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Comparative analysis of antioxidant, antimicrobial, and tyrosinase inhibitory activities of *Centella asiatica* (l.) Urb and *Eichhornia crassipes* (mart.) Solms

Pannapa Powthong*, Pattra Suntornthiticharoen

Department of Medical Sciences, Faculty of Science, Rangsit University, Pathum Thani, Thailand.

Corresponding author: Pannapa Powthong ✉ pannapa.p@rsu.ac.th, **Orcid Id**: https://orcid.org/0000-0002-8757-6533

Department of Medical Sciences, Faculty of Science, Rangsit University, Pathum Thani, Thailand

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ABSTRACT

In the present study, we examined the potential biological activities of two marginal weeds, *Centella asiatica* (L.) Urb and *Eichhornia crassipes* (Mart.) solms, using various solvents (water, methanol, and ethyl acetate). The ethyl acetate and ethanol crude extracts of Centella asiatica exhibited notable antioxidant activity, as indicated by their DPPH radical scavenging ability, with values of 349.33±3.92 and 348.48±2.96 µg Vitamin C equivalents/0.01 g dry matter, respectively. These extracts effectively inhibited free radicals from DPPH at half maximal effective concentrations (EC50) of 160.46±1.62 and 1,330±25.25 µg/mL, respectively. Furthermore, the ethyl acetate crude extract of *E. crassipes (Mart.) solms*, demonstrated the highest content of total phenolic compounds, with 185.88 ± 2.31µg GAE/0.01 g dry matter, as determined by the Folin-Ciocalteu colorimetric assay. Additionally, the ethyl acetate extract of *E. crassipes (Mart.) solms*, displayed potent tyrosinase inhibitory activity, with an IC50 value of 67.93±0.82 µg/mL. Regarding antimicrobial activity, both the ethyl acetate and ethanol crude extracts of *C. asiatica* (L.) Urb exhibited potential against three of the seven tested bacterial strains, as determined by the agar well diffusion method. These findings highlight the significant biological activities of *C. asiatica* (L.) Urb and *E. crassipes (Mart.) solms*, extracts, suggesting their potential for various scientific applications. Further studies are necessary to explore their diverse range of potential applications.

Keywords: *Centella asiatica* (L.) Urb, *Eichhornia crassipes* (Mart.) solms, Anti-tyrosinase, Antibacterial, Agricultural wastes, Cosmeceutical

