



Preliminary Screening for Potential Compounds in Inhibiting Enzyme Tyrosinase from 11 Herbal Extracts

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Considering the emerging trends of using natural ingredients in the cosmetic industry, this study was undertaken to confirm the efficiency of some traditional skin-whitening plants in Vietnam. In this study, 11 popular Vietnamese herbal extracts were investigated in suppressing mushroom tyrosinase activity. The most potent material was then analyzed for phytochemicals profiles and the extraction process was optimized by single-factor experiments. It was found that *Morus alba* leaves extract using ethanol had superior mushroom tyrosinase inhibitory activity. The extract under optimal conditions (extract 2 cycles with ethanol 70%, temperature: 50 °C, time: 40 min and solid/liquid ratio = 1:10) contained polyphenols, flavonoids, saponins and tannins and its biological properties included: antityrosinase IC₅₀ = 99.4 µg/mL, antioxidation IC₅₀ (DPPH assay) = 85.7 µg/mL and total phenolic content TPC (GAE) = 128.6 mg/g extract.

Keywords: Antityrosinase, Antioxidation, Cosmetic, Herbal extract, *Morus alba* leaves.

INTRODUCTION

Melanin is a complex-structured, natural pigment that gives colours to humans' and animals' eyes, hair and skin [1]. However, abnormal accumulations of melanin pigments may result in various skin disorders, including melasma, darker spots, freckles and bruises, called hyperpigmentation [2]. In most treatment methods related to melanin hyperpigmentation, tyrosinase is regarded as the main target, as it plays a key role in mammalian melanogenesis or melanin production [3]. Tyrosinase speeds up the hydroxylation of L-tyrosine to 3,4-dihydroxyphenyl-alanine (L-DOPA), which is then oxidized to the corresponding DOPA quinone. From this point, melanin is eventually formed by a combination of enzymatically catalyzed and chemical reactions [3-5].

Over the past few years, many potent tyrosinase inhibitors, including natural, semi-synthetic and synthetic sources had been discovered. Among them, hydroquinone and its derivatives were once regarded as the standard treatment for hyperpigmentation. However, with reports of causing permanent damage to the melanosomes and potential mutagenicity [6,7], there has been an increase in demand for alternative, natural

antityrosinase sources due to their less toxicity and better bio-availability, especially for cosmetic applications [4].

Herbal extracts are added into the cosmetic formulation for various benefits, such as improving skin tone, texture and appearance while avoiding many side effects and irritation, which are commonly seen in synthetic products [8]. Some of the most well-known herbs *e.g.* ginseng (*Panax ginseng* C.A. Mey.) leaves extract is excellent moisturizing, antiaging and whitening ability [9], polysaccharides from mushroom *Ganoderma lucidum* can reduce melanogenesis through disrupting the cAMP/PKA signals [10], Goji berry (*Lycium chinense* Mill.) root extract helps in curing hyperpigmentation through the MAPK and PKA signaling pathways and its antioxidation activity [11]. Moreover *Piper belte* L. was recently reported as an effective natural tyrosinase inhibitor in 2021 [12] and the litchi extract (*Litchi chinensis* Sonn.) was clinically confirmed as a safe and effective in hyperpigmentation treatment [13].

In Vietnam, several plants are blended for face masks, herbal bathing or herbal steaming as an effective natural skin whitening treatment. Although some of them had been studied and recognized as a potent source of natural enzyme tyrosinase inhibitors *e.g.* *Oxalis corniculata* L. [14], *Piper betle* L. [12],

Camellia sinensis [15], *Perilla frutescens* [16,17]. Therefore, keeping in mind, in this work, 11 popular herbals viz. *Piper betle* L., *Morus alba* L., *Oxalis corniculata* L., *Camellia sinensis* L., *Perilla frutescens* L., *Artemisia vulgaris* L., *Centella asiatica* L. Urban, *Houttuynia cordata* Thunb., *Moringa oleifera* L. and the peel of 2 Vietnamese pomelos i.e. *Citrus grandis* L. Obs. cv. *Nam Roi* and *Citrus grandis* L. Obs. cv. *da xanh* from Vietnam are investigated for their antityrosinase activities. This work will be a preliminary study to broaden the material choices of natural ingredients in the cosmetic industry as well as to enhance the economical values of Vietnamese agriculture products.

