Thai Journal of Pharmaceutical Sciences (TJPS)

Journal homepage: http://www.tjps.pharm.chula.ac.th

Comparative *in vitro* anti-aging activities of *Phyllanthus emblica* L. extract, *Manilkara sapota* L. extract and its combination.

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Keywords: *Phyllanthus emblica* L.; *Manilkara sapota* L.; Combination; Antioxidant; Anti-collagenase; Anti- elastase

Objectives: To investigate *in vitro* anti-aging properties of *Phyllanthus emblica* L. (amla), *Manilkara sapota* L. (sapota) and its combination extracts including an *in vitro* antioxidant, anti-collagenase and anti-elastase activities for cosmetic application.
Methods: The ethanolic extracts of both individual amla and sapota extracts and combination extract at the ratios of 1:1 were prepared. The antioxidant capacity was investigated by DPPH radical scavenging assay. Anti-collagenase and anti-elastase assays properties were evaluated by using the EnzCheck® assay kits (Molecular-Probes, Eugene, OR).
Results: The result of DPPH scavenging assay exhibited that amla showed the most potent activity (IC_{50} 1.84±0.1 µg/ml) while sapota showed the weakest activity (IC_{50} 29.7±129 µg/ml). The combination extract provided activity comparable to standard ascorbic acid and individual amla extract with IC_{50} of 3.13±0.06 µg/ml. Conversely, strongest inhibitions against collagenase and elastase were detected for sapota with IC_{50} of 65.68±3.63 and 36.82±0.72 µg/ml, respectively, followed by combination extract and individual amla extract.
Conclusion: Amla extract showed potent antioxidant activity while sapota showed potent anti-collagenase and anti-elastase activity. The combination of both extracts did not offer additive effect but rather synergistic effect which resulted in the strong antioxidant, anti-elastase and anti-collagenase activities. This suggested that this combination extracts is of interest for being use as cosmetic ingredient.

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Introduction

The natural ingredients especially plant extracts have been widely included in cosmetic products to moisturize and relieve in the sign of skin aging. The research on anti-aging activities of plant extract has been continuously investigated and mostly focused on single extract. *Phyllanthus emblica* L. commonly known as Amla or Makampom in Thai has been used in many traditional medicines. It contains polyphenolic and phenolic compounds such as gallic acid, cinnamic acid, quercetin and ellagic acid[1]. Amla offers effective antioxidant, antimelanogenesis, and anti-inflammation properties[2] with ability to decrease MMP-1 and MMP-2 production in fibroblasts[3-5]. *Manilkara Sapota* L. is called Lamud in Thai. It has been investigated for nutritional benefit. Lamud presented moderate antioxidant property[6]. Its activity was suspected to be due to the presence of polyphenolic compounds such as methyl 4-O-galloylchlorogenate, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and gallic acid[7]. The study within our research group revealed its strong anti-collagenase and anti-elastase activities with moderate antioxidant effect[5].

Skin aging involves several pathways. Active compound with complete anti-aging properties is barely achieved. Amla showed potent antioxidant and efficient anti-collagenase effect while sapota showed efficient anti-elastase and anti-collagenase effects[5]. Thus, incorporation of the extracts as a combination might enhance overall activities. In the present study, the effects of amla and sapota extracts as a combination were investigated for antioxidant, anti-collagenase and anti-elastase properties. The data will provide beneficial information for cosmetic uses.

Materials and Methods

Materials: Dried *Phyllanthus emblica* L. fruit (Amla) was purchased from Chao Phraya Abhaibhubejhr Hospital, Prachinburi province, Thailand. *Manilkara sapota* L. fruit (Sapota) was purchased from Thai vegetable and fruit market in Pathum thani province, Thailand. DPPH (2,2- diphenyl-1-picrylhydrazyl) and EGCG were supplied from Sigma Aldrich (USA). Ascorbic acid was obtained from Carlo Erba (Italy). EnzChek® collagenase /gelatinase assay kit (E-12055) and Enzchek® elastase assay kit (E-12056) were purchased from Molecular-Probes (USA).

***Preparation of plant extract*:** The dried amla fruits were ground and extracted with 95% ethanol for 24 hours. The solvent was filtered and the extraction was repeated for 3 cycles. The solvent was combined and evaporated by rotary evaporator at 40°C until dryness. Fresh sapota fruits was sliced into small pieces and the seeds were discarded. The fresh sapota fruits were macerated with absolute ethanol and followed the extraction process as for amla. Both crude extracts were kept in the dark at 4°C until further used.

