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Total Phenolics, Flavonoids, DPPH Radical Scavenging and Tyrosinase Inhibition Activities of Sacha inchi (*Plukenetia volubilis* L.)

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Abstract

Sacha inchi (*Plukenetia volubilis* L.) or Inca star is a medicinal plant whose fruits and seeds are mainly used in processing. The objectives of this research were to analyze the total phenolic and flavonoid contents of Sacha inchi from leaves, vines and shells and to study the efficiency of DPPH radical scavenging activities and tyrosinase inhibition activities of Sacha inchi. The leaves, vines and shells were extracted with 95% ethanol using the maceration technique. The crude extracts of leaves, vines and shells were analyzed in terms of total phenolic contents using the Folin-Ciocalteu method, whereas the total flavonoid contents were analyzed using aluminium chloride colorimetric method. The results of the percentage yield in leaves, vines and shells were found to be 19.4815 ±0.0617, 4.81 ± 0.0482 and 2.51 ± 0.0943, respectively. The total phenolic contents in leaves, vines, and shells were 26.3441±0.8895, 37.0925±0.8898 and 30.5202±1.9938 mg of gallic acid/g sample, respectively. The total flavonoid contents were 407.6190±2.5036, 171.2516±3.6696, and 179.0318±3.7771 mg of rutin/g sample, respectively. DPPH radical scavenging activities in leaves, vines and shells were shown with EC_{50} as 0.0092±0.0090, 0.0803±0.0063 and 0.2527±0.1105 mg/mL, respectively, compared to standard BHT with EC_{50} as 0.1296±0.0528 mg/ml. The tyrosinase inhibition activities in leaves, vines and shells were shown with IC_{50} as 0.0016±0.0107, 0.4924±0.1500 and 0.5986±0.2751 mg/mL, respectively, compared to standard kojic acid with IC_{50} as 0.0002±0.0011 mg/mL. All selected parts of the Sacha inchi had phenolic and flavonoid contents. The DPPH radical scavenging activities in Sacha inchi extracts were found in leaves, vines and shells. In addition, the tyrosinase inhibition activities were found mainly in leaves, although to a lesser degree in vines and shells of Sacha inchi.

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Introduction

Sacha inchi (*Plukenetia volubilis* L.) is a medicinal as well as rubber plant in the family Euphorbiaceae. This novel vegetable has been researched for its health benefits in terms of its characterizations, phytochemical contents, mineral forms and antioxidant activities. Most of the previous research mainly involves crude extraction from the seed. The efficacy of this herb's active ingredients in other parts such as vines and shells is still, unknown.

The seed is considered an important dietary source of health-promoting phytochemicals that are rich in high contents of total phenolics, total carotenoids and hydrophilic and lipophilic antioxidant capacities (Chirinos et al., 2013; Maurer et al., 2012). The beans of Sacha inchi are roasted, heated and eaten as a snack. The seeds are fragrant, crunchy and have a delicious nutty flavor. Sacha inchi beans can be processed into snack products such as salted roasted nuts and fried beans. They can also be processed into food products such as sauce, soy sauce, soybean paste, processed into Sacha inchi bean flour for cooking and baking. In addition, they can be extracted into oil that can be used for many purposes (Norhazlindah et al., 2022). Sacha inchi seeds could be processed into edible oil as dietary supplement products (Suwanangul et al., 2022). For the leaves, the young shoots are boiled or eaten with chili paste or bamboo shoot soup. The dark old green leaves are chopped into small pieces and dried in the sun to make tea. The bright green leaves are extracted from chlorophyll or extracted to make chlorophyll water. The pod and seed husks are used to make compost or to be compressed into fuel sticks for cooking (Juntawieng, 2020). Kittibunchakul et al. (2022) found that the leaves provide antioxidant effects and phenolic compositions and are a source of health promotion. This will prevent damage to DNA, protein and lipid, which decreases the risk of heart disease and cataracts and results in people's better health (Ames et al., 1993).