EXPERIMENTAL

Eleven plants, including 2 types of pomelo's peel collected at Ben Tre, Vietnam in 10/2020 and 9 herbal leaves collected in Buon Ho, DakLak, Vietnam in 02/2020 were dried until the moisture was < 12%, then ground into powder. The chemicals viz. DPPH, ascorbic acid (99.4%), kojic acid (99.0%), enzyme tyrosinase (from mushroom) and L-DOPA were purchased from Sigma Chemical Co., USA, whereas gallic acid (99.5%) and Folin-Ciocalteu reagent were procured from Merck, Germany.

The sample absorbent was measured by UV-Vis Thermo Genesys 10S UV-Vis and Elisa Microplate Reader 2100-C. The moisture was recorded using Sartorius MA37-1.

General procedure: In this study, the extract of 11 herbals (*Piper betle* L., *Morus alba* L., *Oxalis corniculata* L., *Camellia sinensis* L., *Perilla frutescens* L., *Artemisia vulgaris* L., *Centella asiatica* L. Urban, *Houttuynia cordata* Thunb., *Moringa oleifera* L. and the peel of 2 Vietnamese pomelos i.e. *Citrus grandis* L. Obs. cv. *Nam Roi*, *Citrus grandis* L. Obs. cv. *da xanh*) were prepared in water and absolute ethanol solvent. The most promising sample in terms of mushroom tyrosinase enzyme inhibition will be chosen for the preliminary phytochemicals analysis and its extraction process was optimized using the single variable optimization technique.

Preliminary phytochemicals analysis: The phytochemical profiles of 11 herbals were determined by using following standard methods e.g. alkaloid content [18], flavonoid [19,20], saponin content [21], polyphenol content [22] and tannin content [23].

Antityrosinase activity: The mushroom enzyme tyrosinase inhibitory activity was performed according to the Kamkaen *et al.* [24] method with some modifications. Briefly, 240 µL extract in DMSO and tyrosinase (40 µL, 200 U/mL) were added into tubes. After incubation at 25 °C for 10 min, the absorbance at wavelength 475 nm was measured. DMSO (5%, 240 µL) and kojic acid were used as the blank reference and positive control, respectively. The percent of enzyme tyrosinase being inhibited was calculated at an extract concentration between 0 and 1000 (µg/mL) and the concentration at 50% tyrosinase inhibition was expressed as IC₅₀ value.

Antityrosinase activity: The antioxidation activity was evaluated by the DPPH radical scavenging assay as described by Sharma & Bhat [25]. In brief, DPPH solution (30 µg/mL) in aqueous methanol 80% with the optical density (OD) of 0.8 ± 0.02 and the extract with different concentrations (0-1000 µg/mL in 80% aqueous methanol) were freshly prepared. The

reaction mixtures, including 120 µL of extract solution and 180 µL of DPPH were vortexed and incubated for 30 min at 30 °C, then the optical density at λ = 517 nm was measured. The colour of the extract and blank samples were prepared using 80% aqueous methanol solution. Vitamin C solutions (120 µL, 0-100 µg/mL) were used as positive control and the DPPH radical scavenging activity was evaluated using the following formula:

$$I_{\text{DPPH}} (\%) = \frac{A_b - (A_s - A_c)}{A_b} \times 100$$

where A is the absorbance at λ = 517 nm and A_b, A_s, A_c stands for blank sample, reaction sample and colour samples, respectively. The IC₅₀ value for DPPH radical scavenging activity was also calculated.

Extraction preparation: Dry powder (10 g) of 11 samples was extracted twice by ethanol and water (50 °C, 30 min and solid/liquid ratio = 1:10). The aliquot was collected by vacuum filtration and concentrated by rotavap (60 °C). The dry extracts were weighed to evaluate the extraction yield before any further treatments.

Extraction optimization: The extraction process of the chosen material was optimized using single-variable experiments. The independent factors were solvent concentration, time, number of cycles, temperature and solid material to solvent volume ratio (g/mL). The dependent variables were extraction efficiency and IC₅₀ value of mushroom tyrosinase inhibitory activity (at the concentration of 1000 µg/mL).

RESULTS AND DISCUSSION

Bioactivity screening: Ethanol and water are the two benign solvents that are commonly found in many cosmetic products with direct skin exposure, such as hand sanitizers, hairsprays or mouthwashes [26,27]. Therefore, in this section, the extract of 11 herbs by water (Fig. 1a) and ethanol (Fig. 1b) was compared on antityrosinase activity for further applications in the cosmetic field. Overall, the ethanol extract showed much stronger mushroom tyrosinase inhibitory activity (I) than water, with the maximum recorded I% = 57% (*Morus alba* leaves), compared to its water extract, I% = 30% (*Morus alba* leaves). This finding came as no surprise as ethanol was confirmed to effectively recover natural phenolic compounds in several studies [28,29].

