The Pharmaceutical Journal of Sri Lanka 2024, 14(1): Pages: 11-18

DOI: https://doi.org/10.4038/pjsl.v14i1.98

Research Article

In vitro synergistic evaluation of tyrosinase inhibition and antioxidant activities of *Mimosa pudica* and *Glycyrrhiza glabra* plant extracts for the treatment of hyperpigmentation

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Revised: 10 December 2024; Accepted: 30 December 2024

ABSTRACT

Purpose: This study aimed to evaluate the tyrosinase inhibitory activity of a combination of *Mimosa pudica* and *Glycyrrhiza glabra* extracts to explore potential synergistic effects and to assess the antioxidant activity of the individual extracts. The findings support the development of a polyherbal topical formulation for hyperpigmentation treatment.

Methods: The ethanolic extract of *G. glabra* roots was obtained via reflux condensation at 60°C, while the methanolic extracts of *M. pudica* leaves and seeds were prepared at 50°C. The mushroom tyrosinase inhibition assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay were conducted in triplicate to evaluate the extracts' enzyme inhibitory and antioxidant activities, respectively. *M. pudica* leaves extract, *M. pudica* pods extract, and *G. glabra* roots extract were tested as a formulated mixture (1:1:2 ratio) for tyrosinase inhibition. A brine shrimp lethality assay was conducted to assess toxicity.

Results: The extract mixture exhibited potent tyrosinase inhibition, with a 50% inhibitory concentration (IC_{50}) of 47.47 ± 0.42 µg/mL, surpassing the standard inhibitor, kojic acid (IC_{50} = 76.61 ± 0.80 µg/mL). The *M. pudica* leaves extract exhibited antioxidant activity with an IC_{50} of 34.26 µg/mL, followed by *M. pudica* pods extract at 75.09 µg/mL, and *G. glabra* roots extract at 95.82 µg/mL, compared to the standard inhibitor ascorbic acid at 11.56 µg/mL. All three plant extracts were non-toxic to brine shrimp at concentrations that exhibited tyrosinase inhibition and antioxidant activity.

Conclusion: The combination of *M. pudica* leaves and seed extracts with *G. glabra* root extract exhibited notable tyrosinase inhibition and antioxidant activities at concentrations below 100 µg/mL, supporting its potential as a polyherbal topical formulation for hyperpigmentation management. Notably, *M. pudica* leaf extract demonstrated prominent antioxidant activity.

Keywords: antioxidants, *Glycyrrhiza glabra,* hyperpigmentation, *Mimosa pudica,* tyrosinase inhibition

INTRODUCTION

Hyperpigmentation is a condition characterized by the formation of dark spots on the skin due to the overproduction of melanin. Various topical agents are available for its treatment, with hydroquinone, kojic acid, arbutin, and vitamin C being commonly used. However, these synthetic agents are associated with side-effects such as skin irritation, erythema, and, in rare cases, exogenous ochronosis. (1) Formulations incorporating natural herbs such as *Glycyrrhiza glabra*, *Panax ginseng*, *Emblica officinalis*, *Azadirachta indica*, and *Curcuma longa* have been used to treat skin hyperpigmentation. Furthermore, natural phytoconstituents, including ellagic acid and quercetin, have demonstrated efficacy in managing hyperpigmentation. (2) Understanding the benefits of botanical extracts presents an opportunity for developing novel products to address hyperpigmentation. (3)

The plant *Mimosa pudica*, belonging to the family Fabaceae, is a prostrate or semi-erect subshrub primarily found in tropical regions of America, Australia, and India. (4) Its pharmacological profile suggests its potential as a promising herbal candidate for further investigation, as it has been reported to possess antibacterial, antivenom, antifertility, anticonvulsant, antidepressant, aphrodisiac, and wound-healing properties. (4) Previous research has indicated that leaf and seed extracts of *M. pudica* exhibit moderate antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and contain significant amounts of flavonoids and phenolic compounds. (4-6) Natural antioxidants generally have fewer adverse effects and greater efficacy in disease management.

