Pharmacogn J. 2024; 16(2): 302-306
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Assessment of Anti-tyrosinase, Antioxidant and Cytotoxic Activities of *Trigonostemon reidioides* Extracts on Mouse Fibroblast (L929) Cells

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ABSTRACT

Background: *Trigonostemon reidioides* (Kurz) Craib is a plant traditionally used for its whitening properties, although previous studies have reported some adverse effects associated with its use. **Objectives**: The objective of this study were to investigate the antityrosinase activity, antioxidant activity, and toxicity on Mouse Fibroblast (L929) Cells of the *T. reidioides* extracts. The antityrosinase activity was determined using the dopachrome method, while the antioxidant activity was evaluated using the DPPH method. The cytotoxicity was assessed using the MTT assay. **Results**: The results revealed that the ethanol extract exhibited strong antityrosinase activity, with an IC_{50} value of 5.38 µg/ml. Furthermore, Fraction 3 and Fraction 4, which were further separated from the ethanol extract, demonstrated high antioxidant activity, with IC_{50} values ranging from 1.65 to 2.10 µg/ml. The hexane extract, as well as Fraction 4 and Fraction 5, exhibited low toxicity, with IC_{50} values ranging from 11.82 to 40.12 µg/ml. **Conclusions**: These findings indicate that Fraction 4 and Fraction 5 of *T. reidioides* possess potent antityrosinase and antioxidant activities, while displaying low cytotoxicity against the mouse fibroblast (L929) cell line. Therefore, Fraction 4 and Fraction 5 hold considerable potential for further development as skin whitening agents derived from *T. reidioides* extracts.

Key word: *Trigonostemon reidioides* (Kurz) Craib, Antityrosinase activity, Antioxidant activity, Cytotoxicity activity.

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History

- Submission Date: 14-02-2024;
- Review completed: 21-03-2024;
- Accepted Date: 25-03-2024.

DOI : 10.5530/pj.2024.16.45

Article Available online

http://www.phcogj.com/v16/i2

INTRODUCTION

Currently, several medicinal plants, including *Curcuma longa*, *Aloe vera*, and *Hibiscus sabdariffa*, have gained attention for their use as ingredients in cosmetics products. This has led to the development and research of these medicinal plants for practical application in the market. Natural extracts derived from medicinal plants, such as curcumin from *Curcuma longa*, have been widely incorporated into cosmetics production, indicating the significant potential of medicinal plants in the cosmetics industry. Moreover, natural extracts or products used for skin whitening and anti-aging should possess various biological activities, including anti-tyrosinase and antioxidant properties[1].

Trigonostemon reidioides (Kurz) Craib (Lodthannongdaeng in Thai) is a Thai medicinal plant belonging to the Euphorbiaceae family. In Thai traditional medicine, the root of *T. reidioides* has been used for various medicinal purposes, such as an antidote against mushroom and snake toxins, as well as a whitening agent[2]. Previous studies have reported that *T. reidioides extracts* exhibit various biological activities, including anti-inflammatory, antimicrobial, and antioxidant effects. Phytochemical analysis of *T. reidioides* has revealed the presence of diterpenoids, triterpenoids, lignans, flavonoids, and phenolic compounds[3-4].

For beauty purposes, the traditional practice involved grinding the root of *T. reidioides* into a powder, which was then mixed with lime juice and turmeric powder to apply on the face for the treatment of acne and pigmentation, resulting in a fair and radiant complexion. However, adverse effects such as redness and peeling may occur. Nevertheless, the potential cosmetic application of *T. reidioides* root extracts, specifically for skin whitening and anti-aging purposes, remains understudied.

Natural tyrosinase inhibitors, particularly those derived from herbal plants, have garnered significant interest in current research. Tyrosinase inhibitors play a crucial role in preventing the synthesis of melanin, the pigment responsible for skin coloration. Simultaneously, antioxidants offer protection against oxidative damage caused by free radicals, thus benefiting overall skin health. The combination of these two mechanisms has shown promising effectiveness in preventing or delaying skin pigmentation[5].

Therefore, the objective of this study is to investigate the potential of *T. reidioides* root extract as a cosmetic ingredient by evaluating its anti-tyrosinase and antioxidant activities, as well as its cytotoxicity in mouse fibroblast (L929) cells, which serve as a widely used in vitro model for skin toxicity studies. The findings of this study will provide valuable insights into the potential use of *T. reidioides* extracts as a cosmetic ingredient with antioxidant and anti-tyrosinase activities. Furthermore, this study will contribute to confirming the safety of *T. reidioides* extracts for cosmetic applications.

