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IN VITRO BIOLOGICAL PROPERTIES OF *PHYLLANTHUS EMBLICA*, *MANILKARA ZAPOTA* EXTRACTS AND THEIR COMBINATIONS FOR COSMETIC APPLICATIONS

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การเปลี่ยนแปลงสภาพผิวประกอบด้วยหลายกลไก สารสกัดจากธรรมชาติได้ถูกนำมาใช้เพื่อฟื้นฟูสภาพผิว อย่างไรก็ตามสารสกัดเดี่ยวที่ให้คุณสมบัติหลากหลายนั้นมีจำกัด การผสมสารสกัดหลายชนิดจึงอาจเพิ่มคุณสมบัติทางเครื่องสำอางมากขึ้น การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ทางชีวภาพแบบนอกกาย ของสารสกัดมะขามป้อม สารสกัดละมุด และสารสกัดผสมมะขามป้อมและละมุดที่สัดส่วน 5:1 1:1 และ 1:5 ต่อฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบ ฤทธิ์ต้านเอนไซม์คอนลาจิเนส ฤทธิ์ต้านเอนไซม์อิลาสเทส และฤทธิ์ต้านเอนไซม์ไทโรซิเนส ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านการอักเสบทดสอบโดยวิธีการยับยั้งอนุมูลอิสระดีพีพีเอช และวิธีการยับยั้งอนุมูลอิสระไนตริกออกไซด์ตามลำดับ ฤทธิ์ต้านเอนไซม์คอนลาจิเนส ฤทธิ์ต้านเอนไซม์อิลาสเทส และฤทธิ์ต้านเอนไซม์ไทโรซิเนส ทำการวิเคราะห์ด้วยชุดทดสอบ EnzChek® คอนลาจิเนส ชุดทดสอบ EnzChek® อิลาสเทส และวิธีการวัดสีของสารโดปาโครมตามลำดับ สารสกัดมะขามป้อมด้วยเอทานอลแสดงฤทธิ์การต้านอนุมูลอิสระและฤทธิ์ต้านการอักเสบสูงที่สุด ด้วยค่า IC_{50} เท่ากับ 1.84 ± 0.1 และ 17.31 ± 0.37 ไมโครกรัมต่อมิลลิลิตรตามลำดับ ขณะที่สารสกัดละมุดด้วยเอทานอลแสดงฤทธิ์ต้านเอนไซม์คอนลาจิเนสและฤทธิ์ต้านเอนไซม์อิลาสเทสสูงที่สุดด้วยค่า IC_{50} เท่ากับ 65.68 ± 3.63 และ 36.82 ± 0.72 ไมโครกรัมต่อมิลลิลิตรตามลำดับ สำหรับฤทธิ์ต้านเอนไซม์ไทโรซิเนสนั้นสารสกัดละมุดแสดงฤทธิ์การต้านเอนไซม์ไทโรซิเนสต่ำด้วยค่า IC_{50} เท่ากับ 5862.52 ± 169.5 ไมโครกรัมต่อมิลลิลิตร ในขณะที่สารสกัดมะขามป้อมไม่สามารถระบุฤทธิ์ได้ สารสกัดผสมมะขามป้อมและละมุดที่อัตราส่วน 5:1, 1:1 และ 1:5 แสดงฤทธิ์ต้านอนุมูลอิสระด้วยค่า IC_{50} เท่ากับ 2.21 ± 0.15, 3.13 ± 0.06 และ 7.3 ± 0.73 ไมโครกรัมต่อมิลลิลิตรตามลำดับ และแสดงฤทธิ์ต้านการอักเสบด้วยค่า IC_{50} เท่ากับ 19.72 ± 0.35, 31.14 ± 0.49 และ 68.8 ± 1.35 ไมโครกรัมต่อมิลลิลิตรตามลำดับ สารสกัดผสมที่มีปริมาณของมะขามป้อมเป็นหลักแสดงฤทธิ์การต้านอนุมูลอิสระและฤทธิ์ต้านการอักเสบสูงสุด สำหรับการต้านเอนไซม์โปรตีน สารสกัดผสมมะขามป้อมและละมุดที่อัตราส่วน 5:1, 1:1 และ 1:5 แสดงฤทธิ์ต้านเอนไซม์คอนลาจิเนส ด้วยค่า IC_{50} เท่ากับ 78.9 ± 3.42, 74.48 ± 1.61 และ 68.68 ± 2.69 ไมโครกรัมต่อมิลลิลิตรตามลำดับ และแสดงฤทธิ์ต้านเอนไซม์อิลาสเทส ด้วยค่า IC_{50} เท่ากับ 150.72 ± 7.9, 71.9 ± 5.06 และ 36.22 ± 0.99 ไมโครกรัมต่อมิลลิลิตรตามลำดับ สารสกัดละมุดแสดงฤทธิ์ที่โดดเด่นในสองการทดสอบนี้และสารสกัดผสมที่มีปริมาณของละมุดเป็นหลักแสดงฤทธิ์ต้านเอนไซม์คอนลาจิเนส และฤทธิ์ต้านเอนไซม์อิลาสเทสสูงสุด ค่าดัชนีการผสมสาร (CI) ที่จุดกึ่งกลางของระบบ ส่วนใหญ่แสดงผลกระทบจากการผสมสารเป็นการรวมฤทธิ์ ยกเว้นสารสกัดผสมที่อัตราส่วน 1:1 ในฤทธิ์ต้านอนุมูลอิสระ อัตราส่วน 5:1 และ 1:5 ในฤทธิ์ต้านการอักเสบ อัตราส่วน 5:1 ในฤทธิ์ต้านเอนไซม์คอนลาจิเนส และอัตราส่วน 1:5 ในฤทธิ์ต้านเอนไซม์อิลาสเทส ซึ่งแสดงผลกระทบจากการผสมสารเป็นการเสริมฤทธิ์ เป็นที่น่าสนใจว่าสารสกัดผสมที่อัตราส่วน 1:5 แสดงฤทธิ์ต้านอนุมูลอิสระสูงและฤทธิ์ต้านการอักเสบที่ดีจากผลของสารสกัดมะขามป้อม ยิ่งไปกว่านั้นยังแสดงฤทธิ์ต้านเอนไซม์อิลาสเทสสูงและฤทธิ์การต้านเอนไซม์คอนลาจิเนสที่ดีจากผลของสารสกัดละมุด สารสกัดผสมที่อัตราส่วนนี้จึงอาจสามารถครอบคลุมคุณสมบัติทางเครื่องสำอาง และอาจถูกนำไปใช้เป็นสารออกฤทธิ์ในผลิตภัณฑ์เครื่องสำอางในภายภาคหน้า