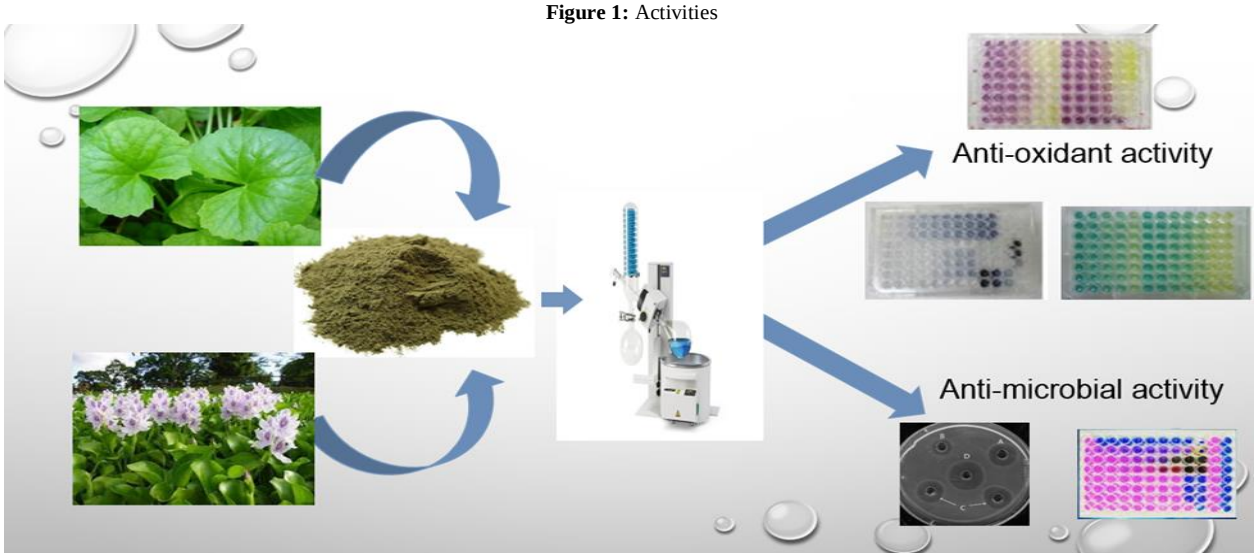
INTRODUCTION

Centella asiatica (L.) Urban is a perennial herb known as Tiger Herbal, pennywort, or gotu kola. Its stem, leaves, and aerial parts have been used in traditional medicine for centuries to treat a wide variety of disorders including insanity, asthma, leprosy, ulcers and eczema, diuretic, and wound healing [1,2]. This plant contains active Terpenes and Terpenoid compounds, as well as triterpene glycosides like centellasaponin, asiaticoside, madecassoside, sceffoleoside, asiatic acid, and madecassic acid [3-5].

The fresh water aquatic plant *Eichhornia crassipes* (Mart.) solms, also known as water hyacinth, is a fresh water aquatic plant. This free-floating perennial plant is native to Brazil's Amazon basin and the Ecuador region. It is one of the world's most invasive aquatic plants, posing ecological, economic, and social threats. It endangers biodiversity, causes significant water resource loss, causes eutrophication, serves as a breeding ground for pests, clogs fresh waterways, has an impact on agriculture and aquaculture, and impedes shipping and recreational activities. However, it has many industrial applications, including bioenergy, biofertilizer production, wastewater treatment (absorption of heavy metals), and animal feed [6,7,8]. Due to its habitat effects, extensive research has been conducted on *E. crassipes* in recent years, although eradicating it will require significant investment. Water hyacinth contains numerous compounds with radical-scavenging activity, including vitamins, terpenoids, phenolic acids, lignin, stilbens, alcaloids, sterols, and other metabolites

with high antioxidant activity [9]. The plant has traditionally been used to treat gastrointestinal issues such as diarrhea, intestinal worms, digestive disorders, and flatulence [10]. Many kinds of plants have proven to be an excellent source of novel biologically active compounds. The biochemical functions of natural antioxidant extracts from plants, which may be candidates for preventing oxidative damage and cosmetic aspects, are gaining attention [11, 12]. Natural cosmetics production should be encouraged by making the best use of domestic resources to replace chemical constituent imports from other countries [13].

Figure 1: Activities



Natural weed plant extracts were interested in including *C. asiatica (L.)* and *E. crassipes* (Mart.) solms [14,15]. As a result, the use of agricultural weeds in the production of natural cosmetics should be encouraged [16]. It also has the potential to raise product standards through the use of natural extracts derived from local community resources for maximum benefit, sustainability, environmental friendliness, and user safety [17,18]. However, there is a limited research focus on the bioactive potential of these agricultural weeds in Thailand, and they remain understudied and overlooked [19]. Therefore, the purpose of this research was to examine *in vitro* biological effect of the bioactive substances from the ethanolic, water, and ethyl acetate crude extract received from weed plant including *C. asiatica (L.)* and *E. crassipes* (Mart.) solms extracts by mean of antioxidant, anti-tyrosinase, and anti-microbial activity. The scientific result obtained here suggested that this variety could be developed as an ingredient in health/medical cosmeceutical purposes or as an alternative treatment to reduce anti-inflammatory drug side effects. Furthermore, the knowledge gained will add value to these weed materials while also reducing pollution and being environmentally friendly.