The crude combination extract which was the mixture of amla and sapota extracts at the ratio of 1:1 was prepared for further investigation on *in vitro* DPPH radical scavenging, anti-collagenase and anti-elastase activities.

***In vitro DPPH radical scavenging assay*:** The DPPH radical scavenging assay was determined based on a modified method from Marinova and Batchvarov (2011)[8]. Briefly, 100 µl of sample in ethanol at different concentration was mixed with 100 µl of 0.06 mM DPPH ethanolic solution in 96 well microplate and was incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 510 nm by using microplate reader. Ascorbic acid was used as a standard. DPPH inhibition was calculated by the following equation:

$$\text{DPPH Inhibition (\%)} = [(A_{control}-A_{sample})/A_{control}] \times 100$$

The antioxidant activities of test extracts were expressed as an IC_{50} value which is concentration of the test extract providing 50% inhibition.

***In vitro anti-collagenase assay*:** The *in vitro* anti-collagenase activity was determined by using EnzChek E-12055 gelatinase/collagenase assay kit[5]. The given protocol was followed. 80 µl of sample solution was mixed with 20 µl of DQ™ gelatin substrate and 100 µl of 0.4 U/ml collagenase in 96-well microplate and incubated for 90 minutes at room temperature in the dark. The fluorescent intensity was measured at excitation and emission wavelength of 485 nm and at 535 nm, respectively, by microplate reader. (-)-Epigallocatechin gallate (EGCG) was used as a standard. Collagenase inhibition was calculated by equation:

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

Where A is fluorescent intensity without test sample, B is fluorescent intensity without test sample and enzyme, C is fluorescent intensity of test sample, D is fluorescent intensity of test sample and no enzyme. The anti-collagenase activities of all test samples were reported as an IC_{50} value which is concentration of the test extract providing 50% inhibition.

***In vitro anti-elastase assay*:** The *in vitro* anti-elastase activity was determined by using EnzChek E-12056 elastase assay kit[5]. The given protocol was followed. 50 µl of sample solution was mixed with 100 µl of 0.4 U/ml elastase enzyme in 96 well microplate and preincubated for 15 minutes. Next, 50 µl of DQ™ elastin substrate was added and incubated for 30 minutes at room temperature in the dark. The fluorescent intensity was measured at excitation and emission wavelength of 485 nm and at 535 nm, respectively, by microplate reader. (-)-Epigallocatechin gallate (EGCG) was used as a standard. Elastase inhibition was calculated by equation:

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

Where A is fluorescent intensity without test sample, B is fluorescent intensity without test sample and enzyme, C is fluorescent intensity of test sample, D is fluorescent intensity of sample and no enzyme.
The anti-elastase activities of all test samples were reported as an IC_{50} value which is concentration of the test extract providing 50% inhibition.

Statistical analysis
The data were reported as mean ± standard deviation of triplicate. IC_{50} values were calculated from linear regression analysis. One-way ANOVA was used to analyze the significant difference between samples ($P<0.05$) by SPSS version 22 software.

Results

***In vitro* DPPH radical scavenging assay**
DPPH radical scavenging activity of both individual amla and sapota extracts and combination extract at the ratio of 1:1 are shown in Figureure 1. Amla presented strong antioxidant activity in comparable to the standard, ascorbic acid, followed by combination extract. sapota extract showed the weakest activity which was at least 10 times inferior than other test samples.

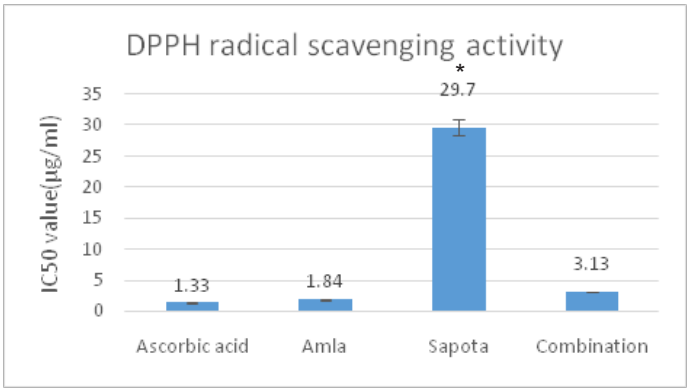

Figure.1 DPPH radical scavenging activities of ascorbic acid, amla extract, sapota extract and combination extract.
* Significantly different from combination extract, p<0.05

***In vitro* anti-collagenase assay**

In vitro anti-collagenase activities of all test samples and EGCG are presented as IC_{50} values (Figureure 2). Amongst the test extracts, sapota showed the most effective in collagenase inhibition followed by combination extract and amla, respectively. However, these activities of all test samples were much lower than that of the standard EGCG.