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต	.....
สาขาวิชา	วิทยาศาสตร์เครื่องสำอาง	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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RAPEEKAN SAKDAWATTANAKUL: *IN VITRO* BIOLOGICAL PROPERTIES OF *PHYLLANTHUS EMBLICA*, *MANILKARA ZAPOTA* EXTRACTS AND THEIR COMBINATIONS FOR COSMETIC APPLICATIONS. ADVISOR: ANYARPORN TANSIRIKONGKOL, Ph.D., CO-ADVISOR: ASST. PROF. VIPAPORN PANAPISAL, Ph.D., 105 pp.

The changes in skin conditions involve several pathways. Natural extract has been included to improve the skin condition. However, single extract with several properties is barely available. The purpose of this study is to investigate the effect of individual amla and sapota fruit extracts and their combinations at the ratio of 5:1, 1:1 and 1:5 on *in vitro* antioxidant, anti-inflammatory, anti-collagenase, anti-elastase and anti-tyrosinase activities. The antioxidant and anti-inflammatory activities were determined by DPPH radical scavenging assay and by nitric oxide radical scavenging assay, respectively. Anti-collagenase, anti-elastase and anti-tyrosinase were analyzed by EnzChek® gelatinase/collagenase assay kit, EnzChek® elastase assay kit and DOPA chrome method, respectively. Ethanolic amla extract exhibited the strongest antioxidant and anti-inflammatory activities with IC_{50} of 1.84 ± 0.1 and 17.31 ± 0.37 µg/ml, respectively, while ethanolic sapota eaxtract revealed the highest anti-collagenase and anti-elastase activities with IC_{50} of 65.68 ± 3.63 and 36.82 ± 0.72 µg/ml, respectively. For anti-tyrosinase activity, sapota extract showed low activity with IC_{50} of 5862.52 ± 169.5 µg/ml whereas it was undetectable in amla extract. Combination amla and sapota extract at the ratio of 5:1, 1:1 and 1:5 provided antioxidant activity with IC_{50} of 2.21 ± 0.15, 3.13 ± 0.06 and 7.3 ± 0.73 µg/ml, respectively, and showed anti-inflammatory activity with IC_{50} of 19.72 ± 0.35, 31.14 ± 0.49 and 68.8 ± 1.35 µg/ml, respectively. Combination containing amla as a main constituent showed the highest activities. For anti-proteinase activity, their combination at the ratio of 5:1, 1:1 and 1:5 exhibited anti-collagenase activity with IC_{50} of 78.9 ± 3.42, 74.48 ± 1.61 and 68.68 ± 2.69 µg/ml, respectively, and revealed anti-elastase activity with IC_{50} of 150.72 ± 7.9, 71.9 ± 5.06 and 36.22 ± 0.99 µg/ml, respectively. Sapota showed a predominant in both activities and the combination which contained sapota as a main constituent exhibited the highest in both activities. The combination index (CI) at median condition of most combination ratios were exhibited additive effect, except combination 1:1 in antioxidant, combination 5:1 and 1:5 in anti-inflammatory, combination 5:1 in anti-collagenase and combination 1:5 in anti-elastase activities which showed synergistic effect. Interestingly, combination 1:5 provided potent antioxidant and good anti-inflammatory activities from the effect of amla extract, moreover, it obtained effective anti-elastase and good anti-collagenase from the effect of sapota extract. This combination, therefore, may enhance overall cosmetic properties and may be further included as an active ingredient in cosmetic product.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Cosmetic Science	Co-Advisor's Signature	.....
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CHAPTER I
INTRODUCTION

