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Effects of sapota part and extracting solvent on *in vitro* anti-aging properties of *Manilkara zapota* extract

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Objectives: The aim of this study is to investigate effects of sapota part and extracting solvent on in vitro anti-aging activities including antioxidant, anti-collagenase and anti-elastase activities of sapota extract.
Methods: Three samples, including fresh pulp, dry pulp, and seed, were prepared and extracted by maceration technique. After obtaining the crude extracts, antioxidant activities of all extracts were investigated by DPPH radical scavenging assay. The selected extracts were, thus, further screened for anti-collagenase and anti-elastase activities by using EnzChek® assay kits.
Results: The results revealed that extracting solvents and sapota parts affected extraction yields and *in vitro* anti-aging biological activities of sapota extracts. Ethyl acetate extracts exhibited low percent yield of roughly less than 2% wt. comparing with ethanolic extracts, which presented more than 10% yields. In addition, the highest yields of 34.53% wt. were observed in 60% ethanolic dried pulp extracts. Ethyl acetate extracts of fresh pulp showed the highest antioxidant activity among other extracts with IC_{50} of 37.78 µg/mL. However, 60% ethanolic and 95% ethanolic fresh pulp extracts were selected for further investigation on anti-proteinase activities due to their moderate antioxidant activities with considerable yield. Both 60% ethanolic and 95% ethanolic fresh pulp extracts at concentration of 140 µg/mL exhibited moderate collagenase inhibition with percent inhibitions of 66.42% and 64.66%, respectively. In contrast, 95% ethanolic fresh pulp extract showed stronger inhibitory effects with 47.74% inhibition on elastase activity than that of 60% ethanolic fresh pulp extracts.
Conclusion: The differences in extraction yields and *in vitro* anti-aging activities of sapota extracts were affected by types of extracting solvents and sapota parts. The present study revealed that fresh pulp extracted with 95% ethanol possessed moderate inhibitory effects on DPPH radical scavenging and collagenase activities, but showed strong elastase inhibition activity among other extracts.

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Introduction

Skin aging is a perpetual phenomenon confronted by all humans throughout their life spans. Aging process is unavoidable, however, skin care products could delay the aging process by maintaining physical appearance and prolonging youth of the skin.

Recently, natural products including green tea, grape seed, and pomegranate were mostly used in anti-aging skin care products because of their antioxidant activities.[1] The tropical fruit *Manikara zapota*, or commonly known as sapota is seasonal fruit in Thailand. It was reported to consist of several polyphenolic compounds including catechin, epicatechin, and quercitrin[2], and to exhibit the stronger antioxidant activity than strawberry, guava, star fruit and grape seed.[3]

There are several factors reported to affect the biological activities of plant extracts such as extracting solvent, maturation stage, and sapota part.[4-6] The aim of this study is, thus, to investigate effects of sapota part and extracting solvent on *in vitro* anti-aging activities including both antioxidant and anti-proteinase activities of sapota extract.

Materials and methods

Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, epigallocatechin gallate (EGCG) were purchased from Sigma (Sigma-Aldrich®, USA). Collagenase and Elastase assay kits were purchased from Molecular Probes (Molecular Probes®,USA). All other reagents and chemicals used were analytical grade and purchased from RCI Labscan (RCI Labscan, Thailand).

Sapota extract preparation

Unripe Malay sapota fruit were purchased from seasonal fruit ground market of Talaad Thai in Phathum Thani, Thailand. Three samples, including fresh pulp, dry pulp, and seed, were prepared. The seeds were removed and the pulps were cut to serve as fresh pulp sample. Dry pulp sample was dried at 45°C in the oven. To prepare seed sample, the seeds were washed, air-dried, and cut into small pieces. Three samples were macerated with three different solvents including 60% ethanol, 95% ethanol, and ethyl acetate at room temperature for 24 hours each cycle. After three cycles of maceration, all collected solvents were combined and evaporated under reduced pressure at 40°C by using rotary evaporator (Büchi, Switzerland). All crude extracts were obtained and percent yield of each extract was calculated.

***In vitro* biological activities of individual extracts**

DPPH radical scavenging assay

DPPH radical scavenging assay was slightly modified from Marinova and his colleagues.[7] The assay mixture contained 100 µL of DPPH in absolute ethanol and 100 µL of test sample at different concentrations. The test mixture was mixed and incubated in light protection for 30 minutes at room temperature, and then the absorbance was measured at 510 nm using a microplate reader (Perkin-Elmer™, USA). L-ascorbic acid was used as a standard inhibitor. All measurements were performed in triplicate. The concentration of test sample showing 50% inhibition of DPPH free radicals (IC_{50}) was calculated.

