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Free Radical Scavenging Activity, Tyrosinase Inhibition Activity and Fatty Acids Composition of Oils from Pupae of Native Thai Silkworm (*Bombyx mori* L.)

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ABSTRACT

Oils were extracted from five native Thai silkworm varieties, Keaw Sakon, Nangnoi Srisaket, Sam Rong, Nang Luang and None Ruesee. The yields of the oils by the Soxhlet and maceration methods were in the ranges 24–29 and 5–7%, respectively. Oils extracted from None Ruesee by the Soxhlet method, and oils extracted from Nang Leung, Sam Rong and None Ruesee by the maceration method showed free radical scavenging activity. Oil extracted from None Ruesee by the maceration method gave the highest free radical scavenging activity. Moreover, oil extracted by the Soxhlet method from None Ruesee gave the highest tyrosinase inhibition activity, but was lower than that of standards of vitamin C and kojic acid. The silkworm pupae oil obtained from Soxhlet extraction had unsaturated fatty acid content in the range 72–79% and alpha-linolenic acid content in the range 32–44%, whereas that obtained from the maceration extraction had unsaturated fatty acid content in the range 75–80% and alpha-linolenic acid content in the range 40–46%. The study indicated that oil from native Thai silkworm pupae could be used as an alternative in the food and cosmetic industries.

Keywords: silkworm pupae oil, fatty acid, antioxidant activity tyrosinase, inhibition activity

INTRODUCTION

In Oriental Asia, male silk cocoon extract has been known for its effectiveness in enhancing male stamina and improving vitality. The main ingredients are reported as protein (51%), essential fatty acid (29%), cholesterol (3%), chitin and vitamins A, B2 and D, with these vitamins being both safe and vital to the human body (Pongsatharg and Parpasrt, 1999; Mi *et al*., 2007). Silkworm pupae contain food nutrients with high nutritional value and are a good and cheap source of protein (Nipha and Arunyakorn, 1997). Silkworm pupae oil contains various essential fatty acids with bioactivity and thus can be used as raw material for cosmetics. Silkworm pupae oil extracted by boiling is used in the cosmetic industries for making soaps and moisturizers (Kotake-Nara *et al*., 2002). In the present study, the active substances in silkworm pupae were extracted by

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organic solvent. Free radical scavenging activity is used to determine the antioxidative property, to prevent aging by many oxidants. Tyrosinase inhibition activity is used to determine the inhibition of melanin formulation in the skin. Antioxidants have been used as anti-aging agents in the cosmetic industry. Tyrosinase is a copper-containing monooxygenase enzyme, which can be found in fungi, higher plants and animals. It is known to be a key enzyme in melanin biosynthesis (Leibovitz and Siegel,1980; Gutierrez *et al*., 2006). Tyrosinase inhibitors have been used as a whitening agent or an antihyperpigment agent because of their ability to suppress dermal-melanin production (Rosa *et al*., 2006). The present study aimed to investigate the chemical composition of silkworm oils obtained from native Thai silkworm pupae by the Soxhlet and maceration methods and to study the free radical scavenging activity and tyrosinase inhibition activity of the silkworm oils from five silkworm varieties in Thailand

MATERIALS AND METHODS

Preparation of the Native Thai silk worm pupae oils (Adapted from Doneanu *et al*., 1997; Nipha and Arunyakorn, 1997).

Five native Thai silkworm varieties (Keaw Sakol, Nangnoi Rrisaket, Nang Leung, Sam Rong and None Ruesee) were used for oil extraction by the Soxhlet and maceration methods using petroleum ether as the solvent. After filtering and evaporating to dryness in a vacuum, all oil samples were stored in the dark at 4 °C until used.

Physical and chemical stability of oil extracted from Thai native silkworms

An amount of 0.1 g of each oil sample was dissolved in a mixture of ethanol and water. The pH of the solution was measured by a pH meter (Model Cyberscan 510, Eutech Instruments, Singapore). The physical and chemical stability of the resulting solution were observed separately in solutions of 10% HCl, 10% CH_3COOH, 10% NaOH, 10% NH_4OH, 10% CH_3COONa, 10% $FeCl_3$ and 10% H_2O_2. Then, 0.1% oil solvent was used to measure pH. Next, a maximum of 50 drops of each of the test solutions was added drop-wise into separate oil sample solutions until a change was observed. Any changes in color, turbidity and sedimentation were noted.