Ultraviolet irradiation from sunlight is categorized into different subtypes including UVA, UVB and UVC. UVB radiation (290-320 nm) can damage superficial skin which causes sunburn and leads to developing of skin cancer. UVA radiation (320-400 nm) could be able to penetrate deeper into the skin which causes skin aging and tanning. Long-term sun exposure can promote production of reactive oxygen species (ROS) and induce oxidative stress which becomes harmful to cells due to the damage of protein, lipid and intracellular structure such as DNA (Herrling, Jung, and Fuchs, 2006). Moreover, it can induce the formation of destructive metalloproteinase which degrades collagen and elastin causing skin wrinkle, sagging and premature skin aging. In addition, stimulation of melanogenesis by UV radiation could increase the production of melanin pigment resulting in skin hyper-pigment and tanning. Chronic UV exposure could generate reactive nitrogen species (RNS) which induces the release of pro-inflammatory cytokines by resident skin cell. The cytokines cause sunburn inflammation and activation of keratinocyte and macrophage that generates ROS leading to cellular damage or severe skin condition such as skin cancer (Cals-Grierson and Ormerod, 2004; Herrling et al., 2006). All these factors lead to the changes in the skin condition and aged skin.

Skin care has been used to prevent and improve various signs of skin aging. Synthetic compounds and/or natural compounds have been incorporated in the product to alleviate or beautify the skin condition. Plant extracts have been widely used for a long time and they became popular as cosmetic ingredient since customers presume that natural compounds are safer than synthetic compounds. This leads to the numerous numbers of researches and efficacy testings toward anti-aging, whitening and moisturising effects of natural skin care. For example, green tea extract has been included as active ingredient in numbers of skin care preparations such as anti-aging or sun care products due to its potent antioxidant property contributed from the presence of high phenolic content, its anti-collagenase and anti-elastase properties due to the high flavonoid such as (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG) and its ability to protect sunburn inflammation caused by UV radiation (Demeule et al., 2000; Elmets et al., 2001; Thring, Hili, and Naughton, 2009).

***Phyllanthus emblica* L.** or ***Emblica officinalis* L.** commonly known as Amla or Makampom in Thai has been used in many traditional medicines for sore throat, diarrhea, haematonic and in skin and hair cares. Amla fruit was reported to be an effective antioxidant with IC_{50} of 1.70±0.07 µg/ml comparable to standard ascorbic acid which is well known potent antioxidant and is widely used as cosmetic ingredient in several types of cosmetic products (Homklob, 2010; Pientaweeratch, Panapisal, and Tansirikongkol, 2016). Gallic acid, ellagic acid, mucic acid, chebulagic acid, mallotusinin,

cinnamic acid, quercetin, geraniin, kaempferol and 5-hydroxymethylfurfural were found in amla extracts and may contribute for antioxidant activity (Liu, Cui, et al., 2008; Luo et al., 2011; Luo et al., 2009). Furthermore, amla was reported to offer comparative anti-inflammatory activity to aspirin and standard diclofinac, known anti-inflammatory agents, in carrageenan induced rat paw edema model (Dang et al., 2011; Jaijoy et al., 2010; Muthuraman, Sood, and Singla, 2010). Moreover, it showed potent analgesic activity than aspirin at beginning time and showed slightly lower than aspirin at later time of treatment (Jaijoy et al., 2010). These effects were suspected to be due to the rich in phenolic compounds such as gallic acid and tannic acid (B. Yang and Liu, 2014). The inflammatory process was reported to implicate with production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which induce pro-inflammatory cytokine (Koblyakov, 2011). Therefore, the anti-inflammatory activity of amla may be related to its potent antioxidant activity. Amla has been used as skin lightening agent in several skin care products. It offered anti-tyrosinase activity with IC_{50} value in the range of 0.151±0.72 to 0.710±0.026 mg/ml. However, its activity is significantly lower than standard kojic acid in which the IC_{50} value is 0.05±0.007 mg/ml (Homklob, 2010). For anti-proteinase activity, amla showed moderate anti-collagenase and poor anti-elastase activities with IC_{50} value of 89.41±3.26 and 520.83±18.71 µg/ml, respectively (Pientaweeratch et al., 2016).