MATERIALS AND METHODS

Plant Material

The roots of *Trigonostemon reidioides* (Kurz) Craib were collected from the Mahasarakham province of Thailand and identified by Dr. Phadungkit M.

Cite this article: Chummalee I, Phadungkit M, Laovachirasuwan P. Assessment of Anti-tyrosinase, Antioxidant and Cytotoxic Activities of *Trigonostemon reidioides* Extracts on Mouse Fibroblast (L929) Cells. Pharmacogn J. 2024;16(2): 302-306.

The voucher specimens were deposited at the Faculty of Pharmacy, Mahasarakham University, Thailand.

Extraction and Isolation

The plant sample was subjected to maceration using hexane, dichloromethane, ethyl acetate, and 95% ethanol as solvents. The resulting extracts were concentrated using a rotary evaporator. All the extracts were evaluated for their biological activities.

The 95% ethanol extract was further fractionated by column chromatography on silica gel Si 60 (Merck® Germany), using a gradient of dichloromethane, methanol, and water as the eluents. The subfractions were monitored by TLC chromatography under UV 254 and 366 nm, using a solvent system of chloroform, ethyl acetate, methanol, and glacial acetic acid (7:1.5:0.5:0.2, Carlo Erba® Italy). All extracts and the collected fractions were evaluated for their antioxidant, anti-tyrosinase, and cytotoxic activities.

Anti-Tyrosinase Activity Assay

The tyrosinase inhibitory activity of the extract was evaluated using L-DOPA. We employed the 96-well microplate method, which is convenient for a screening assay. Masuda and Yamashita's method [6] was used with slight modifications. Briefly, a total of eight wells were designated for A (three wells), B (one well), C (three wells), and D (one well) containing the following reaction mixture: 120 µl of a 1/15 M phosphate buffer (pH 6.8) and 40 µl of tyrosinase (46 unit/ml) (Sigma®, USA) in the same buffer, 40 µl of the sample solution containing 5% DMSO. The contents of each well were mixed and incubated at 25°C for 10 min before adding 2.5 mM of L-DOPA (Sigma®, USA) in the same buffer (40 µl). After incubation at 25°C for 10 min, the absorbance at 492 nm of each well was measured. The percentage inhibition of tyrosinase activity was calculated using the following equation: percentage inhibition of tyrosinase activity = {[(A-B)-(C-D)]/(A-B)}x100. The effective concentration of the sample required to scavenge tyrosinase enzyme by 50% (IC_{50} value) was obtained by linear regression analysis of a dose-response curve plotting % inhibition versus concentration.

Antioxidant Activity Assay

Antioxidant Activity Assay The antioxidant activity of the extracts was evaluated using the DPPH (1,1-diphenyl-2-picryl hydrazyl) assay [7], with some modifications. Briefly, 100 µl of each extract at various concentrations (0.0097-80 mg/ml) was added to 100 µl of 6x10-3 M DPPH ethanolic solution in a 96-well plate. The mixture was then incubated at room temperature in the dark for 20 min, and the absorbance was measured at 517 nm using a UV–VIS microplate reader. The percentage of inhibition was calculated using the following formula: Percentage of inhibition (%) = [(Acontrol-Asample)/Acontrol]x100. The IC_{50} value, defined as the concentration required to scavenge 50% of the free radicals, was determined by plotting a dose-response curve. Vitamin C (Sigma®, USA) was used as a standard in this assay. All tests and analyses were performed in triplicate, and the results were averaged."

Cytotoxicity activity assay

Cytotoxicity was assessed by measuring the effect of the extract on Mouse fibroblast cells (L929) [8]. The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco®, Germany) containing 10% (v/v) fetal bovine serum (FBS) (Gibco®, Germany) and antibiotics (penicillin at 100 U/ml and streptomycin at 100 µg/ml) at 37°C with 5% CO_2. The cells were subcultured every 2-3 days, and cells in the exponential phase were used in all experiments. The cells were seeded at a density of $2x10^5$ cells/ml in 24-well plates and grown for 48 hours prior to treatment. Serum-free DMEM medium was used to treat the cells, and the extract was freshly prepared at various concentrations (0.078-10 mg/ml). After incubation for 24 hours at 37°C with 5% CO_2, the supernatant was removed, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide) (Sigma®, USA) solution (0.5 mg/ml) was added to each well. The formazan crystals that had formed in viable cells were measured at 540 nm using a microplate reader. All experiments were performed in triplicate, and the average data from the triplicates were expressed in terms of killing percentage relative to a negative control. The percentage inhibition (%) of each test sample was calculated using the following formula: Percentage of inhibition (%) = [(Acontrol-Asample)/Acontrol] x 100. Cytotoxicity of each sample was expressed as the IC_{50} value, which is the concentration of the test compound that causes 50% inhibition or cell death. The IC_{50} value was obtained by plotting the percentage inhibition versus the concentration of the test compound.