A community group is currently using various parts of the Sacha inchi including leaves, vines and shells. The processing community enterprise group of Sacha inchi is named Kru Cheun Farm, which is located at 1 Village No. 14, Ban Kaeng Sub-district, Mueang District, Sa Kaeo Province and whose chief entrepreneur is Ms. Patma Sidawong. Leaves are dried and packed in containers for sale as healthy tea while vines and shells of the seeds are dismissed as waste. According to the World Health Organization's Good Agricultural and Collection Practice (GACP) standard of the traditional medicine industry, the safety and quality of raw medicinal plants, materials and finished products depend on factors that may be classified as intrinsic (genetic) or extrinsic (environment, collection method, cultivation, harvest, post-harvest processing, transport and storage practices). Inadvertent contamination by microbial or chemical agents during any of the production stages can also lead to deterioration in safety and quality. Medicinal plants collected from the wild population may be contaminated by other species or plant parts through misidentification, accidental contamination, or intentional adulteration, all of which may have unsafe consequences (Zhang, 2003). The community has grown these plants without using insecticide to prevent the effects of chemicals. Sacha inchi is provided with enough water and kept away from any source of chemicals. Weeds are removed every 1-2 months. At 5-6 months after planting, Sacha inchi trees begin to blossom and bear pods. Then 3 months later, the pods begin to age and are ready to be picked. These should be ripe and drying pods, that is, black and then brown. For leaves, green rather than brown ones should be chosen, which are the younger leaves. The personnel of planting, nursing and harvesting communities must receive training from community government agencies to acquire technical knowledge and expertise. After harvest, the community will keep the crops and pack them in a clean and ventilated greenhouse. They must not be affected by chemicals and insects. In this study, the researchers selected the green leaves, vines and shells of the seeds of Sacha inchi. All of these parts would be discarded by the community members and were not used for their business purpose. Sacha inchi parts needed to be transported to researchers by a temperature-controlled vehicle (Sidawong, 2021). After that, they were extracted and studied in terms of their biological activities.

To meet the needs of the community members who are concerned about the plant safety, they requested an investigation into the activities and properties of the various parts of Sacha inchi, including leaves, vines and shells. Other parts of this medicinal plant that are aforementioned can be considered as an important dietary source that has a high level of phenolics, flavonoids, antioxidants and tyrosinase inhibition activities.

The effect of oxidative activities is related to and correlates with total phenolics (Velioglu et al., 1998). In

this study, the research explored this effect by using the basis from the scavenging of DPPH free radicals. Moreover, the researchers investigated the performance of inhibition tyrosinase activities because prior studies have found that medicinal plants in this same family have anti-tyrosinase activities (Meechai et al., 2010; Momtaz et al., 2010). Baurin et al. (2002) ran a preliminary screening of tropical plants and found many families including Euphorbiaceae having antityrosinase activities. Plants in the same family have the possibility to have the same biological effect. These activities in this research could be considered as an important dietary or raw material source for promoting community natural products.

Materials and methods

1. Preparation of crude extracts by the maceration method

Leaves, vines and shells of Sacha inchi were washed thoroughly and dried at 45°C until they were dry. They were crushed and separated. Five hundred grams of Sacha inchi leaves, vines and shells were baked and crushed. They were wrapped in a thin white cloth, soaked in 95% ethanol 3,000 mL and shaken gently. They were separated and stored in a dark place for five days. Each type was filtered to remove residues. All parts were re-extracted with ethanol and left for 5 days. Then the extracts were stored in the flask for evaporation of the solvent. The solvent was evaporated with a rotary vacuum evaporator and freeze-dryer (triplicates).

The researchers recorded the results and calculated the percentage yield (% yield) of the crude extracts obtained from the formula.

$$\% \text{ yield of crude extracts} = \frac{\text{weight of crude extracts X 100}}{\text{weight of dry plants before extraction.}}$$

The crude extracts of each part were stored in the refrigerator at 5°C to wait for the determination of phytochemical and DPPH radical scavenging and anti-tyrosinase activities (Kotpoohtorn, 2016).