***Manilkara sapota* L.** is called Lamud in Thai. It has been widely investigated for nutritional benefit. Unripe sapota fruit has astringent taste and has some white

latex. Ripe sapota fruit has sweet taste and due to its high in fiber could relive constipation. Sapota fruit presented moderate antioxidant activity with IC_{50} value in the range of 37.63±1.18 to 50.8±4.5 µg/ml. Its property is much lower than standard ascorbic acid (Einbond et al., 2004; Pientaweeratch et al., 2016). Its antioxidant activity is dependent on maturation stage. Increase in storage duration would reduce its antioxidant effect (Shui, Wong, and Leong, 2004). This activity was suspected to be due to the presence of polyphenolic compounds such as methyl 4-O-galloylchlorogenate, 4-O-galloylchlorogenic acid, methyl chlorogenate, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, quercitrin, myricitrin, dihydromyricetin and gallic acid (Ma et al., 2003). Amongst these, the first compound represents the highest antioxidant activity. Pientaweeratch et al. (2016) reported that the ethanolic extract of sapota fruit showed IC_{50} value of 35.73±0.61 µg/ml on anti-elastase activity which is three times more effective than standard EGCG and showed significant inhibition on collagenase activity. Several reports revealed that polyphenols and flavonoids such as catechin, epicatechin, gallocatechin, myricitin, quercetin and kaempferol, which also present in sapota, significantly inhibited elastase and collagenase activities (Demeule et al., 2000; Kanashiro et al., 2014; Wittenauer et al., 2015). Other cosmetic properties such as anti-tyrosinase and anti-inflammatory activities have not been explored.

The changes in skin conditions involve several pathways. Skin cares containing a plant extract as active ingredient are widely available whereas a plant extract with complete all of cosmetic properties is barely achieved. Incorporation of the extracts

as a combination might enhance overall activities. Accordingly, in cosmetic science industries, numbers of active compounds are combined in a product to cover many cosmetic properties. Using active as a combination may enhance particular cosmetic property or enhance overall cosmetic effects. Synergistic effect of plant extract mixtures were reported. The combination of *Camellia sinensis* (green tea), *Vitis vinifera* (grape seed), *Phyllanthus emblica* (amla), *Punica granatum* (pomegranate), *Cinnamomum cassia* (cinnamon) and *Ginkgo biloba* at the ratio of 5:3:3:3:3:3 exhibited the highest antioxidant activity compared with each individual extract and showed comparable effect to standard ascorbic acid (Jain, Pancholi, and Patel, 2011). Yang et al. (2009) studied antioxidant activities of traditional chinese herb as a pair of *Astragalus membranaceus* with *Glycyrrhiza uralensis, Astragalus membranaceus* with *Paeonia lactiflora* and *Astragalus membranaceus* with *Atractylodes macrocephala*. The combinations demonstrated significant synergistic effect in DPPH scavenging, hydroxyl radical and superoxide anion radical scavenging assays. In addition, the combination of plant extracts which offered combined cosmetic effects has been revealed. *Astragalus membranaceus*, *Bupleurum falcatum* and *Atractylodes macrocephalae* has been investigated and the result demonstrated that individual extracts have little or no effect on *in vitro* collagen type I and collagen type IV synthesis by normal dermal human fibroblasts, anti-melanogenesis, reducing oxygenate free radical production and inhibition of lipid peroxidation. However, the combination of three plant extracts at ratio of 1:1:1 showed synergistic effect on these tests (Grizaud

and Mondon, 2014). These results demonstrated the superior effect of combination extract in enhancing overall cosmetic properties. Several researches have studied the activity of mixed extracts but information about combination ratio and concentration used which could influence the result of combination is limited. From the research of Pientaweeratch (2014), the effects of combination of amla and sapota were studied. Amla showed potent in antioxidant activity while sapota showed potent in anti-collagenase and anti-elastase activities. Its combinations were prepared by 1.) fixed concentration of amla with varied concentrations of sapota extract and 2.) combination with vice versa. The results showed additive effect in DPPH assay and synergistic effect in anti-collagenase assay for both combinations and all test ratios. The combination with fixed concentration of amla and varied in sapota concentrations presented synergistic effect in anti-elastase activity at all ratios. However, the other combination with fixed concentration of sapota showed antagonistic effect when the concentration of amla was more than 300 µg/ml. The antagonistic effect might be due to high total concentration of extract which could influence the reaction where available substrate is limited or saturation is occurred. Therefore, ratio and concentration of combination are suspected to have an influence on biological activities. Consequently, the overall response curve of each ratio shall be constructed in order to retain the value at the midpoint and hence, to exclude the bias caused by concentration factor.

Several plant extracts, for example amla, have effective antioxidant but poor anti-collagenase and anti-elastase effects. In contrast, sapota was reported to offer

potent anti-elastase but moderate antioxidant effects (Pientaweeratch et al., 2016). By having one extract as the main constituent, the effect of combination could be either suppressive or supportive response which yet to be investigated. Therefore, in the present study, both individual extracts and the combinations of amla and sapota at the ratios of 5:1 (amla as main constituent), 1:5 (sapota as main constituent) and 1:1 (median mixture) will be chosen to investigate for *in vitro* antioxidant, anti-inflammatory, anti-collagenase, anti-elastase and anti-tyrosinase activities. The effect of combination ratio and concentration will be then evaluated. The proper ratio will provide beneficial information for cosmetic uses.

CHAPTER II
LITERATURE REVIEW

Phyllanthus emblica Linn.