The plant *G. glabra*, also known as "licorice" or "sweet wood," is a perennial shrub belonging to the Fabaceae family. It is native to the Mediterranean region and parts of Southwest Asia, particularly the Indian subcontinent. Previous studies have demonstrated that *G. glabra* root extract exhibits potent antioxidant activity in the DPPH assay. (7) The phenolic content of this plant is responsible for its strong antioxidant activity, which has been shown to be beneficial in protecting biological systems against oxidative stress and preventing various types of skin damage. Additionally, its tyrosinase-inhibitory properties are crucial in reducing hyperpigmentation. Studies have suggested that compounds with significant tyrosinase-inhibitory potential could be used to assess the whitening efficacy of cosmetics and monitor color changes associated with skin conditions. (8)

A study on *G. glabra* extracts revealed that the root extract exhibited a stronger inhibitory effect on mushroom tyrosinase than the leaf extract. (9) The bioactive compounds responsible for this inhibition include glabridin, glabrene, isoliquiritigenin, licochalcone A, and liquiritin. (10-12) Furthermore, a tyrosinase inhibition assay conducted on *M. pudica* seed extract demonstrated significant inhibitory activity against mushroom tyrosinase. (6) Given the antioxidant and tyrosinase-inhibitory activities of these two plant extracts, this study aimed to evaluate their synergistic potential in reducing hyperpigmentation. The objective of this research was to assess the tyrosinase inhibitory activity of a combination of *M. pudica* and *G. glabra* plant extracts to explore potential synergistic effects, while also independently determining the antioxidant activity of the individual extracts. This study was conducted with the goal of supporting the development of a polyherbal topical formulation for hyperpigmentation treatment.

METHODS

Plant sample preparation and authentication

The roots of *G. glabra* and the leaves and pods of *M. pudica* were thoroughly washed, oven-dried at 40°C, and ground into a fine powder. The prepared herbarium specimen of *M. pudica* was authenticated by the National Herbarium at the Peradeniya Botanical

Gardens, Sri Lanka. Commercially available *G. glabra* root samples were obtained from '*Bowatte Beheth Shalawa*' in Kandy, Central Province, and identified through morphological analysis.

Plant extraction

Each powdered plant material was subjected to reflux condensation separately using a reflux condenser. Initially, 10 g of powdered *M. pudica* leaves were refluxed with 30 mL of methanol at 50 °C for one hour. Subsequently, 10 g of powdered *G. glabra* roots were refluxed with 50 mL of ethanol at 60 °C for one hour. Finally, 10 g of powdered *M. pudica* pods were refluxed with a 4:1 mixture of ethanol (40 mL) and water (10 mL) at 50 °C for one hour. The resulting extracts were filtered using Whatman grade 1 filter paper and a Büchner funnel under vacuum filtration. The filtrates were then evaporated using a water bath at 40 °C to obtain dried extracts.

Plant extraction yield

The extraction yields of *Mimosa pudica* and *Glycyrrhiza glabra* were determined by calculating the proportion of the extracted weight obtained from the initial weight of the powdered plant material. The percentage yield was derived by dividing the weight of the dried extract by the total weight of the powdered plant material used for extraction and then multiplying by 100 to express the value as a percentage. Each dried extract was weighed, and the percentage yield was recorded for further analysis.

***In vitro* DPPH assay**

The DPPH assay was performed following the method described previously. (7) The antioxidant activity of each plant extract was evaluated using a DPPH solution. Different concentrations (500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, and 15.625 ppm) of each extract were prepared in absolute methanol (100%), and 1 mL of each concentration was added to 1 mL of 0.1 mM DPPH radical solution in methanol. The reaction mixture was incubated in test tubes for 30 minutes, during which the color change from deep violet to light yellow was monitored. Absorbance was measured at 517 nm against a blank solution using a UV-Vis spectrophotometer. Ascorbic acid was used as the standard antioxidant. Each assay was performed in triplicate, and the percentage inhibition of free radical DPPH was calculated.