The extract from *Morus alba* leaves and *Camellia sinensis* leaves provided the exceptional results among the 11 herbals when inhibited more than 50% of tyrosinase at the 1000 µg/mL concentration. However, their activity was relatively comparable, therefore, further investigations were conducted to determine the most potential material.

Fig. 2 depicted the IC₅₀ values of *Morus alba* and *Camellia sinensis* leaves against mushroom tyrosinase. Accordingly, the extract of *Morus alba* leaves had stronger antityrosinase activity (IC₅₀ = 397.7 µg/mL compared to kojic acid as positive control IC₅₀ = 9.3 µg/mL). It means that *Morus alba* leave was the most superior in suppressing enzyme tyrosinase among 11 herbals, hence it will be the target for the preliminary phytochemicals analysis and extraction optimization.

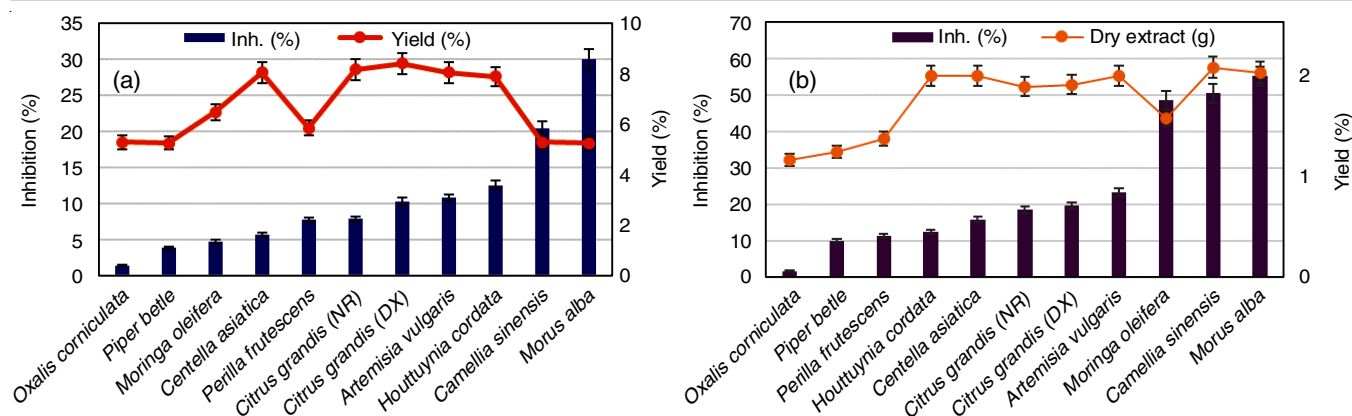
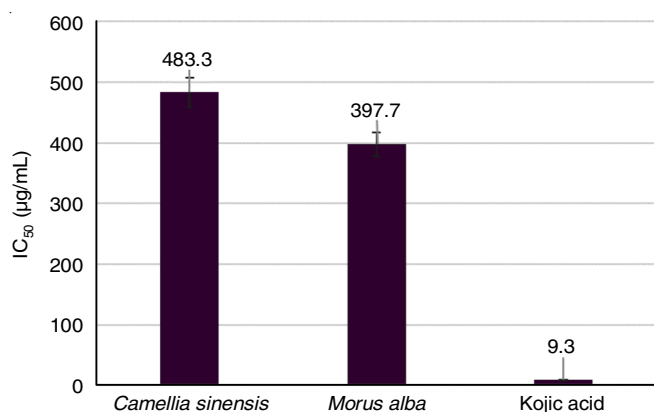


Fig. 1. (a) Water and (b) ethanol extracts of 11 herbs on antityrosinase activity

Fig. 2. IC₅₀ value of the 2 most potential materials on antityrosinase activity

Preliminary phytochemical studies: The phytochemical profile of *Morus alba* leaves extract was studied using standard methods. The results confirmed the presence of polyphenol, flavonoid, saponin and tannin in the extract. There was no sign of presence of alkaloids and the signals for the carotenoid class were not clear (Table-1).