Phyllanthus emblica Linn. or *Emblica officinalis* Gaert. was classified in Euphorbiaceae/Phyllanthacae family. It is commonly known as amla or Makhampom in Thai. Amla is small to medium tree, 15-20 m in height with brown-gray bark. Its leave is in monocot, pinnate and numerous leaflets pattern with light green in color. Inflorescence greenish-yellow color is observed. The fruit is greenish, spherical, and smooth surface with 6 vertical lines. Amla has been used in many traditional medicines especially its fruit. It is most widely used to relieve the symptoms of sore throat, dyspepsia, diarrhea, constipation and used as expectorant, diuretic and digestion enhancer (Khan, 2009). In addition, amla fruit is an excellent source of ascorbic acid and minerals which are popular used as active ingredient in cosmetic products (Gaire and Subedi, 2014; Singh et al., 2011).

Biological activities of *Phyllanthus emblica* Linn.

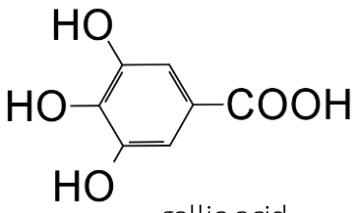
Many researchers reported that amla fruit extract had potent antioxidant property. Sripanidkulchai and Junlatat (2014) revealed effective antioxidant property of ethanolic fruit extract with EC_{50} value of 3.65 µg/ml in DPPH radical scavenging assay. The result was supported by Pientaweeratch and her group (2016) showing IC_{50} of 1.70±0.07 µg/ml in which its activity was comparable to standard ascorbic acid. While

ethanolic fruit extract presented potent antioxidant property, water fruit extract in study of Charoenteeraboon et al. (2010) showed IC_{50} of 51.3±16.5 μg/ml which was approximately 6 folds much lower than standard gallic acid. The different results in activity may be due to different extracting solvents. Amla fruit was reported to contain various phytochemical components and hence, ethanol could dissolve both polar and non-polar phytochemical compositions in amla better than water. Apart from the effect of extracting solvent, part of amla is another considerable factor. Amla fruit possessed high total phenolic content compared to its leaves and branches. Since the total phenolic content of amla extract exhibited good relationship with antioxidant activity (Bajpai et al., 2005; Pientaweeratch et al., 2016), the fruit showed higher antioxidant activity than other parts (Bajpai et al., 2005; Sripanidkulchai and Junlatat, 2014).

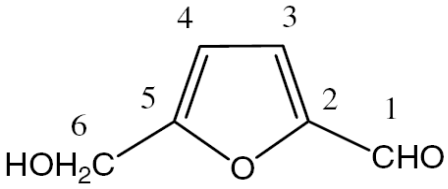
Various phytochemical constituents of amla fruit were reported including gallic acid, ellagic acid, ascorbic acid, chebulagic acid, cinnamic acid, mallotusinin, phyllantidine, quercetin, geraniin, kaempferol, 5-hydroxymethylfurfural and etc. (Figure 1). Phenolic compounds in amla fruit were partly investigated for DPPH radical scavenging and lipid peroxidation activities by Liu and her group (2008). Geraniin exhibited as the most potent of both activities with IC_{50} of 4.7±0.5 and 65.7±2.5 μM, respectively, followed by quercetin and keampferol. Luo and his group (2009) reported the DPPH and ABTS radical scavenging activities of other phenolic compounds presenting in amla fruit including gallic acid, ellagic acid, cinnamic acid and quercetin.

The results revealed that gallic acid and ellagic acid which were found as main compounds in amla showed the highest activities in both studies followed by quercetin and cinnamic acid, respectively. Gallic acid and ellagic acid showed IC_{50} values of 3.18 and 4.17 µg/ml, respectively on DPPH assay and TEAC values of 3.62±0.08 and 3.91±0.09 µg/ml, respectively on ABTS assay. The result revealed that both compounds exhibited higher antioxidant activities than standard ascorbic acid, butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) (Luo et al., 2009). The effective activity may be due to the presence of OH groups on the structure of gallic acid and ellagic acid. This structure was showed to be responsible for interacting and scavenging free radical leading to the potent antioxidant property (Charoenteeraboon et al., 2010).

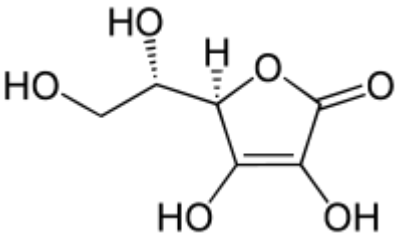
Furthermore, *in vitro* and *in vivo* anti-inflammatory effects of amla were investigated by several researchers. Kumaran and Karunakaran (2006) revealed *in vitro* nitric oxide radical scavenging property of amla extract. The methanolic extract showed anti-inflammatory property with IC_{50} of 39.27±1.27 µg/ml which was stronger than the standard curcumin. Purified compounds presented in the extract such as geraniin and gallic acid were also checked for such activity and the results showed IC_{50} of 3.81±0.88 and 28.97±1.57 µM, respectively. Anti-inflammation of amla extract was, therefore, contributed by the mixture of several phenolic compounds. *In vivo* anti-inflammation activity was evaluated by Jaijoy and her group (2010). The result presented that