Statistical analysis

The results are presented as the mean ± standard deviation (SD). One-way ANOVA was performed to assess the significant differences between the means of the samples and the standard, with a significance level set at $p \leq 0.05$. The post-hoc Scheffe test was employed to compare the means following the one-way ANOVA.

RESULTS

Extraction and Isolation

From the maceration extraction method using various solvents, the brown viscous extracts of hexane, dichloromethane, ethyl acetate, and 95% ethanol extracts were obtained.

Subsequently, a portion of the ethanol extract was separated using column chromatography, which utilized a stationary phase of silica gel and was eluted with a gradient of dichloromethane, methanol, and water. This yielded a total of 1,661 fractions. The fractions that exhibited similar characteristics as determined by thin layer chromatography were combined, resulting in a total of five fractions (Fr1-Fr5). The entire extracts and the five fractions were then subjected to biological activity testing.

Anti-tyrosinase activity assay

The inhibitory activity of the extracts and fractions from *T. reidioides* against tyrosinase was evaluated by determining their IC_{50} values. The IC_{50} values represent the concentration required to inhibit 50% of the enzyme's activity, with lower values indicating stronger inhibitory activity.

Among the extracts, the ethanol extract exhibited the strongest antityrosinase activity, with an IC_{50} value of 5.38 ± 0.76 µg/ml. This suggests that the ethanol extract contains potent tyrosinase inhibitory compounds. The hexane and dichloromethane extracts also showed significant antityrosinase activity, with IC_{50} values of 13.80±0.53 µg/ml and 15.18±2.01 µg/ml, respectively.

Further fractionation of the ethanol extract resulted in five fractions (Fr1-Fr5), which were also tested for their antityrosinase activity. Fraction 2 showed the highest activity among the fractions, with an IC_{50} value of 15.9±1.13 µg/ml. Fractions 1, 3, 4, and 5 also exhibited moderate antityrosinase activity, with IC_{50} values ranging from 18.19±0.24 to 24.81±2.94 µg/ml.

Antioxidant activity assay

The antioxidant activity is summarized in Table I. The antioxidant activity of the various extracts and fractions from the plant material was evaluated using the IC_{50} values, which represent the concentration required to scavenge 50% of the free radicals in the assay. The lower the IC_{50} value, the higher the antioxidant activity.

Table 1. Biological activity of the *T. reidioides* extract and fraction from column chromatography.

	Antityrosinase activity (IC_{50}, µg/ml)	Antioxidant activity (IC_{50}, µg/ml)	Cytotoxic activity (IC_{50}, µg/ml)
Hexane extract	13.80±0.53 [b]	128.46±9.02 [c]	40.12±1.98 [a]
Dichloromethane	15.18±2.01 [b]	38.40±0.81 [b]	1.71±0.08 [b]
Ethyl acetate extract	37.19±1.83 [d]	1.93±0.17 [a]	1.77±0.09 [b]
Ethanol extract	5.38±0.76 [a]	1.93±0.018 [a]	4.63±0.03 [b]
Fraction 1	23.34±2.00 [c]	169.39±6.10 [c]	2.57±0.09 [b]
Fraction 2	15.9±1.13 [b]	22.96±1.42 [b]	2.07±0.06 [b]
Fraction 3	18.19±0.24 [b]	1.65±0.09 [a]	2.01±0.11 [b]
Fraction 4	24.81±2.94 [c]	1.74±0.26 [a]	11.82±0.59 [a]
Fraction 5	13.52±0.93 [b]	15.45±0.53 [b]	35.26±4.43 [a]
Kojic acid	0.047±0.01 [a]	nd	nd
Ascorbic acid	nd	1.48 ± 0.19[a]	nd

Means with different letters in the same column indicate signifi cant difference ($p < 0.05$)
nd = not determined.