MATERIALS AND METHODS

Plant material and Preparation of crude extract

Two weeds including *Centella asiatica (L.) Urb* (Tiger's herb), and *Eichhornia crassipes* (Mart.) solms (Water Hyacinth) were randomly and aseptically collected from different areas of Pathum Thani, Thailand. A taxonomist from Drug and Herbal Product Research and Development Center, College of Pharmacy, Rangsit University validated the botanical identity of each plant specimen. It authenticated to be *C. asiatica (L.)* Urb. belonging to family Umbelliferae and *E. crassipes (Mart.)* solms belongs to the Pontederiaceae family. The plant material (leaves, root, shoot) was then washed with distilled water, cut into small pieces, and dried under shadow. The dried material was crushed into powder and stored in an airtight plastic bag in a desiccator at room temperature for subsequent examination.

Plant materials (leaves, root, shoot) extracts were prepared using a solvent equivalent to a 1:10 ratio of ethanol, water, and ethyl acetate for 10 days at 25°C in the solid was extracted by filtering it through Whatman No. 1 filter paper. The extracts were dried by using rotor evaporator. The crude was redissolved in dimethyl sulfoxide (DMSO) to 1 g/mL and stored at -20°C in a glass container until needed for analysis.

Microorganisms and Chemicals reagents

In this study, a diverse range of pathogenic microorganisms were employed, including Gram-positive and Gram-negative bacteria, such as *Pseudomonas aeruginosa* multi drug resistant (MDR), Vancomycin resistant *Enterococcus faecalis* (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* clinical isolate, *Acinetobacter baumannii* multi drug resistant (MDR), *Klebsiella pneumonia* extended-spectrum beta-lactamases (ESBLs), *Escherichia coli* clinical isolate, *Escherichia coli* extended-spectrum beta-lactamases (ESBLs). These microbes were kindly provided by the faculty of Medical Technology at Rangsit University, Thailand.

To conduct the experiment, several chemical compounds were used, including Gallic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Folin-Ciocalteu phenolic reagent, Ethylenediamine tetra acetic acid (EDTA), L-dopa, and dimethyl sulfoxide (DMSO) that

were procured from Sigma Chemicals Co. (St. Louis, MO, USA). All other basic reagents were of analytical grade.

Anti-oxidant assay of crude extract

Scavenging effect on 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging assay

One of the mechanisms in inhibiting oxidation that is commonly used to estimate antioxidant activity is free radical scavenging. According to the previous report, the free radical scavenging activities of agricultural waste extracts were determined in the present study using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) [20]. Briefly, 100 µl of plant extracts, obtained from *C. asiatica (L.)* and *E. crassipes* (Mart.) solms using ethanol, water, and ethyl acetate as solvents (at concentrations ranging from 625 to 10,000 µg/ml) resail 2-fold dilution in methanol, were mixed with 100 µl of a methanolic solution containing 0.2 mM of 2,2-diphenyl-1-picryl-hydrazyl (DPPH, Sigma). As a control, DPPH solution and distilled water equivalent to extract were used. The mixture was vigorously shaken and allowed to stand for 30 minutes in the dark before measuring absorbance at 517 nm against a blank. The calibration curve was also established using L-ascorbic acid solution in methanol to determine vitamin C equivalent antioxidant capacity (VCEAC). The ability to scavenge the DPPH radical, as calculated by the equation:

$$\textbf{DPPH scavenging effect (\%)} = [1-(A_1/A_0)] \times 100$$

Where A_0 represented the absorbance of the control reaction and A_1 represented the absorbance in the presence of the sample. The test was performed in triplicate, and the half maximal effective concentration (EC50) value was calculated. Furthermore, the total antioxidant content was calculated using the basic of the ascorbic acid calibration curve and the results were expressed as g of vitamin C equivalent/0.01 g dry matter.

Folin-Ciocalteu colorimetric assay

The total phenolic phytochemical content of all interested extracts was determined using Folin- Ciocalteau's method [21]. Briefly, 100 µl of crude extracts were dissolved in methanol to achieve concentrations ranging from 625 - 10,000 µg/mL. After that, the extract was mixed with 100 µl of Folin- Ciocalteu's reagent. The resulting mixture was treated with 80 µl of 2% aqueous sodium carbonate. The mixture was incubated at room temperature for 30 minutes. The absorbance was measured at 765 nm against a blank. The total phenol content was determined using the basic of the gallic acid calibration curve and expressed as µg of gallic acid equivalent (GAE)/0.01 g dry matter.