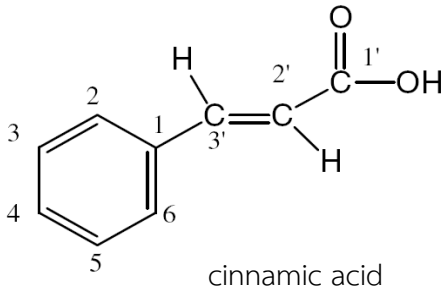
gallic acid



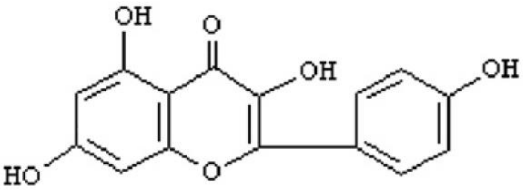
5-hydroxymethylfurfural



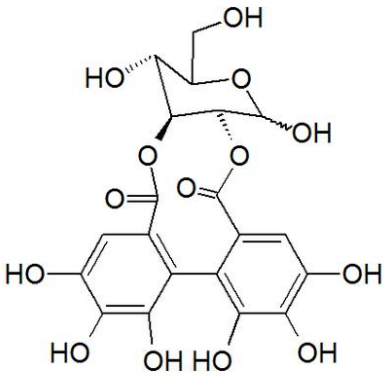
ascorbic acid



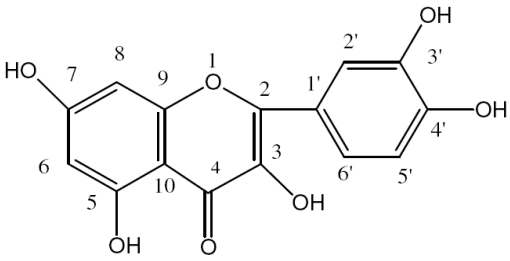
cinnamic acid



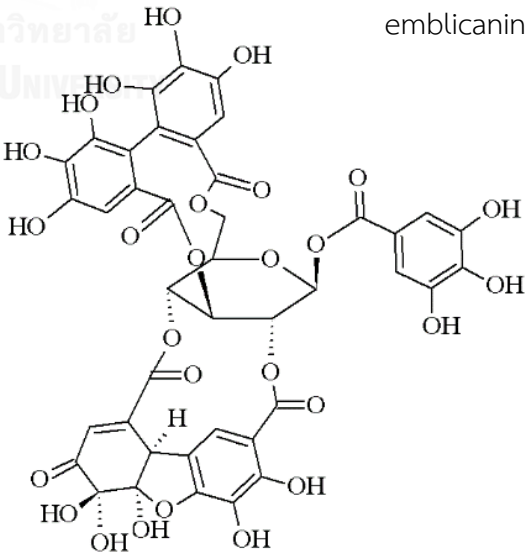
kaempferol



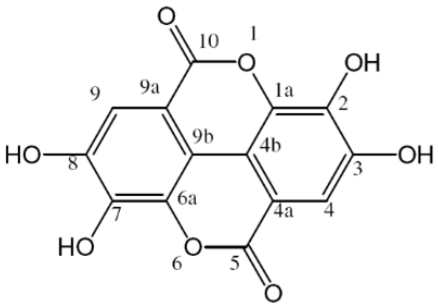
emblicanin



qeurcetin



geraniin



ellagic acid

Figure 1 Structures of bioactive compounds presenting in emblica fruit

inflammatory inhibition of aqueous amla extract at 600 mg/kg body weight on carrageenan induced rat paw edema was comparable to standard aspirin. In addition, it was more potent on analgesic effect at beginning time of treatment than the standard aspirin. Moreover, oral administration of water fraction of butanolic amla fruit extract at concentration of 100 mg/kg body weight showed protective effect against indomethacin inducing gastric ulcer (Bandyopadhyay, Pakrashi, and Pakrashi, 2000) which might be caused by production of reactive oxygen species (ROS). Decreases in lipid peroxidation and superoxide anion and increases in mucin and hexosamin contents by amla extract, therefore, led to an increase in gastric barrier related with gastric ulcer (Bandyopadhyay et al., 2000). The inflammatory process was reported to implicate with productions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which induce the release of pro-inflammatory cytokine (Fubini and Hubbard, 2003; Koblyakov, 2011). Hence, the anti-inflammatory activity of amla may also be related to its potent antioxidant activity.

In addition to antioxidant and anti-inflammation properties, amla has been reported to possess skin lightening effect and has been used as an active agent in several skin care products. Anti-tyrosinase activity of amla was evaluated by Homklob (2010). Ethyl acetate amla extracts presented moderate anti-tyrosinase activity with IC_{50} value in the range of 0.151±0.72 to 0.710±0.026 mg/ml. However, its activity were significantly lower than standard kojic acid in which the IC_{50} value is 0.05±0.007 mg/ml (Homklob, 2010). Lightening effect of amla may due to ß-glucogallin or emblicanin A

presenting in amla fruit. Emblicanin is a hydrolysable tannin which possesses potent antioxidant activity with metal chelating ability and it may interrupt melanogenesis process (Chaudhuri, 2002). It showed a dose dependence in melanin reduction in B16F1 mouse melanoma cells (Majeed, Bhat, and Anand, 2010). However, ß-glucogallin and emblicanin A was not the major compounds in amla fruit. Thus, only moderate anti-tyrosinase activity is achieved.