2. Determination of total phenolic and total flavonoid contents in leaves, vine and shells of Sacha inchi extracts

2.1 Total phenolic contents (TPC)

Following a modified method by Wattanuruk et al. (2020b), the total phenolic contents (TPC) were determined by Folin-Ciocalteu method. Ten milligrams of extracts from Sacha inchi leaves, vines and shells were dissolved with 99.99 % ethanol, an adjusted volume of 5 mL. After that, 100 µL of diluted extracts in 8.4 mL distilled water was mixed with 500 µL of freshly prepared diluted Folin-Ciocalteu. One minute after, 1 mL of 20% Na_2CO_3 was added. Mixtures were incubated at room temperature for two hours in the dark. The standard solution of gallic acid (10 mg) was dissolved with 99.9% ethanol, adjusted volume 5 mL and diluted in various concentrations (1.0, 0.8, 0.4, 0.2, 0.1 and 0.05 mg/mL.). The absorbance was measured at a wavelength of 760 nm by UV-Vis spectrophotometer (triplicates).

The basis of the calibration curve of gallic acid was brought to calculate the total phenolic contents, which were expressed as gallic acid equivalent (GAE) in milligrams per gram of the sample (mg GAE/g dried extract).

2.2 Total flavonoids contents (TFC)

Following a modified method by Wattanuruk et al. (2020b), the total flavonoids contents (TFC) were determined using rutin as a standard. Ten milligrams of extracts from Sacha inchi leaves, vines and shells were dissolved in 80% ethanol and adjusted to volume 10 mL. After that, 1 mL of extracted samples/standard solution was put in a 10 mL volume metric flask, with an addition of 4 mL of distilled water for 0 min, a mixing of 0.3 mL of 5% $NaNO_2$ for 5 min, an addition of 0.3 mL of 10% $AlCl_3$ for 6 min, an addition of 2 mL of 1 M NaOH, an addition of 10 mL distilled water and everything was shaken well. The absorbance was measured at a wavelength of 510 nm using a UV-Vis spectrophotometer (triplicates).

The qualification was done using rutin as the standard and the results were expressed as milligrams of rutin equivalent (mg RE) per gram of the sample (mg RE/g dried extract).

3. Determination of DPPH radical scavenging activities in leaves, vine and shells of Sacha inchi extracts

Following a modified method by Senajuk et al. (2020) and Sharma & Bhat (2009), the DPPH radical scavenging activities were determined. One milligram of extracts from Sacha inchi leaves, vines and shells with concentrations of 500, 250, 125, 62.5 and 31.25 mg/mL was pipetted into a test tube and 3 mL of 0.2 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) was added. Everything was shaken well. After that, it was incubated at room temperature for 30 min in the dark. The absorbance was measured at a wavelength of 517 nm by a UV-Vis

spectrophotometer, using 95 % ethanol as blank, 0.2 mM DPPH as control and BHT (butyl hydroxytoluene) as a standard (triplicates). The efficiency of antioxidant activities is as follows (Pukumpuang et al., 2012):

$$\% \text{ DPPH Scavenging} = \frac{(\text{Abs control} - \text{Abs sample} / \text{Abs control}) \times 100}{\text{Abs Control}}$$

Graphs were plotted for EC_{50} values and were compared to BHT for each extraction of leaves, vines and Shells of Sacha inchi. Values were reported as EC_{50}, obtained by graphing the relationship between % DPPH inhibitions and sample concentrations.

4. Determination of tyrosinase inhibiting activities in leaves, vines and Shells of Sacha inchi extracts

Following a modified method by Kleangjan & Ponmai (2017), the tyrosinase inhibiting activities were determined. The sample solution was prepared by weighing extracts from leaves, vines and shells of Sacha inchi (0.01 g each), dissolved with 99.99% ethanol, shaken for 30 min. The extract volume was then adjusted to 10 mL and diluted in various concentrations (500, 250, 125 and 62.5 mg/mL). The samples were compared with kojic acid standard solution.

A, B, C and D types of solution were added into a multi-well dish of microplate leader.