***In vitro* mushroom tyrosinase inhibitory assay**

The tyrosinase inhibition assay was conducted following the method described in a previous study, using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. (13) A single mixture of *M. pudica* leaf extract, *G. glabra* root extract, and *M. pudica* pod extract in a 1:2:1 ratio was subjected to the assay. This ratio was determined based on previous studies. The assay mixture contained 10 µL of aqueous mushroom tyrosinase solution (50 units/mL), 80 µL of phosphate buffer (pH 6.8), and the plant extract mixture. This was pre-incubated at 37°C for five minutes, followed by the addition of 90 µL of L-DOPA (2 mg/mL). The reaction mixture was then incubated for an additional 20 minutes at 37 °C. The formation of dopachrome was measured at 475 nm. Kojic acid was used as the standard tyrosinase inhibitor. The percentage inhibition of tyrosinase activity was then calculated.

***In vitro* lethal toxicity assay**

The brine shrimp lethality assay was conducted for *M. pudica* leaf, pod, and *G. glabra* root extracts. Artificial seawater (1 L) was prepared in the laboratory by dissolving sodium chloride (24.6 g), potassium chloride (0.67 g), calcium chloride dihydrate (1.36 g), magnesium sulfate heptahydrate (6.29 g), magnesium chloride hexahydrate (4.66 g), and sodium bicarbonate (0.18 g) in distilled water (q.s.). The solution was transferred to a glass tank, and the pH was adjusted to 8.0 (14) The medium was aerated using an air pump, and brine shrimp eggs were added to the surface. After 24 hours under continuous illumination with a 60 W bulb, hatched nauplii were collected. Ten nauplii were transferred to each test tube containing 5 mL of *M. pudica* leaf, pod, and *G. glabra* root extracts at different concentrations (500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm, and

15.625 ppm) using a Pasteur pipette. The number of surviving nauplii was counted after 24 hours, and the lethality percentage at each concentration was calculated. (15)

RESULTS

The yield of *M. pudica* leaf extract was 8.7%, obtained by extracting 0.87 g from 10 g of powdered plant material. Similarly, the yield of *M. pudica* pod extract was 7.1%, with 0.71 g extracted from 10 g of powder. The *G. glabra* root extract had a yield of 16.9%, based on an extracted weight of 1.69 g from 10 g of powdered material.

DPPH assay

The DPPH assay results indicated that the *M. pudica* leaf extract exhibited the highest antioxidant activity, with a 50% inhibitory concentration (IC_{50}) of 34.26 µg/mL, followed by the *M. pudica* pod extract (IC_{50} = 75.09 µg/mL) and *G. glabra* root extract (IC_{50} = 95.82 µg/mL). In comparison, the standard inhibitor, ascorbic acid, demonstrated a significantly lower IC_{50} of 11.56 µg/mL. The DPPH inhibition curves corresponding to these extracts are presented in Figure 1.

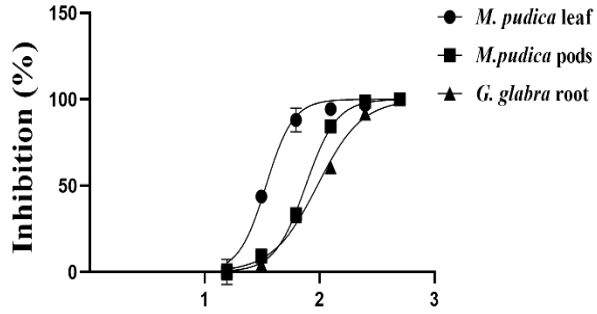

Figure 1. DPPH inhibition curves of plant extracts

Mushroom tyrosinase inhibitory assay

In the mushroom tyrosinase inhibitory assay, the mixture combining all three plant extracts in a 1:2:1 ratio exhibited substantial tyrosinase inhibitory activity, with an IC_{50} of 47.47 ± 0.42 µg/mL. This inhibition was notably greater than that of the standard inhibitor, kojic acid, which displayed an IC_{50} of 76.61 ± 0.80 µg/mL. The tyrosinase inhibition profile for the combined extract is presented in Figure 2.