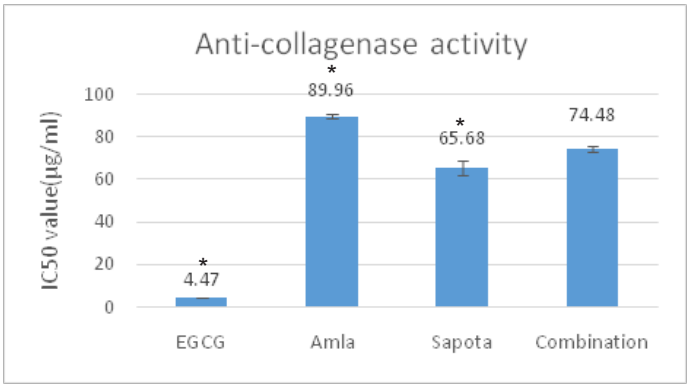

Figure.2 Anti-collagenase activities of EGCG, amla extract, sapota extract and combination extract.
* Significantly different from combination extract, p<0.05

***In vitro* anti-elastase assay**

In vitro anti-elastase activities of all test samples and EGCG are shown as IC_{50} values in Figureure 3. Sapota showed the highest anti-elastase activity followed by combination extract and standard EGCG, respectively, and amla showed the weakest activity which was about 14 times less than sapota.

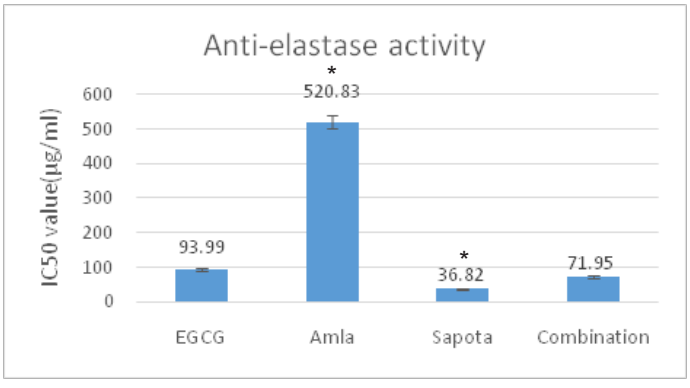

Figure.3 Anti-elastase activities of EGCG, amla extract, sapota extract and combination extract.
* Significantly different from combination extract, p<0.05

Discussion

Several pathways are involved in skin aging. Single extract usually provides high efficacy on one or two specific pathway(s). Application of combining two or more extracts is proposed to fulfil the anti-aging activities by attacking on several pathways. Using of combination of two extracts which have different potential activities may cover more pathways of skin aging. The result of this study showed that amla was very potent to scavenge DPPH radical comparable to ascorbic acid but amla showed the weakest anti-elastase effect. Amla extract was previously found to contain high

phenolic contents which mainly were gallic acid and ellagic acid. Both compounds showed high DPPH scavenging activity[9] and poor anti-elastase activity[10]. Where sapota showed significantly lower in antioxidant effect than ascorbic acid and amla extract. However, it presented the strongest anti-aging activity. The results were in agreement with previous study which showed IC_{50} 37.63±1.18 and IC_{50} 35.73±0.61 µg/ml in DPPH radical scavenging and anti-elastase activities, respectively[5]. Both extracts had their own dominant effect; therefore, the combination may be able to obtain several anti-aging effects. Surprisingly, combination between amla and sapota at ratio of 1:1 did not offer the addition effect but rather provided 9.5 folds increase in antioxidant activity compared to individual sapota and 7 folds increase in anti-elastase activity compared to individual amla. Combination extract showed equivalent antioxidant and anti-elastase properties to the standards. In DPPH assay, predominant effect was dependent on amla which was a powerful antioxidant. Therefore, it improved antioxidant activity of sapota in combination extract. The sapota extract showed effective in anti-elastase activity, thus, it predominated this effect when using as a combination. For anti-collagenase activity, sapota showed slightly potent than amla and the result of combination extract presented slightly change in such activity as expected.

Conclusion

Amla extract showed potent antioxidant activity while sapota showed potent anti-collagenase and anti-elastase activities. The mixture of two extracts at ratio of 1:1 did not suppress the potential activities of each extract. It provided good antioxidant, anti-collagenase and anti-elastase activities. These results suggested that the combination of amla and sapota could be used as an ingredient in cosmetics since it provided several activities in one product. However, futher investigations are required to compare the effects of different ratios and to find the proper ratio which provides the best anti-aging effects for cosmetic uses.

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