search for new biological activities with responsible compounds should be actively continued. In this paper, we report a bioassay-guided isolation of the cytotoxic and anti-oxidative constituent from the rhizome of *C. comosa*.

Materials and Methods

Materials

1. Plant material

Fresh rhizomes of *Waan chak mod luuk* were collected from a plantation in Nakhon Pathom province, Thailand in November 2000 and identified, based on the plant taxonomy, as *Curcuma comosa* Roxb.[1,9,10] The voucher specimens (Department of Medical Sciences Herbarium No. 1464) were deposited at the Department of Medical Sciences Herbarium. The fresh rhizomes were washed, sliced and dried in an oven at 40-50°C. The dried rhizomes were ground and kept at room temperature in well-sealed closed vessels.

2. Chemicals

2.1 Solvents - chloroform, ethyl acetate, ethanol were of commercial grade and were purified by distillation before use.

- methanol was of analytical reagent grade.

2.2 Chemicals used - anisaldehyde-sulfuric acid spraying reagent[11] (0.5% ethanolic solution of anisaldehyde with 5% sulfuric acid).

2.3 Siliga gel 60 for column chromatography with particle sizes 0.063-0.200 mm, siliga gel GF_{254} precoated plate for thin-layer chromatography and siliga gel PF_{254} for preparative-layer chromatography were obtained from E. Merck, Germany. Cosmosil $75C_{18}$-OPN (nacalai tesque) for reversed-phase column chromatography was used.

3. Instruments

3.1 Ultraviolet (UV) spectrophotometer (Jasco, Uvidec 650)

3.2 Gemini 2000 NMR Spectrometer (Varian, USA) for ^{1}H and ^{13}C-NMR

3.3 JASCO A 302 Spectrophotometer for IR determination

3.4 Seisakusho micro melting point

3.5 High performance liquid chromatography : Waters 600 Controller

Detector: Waters 996 Photodiode Array Detector

3.6 Plate reader (Biorad model 550)

Methods

1. Extraction of *C. comosa* rhizomes

Pulverized rhizome (1 kg) was extracted successively with n-hexane and with ethanol in a Soxhlet extractor to yield dried extract of 41.42 g and 59.05 g, respectively.

2. Separation of biologically active compound A

The ethanol extract (20 g) was chromatographed on silica gel 60 using a gradient of ethylacetate-methanol as the eluting solvent to yield two fractions of Zcc(R)-E-1 and Zcc(R)-E-2. Biologically guided separation led to the re-chromatography of fraction Zcc(R)-E-1 (11.40 g) with silica gel 60 twice and finally with Cosmosil 75C18-OPN reversed-phase column chromatography. The active compound was obtained in methanol water fraction. After recrystallization in methanol, the purification of the active compound was confirmed by subjecting it to HPLC using X Terra™ RP_{18} column (5μm, 4.6 × 150 mm), 40 per cent methanol in water as the eluent and the Waters 996 photodiode array as the detector.

3. Structure elucidation of the active compound A

The structure of the active compound was elucidated by spectroscopic techniques using ^{1}H-NMR, ^{13}C-NMR and IR. The melting point of the compound was also determined and uncorrected.

4. Free Reducing/Antioxidant Power (FRAP) Assay

The FRAP assay[12] was conducted at room tem-

perature under pH 3.6 where the reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous form (blue color) can be monitored for 0-4 min absorbance change at 595 nm with a plate reader (Biorad model 550). The FRAP reagent contained 10 mM TPTZ (Fluka), 40 mM HCl (Merck), and 20 mM $FeCl_3.6H_2O$ (Merck) at the ratio of 10:1:1. The standard solution of $FeSO_4.7H_2O$ (Merck) and antioxidant ascorbic acid (Sigma) was used as the standard. The antioxidant capacity of the plant extracts was presented as μM vitamin C equivalence.

5. Cell Culture and Treatment Protocol

Human cervix adenocarcinoma cells (HeLa) were obtained from the National Cancer Institute (Thailand) and cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1.5 g/L sodium bicarbonate, 1.0 mM sodium pyruvate, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/ml streptomycin (Gibco BRL). The cultures were maintained at 37˚C in a 5 per cent CO_2 humidified atmosphere. At subconfluence, HeLa cells were trypsinized and seeded to a 96-well plate at 1×10^5 cells/well and allowed to attach overnight. Cells were then exposed to increasing concentrations of doxorubicin (DOX) (10^{-9} M to 10^{-5}M) or plant extracts for 48 hours, in triplicate.