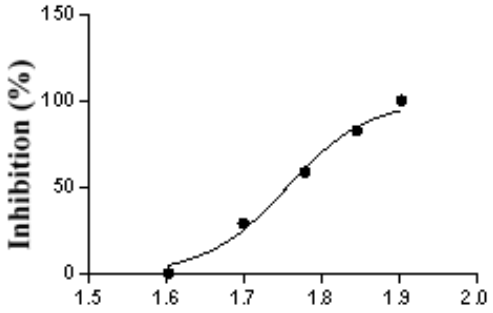

Figure 2. Tyrosinase inhibition curve of *M. pudica* leaves extract, *G. glabra* roots extract, and *M. pudica* pods (1:2:1) mixture

Lethal toxicity assay

All three plant extracts were non-toxic to brine shrimp at the concentrations that exhibited tyrosinase inhibition and antioxidant activities. The 50% lethality (LC_{50}) was determined as 383.8 µg/mL for *M. pudica* pod hydroethanolic extract and *G. glabra* root ethanolic extract, while *M. pudica* leaf methanolic extract exhibited 50% lethality at 192.1 µg/mL.

DISCUSSION

The production of reactive oxygen species can lead to oxidative stress, causing damage to cells, DNA, lipids, and proteins. This imbalance between reactive oxygen species and antioxidants can have deleterious effects on biological systems. Antioxidants play a crucial role in inhibiting reactive oxygen species production, scavenging free radicals, and preventing oxidative damage. Research has shown that plants like *M. pudica* and *G. glabra* contain polyphenols and flavonoids, making them potential sources of antioxidants. (5, 6, 9, 16-19) Studies have demonstrated moderate antioxidant activity in these plants as measured by assays such as the DPPH scavenging assay. (5,6,9)

In this study, the ethanolic extract of *G. glabra* roots showed an IC_{50} value of 95.82 µg/mL, indicating its antioxidant potency. This value was lower than those reported in a previous study for the methanol/water extract of *G. glabra* roots (IC_{50} = 112 µg/mL). (17) A comparable IC_{50} value was observed in another study, where the methanolic extract of *G. glabra* roots exhibited an IC_{50} of 116.17 µg/mL. (3) However, the obtained concentration was higher than the values reported in another study for aqueous (IC_{50} = 62 µg/mL) and ethanol (IC_{50} = 50 µg/mL) extracts of Turkish licorice roots. (7) Similarly, the methanolic extract of *M. pudica* leaves exhibited an IC_{50} value of 34.26 µg/mL, indicating potent antioxidant activity compared to previously reported values. However, this value was lower than the IC_{50} recorded in other studies for the methanolic extract (IC_{50} = 67.85 µg/mL) (5) and for the aerial parts of the plant, which reported IC_{50} values of 126.71 µg/mL (19) and 296.92 µg/mL (4). Additionally, the ethanol:water (4:1) extract of *M. pudica* seeds demonstrated an IC_{50} value of 75.09 µg/mL (6), further supporting its antioxidant activity. Overall, these findings underscore the potential of *M. pudica* and *G. glabra* as sources of antioxidants, reinforcing their therapeutic applications against oxidative stress-related conditions.

Mushroom tyrosinase from species such as *Agaricus bisporus* is used to evaluate tyrosinase inhibition, which is involved in melanogenesis in humans. In the mushroom tyrosinase assay, dopachrome formation was monitored in the reaction mixture. (20) In this study, *M. pudica* leaves extract, *G. glabra* roots extract, and *M. pudica* pod extract were subjected to a tyrosinase inhibitory assay as a combined mixture in a 1:2:1 ratio. The resulting IC_{50} value was 47.47 ± 0.42 µg/mL. In a previous study, IC_{50} values of 114 ± 5.7 µg/mL and 74 ± 1.1 µg/mL were reported for the 70% and 95% ethanolic extracts of *G. glabra* roots, respectively, in the mushroom tyrosinase inhibition assay. (21) Similarly, specific components such as glabrene, isoliquiritigenin, and glabridin in *G. glabra* root extract were identified as tyrosinase inhibitors, with an IC_{50} value of 53 µg/mL. (10)

For *M. pudica*, a hydro-alcoholic extract of the seeds demonstrated 90% mushroom tyrosinase inhibition at a concentration of 1 mg/mL. (6) In contrast, a methanol: water (1:1) extract of *M. pudica* leaves showed only 15% inhibition when tested at 50 µg/mL. (22)

However, research on the tyrosinase-inhibiting activity of *M. pudica* leaves and pods remains limited. Therefore, the current study focused on a combined extract containing all three plant components, which exhibited significant tyrosinase-inhibitory activity, with an IC_{50} value of 47.47 ± 0.42 µg/mL.