Collagenase inhibition activity

The collagenase inhibition activity was performed using the EnzChek® E-12055 Collagenase assay kit as stated in the product leaflet. Crude extracts were dissolved in deionized water at concentration of 140 µg/mL. DQ™ gelatin substrate and collagenase enzyme from Clostridium histolyticum (ChC) were prepared in buffer solution. The final reaction mixture contained the sample, DQ™ gelatin, and collagenase enzyme. After 90 minutes incubation with light protection, the fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 535 nm using a microplate reader (Perkin-Elmer™, USA). EGCG and 1,10-Phenanthroline were used as positive control and a standard inhibitor, respectively. The percentage of collagenase inhibition activity was calculated, and background fluorescence was subtracted from each value. All measurements were performed in triplicate.

Elastase inhibition activity

The elastase inhibition activity was performed using the EnzChek® E-12056 elastase assay kit as stated in the product leaflet. Crude extracts were dissolved in deionized water at concentration of 80 µg/mL. DQ™ elastin substrate and elastase enzyme from pancreatic elastase were prepared in buffer solution. Test sample and elastase enzyme were pre-incubated for 15 minutes prior to addition of DQ™ elastin in a 96-well plate and protected from light. After 30 minutes incubation, the fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 535 nm using a microplate reader (Perkin-Elmer™, USA). EGCG and N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone were used as positive control and a standard inhibitor, respectively. The percentage of elastase inhibition activity was calculated, and background fluorescence was subtracted from each value. All measurements were performed in triplicate.

Results

Sapota extract preparation

The percent yields of crude extracts are shown in Table 1. More than 10% yields were observed in 60% ethanolic and 95% ethanolic pulps extracts. 60% ethanolic dried pulp extract exhibited the highest yield of 34.53% wt. In contrast, ethyl acetate extracts and both extracts from the seeds exhibited low percent yield of roughly less than 2% wt.

Table 1. Effects of extracting solvent on the crude extract yields (% wt.) of *M. zapota* pulps and seeds

Crude extracts	Extract percent yield (% wt.)		
	60% Ethanol	95% Ethanol	Ethyl acetate
Fresh sapota pulps	14.02	12.76	0.31
Dry sapota pulps	34.53	11.72	1.00
Sapota seeds	N/A	1.85	2.09

***In vitro* biological activities of individual extracts**

DPPH radical scavenging assay

The DPPH radical scavenging activity of sapota extracts are shown in Table 2. Ability to scavenge DPPH free radical was observed in pulp extracts. Ethyl acetate extract of fresh pulp showed the strongest anti-free radical property amongst test samples with IC_{50} of 37.78 µg/mL. Dry pulp extracted with 95% ethanolic solvent offer significantly higher in DPPH free radical scanvenging effects than other two dried samples. In consideration with percent yield and physical appearance, the 60% ethanolic and 95% ethanolic fresh pulp extracted were, therefore, selected for further investigation on anti-collagenase and anti-elastase activities.

Table 2. DPPH radical scavenging activities of *M. zapota* crude extracts

Crude extracts	DPPH scavenging assay IC_{50}(µg/mL)		
	60% Ethanol	95% Ethanol	Ethyl acetate
Fresh sapota pulps	63.72±2.61	80.00±1.88[a]	37.78±1.68
Dry sapota pulps	128.67±6.96	77.32±1.3[a]	≥1700
Sapota seeds	N/A	285.77±21.69	2542.31±84.82

All values are expressed as mean ± standard deviation (n=3).
*Values with the same letter are not significantly different ($p < 0.05$)

Collagenase inhibition assay

The results of collagenase inhibition assay, as shown in Figure 1, revealed that 60% ethanolic and 95% ethanolic extracts of fresh pulps at concentration of 140 µg/mL showed strong inhibition on collagenase activity with 66.42% and 64.66% inhibitions, respectively. However, the anti-collagenase effects of extracts were much lower than that of EGCG which exhibited 98.43% inhibitory effects at concentration of 20 µg/mL.

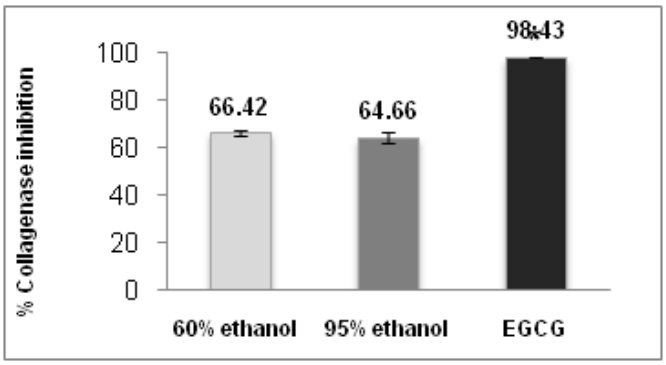

Figure 1. The percent inhibition on collagenase activity of 60% ethanolic and 95% ethanolic fresh pulp extracts at concentration of 140 µg/mL, and EGCG at concentration of 20 µg/mL. The bars presented as mean ± standard deviation (n=3). Asterisk indicated significant differences ($p<0.05$) among samples.