Another potential effect in anti-proteinase activity of amla has been explored. This property would support anti skin aging of amla extract since such enzymes are responsible for degradations of major fibers in dermis; collagen and elastin. Chanvorachote et al. (2009) reported anti-collagenase property of water amla extract which was determined by Enzcheck® test kit. It exhibited collagenase inhibition activity in a dose dependent manner with approximately 15% to 78% inhibition at the concentrations of 0.01-1 mg/ml. Pientaweeratch et al. (2016) also reported anti-proteinase activities of ethanolic amla extract. It showed IC_{50} value of 89.41±3.26 and 520.83±18.71 µg/ml on anti-collagenase and anti-elastase activities, respectively. Amla demonstrated a more favorable in collagenase inhibition compared to elastase inhibition property.

***Manilkara zapota* Linn.**

***Manilkara zapota* Linn.** or *Achras sapota* is called Lamud in Thai. It is classified in Sapotaceae family. Sapota is medium-size tree, 15-30 meters in height and wind resistance. Leaves are glossy and evergreen, elliptic shape and alternate arrangement. The fruit is a berry, light or dark brown and velvet smooth skin (Moore and Stearn, 1967). Unripe sapota fruit has astringent taste and has some white latex. Ripe sapota fruit is sweet and could relive constipation due to its high fiber. The fruit is nutritious and mostly eaten as fresh fruit. Sapota has been widely investigated for nutritional benefits and for other possible applications.

Biological activities of *Manilkara zapota* Linn.

Sapota has been mainly evaluated for nutritional purpose and antioxidant property has been focused. Antioxidant activities of sapota leaf, seed and fruit were evaluated in previous studies. Ethanolic and aqueous extracts of sapota leaf provided antioxidant activities with IC_{50} of 68.27 and 160 µg/ml on DPPH radical scavenging assay which was 4 and 14 folds lower than standard ascorbic acid, respectively (Chanda and Nagani, 2010; Islam et al., 2011). Ethanolic and methanolic sapota fruit extracts showed antioxidant activities with IC_{50} of 37.63±1.18 and 50.8±4.5 µg/ml, respectively (Einbond et al., 2004; Pientaweeratch et al., 2016). Whereas effective activity was observed in ethanolic seed coat extract. It exhibited IC_{50} of 2.387±0.023 µg/ml comparable to standard ascorbic acid (Kanlayavattanakul and Lourith, 2011). Although, sapota seed coat provided antioxidant activity better than other parts, fruit portion has gained more

attention of its higher volume and its moderate antioxidant property. Antioxidant activity of sapota fruit extract was reported by several groups of researches. In comparison with strawberry, plum, star fruit, guava, grape seed and other fruits, higher antioxidant activity of ethanolic sapota fruit extract evaluated by ABTS and DPPH was observed with approximately 3500 and 4700 mg of L-ascorbic acid equivalent antioxidant (AEAC) per 100 g of sample, respectively (Leong and Shui, 2002). In addition, ethanolic and methanolic sapota pulp extracts inhibited lipid peroxidation activity with IC_{50} of 6.63 and 6.62 mg/ml, respectively, and IC_{50} of 1.92 and 1.98 mg/ml were observed for ethanolic and methanolic sapota peel extracts, respectively (Gomathy, Baskar, and Kumaresan, 2013). The result showed no difference in activities between using ethanol and methanol as an extracting solvent. In study of Woo and his group (2013), ethanol and water were used as solvents for sapota fruit extraction. The result revealed that ethanolic fruit extract showed higher antioxidant activity and higher total phenolic content (TPC) than water extract. Lower activity of water extract might be due to ability of water to dissolve other inactive materials such as polysaccharides leading to the reduction in antioxidant activity. Therefore, ethanol was used as a solvent for sapota extraction in the present study to provide an effective activity and to avoid the toxicity of methanolic solvent.

The activities of sapota fruit were suspected to be due to the presence of (-)-epigallocatechin as a major compound and (+)-epigallocatechin gallate as a minor compound (H. Wang et al., 2012) and other polyphenolics, flavonoids and tannins

including methyl 4-O-galloylchlorogenate, 4-O-galloylchlorogenic acid, methyl chlorogenate, quercetin, myricitrin, dihydromyricetin and gallic acid (Figure 2) (Ma et al., 2003). Shui et al. (2004) revealed that tannins in unripe sapota fruit might be possessed high antioxidant activity. However, antioxidant activity was reported to be affected by storage time of sapota fruit. The activity decreased when the fruit became ripen or over-ripen. The astringency of unripe sapota might be due to the high tannin content which will be decreased and replaced by sweetness when the fruit begins to ripe (Brito and Narain, 2002).

