In vitro cytotoxic, genotoxic, and antityrosinase activities of Clitoria macrophylla root

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J. Adv. Pharm. Technol. Res.

INTRODUCTION

Clitoria macrophylla Wall. (Leguminosae), locally known as Non-tai-yak or An-chan-pa, commonly distributed in tropical nations and Southeast Asia. Regarding traditional Thai medical system, C. macrophylla roots carry out a potential in dermatology. Its roots are also used as insecticide in agriculture and animal farming. Moreover, clitoriacetal is the major component that can be detected in C. macrophylla root. This research aimed to assess the efficacy of C. macrophylla root extract and clitoriacetal for its anticancer and antityrosinase activities as well as to assess in vitro safety potential for its cytotoxic and genotoxic effects. C. macrophylla root and clitoriacetal were tested by brine shrimp lethality, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, comet assay, and antityrosinase activity. C. macrophylla root, clitoriacetal, and rotenone demonstrated the toxicity against brine shrimp nauplii with LC50 of 332.15, 136.54, and 0.15 µg/mL, respectively. C. macrophylla root and clitoriacetal showed cytotoxic potential against breast ductal carcinoma (BT-474), liver hepatoblastoma (Hep-G2), and colon adenocarcinoma (SW-620). At 100 µg/mL, the percent DNA damage of C. macrophylla root and clitoriacetal was 37.84% and 36.01%, respectively. C. macrophylla root and clitoriacetal were able to inhibit the tyrosinase enzyme with IC50 of 12.27 and 7.30 µg/mL, respectively, which less effective than glutathione (positive control). The present study revealed the in vitro biological activities of C. macrophylla root and its clitoriacetal constituent which proposed the scientific evidences in efficacy and safety evaluation including in vitro cytotoxicity, DNA damage as well as antityrosinase activities.

Key words: Antityrosinase activity, brine shrimp toxicity, Clitoria macrophylla Wall, clitoriacetal, comet assay, MTT assay

Abstract

Clitoria macrophylla Wall. (Leguminosae), locally named Non-tai-yak or An-chan-pa, commonly distributed in tropical nations and Southeast Asia. Regarding traditional Thai medical system, C. macrophylla roots carry out a potential in dermatology. Its roots are also used as insecticide in agriculture and animal farming. Moreover, clitoriacetal is the major component that can be detected in the root part in tropical nations and Southeast Asia. Regarding traditional Thai medical system, C. macrophylla roots carry out a potential in dermatology. Its roots are also used as insecticide in agriculture and animal farming. Tuber juice and root juice of C. macrophylla were reported to get rid of green flies on vegetable and clear up worms on the buffalo’s back. For the phytochemical investigation of C. macrophylla root, the main compositions of rotenoid were isolated and identified such as clitoriacetal, 6-deoxyclitoriacetal, and stemonacetal. Especially, clitoriacetal (C16H18O9) is the major component that can be detected in the root part.

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How to cite this article: Pitakpawasutthi Y, Suwatronnakorn M, Issaravanich S, Palanuvej C, Ruangrungsi N. In vitro cytotoxic, genotoxic, and antityrosinase activities of Clitoria macrophylla root. J Adv Pharm Technol Res 2021;12:8-13.
which demonstrates antipyretic, anti-inflammatory, and antioxidant activities [Figure 1].

Most herbal medicine still needs to be studied scientifically such as standardization, biological activities, and efficacy of each plant material to become important concerns for both authorities and the public. Moreover, the biological experimental assessments have been used as standard safety studies together with the efficacy tests. The herbal medicines and natural products are mostly comprised complex compounds. It is important to investigate the biological studies to get scientific information before clinical trials. Thus, this research aimed to assess the efficacy of C. macrophylla root ethanolic extract and standard clitoriacetal for its anticancer and antityrosinase activities as well as to assess in vitro safety potential for its cytotoxic and genotoxic effects by brine shrimp lethality and comet assays.

MATERIALS AND METHODS

Plant materials

C. macrophylla roots were obtained from traditional Thai drug store in Thailand and authenticated by the expert (Ruangrungsi N). The voucher specimens were prepared and kept at College of Public Health Sciences, Chulalongkorn University.

Plant extraction

C. macrophylla roots were cleaned, dried, and ground into powder before exhaustive extraction with 95% ethanol through Soxhlet apparatus. The yields after filtration and evaporation under vacuum were kept for bioactivity tests.