6. Cytotoxicity assay

HeLa cytotoxicity was determined by crystal violet method, as described by Takahashi[13]. Briefly, after 48 hours incubation, cells were washed twice with PBS, and fixed with 10 per cent buffered formalin. Prefiltered 0.1 per cent crystal violet solution in water/MeOH was used to stain the live cells. Cell survival was quantified by lysing the cells in 50 mM sodium citrate solution in water/EtOH and measured the absorbance at 595 nM with the plate reader. To determine the cytotoxic effect of the medicinal plant extracts, IC_{50} of doxorubicin (Sigma) were evaluated in comparison with the plant extracts.

Results

1. Extraction of *C. comosa* rhizomes

The n-hexane extract of *C. comosa* rhizomes was obtained as a pale yellowish viscous oil in a 4.14 per cent yield. The ethanol extract was obtained as a dark reddish-brown mass in a 5.9 per cent yield.

2. Separation of biologically active compound A

Chromatographic separation of ethanol extract (20.0 g) of *C. comosa* rhizome yielded the fractions Zcc(R)-E-1 and Zcc(R)-E-2 of 11.40 g and 3.61 g, respectively. Repeated separation of fraction Zcc(R)-E-1 using chromatograph techniques afforded active fraction Zcc(R)-E-15. In continuation of recrystallization, the crystalline compound A was obtained (0.16 g).

The HPLC -retention time of compound A was 3.403 min [mobile phase: 40% MeOH:H_2O, wavelength of detection 254 nm].

3. Structure elucidation of the active compound A

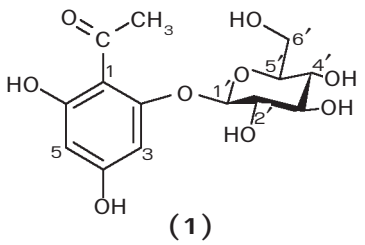
Compound A was obtained as a colorless needle, mp 210-212 ˚C

^{1}H-NMR : δ (CD3OD) 5.95(1H, *d*, *J*= 2.0 Hz), 6.17 (^{1}H, *d*, *J*= 2.0 Hz), 2.69(3H, s, $OCCH_3$).

^{13}C-NMR: δ (CD3OD): 32.7(CH3), 63.0, 67.4, 70.9, 73.9, 74.6, 102.2(C-6′,C-4′,C-3′,C-2′,C-5′,C-1′)[a], 91.6(C-3), 94.9(C-5), 104.5(C-1), 160.3(C-6), 165.1(C-2), 165.4(C-4), 202.7(CO).

IR: ν_{max}^{KBr} 3434, 2920, 1637, 1604, 1466, 1367, 1288, 1077, 834 cm^{-1}.

Based on spectroscopic data, compound A was identified as 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone **(1)**, Figure 1.



(1)

Figure 1 Compound A, 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone

4. Ferric Reducing/Antioxidant Power (FRAP) Assay

The total antioxidant capacity of the plant extracts (1 mg/mL) presented in a vitamin C equivalent unit is shown in Table 1. Crude extract-Zcc(R) showed the highest antioxidant power among the samples

Table1 Total antioxidant capacity of the plant extracts evaluated by ferric reducing/antioxidant power (FRAP) assay.

Curcuma comosa **Roxb. extracts (1 mg/mL)**	**Total antioxidant capacity (μM vitamin C equivalence)***
Zcc(R)	6931±407.70
Zcc(R)-E-1	2023.12±154.73
Zcc(R)-E-2	854.11±42.45
Zcc(R)-E15	1064.73±60.96

*Data are presented as Mean ± SEM

Table 2 Cytotoxicity of doxorubicin and plant extract in HeLa cells.

Compound	**IC_{50}(μg/mL)***
Doxorubicin	0.11 0.01
Zcc(R)	1.70 0.21
Zcc(R)-E-1	1.47 0.46
Zcc(R)-E-2	11.56 2.13
Zcc(R)-E15	4.44 0.85

*Data are presented as Mean ± SEM

tested. The assay showed intra and inter-day variability at less than 10 per cent.

5. Cytotoxicity Effect of Plant Extracts

Table 2 shows the cytotoxic effects of doxorubicin and the plant extracts on HeLa cells. The IC_{50} (concentrations that kill 50% of the cells) demonstrated that doxorubicin was (15-fold more potent than Zcc(R) (IC_{50}=0.11 ± 0.01 vs 1.70 ± 0.21 g/ml, respectively). Further fractionation did not increase the potency.