TABLE-1 PHYTOCHEMICAL PROFILES OF <i>Morus alba</i> LEAVES EXTRACT BY ETHANOL		
Class	Reagents	Results
Carotenoid	Concentrated H ₂ SO ₄	+
Alkaloid	Dragendorff	–
	Bouchardat	–
Polyphenol	FeCl ₃ 5%	+++
Flavonoid	Cyanidin reaction	+++
	Lead(II) acetate in basic	++
Saponin	Foam test	+++
	Liberman-Burchardt	+++
Tannin	Gelatin 1%	++
– = Absent; + = Trace amount; ++ = Moderate amount; +++ = Appreciable amount.		

According to Dzialo *et al.* [30], as phenolic compounds possess similar chemical structures to tyrosinase, so likely they could be oxidized by tyrosinase and act as an analog inhibitor against melanogenesis. Therefore, this result emphasized the potential of *Morus alba* leaves as a potent source of anti-melanogenic agents.

Single-factor optimization of *Morus alba* leaves extract (MLE): To recover bioactive compounds effectively from plant materials, understanding the influences of different factors on the extraction process are of great value. Munekata *et al.* [31] suggested a list of factors associated with the recovery of natural phenolic compounds, including temperature, time, solvent concentration and solid/liquid ratio. These variables were optimized by single-factor experiments.

Ethanol concentration: A mixture of water-alcohol was commonly used to recover large amounts of polar and medium to low-polar polyphenol compounds from the fibrous matrix [28,32]. While the alcohol part facilitates the extraction by enhancing cell wall permeability [33], the water constituents can cause the swelling of pectin, which increases the viscosity of sample and interrupts the mass transfer process. Since ethanol was an appropriate solvent for this research, Fig. 3 clarified the effect of ethanol concentration. The I% against tyrosinase increased from 28% to 69.5% as ethanol concentration increased to 70%, followed by a steep decline to I% = 52%. Moreover, the extraction yields also reached its peak at this point (H% = 9%). Thus, it can be concluded that the polyphenol profiles of *Morus alba* leaves were at medium to high polarity and for recovering polyphenols from *Morus alba* leaves, 70% aqueous ethanol was the best solvent.

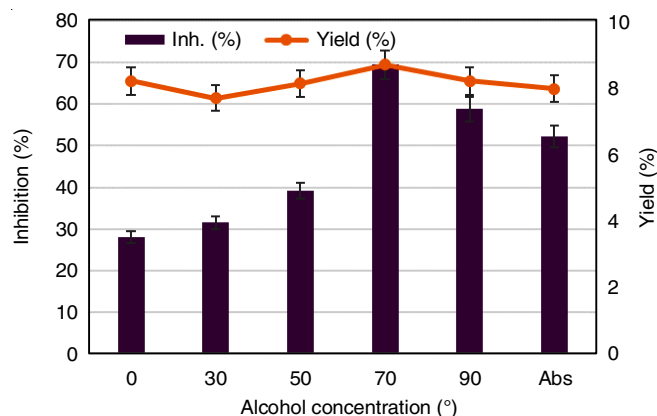


Fig. 3. Effects of different ethanol concentrations

Effect of extraction time: The effects of extraction time were investigated in the range of 20-120 min. It was noted

that a prolonged extraction period leads to the increase of undesired compounds and thermal degradation of target substances, while limited extraction time provides low yields as the mass transfer process is not completed [34]. The I% value prominently increased from 28% to 70% when extracted up to 40 min, then gradually decrease to just above 50% at longer extraction periods (Fig. 4). This can be explained that the mass transfer process of the desired products reached the equilibrium stage, where there is no escape of matter from the fibrous materials after 40 min of extraction [35] and the minor reduction in I% value was possible because of thermal degradation of phenolic compounds during the prolonged heating process. Thus, 40 min were chosen as the most optimal condition.

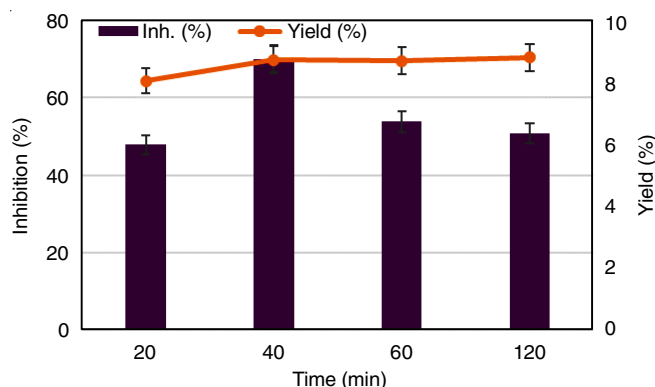


Fig. 4. Effects of different extraction time

Effect of temperature: The effects of varying temperature (40–80 °C) were investigated. The highest performance was obtained at 50 °C (I% = 70%) and then dramatically declined to 34% when further increase to 80 °C (Fig. 5). It can be due to thermal degradation as phenolic compounds are mostly heat-sensitive and easily oxidized to form quinones at high temperatures [36,37]. Therefore, the upper limit for extraction temperature was deemed to be 50 °C.