Determination of linoleic acid

The linoleic acid content in the oil samples was determined by high performance liquid chromatography (HPLC) using a Luna® C18 connected to a micron 250 × 4.0 nm Phenomenex USA Column, LC1200 UV/VIS Detector and LC1100 HPLC pump, with 90% acetonitrile and 10% trifluoroacetic acid (0.1% v/v) as a mobile phase, injection volume of 10 µl, flow rate 1 mL/min and the UV detector at 210 nm. The linoleic acid content was calculated by comparing with a standard of linoleic acid (Sigma, Co., St. Louis, USA).

Determination of fatty acids

Fatty acids were measured following the analytical methods described in the regulations of the European Union Commission (1992).

Determination of tocopherol and cholesterol

Tocopherol and cholesterol were measured following the IUPAC Standard Method (IUPAC, 1992).

Free radical scavenging assay

Oil samples at 200, 100, 50, 25 and 12.5 mg/mL and the standard antioxidants: vitamin C , vitamin E, butylhydroxytoluene (BHT) and linoleic acid in a mixture of 95% ethanol and 10%(v/v) DMSO (1:1) were assayed for free radical scavenging activity by the DPPH method (Jung *et al*., 2006). Briefly, 100 µL of samples or standards, 25 µL of 2mg/mL DPPH in 95% ethanol and 25 µL of 95% ethanol were mixed in 96-well microplates and incubated at room temperature (25 °C) for 30 min. The absorbance was measured at

515 nm. The percentages of DPPH radical scavenging activity were calculated according to Equation 1:

$$\% \text{ DPPH radical scavenging activity} = (A - B) / A \times 100 \quad (1)$$

where: A = the absorbance of the control reaction,

B = the absorbance of the test samples.

Sample concentrations providing 50% scavenging (SC_{50}) were calculated from the graph plotted between free radical scavenging inhibition percentages and the sample concentrations.

Tyrosinase inhibition assay

Oil samples at 200, 100, 50, 25 and 12.5 mg/mL and the standard antioxidants: vitamin C and kojic acid were mixed with 5% (v/v) DMSO and assayed by the modified dopachrome method using tyrosine as a substrate as previously described (Piao *et al*., 2002). Briefly, 40 µL of samples or standards, 40 µL of 0.1 mg/mL L-tyrosine, 50 µL of 0.1 mg/mL mushroom tyrosinase and 80 µL of 0.1M phosphate buffer were added in 96-well microplates. A 5% (v/v) DMSO solution was used as a negative control. The mixture was incubated at 37 °C for 60 min. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 450 nm. The experiment was carried out in triplicate. The percentages of tyrosinase inhibition were calculated according to the Equation 2:

$$\% \text{ inhibition activity} = [(A - B) - (C - D)] / (A - B) \times 100 \quad (2)$$

where: A = the absorbance of the blank after incubation,

B = the absorbance of the blank before incubation,

C = the absorbance of the sample after incubation,

D = the absorbance of the sample before incubation.

Sample concentrations providing 50% inhibition (IC_{50}) were calculated from the graph plotted between tyrosinase inhibition activity percentages and the concentrations.

RESULTS AND DISCUSSION

Extraction and physico-chemical stability

The percentage yields of the extract by the Soxhlet and maceration methods from native Thai silkworm pupae oils (Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong, and None Ruesee varieties) were in the range 24–29% and 4–7%, respectively (Table 1). The Soxhlet extraction method gave yields about five times higher than those obtained from maceration extraction. The oils were soluble in ethanol and had a pH range of 6.34–6.91. All oils were precipitated in acids (10% HCl and 10% CH_3COOH), bases (10% NaOH, 10% NH_4OH and 10% CH_3COONa), reducing agent (10% $FeCl_3$) and oxidizing agent (10% H_2O_2), which showed that the oils were not stable.

Table 1 Percentage yields (mean ± standard deviation; n = 3) of ative silkworm oil extracted by Soxhlet and maceration methods.