Isolation of clitoriacetal

Dried powders of C. macrophylla roots (500 g) were successively undergone 95% ethanol maceration for 60 days and filtered. The combined filtrates were collected and evaporated to obtain a crude extract. The extract was subjected to column chromatography packed with silica gel 60G and eluted with the solvent system, chloroform-ethanol (19:1). Each fraction (10 ml) was collected and observed by thin-layer chromatography. The homogenous fractions were evaporated to give a pale yellow solid that was further purified by recrystallization. Clitoriacetal was obtained as a pale yellow powder and confirmed by NMR (Bruker Avance III™ HD 500 MHz).

Brine shrimp nauplii lethality assay

According to Meyer et al., 1982,[6] artificial sea water at the concentration of 36.66% (w/v) was prepared and aerated for 24 h in brine shrimp hatching box under illumination. Artemia salina cysts were added and incubated at room temperature. Ten brine shrimps were transferred after 48 h using pasture pipette to individual vial containing 5 ml saline water. Various concentration of C. macrophylla root ethanolic extract, clitoriacetal, and positive control (rotenone) in methanol were pipetted into a small filter paper and left until methanol was dried. Then, the prepared filter paper was placed into each vial containing the brine shrimp. Each concentration was performed in five replicates. The percent death of nauplii at 6, 12, 18, and 24 h was counted, recorded, and calculated for the LC50.

Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the modified method as described by Masmann.[7] Five human cancer and one human normal cell lines including BT-474 (breast ductal carcinoma), CHAGO-K1 (undifferentiated lung carcinoma), SW-620 (colon adenocarcinoma), KATO-3 (gastric carcinoma), Hep-G2 (liver hepatoblastoma), and Wi-38 (Lung fibroblast) were studied. Tested cell lines were incubated in tissue culture flask in RpMI-1640 supplemented with 5% (v/v) fetal calf serum at 37°C in 5% CO2 for 3 days. Two hundred microliters of these cells were transferred to 96 well culture plates (about 1 × 104 cells/well) and incubated for 24 h at 37°C, 5% CO2 and 100% relative humidity condition.

The sample solution (2 µl) was dispensed into the appropriate wells. This analysis was performed in four replicates. The plates were further incubated for 72 h then added with 10 µl of MTT solution. After 4 h incubation, the supernatant medium was removed, dimethyl sulfoxide (DMSO) (150 µl) was added to solubilize the formazan crystal in the cells, mixed for 2–3 min on an orbital shaker. The absorbance was measured at 540 nm. The IC50 was determined from % cell survival as follows:

\[
\% \text{ cell survival} = \frac{\text{Mean of sample absorbance}}{\text{Mean of negative control absorbance}} \times 100
\]

DNA damage

Lymphocytes isolation

Fresh blood specimen from healthy donor was aseptically collected in sterile tube containing heparin. Six milliliters
of diluted fresh blood was layered over 3 mL of Ficoll-Histopaque 1077 in a conical centrifuge tube, then centrifuged at 1800 rpm, 4°C for 30 min. The lymphocyte cells were 3-time rinsed in phosphate buffer saline (PBS, pH 7.4) and added with 10 ml of incomplete RPMI-1640 medium to discharge the buffer. Each step was centrifuged at 1600 rpm, 4°C for 10 min. Then, complete RPMI-1640 medium was added to get the lymphocyte suspension about 4 × 10⁵ cells/mL using hemocytometer. Four hundred microliters portions were aliquoted into microcentrifuge tube and kept at −80°C.

Comet assay

According to Singh et al.,⁹ each lymphocyte suspension was rinsed in PBS, pH 7.4 for 3 times, and added incomplete RPMI-1640 medium to obtain 4 ml as suspension. C. macrophylla root ethanolic extract and citoriacetal at concentration of 50 and 100 µg/mL were dissolved in 2% DMSO. Hydrogen peroxide and PBS (pH 7.4) were used as positive and negative controls, respectively. One hundred microliters of lymphocyte suspension were added into microcentrifuge tube that containing 100 µl of sample and incubated at 37°C for 1 h, centrifuged at 3000 rpm, 4°C for 5 min, and the supernatant was discarded.

The slides and coverslips were cleaned with ethanol and air dried before used. The slide was coated with 1% normal agarose which melt in PBS (pH 7.4) as the 1st layer and placed in low humidity before use to ensure the agarose adhesion. The treated samples were mixed with 1% low melting agarose which melt with PBS (pH 7.4) as ratio 1:1 at 37°C and spread onto the precoated slide, placed the coverslip over the second layer, and kept on ice until agarose gel solidified. After agarose gel has hardened, the coverslip was slid off and spread with 0.5% low melting agarose which melt with PBS (pH 7.4) as the third layer, cover with coverslip, and kept in a cool temperature until agarose forming harden. Then, the freshy lysis solution was prepared by mixing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10) with 10% DMSO. 1% Triton X-100 was added just before use. The coverslip was slid off, and the slide was immersed into a cool freshly lysis solution at 4°C for 1 h. After that, the slides were placed in horizontal gel electrophoresis chamber. The electrophoresis solution was 200 mM EDTA and 10 N NaOH, pH 13. For electrophoresis process, the slide was placed horizontally and washed three times with the neutralization buffer containing 0.4 M Tris buffer (pH 7.5) for 5 min.