Elastase inhibition assay

The results of elastase inhibition assay were shown in Figure 2. 60% ethanolic and 95% ethanolic extracts of fresh pulps at concentration of 80 µg/mL exhibited 19.35% and 47.74% elastase inhibition, respectively. At the same concentration of 80 µg/mL, the 95% ethanolic extracts of fresh pulp exhibited strong inhibitory effects on elastase activity comparable to that of EGCG, which showed 45.54% inhibition.

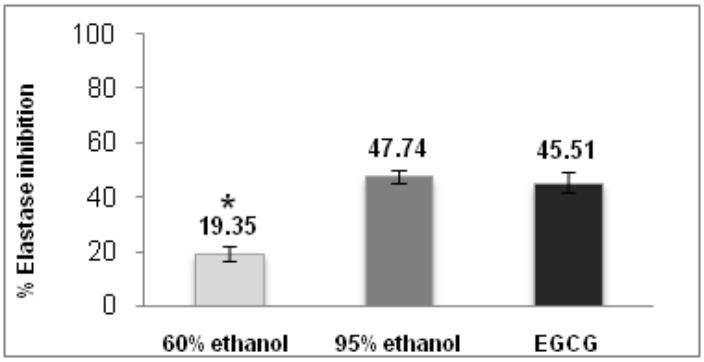

Figure 2. The percent inhibition on elastase activity of 60% ethanolic and 95% ethanolic fresh pulp extracts and EGCG at concentration of 80 µg/mL. The bars presented as mean ± standard deviation (n=3). Asterisk indicated significant differences ($p<0.05$) among samples.

Discussion

Natural polyphenolic compounds, particularly presented in fruit, drew more interest in the scientific researches because of their antioxidant properties[1] and economics. However, the antioxidant activities of natural extracts might be affected by types of extracting solvents and natural parts.[8-10] The results of present study on sapota extracts were in agreement with those previous reports, in which the extraction yields and *in vitro* biological activities of sapota extracts were influenced by sapota parts and extracting solvents. Seed extracts showed low percent yield among other extracts. The results could be implied that sapota seed extract might have significantly lower in amount of active components than that of pulp extract and presented low antioxidant activity. These data are in conflict with previous study on seed coat

extract reported by Kanlayavattanakul and his colleagues[11], showing potent inhibition effects on DPPH radical scavenging activity. The strong inhibition effects may be due to the method of sample preparations, which only seed coats were used and ground into powder before being extracted. The extracting solvent also showed an impact on sapota extraction on both yields and activities. The difference in solvent polarity may extract different types and amounts of active compounds. Sapota extracts have been reported to contain catechin, epicatechin, gallocatechin, and quercitrin.[2, 12] Among these active compounds were considered toward non-polar property[13]. Extraction of sapota fresh pulp by ethyl acetate dissolved or withdrew only selective compounds due to its non-polar effects, leading to low percent yield and unsurprisingly strong antioxidant activity compared to ethanolic extracts. In contrast, the ethanolic extracts showed higher percent yields than that of ethyl acetate due to an increasing in polarity of solvent. The ethanolic extracts were capable to extract more substances including inactive compounds, resulting in low antioxidant activity. In addition, 60% ethanolic dried pulp extract had highest percent yield which might be due to its 4 times higher in initial weight of fresh samples and its ability to dissolve more on non-selective compounds. Thus, this fraction offered low antioxidant activity. The extracts with potential antioxidant effects in consideration with yield and suitable appearance were selected for further study. Therefore, 60% and 95% ethanolic fresh pulp extracts were chosen for investigation on anti-proteinase activities.

The inhibitory effects on collagenase activities showed that 60% ethanolic fresh pulp extract at concentration of 140 µg/mL possessed moderate inhibition comparable to that of 95% ethanolic fresh pulp extracts, but had lower inhibitory effects than that of EGCG at concentration of 20 µg/mL. In contrast, the 95% ethanolic fresh pulp extract at concentration of 80 µg/mL showed stronger elastase inhibitory activity than that of 60% ethanolic extracts. It can be seen that the inhibitory effects on each pathways were, hence, not correlated. The 95% ethanol may extract more specific compounds for anti-elastase activity, which its activity was comparable to that of EGCG.

Conclusion

The present study revealed that the extracting solvents and sapota parts had an influence on extraction yields and *in vitro* anti-aging biological properties of sapota extracts. The results showed that fresh pulp extracted with 95% ethanol exhibited moderate antioxidant and anti-collagenase activities, but showed strong anti-elastase activity among others. Further studies on active compound identifications may be required to obtain information on active component(s) presenting in sapota extract.

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