A (control): tyrosinase solution 50 µL, 0.02 M sodium phosphate buffer solution (pH 6.8) 150 µL, and 99.99% ethanol 50 µL.

B (blank of A): 0.02 M sodium phosphate buffer solution (pH 6.8) 150 µL and 99.99% ethanol 50 µL.

C (test sample): tyrosinase solution 50 µL, 0.02 M sodium phosphate buffer solution (pH 6.8) 150 µL and sample/standard in 99.99% ethanol 50 µL.

D (blank of C): 0.02 M sodium phosphate buffer solution (pH 6.8) 150 µL and sample solution 50 µL.

The samples were compared with kojic acid standard solution. After the substance was added to a multi-well dish and shaken to mix well, the tests were incubated at room temperature (25°C) for 10 min. After that, 50 µL L-DOPA solution was added into a multi-well dish and shaken to mix well. The absorbance was measured at a wavelength 492 nm with a UV-Vis spectrophotometer. Then they were incubated at the room temperature for 2 min and absorbance was measured again at the same wavelength (triplicates). The calculation of % tyrosinase inhibition is as follows:

$$\% \text{ Tyrosinase Inhibition} = \frac{(A-B)-(C-D) \times 100}{A-B}$$

A. B, C and D were different types of absorbance at 492 nm wavelength between the measured values before and after 2 min of the incubation period.

5. Statistical analysis

All treatments and determination were implemented in triplicates and data were analyzed in terms of mean ± standard deviation.

Results and discussion

1. The extraction of substances in leaves, vines, and shells of sacha inchi

It was found that the percentage yield in leaves, vine and shells of sacha inchi extracts was 19.4815±0.0617, 4.8100 ± 0.0482 and 2.5100 ± 0.0943, respectively. The percentage yield and other physical properties of Sacha inchi extracts are shown in Table 1.

Table 1 Yield and other physical properties of Sacha inchi extracts

Sample (from different parts of Sacha inchi)	Yield (%)	Color and consistency
Leaves	19.4815±0.0617	Deep green, Gummy solid
Vines	4.8100± 0.0482	Light brown, solid in form of powder
Shells	2.5100± 0.0943	Light brown, solid in form of powder

2. Total phenolic contents (TPC) and total flavonoid contents (TFC) of Sacha inchi extracts

The total phenolic contents in Sacha inchi leaves, vines and shells were 26.3441±0.8895, 37.0925±0.8898 and 30.5202±1.9938 of gallic acid/g sample, respectively. The total flavonoid contents were 407.6190±2.5036, 171.2516±3.6696 and 179.0318±3.7771 mg of rutin/g sample, respectively. Total phenolic and total flavonoid contents of Sacha inchi extracts are shown in Table 2.

Table 2 The amounts of total phenolics and total flavonoids of Sacha inchi extracts

Sample (from different parts of Sacha inchi)	Phenolic (mg GAE/g)	Flavonoid (mg Rutin/g)
Leaves	26.3441±0.8895	407.6190±2.5036
Vines	37.0925±0.8898	171.2516±3.6696
Shells	30.5202±1.9938	179.0318±3.7771

Chirinos et al. (2013) investigated Sacha inchi seeds and found a high amount of nutrients in them including phenolic properties. Other parts also had large amounts of phenolics, namely vines, followed by shells and leaves, respectively. The total flavonoid contents were highest in leaves, followed by shells and vines, respectively. Both phenolic and flavonoid compounds

are great activities of antioxidants (Atoui et al., 2005; Velioglu et al., 1998).

3. DPPH radical scavenging activities

Regarding the evaluation of hydrogen donation of the antioxidants, EC_{50} values represented the concentration of antioxidants that decreased the DPPH radicals to half of their initial concentration (Wattanuruk et al., 2020a). The results of this measure in leaves, vines and shells of Sacha inchi showed EC_{50} values of 0.0092±0.0090, 0.0803±0.0063 and 0.2527±0.1105 mg/mL, respectively, compared with the BHT standard with an EC_{50} of 0.1296±0.0528 mg/mL. The DPPH radical scavenging activities are shown in Table 3.