Anti-tyrosinase activity assay

The degree of inhibition of mushroom tyrosinase-catalyzed L-DOPA oxidation was used to measure the inhibitory effect of all interested extracts on tyrosinase activity. The anti-tyrosinase activity was measured using L-DOPA as a substrate, according to Batubara and colleagues [22]. The interested extracts were dissolved in dimethyl sulfoxide (DMSO) to achieve concentrations ranging from 625 - 10,000 µg/mL. One hundred µl of L-dopa solution (1 mg/ml in phosphate-buffered saline (PBS) at pH 7.34) was previously incubated at 30 °C in PBS buffer (pH 6.8). The 60 µl of sample was then added with L-dopa solution. After 1 minute, 40 µl of Tyrosinase (100 U/mL in PBS 0.1 M pH 6.8) was added to the mixture. After 1 hour of incubation at 37 °C, the absorbance was measured at 475 nm. Instead of crude extracts, PBS was used as a control. Anti-tyrosinase activity was assessed using the inhibitory concentration 50 (IC50). Each sample was examined three times and the average was calculated. The inhibitory rate was calculated using the following formula:

$$\textbf{Inhibitory rate (\%)} = [(S_0 - S_1)/S_0] \times 100\%$$

Where S_1 is the absorbance value with samples and S_0 is the absorbance value of control.

Quantitative antimicrobial assay by agar well diffusion method

The agar well diffusion method was adapting form prior report [23] used to test antimicrobial activity to all of the microorganisms mentioned above. Briefly, re-culture the microorganisms from the freezing vials on tryptic soy agar and adjust the turbidity to 0.5 McFarland standards to achieve a concentration of 1.5×10^8 CFU in a sterile 0.85% NaCl solution. Using a three-way cotton swab technique, the test organism was applied to the surface of Muller-Hinton agar. A sterile cork borer was used to make six mm diameter wells in agar. To test extract, concentrations of 10,000 g/100 l in sterile broth were made. Thirty microliters of crude extract were added into the wells. Following that, sterile broth and gentamycin (1mg/mL) were used as negative and positive controls, respectively. The plates were incubated for 24 hours at 37 °C, and the diameter of the inhibition zone around the well was measured using a scale and compared to that of the control groups. All experiments were carried out three times.

Statistical Analysis

All experiments were carried out and studied in triplicate. The results are the mean ± standard deviation.

Total phenolic compounds

The Folin-ciocalteu colorimetric results were shown in Table 1. For *C. asiatica (L.)* extract, it was observed that ethyl acetate and ethanol crude extracts showed the high value as 111.32± 5.92 and 65.37± 6.07 µg GAE/0.01 g dry matters respectively. For *E. crassipes* (Mart.) solms, the highest level of was found in ethyl acetate as 185.88 ± 2.31 µg GAE/0.01 g dry matters. Secondly, ethanol crude extracts showed level of 57.11± 8.05 µg GAE/0.01 g dry matters.

Anti-tyrosinase activity

The ethanol extract of *C. asiatica (L.)* had the greatest anti-tyrosinase value of 211.39±4.50 µg/ mL. Second, the ethyl acetate extract yielded a value of 638.83±6.30 µg/ mL. The best anti-tyrosinase IC50 value of *E. crassipes* (Mart.) solms was ethyl acetate

extract as 67.93±0.82 µg/ mL. Follow by the aqueous extract at 1,119.36±0.65 µg/ mL as shown in Table 2.

Table 1: Anti-oxidant assays (µg Vitamin C equivalents/0.01 g dry matters) and Total phenolic compound (µg gallic acid equivalents (GAE) of aqueous, ethanol, and ethyl acetate extracts of *C. asiatica (L.)*, and *E. crassipes* (Mart.) solms.