Discussion

The ethanol extract of *C. comosa* rhizomes possesses high antioxidant power and cytotoxic effect on HeLa cells. These results prompted us to carry out the bioassay-guided separation of the active constituent from the rhizomes of *C. comosa*. Chromatographic isolation of the ethanol extract resulted in the isolation of a phloracetophenone glucoside, compound A. The ^{1}H-NMR spectrum revealed the presence of two aromatic protons δ 5.95 *d, J*= 2.0 Hz ; 6.17 *d, J*= 2.0 Hz. The coupling constant value showed these two protons to be *meta* to each other. A three-proton singlet at δ 2.69 accounted for a methyl group attached to an aromatic keto group. The rest of the ^{1}H-NMR spectrum clearly revealed the presence of a glucoside moiety in 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone (mp 210-212˚C) as shown in Table 3. The signal of a quaternary carbon at 202.7 in the ^{13}C-NMR spectrum confirmed the presence of a keto functional group in the molecule as shown in Table 4. Its IR spectrum (KBr) showed absorption at 3434 cm^{-1} indicating the presence of hydroxyl groups (O-H); the broad, intense absorption band at 1637 cm^{-1} was indicative of a conjugated

Table 3 ^{1}H-NMR Spectral Data of 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl) acetophenone in CD3OD.

Position	**δ_H(ppm), *J*(Hz)**
3	6.17(1H, *d*,2.0)
5	5.95(1H, *d*, 2.0)
$OCCH_3$	2.69

Table 4 ^{13}C-NMR Data for 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone in CD_3OD

Position	**4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl) acetophenone**
1	104.5
2	165.1
3	91.6
4	165.4
5	94.9
6	160.3
1′	102.2
2′	73.9[a]
3′	70.9[a]
4′	67.4[a]
5′	74.6[a]
6′	63.0[a]
C=O	202.7
CH_3	32.7

[a]Assignments within a column may be interchanges.

keto group (C=O). All these described spectroscopic data implied that compound A was 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone.

The phloracetophenone glucoside fraction Zcc(R)-E15, showed total antioxidant capacity of 1064.73 ± 60.96 μM equivalent to vitamin C. Furthermore, it was found to be cytotoxic to HeLa cells and had an IC_{50} value of 4.44 ± 0.85 μg/ml. These results showed clearly that the isolated phloracetophenone glucoside fraction was relatively less potent than the ethanol extract, Zcc(R) as shown in Tables 1 and 2. Therefore, *C. comosa* may contain more than one active antioxidative/cytotoxic constituent and further studies to isolate other active constituents are recommended.

Conclusions

Ethanol extracts of *Curcuma comosa* rhizomes were evaluated in the cytotoxicity assay in HeLa cells and antioxidant activity by ferric reducing/antioxidant power assay (FRAP). The isolation and identification of one of the responsible active compounds was found to be 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl) acetophenone.

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บทคัดย่อ

สารสกัดว่านชักมดลูกที่มีฤทธิ์ต้านอนุมูลอิสระและเป็นพิษต่อเซลล์มะเร็งคอมดลูก

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สารสกัดเหง้าว่านชักมดลูกโดยได้รับการตรวจทางพฤกษอนุกรมวิธานที่ห้องปฏิบัติการพิพิธภัณฑ์พืช สถาบันวิจัยสมุนไพร กรมวิทยาศาสตร์การแพทย์ (Voucher specimen: No. 1464) พบว่ามีฤทธิ์ต้านอนุมูลอิสระและยับยั้งการงอกเจริญของเซลล์มะเร็งปากมดลูก ไปแยกหาสารสำคัญที่ออกฤทธิ์โดยเทคนิคโครมาโตกราฟฟีออกเป็น ๒ ส่วนคือ Zcc(R)-E-1 และ Zcc(R)-E-2 พบว่า ส่วนสกัด Zcc(R)-E-1 เป็นส่วนสารสกัดที่ออกฤทธิ์ดังกล่าว จึงทำการแยกต่อเป็นส่วนสกัดย่อยควบคู่กับการทดสอบฤทธิ์ของแต่ละส่วนสกัด พบว่าส่วนสกัดย่อยที่มีฤทธิ์คือ Zcc(R)-E-15 ซึ่งเมื่อตกผลึกจะได้สารคอมเปานด์ เอ ที่มีฤทธิ์ต้านอนุมูลอิสระซึ่งมีค่า ๑๐๖๔.๗๓ ± ๖๐.๕๖ μM สมมูลของวิตามินซี (vitamin C equivalence) และมีฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งปากมดลูกโดยแสดงค่า IC_{50} ๔.๔๔ ± ๐.๘๕ มคก./มล. จากการศึกษาสูตรโครงสร้างของสารโดยเทคนิคทางสเปกโตร สโคปี พบว่า Compound A คือ 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl) acetophenone

คำสำคัญ : **ว่านชักมดลูก, ฤทธิ์ต้านอนุมูลอิสระ, 4,6-dihydroxy-2-*O*-(β-D-glucopyranosyl)acetophenone**