The brine shrimp lethality assay using *Artemia salina* was employed to assess the toxicity of plant extracts. This assay serves as a preliminary cytotoxicity evaluation, measuring the ability of plant extracts to induce lethality in laboratory-cultured brine shrimp nauplii. Approximately 22 mm in length, brine shrimp nauplii serve as a convenient test subject due to their observable size without high magnification and their ability to hatch in large numbers within limited laboratory space. (15)

In this study, the lethality of various plant extracts was evaluated at different concentrations over a 24-hour exposure period. The results indicated 50% lethality at 383.8 µg/mL for the hydro-ethanolic extract of *M. pudica* pods and the ethanolic extract of *G. glabra* roots. For the methanolic extract of *M. pudica* leaves, 50% lethality was observed at 192.1 µg/mL.

However, contrasting results were reported in a previous study, (19) which observed 80% lethality for the methanolic extract of *M. pudica* leaves at 500 µg/mL, with an LC_{50} value of 282.35 µg/mL. This disparity in findings could be attributed to variations in the composition of artificial seawater solutions used in the assays. Factors such as salinity levels, pH variations, dissolved oxygen levels,

and water purity may influence brine shrimp metabolic rates and stress responses, potentially affecting lethality outcomes. (23, 24)

Additionally, a previous study reported an LC_{50} value of 318.43 µg/mL for the ethanolic extract of *G. glabra* roots, which is comparable to the LC_{50} value of 383.8 µg/mL recorded in the current study for the same extract. (25) Thus, the similarity in LC_{50} values suggests a consistent toxicity profile for *G. glabra* root extracts across studies.

In summary, the brine shrimp lethality assay provided insights into the toxicity of plant extracts, with notable variations observed between different extracts and concentrations. These findings underscore the importance of considering assay conditions and experimental variations when interpreting cytotoxicity results.

CONCLUSION

This study demonstrated the potential of *M. pudica* and *G. glabra* extracts as effective natural agents for hyperpigmentation management. The combination of these extracts exhibited significant tyrosinase-inhibitory activity, with an IC_{50} value of 47.47 ± 0.42 µg/mL. Furthermore, *M. pudica* leaf extract showed the most potent antioxidant activity (IC_{50} = 34.26 µg/mL), followed by *M. pudica* pod and *G. glabra* root extracts, highlighting their ability to neutralize free radicals and mitigate oxidative stress. The low cytotoxicity of these extracts, as determined by the brine shrimp lethality assay, further supports their suitability for dermatological applications.

The findings suggest a potential synergistic interaction between the plant extracts in tyrosinase inhibition, making them promising candidates for the development of polyherbal formulations aimed at treating hyperpigmentation. This research not only underscores the cosmeceutical potential of *M. pudica* and *G. glabra* but also lays the groundwork for future studies to explore the efficacy, physicochemical stability, and long-term safety of topical formulations derived from these extracts. Such formulations could offer a plant-based, effective, and safer alternative to synthetic treatments for hyperpigmentation, addressing the growing demand for natural skincare solutions.

ACKNOWLEDGEMENT:

The authors express their profound gratitude to the Faculty of Health Sciences, CINEC Campus, for their institutional support. Special appreciation is extended to Senior Professor Menik Hettihewa, Dean of the Faculty of Health Sciences, CINEC Campus, for her unwavering encouragement and academic guidance. Furthermore, the authors acknowledge the invaluable expertise and technical contributions of Dr. Sumudu N. Warnakulasuriya, Dr. Chinthaka De Silva, and Dr. Gavini Liyanaarachchi of the Industrial Technology Institute of Sri Lanka.

AUTHOR'S DECLARATION:

The authors declare that all persons listed as authors have read and given approval for the submission of this manuscript.

CONFLICTS OF INTEREST:

The authors declare that they have no conflict of interests.

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