Agricultural wastes extracts	Anti-oxidant assays (µg Vitamin C equivalents/0.01 g dry matters)	Total phenolic compound (µg gallic acid equivalents (GAE) /0.01 g dry matters)
C. asiatica (L.)		
Aqueous extract	342.49±3.63	32.13±2.26
Ethanol extract	348.48±2.96	65.37± 6.07
Ethyl acetate extract	349.33±3.92	111.32± 5.92
E. crassipes (Mart.) solms		
Aqueous extract	235.32±4.13	36.77±6.20
Ethanol extract	346.77±1.48	57.11± 8.05
Ethyl acetate extract	336.08±1.81	185.88 ± 2.31

Table 2: EC50 Values for DPPH anti-oxidant and anti-tyrosinase assays of different concentrations of aqueous, ethanol, and ethyl acetate extracts of *C. asiatica (L.)*, and *E. crassipes* (Mart.) solms extracts. Data are given as Mean ± SEM (n=3)

Agricultural wastes Extract	EC50 Values for DPPH anti-oxidant activity ± SEM (µg /mL)	IC50 Values for anti-tyrosinase activity ± SEM (µg /mL)
C. asiatica (L.)		
Aqueous extract	1,648 ±40.70	895.06±19.13
Ethanol extract	1,330±25.25	211.39±4.50
Ethyl acetate extract	160.46±1.62	638.83±6.30
E. crassipes (Mart.) solms		
Aqueous extract	5,237±5.24	1,119.36±0.65
Ethanol extract	143.70±3.30	2,076.83±2.79
Ethyl acetate extract	176.50±6.77	67.93±0.82

Antimicrobial assay by well diffusion

Table 3 shows the results of the antibacterial activity of 2 interested crude weed plant extract using the well diffusion technique in comparison to the reference drug (Gentamycin). It was denoted that at a concentration of 10,000 µg/ mL, ethanol and ethyl acetate extract of *C. asiatica (L.)* expressed potential antimicrobial activity against the 3 respective tested bacterial strains; *Acinetobacter baumannii* MDR, *Staphylococcus aureus* MRSA, and *Staphylococcus aureus* clinical isolate with the range of an inhibition zone from 8.0±0.0 - 11.0±0.0 mm compared with that of the standard drug (1 mg/mL of Gentamycin) with an inhibition zone range from 24.5±0.5 - 26.5±0.5 mm.

For *E. crassipes* (Mart.) solms extraction, it was revealed that ethanol and ethyl acetate crude extracts at a concentration of 10,000 µg/ mL showed the antimicrobial activity against 1 tested bacterial strain; *Acinetobacter baumannii* MDR with an inhibition zone 9.0±0.0 and 10.5±1.0 mm respectively.

RESULTS AND DISCUSSION

Anti-oxidative activity

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) assay were used to screen crude extracts of two plants for anti-oxidative activity. The extracts' radical scavenging activities were proportional to their concentrations. The inhibitory activity of ethyl acetate and ethanol crude extracts of *C. asiatica (L.)* on DPPH radicals is presented in Table 1. Both extracts showed substantial antioxidant activity, with values of 349.33±3.92 and 348.48±2.96 µg Vitamin C equivalents per 0.01 g dry matter, respectively. Furthermore, these extracts exhibited effective inhibition of DPPH radicals at half maximal effective concentrations (EC50) of 160.46±1.62 and 1,330±25.25 µg/mL, respectively. The ethanol extract of *E. crassipes* (Mart.) solms had the highest antioxidant content 346.77±1.48 µg Vitamin C equivalents /0.01 g dry matter. Follow by ethyl acetate of Water Hyacinth 336.08±1.81 µg Vitamin C equivalents /0.01 g dry matter. They can inhibit DPPH (EC50) of 143.70±3.30 and 176.50±6.77 µg/ mL respectively as shown in Table 1 and 2.

Table 3: Anti- microbial activity of aqueous, ethanol, and ethyl acetate extracts of *C. asiatica (L.)*, and *E. crassipes* (Mart.) solms extracts.