Each slide was stained with 20 µg/mL ethidium bromide for 5 min, washed with water, and covered with coverslip, kept in a cool temperature. The migrated DNA (comet) was observed under fluorescent microscope (Axio Imager A2; Carl Zeiss, Germany) with the magnification of ×400. The degrees of damage were categorized into four classes of visual scoring depended on the size and intensity of the comet tail. Each comet was authorized a value of 0–3 conforming to its class; Class 0 interpreting comets with no or barely detectable tails (undamaged cells) and Class 1–3 interpreting increasing relative tail intensities [Figure 2].⁹ DNA damage was calculated as equation below.

\[
\text{%DNA damage} = \frac{\text{sum score of count cells}}{\text{sum score of all cells}} \times 100
\]

\[
\text{Antityrosinase activity}
\]

Antityrosinase activity was evaluated by dopachrome method in 96-well microplate.¹⁰ The C. macrophylla root extract, citoriacetal, and positive control (L-glutathione) were dissolved in 1 ml of DMSO (50% DMSO in water) and diluted to different concentrations. Each sample (40 µl) was mixed with 80 µl of 0.1 M sodium phosphate (pH 6.8) and 40 µl of L-DOPA substrate solution (19.7 mg in 0.1 M sodium phosphate, pH 6.8). Mushroom tyrosinase solution (31 U/mL) 40 µl was added in the reaction. The dopachrome was measured at 475 nm. The tyrosinase inhibitory activity was determined as equation below and IC₅₀ was calculated.

\[
\text{%Inhibition} = \left(\frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}}\right) \times 100
\]

RESULTS AND DISCUSSION

Brine shrimp lethality activity

The toxicity investigation on brine shrimp nauplii has been reported by Meyer et al., 1982.⁶ Brine shrimp lethality assay is always set for toxicological activity test of the natural products because it is a sensitive indicator species. It is rapid, reliable, nonexpensive, and convenient preliminary test for plant extracts which correlates reasonably well with cytotoxic properties. In several studies, brine shrimp

![Figure 2: DNA comet classification. Class 0 level is normal nucleus. Class 1 level has halo around the nucleus. Class 2 level increases in the length of the comet tail and decreases in the nuclear DNA content. Class 3 level has not nucleus](image-url)
lethality assay has been an authentic assay to estimate toxicity of the compounds or the extracts.[12-14]

The results of brine shrimp lethality activity of *C. macrophylla* root ethanolic extract, clitoriacetal, and positive control (rotenone) were evaluated and expressed as LC\(_{50}\) values: LC\(_{50}\) values <500 µg/mL (toxic), ≥500 ≤1000 µg/mL (weak toxicity), and >1000 µg/mL (nontoxicity).[15] It was found that *C. macrophylla* root ethanolic extract, clitoriacetal, and rotenone (positive control) were toxic against brine shrimp nauplii with LC\(_{50}\) of 332.15, 136.54, and 0.15 µg/mL, respectively [Figure 3]. On the contrary, clitoriacetal that was one of rotenoids isolated from the roots of *Clitoria fairchildiana*. Howard[16] was reported as weak toxic with the LC\(_{50}\) of 515.3 µg/mL.[17] The methanolic extract of the root of *C. fairchildiana* was found to be more toxic than *C. macrophylla* root ethanolic extract due to the LC\(_{50}\) of 158.0 µg/mL.[17] Brine shrimp lethality assays on *Clitoria ternatea* L. leaf extracts by two studies showed different LC\(_{50}\) as well. Kamilla *et al*. studied the exposure of 24 h aged nauplii to the methanolic extract of *C. ternatea* leaf and LC\(_{50}\) were found to be 1.46 and 0.49 mg/mL for 24 and 48 h of incubation periods, respectively.[18] Das and Chatterjee studied the effect of *C. ternatea* leaf extracted with 50% aqueous ethanol on 48 h aged nauplii. The LC\(_{50}\) after 24 h incubation period was 3.25 mg/mL.[19] Brine shrimp cytotoxic potency of *C. macrophylla* and clitoriacetal in this study was determined using 48 h aged nauplii and 24 h exposure period.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability activity**

The MTT assay has been used successfully to quantitate cell survival and proliferation in macrophage-mediated cytotoxicity. This assay is used depending on the ability of succinate dehydrogenase in mitochondria of viable cells capable to transform the MTT tetrazolium salt into intracellular purple formazan that can be solubilized by DMSO and quantified by spectrophotometry.