Variety	% yield	
	Soxhlet extraction	Maceration extraction
Nangnoi Srisaket	24.00 ± 1.60	3.81 ± 0.02
Nang Leung	28.75 ± 1.52	7.00 ± 0.45
None Ruesee	24.18 ± 1.22	5.10 ± 0.67
Keaw Sakol	28.98 ± 1.65	5.77 ± 0.04
Sam Rong	27.22 ± 1.75	5.42 ± 0.89

Linoleic acid assay

The amounts of linoleic acid extracted from Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong, and None Ruesee oil samples by the Soxhlet method were 2.19, 1.60, 5.89, 5.27 and 4.63%, respectively, whereas those of Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong, and None Ruesee oils extracted by the maceration method were 2.10, 2.25, 2.57, 2.02 and 2.50%, respectively. Total lipid extracted from silk worm pupae mainly consisted of triacyglycerol, phosphatidylethanolamine and phosphatidylcholine; however, the quantities of linoleic acid in the triacylglycerol and total lipids were very small (Kotake *et al*., 2002).

Determination of fatty acids

Fatty acids were separated from the silkworm pupae oil by the Soxhlet and maceration methods. The fatty acid composition of the oils (Figures 1A, 1B, 1C, 1D and 1E) was relatively determined by gas chromatography (GC), and showed that the silkworm pupa oils comprised both saturated and unsaturated fatty acids. The unsaturated fatty acids (alpha linolenic acid and linoleic acid) are essential fatty acids and were more abundant in maceration extracts than Soxhlet extracts; the maceration extraction did not involve any heat treatment and so the chemical compounds were not destroyed and were more stable than those extracted by the Soxhlet method. The silkworm pupa oils obtained from maceration extraction of the None Ruesee variety contained the highest amount of oleic acid (30.17% as the main component) and also contained 42.31% alpha linolenic acid. The oil from Soxhlet extraction of the Keaw Sakol variety contained the highest amount of oleic acid (38.82% as a component) and also contained 32.06% alpha linolenic acid, which was more than in avocado oil (Alicia *et al*., 2003). These two fatty acids are essential for humans. The lack of fatty acid would affect the growth rate and cause skin ulcers. The human body cannot produce linoleic acid and therefore it must be obtained from food (Sappayatosok, 1988). Silkworm pupae contain more oil than soybean, with the latter having only 18.7% (Manit and Nattripop *et al*, 2000). However, soybean also contains 51% linoleic acid which was more than that in silkworm pupae oil. However, soybean has 7% linolenic acid which was less than that in silkworm pupae oil. Thus, silkworm pupae can be a good source of the functional fatty acid, α-linolenic acid; thus it has the potential to be included in functional foods and cosmetics. Silkworm pupae would be a good source of the functional fatty acid, α-linolenic acid, thus making it potentially useful for the food and cosmetic industries.

Determination of tocopherol and cholesterol

The amount of vitamin E in Sam Rong and None Ruesee silkworm pupa varieties extracted by the Soxhlet method was 16.65 and 14.54 mg/mL, respectively, whereas from maceration extraction the amount was only 9.32 and 7.31 mg/mL, respectively. The oil from Nangnoi Srisaket and Nang Leung silkworm pupa varieties obtained from the maceration method contained high levels of cholesterol of 752 and 608 mg/mL, respectively. For the same varieties, the amounts obtained by the Soxhlet method were 161.96 and 170.9 mg/mL, respectively; however, these values were less than the total cholesterol found in bovine liver (273.9 mg/mL) and common sausage (262.1 mg/mL), as revealed by Rowe *et al*. (1997).

Free radical scavenging activity

The free radical scavenging activities of the oils were assayed by the DPPH method (Jung *et al*., 2006). The IC_{50} values (mg/mL) of the samples of native Thai silkworm pupae oil are shown in Figure 2. The SC_{50} mLof the Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong, and None Ruesee oils extracted by the Soxhlet

method were 14.81, 18.25, 19.40, 18.22 and 17.01 mg/mL, respectively, and for the Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong, and None Ruesee oils extracted by the maceration method were 17.96, 18.77, 18.22, 12.51 and 10.08 mg/mL, respectively. The results indicated that the None Ruesee oil extracted by the maceration method showed higher DPPH scavenging activity than the other native Thai silkworm pupae oils.