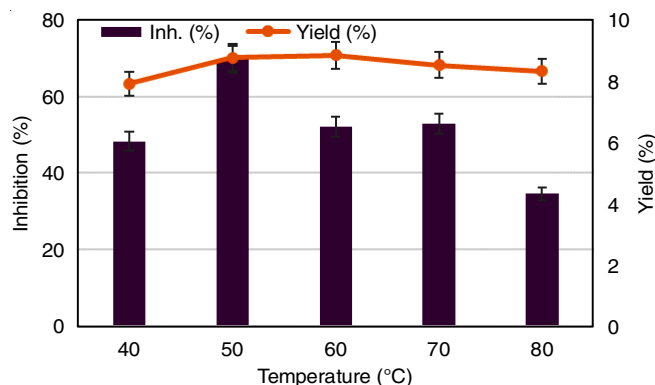


Fig. 5. Effects of different extraction temperature

Solid/Liquid ratio: The solvent volume is one of the most important factors to investigate in the extraction process of natural materials. Overall, higher solvent volume provides better yields as it facilitates the mass transfer process. However, the excessive usage of solvent results in wastage and a longer concentration process [36]. Fig. 6 depicts the I% value of extract

samples concerning solid/liquid ratios (1:6 to 1:12 g/mL). The results indicated that the ratio of 1:10 gave the strongest antityrosinase activity (I% ~ 70%) and this value remained relatively stable as further increasing solvent volume. Moreover, this factor has minor impacts on the extraction yield as these values varied within a small range (from 8.3% to 9.0% even double the solvent volume), indicating a nearly completed mass transfer process. As a result, the solid/liquid ratio of 1:10 g/mL was considered the most optimal ratio.

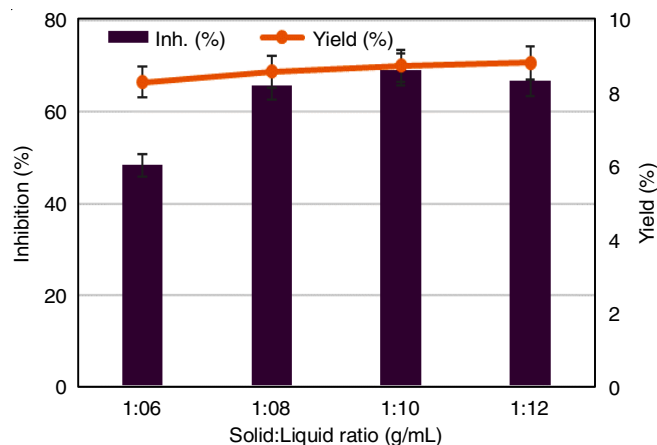


Fig. 6. Effects of different solid/liquid ratios (g/mL)

Successive extraction: To improve the yield of process, *Morus alba* leave was extracted several times and accumulated for bioactivity analysis. Fig. 7 shows that the yield and I% values reached their peaks when extracting for two cycles, then came to the steadystate as further extraction did not advance the yield and bioactivity of the extract. Furthermore, the third extraction cycle experienced a minor downturn in antityrosinase activity, while the extraction yields slightly increased. According to Houck [38], this phenomenon can be possibly attributed to the escape of unwanted pyrolysis products and matrix fibrous materials when extracting for an extended period. Moreover, the third cycle extraction leads to excessive work in the concentration process, hence, thermal degradation of heat-sensitive compounds. Therefore, the materials were extracted in two cycles for economical purposes, in terms of time and solvent volume.