Among the extracts, the ethyl acetate and ethanol extracts exhibited the most potent antioxidant activity, with IC_{50} values of 1.93±0.17 µg/ml and 1.93±0.018 µg/ml, respectively. These results indicate that these extracts have strong free radical scavenging abilities and can effectively inhibit oxidative damage.

Fraction 3 and Fraction 4 exhibited significant antioxidant activity, with IC50 values of 1.65±0.09 µg/ml and 1.74±0.26 µg/ml, respectively. These fractions demonstrated comparable antioxidant potential to the ethyl acetate and ethanol extracts, as well as to the standard antioxidant ascorbic acid, further emphasizing their effectiveness in neutralizing free radicals.

Cytotoxicity activity assay

The cytotoxicity of the *T. reidioides* extracts and fractions was evaluated using mouse fibroblast cells (L929), which are widely utilized as an in vitro model for skin toxicity studies (as shown in Table I). The results indicated that the extracts and fractions with the least cytotoxicity (higher IC50 values indicating lower toxicity) were the hexane extract, Fraction 5 (Fr. 5), and Fraction 4 (Fr. 4). These samples demonstrated IC_{50} values of 40.12±1.98 µg/ml, 35.26±4.43 µg/ml, and 11.82±0.59 µg/ml, respectively.

DISCUSSION

Anti-tyrosinase activity assay

The outcomes of our anti-tyrosinase assays contribute significant insights into the potential of *T. reidioides* as a source of bioactive compounds for dermatological applications. Our results demonstrate the noteworthy inhibitory activity against tyrosinase, a key enzyme in melanin biosynthesis. Specifically, the ethanol extract exhibited robust anti-tyrosinase activity, with an IC50 value of 5.38 ± 0.76 µg/ml. This aligns with the traditional use of *T. reidioides* for skin whitening, substantiating its efficacy in mitigating melanin production.

Our findings also extend to fractionation, where Fraction 2 emerged as particularly potent, displaying the highest anti-tyrosinase activity with an IC_{50} value of 15.9 ± 1.13 µg/ml. Additionally, Fractions 1, 3, 4, and 5 exhibited moderate inhibitory activity. This underscores the complexity of *T. reidioides'* chemical composition, with different fractions potentially harboring distinct bioactive compounds. These observations resonate with the study by Srichayanurak C [9] supporting the assertion that *T. reidioides* is a promising candidate for anti-hyperpigmentation formulations.

Our discussion aligns with the broader literature on plant-derived anti-tyrosinase compounds. Studies by Kim et al.[10] and Sasaki et al. [11] have reported tyrosinase inhibitory effects in various plant extracts, attributing these effects to phenolic compounds like flavonoids and tannins. Consistent with these reports, our study identifies similar phenolic compounds in the *T. reidioides extract*, suggesting a potential mechanism for its anti-tyrosinase activity.

Antioxidant activity assay

The findings from our antioxidant activity assay resonate with established literature, notably the work of Srichayanurak C[9] affirming the robust free radical-scavenging properties of *T. reidioides* root extracts. The conspicuous antioxidant activity observed in our study can be attributed to the presence of bioactive compounds, such as flavonoids and phenolics, consistent with the antioxidant potential demonstrated in previous investigations[12].

Our results highlight the ethyl acetate and ethanol extracts as particularly potent in neutralizing free radicals, with IC_{50} values of 1.93±0.17 µg/ml and 1.93±0.018 µg/ml, respectively. These observations align with other studies, such as Mustarichie et al [13]. which reported strong antioxidant effects in ethyl acetate and ethanol extracts from medicinal plants. Additionally, the antioxidant potential of these extracts may be linked to specific phenolic compounds, as evidenced by the research of Sergio et al.[14] who emphasized the contribution of phenolics to robust antioxidant activity.

Fraction 3 and Fraction 4 demonstrated noteworthy antioxidant activity, with IC_{50} values of 1.65±0.09 µg/ml and 1.74±0.26 µg/ml, respectively. These fractions exhibited antioxidant potential comparable to the ethyl acetate and ethanol extracts, as well as the standard antioxidant ascorbic acid. This aligns with the findings of Deswati et al.[15] who identified potent antioxidant effects in specific fractions of natural extracts. The efficacy of Fractions 3 and 4 in neutralizing free radicals underscores the multifaceted antioxidant profile of *T. reidioides*.