However, the SC_{50} values of the native Thai silkworm pupae oils were higher than those of standard vitamin C, vitamin E and BHT (0.42,

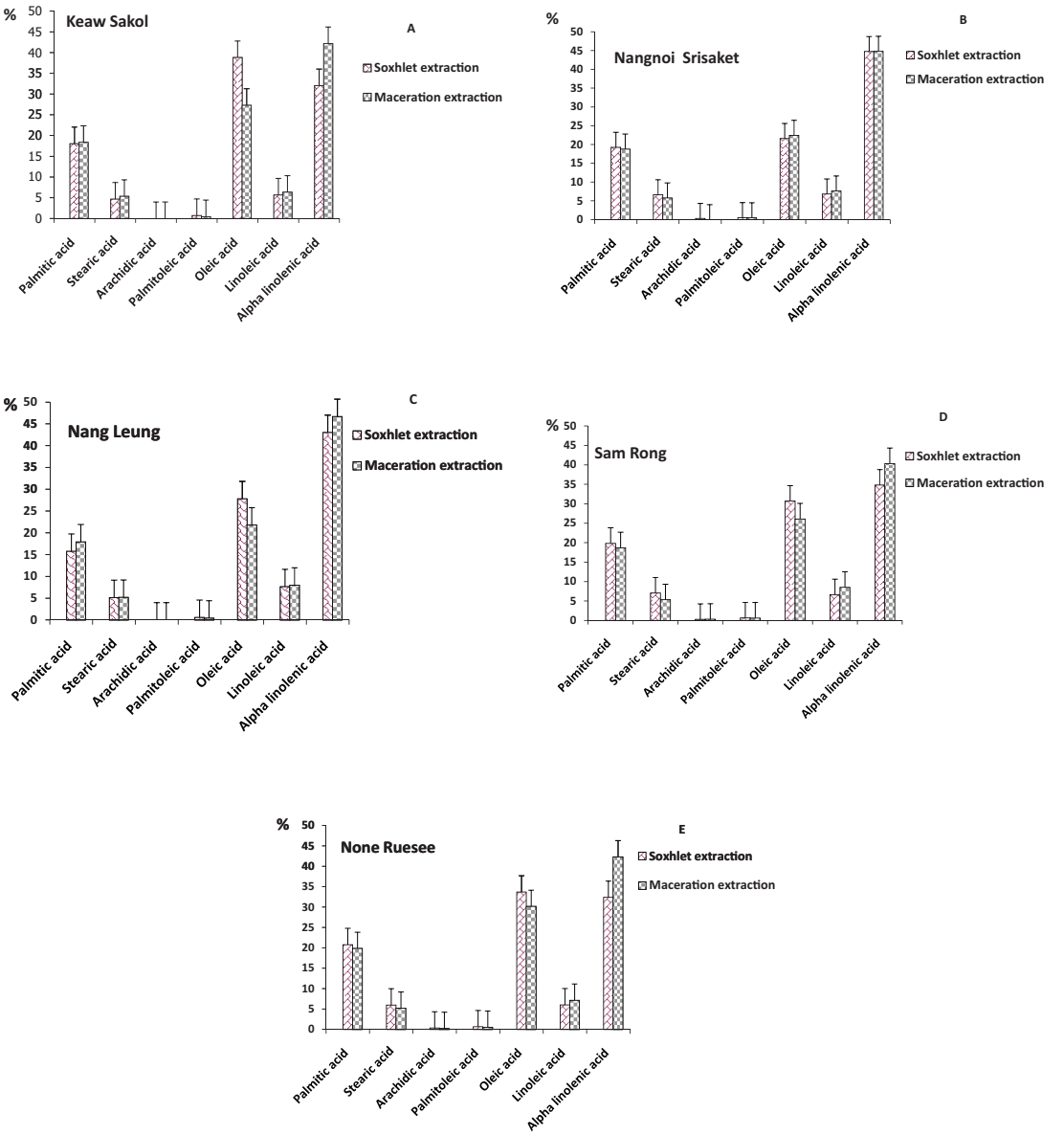

Figure 1 Percentage of fatty acids in silkworm oil of native Thai varieties extracted by Soxhlet and maceration methods (A = Keaw Sakol, B = Nangnoi Srisaket, C = Nang Leung, D = Sam Rong and E = None Ruesee varieties).