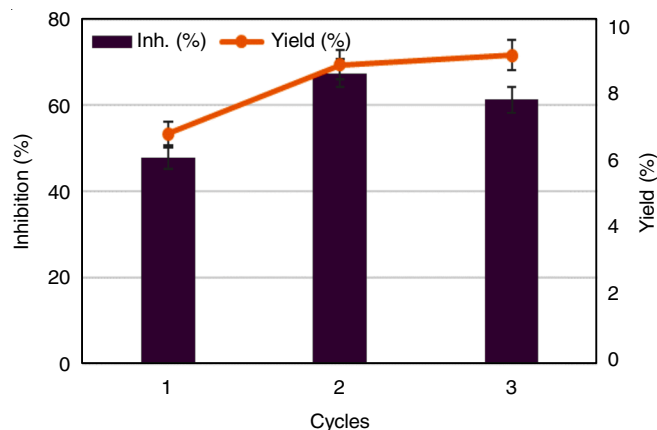


Fig. 7. Effects of different extraction cycles

After the optimization process, the extract of *Morus alba* leaves was carried out under optimal conditions (ethanol 70%, 50 °C, 40 min, solid/liquid ratio = 1:10 and 2 cycles) and then its bioactivities were evaluated for confirmation. *Morus alba* extract under optimal conditions exhibited 5 times stronger in suppressing mushroom tyrosinase and 1.5 times higher in antioxidation activity (Figs. 8 and 9). The total phenolic content was also enhanced from 92.11 GAE ($\mu\text{g/g}$) up to 128.63 ($\mu\text{g/g}$). This result agreed with the studies of Abdullah *et al.* [39] and Gruz *et al.* [40]. Compared to other reports, although the total polyphenol constituents of the extract in this study were insignificant, the DPPH free radical scavenging ability was almost quintuple (total polyphenol content varied between 23.3 to 55.4 mg gallic acid equivalent/g and the radical scavenging ability I_{50} ranged between 584 and 139 $\mu\text{g/mL}$) [41]. This can be explained by the differences in growing conditions, locations of the materials and the extraction techies.

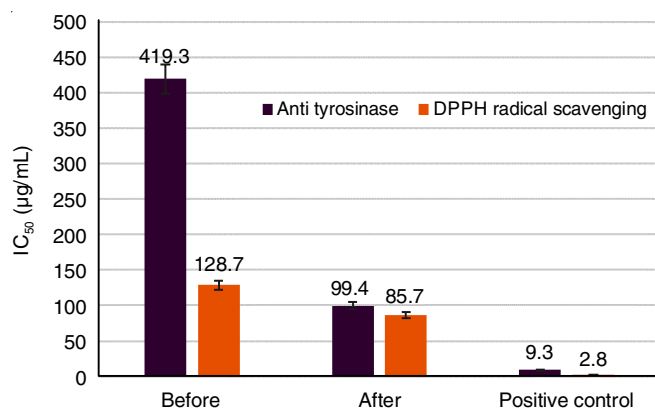


Fig. 8. I_{50} value on the antityrosinase activity and DPPH radical scavenging assay before and after optimization

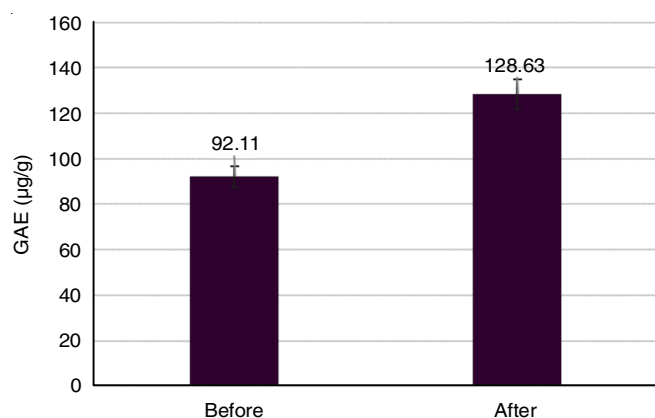


Fig. 9. Total polyphenol constituents before and after the optimization

Additionally, the relationship between oxidative stress and hyperpigmentations initially was verified by many other reports. According to Nahhas *et al.* [42], when the balance between pro and antioxidant systems is broken, oxidative stress can cause biological dysfunctions and increase the number of melanocytes. Therefore, with a competitive antioxidation activity, this confirmed the potential of *Morus alba* leaves to alleviate pigmentation disorders in commercial cosmetic applications.

Conclusion

Morus alba leave was the most potential material among 11 popular herbs in Vietnam in terms of suppressing enzyme tyrosinase. The extraction of this material was optimized using single-variable method and the results were as follows: extraction temperature of 50 °C, ethanol concentration of 70%, extraction time of 40 min and liquid/solid ratio of 1:10 g/mL. The yield under these optimized conditions was 21.9%, anti-tyrosinase IC_{50} = 99.4 $\mu\text{g/mL}$, antioxidation IC_{50} = 85.7 $\mu\text{g/mL}$ and total phenolic content TPE (GAE) = 128.6 mg/g extract.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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