Agricultural wastes extracts	Inhibition zone (mm.)							
	Pseudomonas aeruginosa MDR	*Enterococcus faecalis* VRE	*Staphylococcus aureus* MRSA	*Staphylococcus aureus* clinical	*Acinetobacter baumannii*	*Klebsiella pneumonia*	*Escherichia coli* clinical isolate	*Escherichia coli* ESBL
C. asiatica (L.)								
Aqueous extract	8.5±0.5	-	-	-	-	-	-	-
Ethanol extract	-	-	9.5±0.5	11.0±0.0	9.5±0.5	-	-	-
Ethyl acetate extract	-	-	10.5±0.5	8.0±0.0	8.5±0.5	-	-	-
E. crassipes (Mart.) solms								
Aqueous extract	-	-	-	-	-	-	-	-
Ethanol extract	-	-	-	-	9.0±0.0	-	-	-
Ethyl acetate extract	-	-	-	-	10.5±0.5	-	-	-
Gentamicin	40.0±1.0	34.0±2.0	25.0±0.0	26.5±0.5	24.5±0.5	35.0±0.0	42.0±1.0	33.0±2.0

Weeds plant are undesirable plants that compete with desired agriculture or have a negative impact on agriculture and aquaculture. They have high vigor and tolerance, produce more seeds, and can spread rapidly. However, some weeds are used medicinally. There have been numerous studies conducted on the medicinal properties of weed [24]. One of the good approaches is the recovery of valuable compounds. The present study focused on weed plants that can be investigated as a beneficial source of bioactive ingredients for use in clinical, beauty care, pharmaceutical, or agribusiness industries, which is critical for increasing their added value and reducing severe environmental potential dangers. In the present study, organic solvent extracts of weed plants were tested for free radical scavenging efficacy using the DPPH method. At the tested concentration (625-10,000 g/mL), the ethyl acetate of *C. asiatica (L.)* and the ethanol extract of

E. crassipes (Mart.) solms demonstrated high anti-oxidative activity by DPPH (349.33±3.92 and 346.77±1.48 µg Vitamin C equivalents /0.01 g dry matter with EC50 of 160.46±1.62 and 143.70±3.30 µg/ mL respectively). This result was higher than previous reported that the DPPH radical scavenging activities of ethanolic crude *C. asiatica (L.)* extract have an IC50 of 62.30±1.38 µg/ mL [25].

Meanwhile, using the Folin- Ciocalteau method, the highest level of total phenolic compound was found in ethyl acetate crude extracts of *C. asiatica (L.)* and *E. crassipes* (Mart.) solms (111.32± 5.92 and 185.88 ± 2.31µg GAE/0.01 g dry matters, respectively). These findings are consistent with those reported by Rufchaei and colleagues, who found that ethanol extracts of *E. crassipes* (Mart.) solms have the highest total phenolic contents (620 ± 0.10 mg of GAE g/g dry mass) [26]. Moreover, the values found in this study agree with recent findings that ethanolic crude extracts of *C. asiatica (L.)* had a total phenolic content of 28.6 mg GAE/g dry matters [27]. The fact that *E. crassipes* (Mart.) solms plant is a source of many compounds with radical scavenging activity, such as phenolic acids, sterols, terpenoids, and other metabolites with high antioxidant activity was report by previous study [19].

However, the most abundant major compounds demonstrate by Gas Chromatography Mass Spectrometry (GC-MS) are linolenic acid ethyl ester and stigmasterol which have numerous biological properties such as antioxidant, hypocholesterolemic, nematicide, pesticide, and lubricant activities, as well as acting as a precursor in the production of semi synthetic progesterone, a valuable human hormone [28,29-31]. The *C. asiatica (L.)* plant is a treasure trove of flavonoids, including quercetin, kaempferol, catechin, rutin, apigenin, triterpenoids, asiaticoside, and naringin, which are excellent sources of antioxidants and anti-inflammatory compounds [32, 33]. Besides that, GC-MS analysis revealed 33 major compounds, three of which are unique in methanolic *C. asiatica (L.)* extract: octadecatrienoic acid, hexadecanoic acid, and norreticuline [34].