0.54 and 0.53 mg/L, respectively), but lower than that of standard linoleic acid (data not shown). The native Thai silkworm pupae oil contains the antioxidant, tocopherol (Pises *et al.*, 2006).Therefore, the oil samples might also contain some fatty acids that were more potent antioxidants than linoleic acid (Kotake *et al.*, 2002). This could explain that fact that the silkworm pupae oils contained phospholipids and tocopherol, which play an important role in protecting the lipids against oxidation, and carotenoids, such as lutein and neoxanthin, and might act as antioxidants in the oils.

Tyrosinase inhibition activity

The tyrosinase inhibition activities of the oils were assayed by the modified dopachrome method using tyrosine as a substrate (Piao *et al.*, 2002). The IC_{50} values of the native Thai silkworm pupae oils are shown in Figure 3. The IC_{50} values of the None Ruesee oil extracted by Soxhlet method and Nang Leung, Sam Rong, and None Ruesee oils extracted by the maceration method were 7.36, 31.56, 16.01, and 26.02 mg/L, respectively. However, oil from Keaw Sakol, Nangnoi Srisaket, Nang Leung, Sam Rong extracted by the Soxhlet method, and in Keaw Sakol and Nangnoi Srisaket extracted by the maceration method, showed no activity.

Table 2 Amounts (mean ± standard deviation; n = 3) of vitamin E and cholesterol of silkworm oil from Thai native varieties extracted by the Soxhlet and maceration methods.

Variety	Vitamin E (mg/mL)		Cholesterol (mg/mL)	
	Maceration extraction	Soxhlet extraction	Maceration extraction	Soxhlet extraction
Nangnoi Srisaket	9.32 ± 0.03	5.54 ± 0.03	752 ± 0.03	134.33 ± 0.03
Nang Leung	7.91 ± 0.03	9.53 ± 0.03	6.08 ± 0.03	87.82 ± 0.03
None Ruesee	7.15 ± 0.03	14.54 ± 0.03	173.52 ± 0.03	170.9 ± 0.03
Keaw Sakol	7.06 ± 0.03	6.85 ± 0.03	442 ± 0.03	71.69 ± 0.03
Sam Rong	4.72 ± 0.03	16.65 ± 0.03	165.03 ± 0.03	161.96 ± 0.03

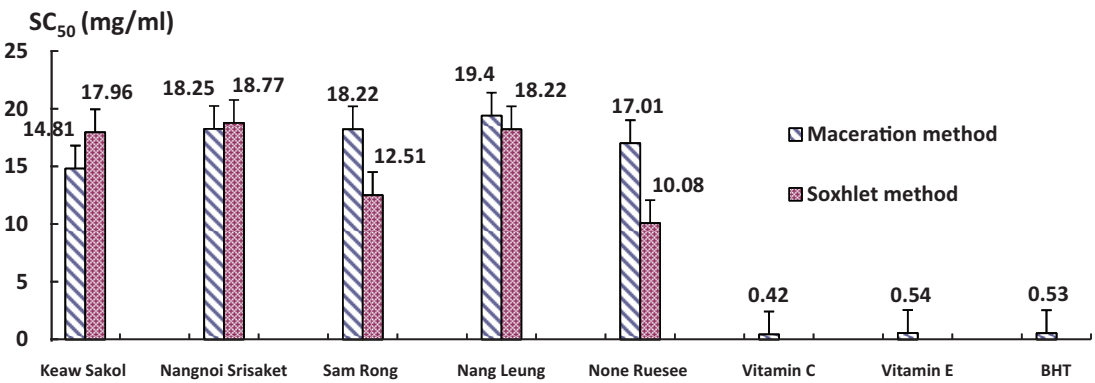

Figure 2 SC_{50} values (mean with standard deviation bar; n = 3) by DPPH scavenging assay of oils from various Thai native silkworm pupae prepared by the Soxhlet and maceration methods compared to the standard antioxidants (vitamin C, vitamin E and BHT).

The native Thai silkworm pupae oil extracted by the maceration method showed higher tyrosinase inhibition activity than that obtained from the Soxhlet method, since the substances that inhibited tyrosinase might be decomposed by heat. However, the IC_{50} of the native Thai silkworm pupae oils were higher than that of standard vitamin C and kojic acid (0.36 and 0.15, respectively).