In a broader context, the antioxidant properties observed in *T. reidioides* extracts and fractions underscore their potential significance in addressing oxidative stress-related conditions. Furthermore, their application in the development of whitening cosmetics is noteworthy.

Cytotoxicity activity assay

Our investigation into the cytotoxicity of *T. reidioides* extracts and fractions, assessed using mouse fibroblast cells (L929), provides critical insights into their safety profile, especially in the context of skin toxicity (as depicted in Table I). The results reveal that the hexane extract, Fraction 5 (Fr. 5), and Fraction 4 (Fr. 4) exhibit the least cytotoxicity, as evidenced by their higher IC_{50} values (indicating lower toxicity): 40.12±1.98 µg/ml, 35.26±4.43 µg/ml, and 11.82±0.59 µg/ml, respectively. This aligns with prior research highlighting the cytotoxic properties of Rediocide G, a compound isolated through column chromatography and eluted with dichloromethane[8]. The consistent findings between our study and existing literature underscore the potential toxicity associated with extracts obtained using dichloromethane as a solvent. Notably, our study identifies the dichloromethane extract and the ethyl acetate extract (solvents with similar polarity) as demonstrating significant toxicity toward mouse fibroblast cells (L929) at elevated concentrations.

CONCLUSION

In summary, this study has elucidated that Fraction 4 and Fraction 5, extracted from *T. reidioides*, manifest robust free radical-scavenging capabilities, noteworthy antityrosinase activity, and minimal cytotoxicity towards mouse fibroblast (L929) cells. These compelling results imply that Fraction 4 and Fraction 5 harbor promising attributes

for prospective development as skin-whitening agents derived from *T. reidioides* extracts. The multifaceted efficacy demonstrated by these fractions underscores their potential significance in cosmetic formulations targeted at skin health and pigmentation modulation. Further investigations, including in-depth characterization of the active compounds within these fractions, will contribute to harnessing the full therapeutic potential of *T. reidioides* extracts in skincare applications.

ACKNOWLEDGMENTS

This research received financial support from the state budget of Mahasarakham University.

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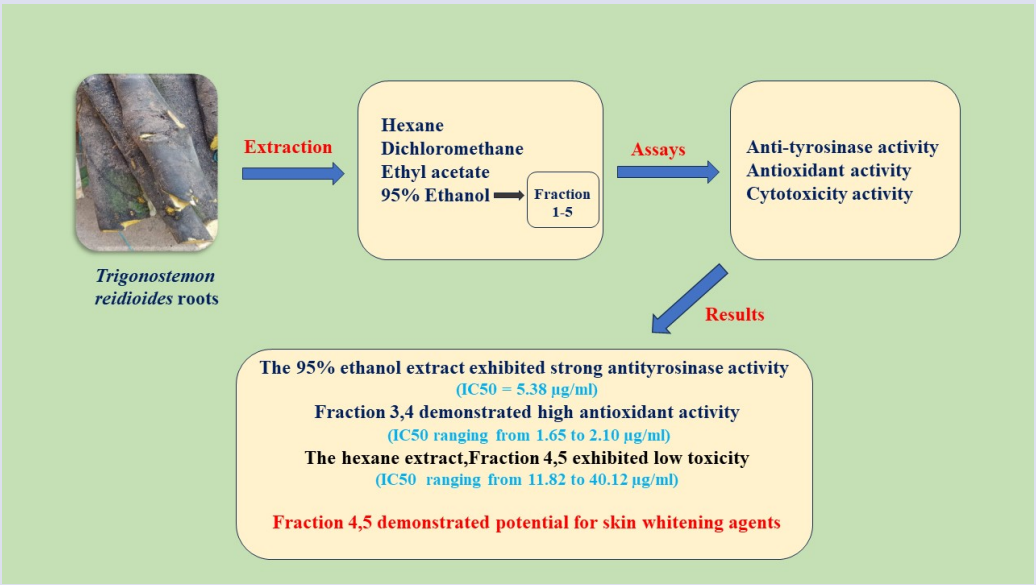
GRAPHICAL ABSTRACT



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Cite this article: Chummalee I, Phadungkit M, Laovachirasuwan P. Assessment of Anti-tyrosinase, Antioxidant and Cytotoxic Activities of *Trigonostemon reidioides* Extracts on Mouse Fibroblast (L929) Cells. Pharmacogn J. 2024;16(2): 302-306.