*C. macrophylla* root ethanolic extract, clitoriacetal, and doxorubicin (positive control) were evaluated for cytotoxic activity against five human cancer cell lines and one normal cell line. The results were shown in Table 1. Cytotoxicity cutoff criteria of crude extract and pure compound are IC\(_{50}\) <20 µg/mL and <4 µg/mL, respectively.[20] As the result, *C. macrophylla* ethanolic extract showed more cytotoxic potential against breast ductal carcinoma (BT-474), liver hepatoblastoma (Hep-G2), and colon adenocarcinoma (SW-620) with IC\(_{50}\) of 6.6, 5.8, and 12.1 µg/mL, respectively. Similarly, clitoriacetal showed cytotoxic potential with IC\(_{50}\) of 4.1, 1.4, and 3.0 µg/mL, respectively. Furthermore, clitoriacetal was the most potent anticancer compound against HEP-G2 cells which showed IC\(_{50}\) less than positive control. Another rotenoid, 6-deoxyclitoriacetal isolated from *C. macrophylla* roots demonstrated strong cytotoxic activity against cultured P-388 lymphocytic leukemia cells and marginal active against multidrug-resistant KB-V1 oral cavity cancer cells. Vinblastin enhanced the cytotoxicity against KB-V1. The mechanism might involve P-glycoprotein affinity with rotenoids.[2] Sangthong *et al*. studied the cytotoxic activity of 6-deoxyclitoriacetal and its derivatives against various cancer cell lines. The results showed promising cytotoxic property that might occur through inhibition of topoisomerase IIα and DNA intercalation.[21]

**DNA damage (comet assay)**

Comet assay is a rapid standard procedure to observe DNA damage in eukaryotic cells depended on the detection of denatured DNA fragments migrating out of the cell nucleus.

![Figure 3: Cytotoxic activity of (a) *Clitoria macrophylla* root ethanolic extract, (b) clitoriacetal, and (c) rotenone due to brine shrimp lethality testing](image-url)
The percent of DNA damage was shown in Figure 4. Hydrogen peroxide was used as positive control and PBS (pH 7.4) was used as negative control. At 100 µg/mL, the percent DNA damage of *C. macrophylla* root ethanolic extract and clitoriacetal was 37.84% and 36.01%, respectively. *C. macrophylla* root ethanolic extract and clitoriacetal showed DNA damage potential with a dose-dependent relationship between the intensity of DNA damage and concentration of the sample. The aromatic methoxylation was reported as one of toxophores of the rotenoid molecule that contributed to the oxygen radical production, leading to DNA damage.\[^{23-25}\]

**Antityrosinase activity**

Tyrosinase is the enzyme in melanosomes of melanocyte, which involves in melanin biosynthesis. Antitryosinase activities were examined using dopachrome method.\[^{10,11}\] L-dopa was used as a substrate of tyrosinase *in vitro*. Dopaquinone product chemically changed to color substance, dopachrome. As the result, *C. macrophylla* root ethanolic extract and clitoriacetal were able to inhibit the tyrosinase enzyme with IC\(_{50}\) of 12.27 and 7.30 mg/mL, respectively, which less effective than positive control (glutathione) with IC\(_{50}\) of 0.01 mg/mL [Figure 5]. Chen *et al.* revealed that *Clitoria ternatea* Lindl. (Butterfly pea) flower extract increased whitening effect of the mask preparation.\[^{26}\] Ruksounjik and Khunkitti studied the tyrosinase inhibition of *C. ternatea* flower ethanolic extract. It appeared that 0.2 mg/mL of the extract and glutathione could inhibit 22.04 ± 2.42% and 95.72 ± 2.00% of tyrosinase activity, respectively.\[^{27}\] The marginal activity on tyrosinase inhibition of *C. macrophylla* root and a rotenoid, clitoriacetal was revealed in this study.

**CONCLUSION**

The *in vitro* biological activities of *C. macrophylla* root ethanolic extract were demonstrated with reference to its rotenoid, clitoriacetal. Cytotoxicity against *A. salina* nauplii...
as well as various cancer cell lines was revealed. DNA damage effect was studied using comet assay. Tyrosinase inhibitory property was also reported.

Financial support and sponsorship
This study was financially supported by Ratchatapisek Somphot Fund for Postdoctoral Fellowship, Chulalongkorn University. The authors wish to thank College of Public Health Sciences, Chulalongkorn University and all staff members for necessary assistance and instrument supports.

Conflicts of interest
There are no conflicts of interest.

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