CONCLUSION

Silkworm pupae oil is an interesting sub-product obtained after the extraction procedure of silk threads. The percentage yields of oils from the Soxhlet and maceration methods were 24–29% and 5–7%. Analysis of oil properties showed high values for fatty acids, such as alpha linolenic acid, whose content was as high as 32–47%. This fatty acid is an essential fatty acid that cannot be naturally synthesized in the body and so has to be acquired from nutritients. All oils extracted by the Soxhlet and maceration methods were unstable in acid, salt, oxidizing agent and reducing agent and thus were precipitated. There was a very small amount of linoleic acid in the oils. The DPPH assay of the native Thai silkworm pupae oils showed some scavenging activity, but it was lower than that of standard vitamin C, vitamin E and BHT. The silkworm pupae oils contained phospholipids and tocopherol, which play an important role in protecting the lipids against oxidation, and against carotenoids, such as lutein and neoxanthin, and might act as antioxidants in the oils. The tyrosinase inhibition assay of native Thai silkworm pupae oils, such as None Ruesee oil, extracted by the Soxhlet method and Nang Leung, Sam Rong and None Ruesee oils, extracted by the maceration method, showed tyrosinase inhibition activity, but it was lower than that of standard vitamin C and kojic acid. The results of the study showed the possibility of selecting suitable varieties of silkworm pupae as sources of protein and fat that could be developed for food and cosmetic products. Thus, it is possible to infer that native Thai silkworm oil could be used to balance human nutrition needs, as a supplementary food, as an alternative antioxidant, whitening agent and as a

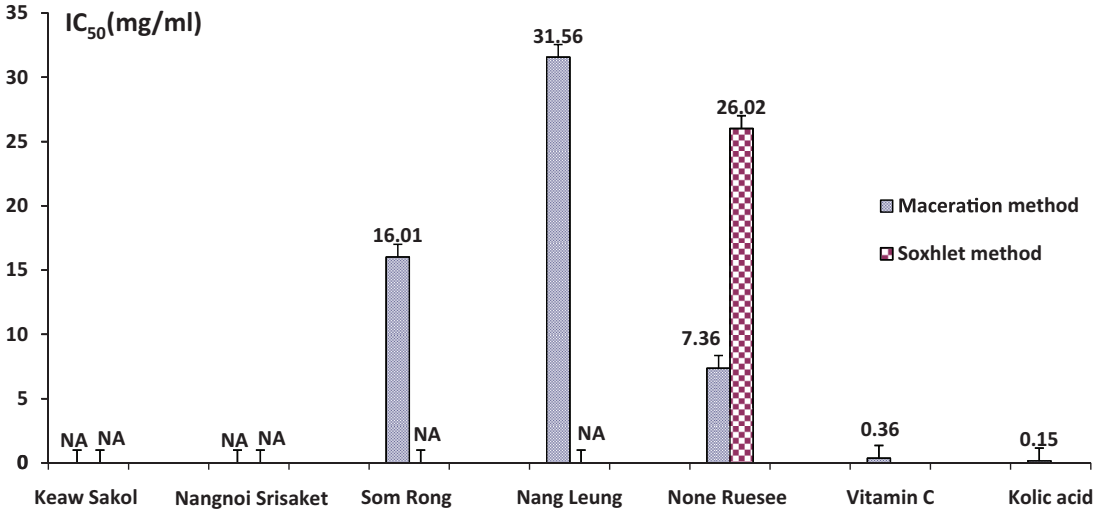

Figure 3 IC_{50} values (mean with standard deviation bar; n = 3) by tyrosinase inhibition activity assay of oil from various Thai native silkworm pupae extracted by the Soxhlet and maceration methods compared to the standard antioxidants (Vitamin C and kojic acid). NA = no activity.

moisturizer ingredient in the cosmetic industry. The results suggest the possible application in cosmetic products of the oil from None Ruesee prepared by maceration extraction, since functional fatty acid, alpha linolenic acid, free radical scavenging and tyrosinase inhibition activities were demonstrated. However, because of the stability problem with heat, even in a basic environment, further study to develop an appropriate form, such as entrapment in nanovesicles, is recommended.

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