The best anti-tyrosinase EC50 value was determined by the anti-tyrosinase assay results for ethanol extract of *C. asiatica (L.)* and ethyl acetate extract of *E. crassipes* (Mart.) solms (211.39±4.50 and 67.93±0.82 µg/ mL). Prior research has found that the tyrosinase inhibition activity of ethanolic crude *C. asiatica (L.)* extract was 31.25±0.33 µg/ in 1.67 mg/mL of extract [35]. Anti-tyrosinase activity in herbal extracts could be due to flavonoid content [25]. The flavonoids chelate two coppers at the active site of the tyrosinase enzyme [36,37]. Antimicrobial property of two interested crude weed plant has been test against both resistant type gram negative and gram-positive microorganism by agar well diffusion method where gentamicin was used as positive control. Results found that at a concentration of 10,000 µg/ mL, ethanol and ethyl acetate extract of *C. asiatica (L.)* expressed potential antimicrobial activity against the 3 from 8 tested bacterial strains. While ethanol and ethyl acetate crude extracts of *E. crassipes* (Mart.) solms showed the antimicrobial activity against only 1 from 8 tested bacterial strains. These findings are consistent with previous report which revealed that methanolic crude extracts of *C. asiatica (L.)* exhibited moderately effective antimicrobial activities on inhibition of *Staphylococcus aureus* and methicillin-resistant *S. aureus* [38]. This finding was corresponding to the result from Ondeko and coworker which reported that methonolic extracts of *C. asiatica* were active against *S. aureus, K. pneumonia, E. coli* and *C. albicans* [34].

A variety of substances (aqueous, 95% ethanol, and ethyl acetate) were used to extract bioactive compounds from plants, each of which demonstrated differential activity throughout this investigation. According to the findings of this study, a single solvent may not be able to extract all of the useful bioactive compounds from a plant. To obtain the highest yields of specific compounds, several solvents may be required. From this study, by using ethanol and ethyl acetate as extraction solvents yielded highest amount of reducing ability in which significantly different from aqueous extracts. Previous research has shown that the extraction method has an effect on *C. asiatica (L.)* 's antioxidant activity. Ethanol exhibited the highest antioxidant activity among the three solvents tested, followed by aqueous, while light petroleum demonstrated negative antioxidant activity [39]. However, the choice of solvent for extraction is a critical factor that can influence the yield and potency of bioactive compounds. In their research study, Kumar and Sharma found that using multiple solvents for extracting important compounds from selected plants resulted in higher extract yields and more potent bioactive compounds. Therefore, the selection of solvent type should be carefully considered for future investigations aimed at extracting significant compounds from target plants.

CONCLUSION

In conclusion, the ethanolic and ethyl acetate extracts of *C. asiatica (L.)* have exhibited the most promising bioactive activity, as revealed by their pharmacological properties in *in vitro* evaluations. These crude extracts have demonstrated remarkable antioxidant, anti-tyrosinase, and antimicrobial activities, indicating that *C. asiatica (L.)* is a rich source of bioactive compounds or phenols. Despite these favorable characteristics, further research is needed before it can be incorporated into health/medical cosmeceuticals. In particular, additional chemical studies are necessary to isolate and identify the active ingredients responsible for these properties. However, the potential of weed plants as a natural ingredient in health/medical cosmeceutical products or as an alternative treatment cannot be ignored. By harnessing the bioactive compounds present in weed plants such as *C. asiatica (L.)*, novel therapeutic formulations with

enhanced efficacy, ecological compatibility, and long-term viability can be developed.

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Author's contribution

All authors contributed extensively to the work presented in this paper. Pannapa Powthong designed, performed experiments, analysed data and wrote the paper; Pattra Suntornthiticharoen gave conceptual advice and revised the manuscript and analyses data. All authors discussed the results and implications and commented on the manuscript at all stages.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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