Table 3 DPPH radical scavenging activities of Sacha inchi extracts

Sample (from different parts of Sacha inchi) and standard	EC_{50} (mg/mL)
Leaves	0.0092±0.0090
Vines	0.0803±0.0063
Shells	0.2527±0.1105
BHT (standard)	0.1296±0.0528

Sacha inchi seeds were reported to have antioxidant capacity (Chirinos et al., 2013). Kittibunchakul et al. (2022) studied antioxidant activities in Sacha inchi leaves and found Sacha inchi young leaves had significantly higher antioxidant activities than mature leaves, exhibiting 1.1-1.2-fold. No studies have reported that other parts such as vines and shells have this effect. In this research, the results of EC_{50} showed that leaves of sacha inchi were rich in flavonoids and their phenolic compounds and had the greatest amounts of antioxidants, followed by vines, BHT (standard test) and shells, respectively. In this research, the results are in line with those in Senajuk et al. (2020). They argued that the extracts with a low EC_{50} have high free radical scavenging activities. The relationship between total phenolic contents and antioxidant activities shows that the amounts of total phenolics vary with antioxidant activities (Yan & Asmah, 2010). It is very interesting to develop a product related to anti-aging that will prevent the body from oxidative stress.

4. Tyrosinase inhibition activities of sacha inchi extracts

The inhibition of tyrosinase activities in leaves, vines and shells of sacha inchi extracts is shown in Table 4.The IC_{50} were 0.0016±0.0107, 0.4924±0.1500 and 0.5986± 0.2751 mg/mL, respectively, compared to standard kojic acid, IC_{50} as 0.0002±0.0011 mg/mL.

Table 4 Tyrosinase inhibition activities of sacha inchi extracts

Sample (from different parts of sacha inchi) and standard	IC_{50} (mg/mL)
Leaves	0.0016+0.0107
Vines	0.4924±0.1500
Shells	0.5986±0.2751
Kojic acid (standard)	0.0002±0.0011

Leaves of Sacha inchi showed high efficacy of tyrosinase inhibition, followed by vines and shells. Kojic acid, which is a standard solution, showed better efficacy than others in terms of inhibitory effects. Even though there is no research about the tyrosinase inhibition of Sacha inchi plants, the three parts of Sacha inchi explored in this study might have the effects of anti-tyrosinase activities.

Even when Meechai et al. (2010) studied the anti-tyrosinase activities of 77 Thai medicinal plant extracts and found about 11 plant extracts, including plants in the Euphorbiaceae family, showed the potential for these activities, Sacha inchi was not mentioned in this study. However, if the fact that the extracts of Sacha inches leaves, vines and shells contain phenolics and flavonoids is taken into consideration, then there are a number of studies confirming that plants containing phenolics and flavonoids usually have an inhibitory effect on tyrosinase activities. (Tidchai, 2019; Chumchaiyapurk, 2015; Momtaz et al., 2010)

As revealed in this study, the leaves of Sacha inchi have inhibitory effects on tyrosinase activities even if these effects are lesser than those of kojic acids. Therefore, the effects of leave extract are more interesting if compared to vines and shells. What has been found in this study will be useful for future research that involves the development of whitening products.

Conclusion

The extracts of Sacha inchi leaves, vines and shells have phenolic and flavonoid contents. The obtained results of each sample extract have shown that phenolic and flavonoid compounds are a source of antioxidant effects. All of them have some anti-tyrosinase effects. The leaf extracts have the highest content of flavonoids and a medium of phenolics. They exhibit a high amount of-enriched antioxidant activities. The extracts of leaves also have the effects of inhibitory tyrosinase activities. Vines and shells are the parts that showed the second and third highest amounts of antioxidants, respectively. Vine and shell extracts have a much lower tyrosinase

inhibitory effects than leaf extracts. All these properties can be further researched in the future to examine more efficacy and safety in vivo and do clinical studies. This would allow the local medicinal plant to be a new source of commercial value as it can be the main ingredient in the creation of new products related to anti-aging, health, and beauty.

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