Additionally to antioxidant activity, anti-inflammation activity of ethanolic fruit extract was reported. Jayakumar and Kanthimathi (2011) revealed that sapota at concentration of 600 µg/ml exhibited more than 80% inhibition on nitric oxide-induced cell proliferation in human breast cancer cell line and at concentration of 640 µg/ml showed 88.4% inhibition on nitric oxide (NO) radical scavenging activity. It showed the highest activity compared to dragon fruit, litchi, pomegranate and other fruits. This result may be due to (+)-epigallocatechin gallate (EGCG) and phenolic compounds which have ability to scavenge nitric oxide in a dose dependent manner (Jayakumar and Kanthimathi, 2011; Jung et al., 2007).

In addition, catechins especially epigallocatechin gallate (EGCG) were reported to inhibit of melanogenesis. EGCG demonstrated the strongest inhibition on B16 melanoma cell proliferation and on melanin formation followed by epigallocatechin, gallic acid and catechin, respectively (Sato and Toriyama, 2009). Skin lightening effect

of sapota fruit may be, thus, expected. However, lightening activity of sapota fruit have not been explored.

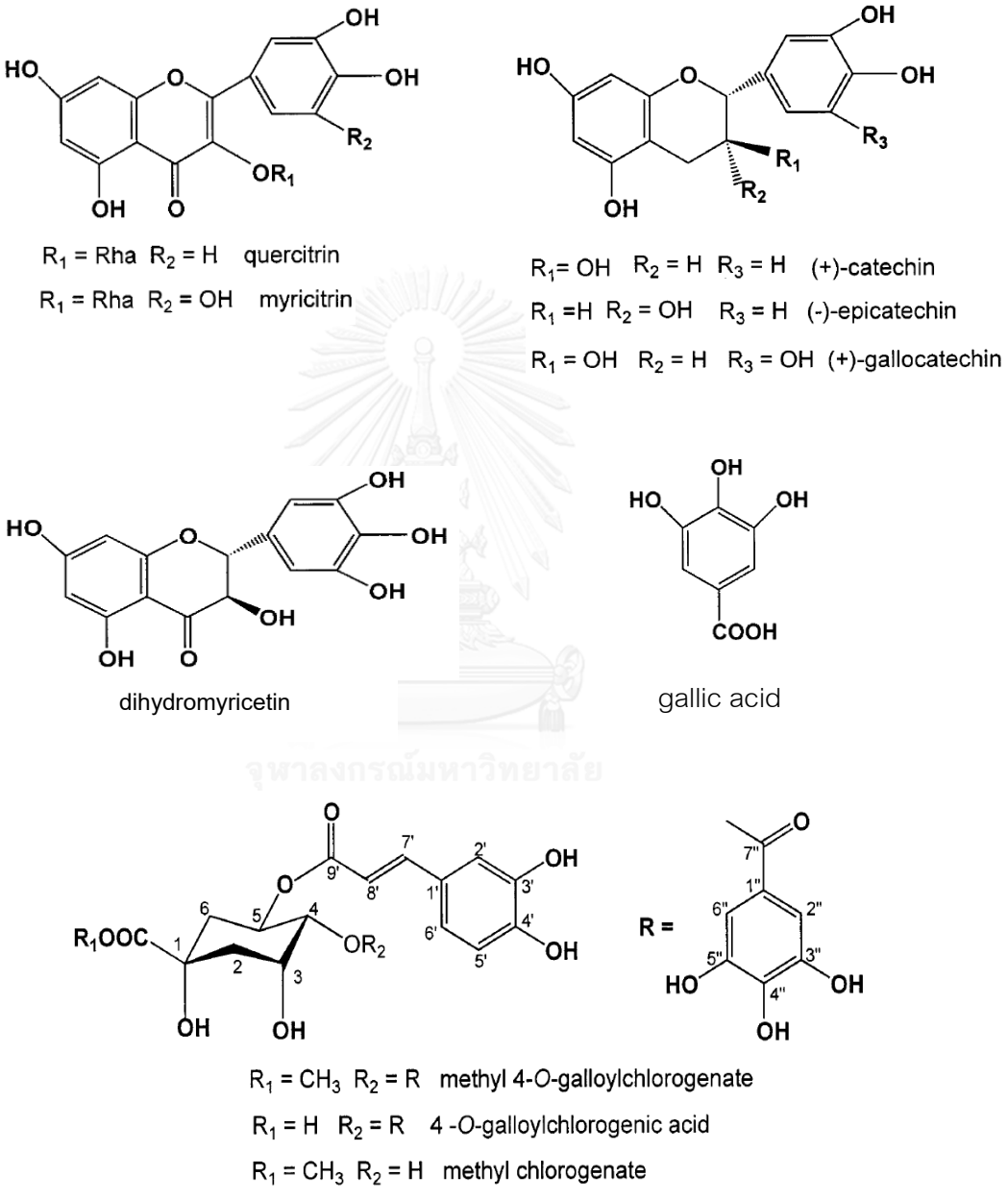


Figure 2 Structures of bioactive compounds presenting in sapota fruit

Other possible properties of sapota have been investigated which might be applied for other application such as for cosmetic purpose. Anti-proteinase activities of sapota were evaluated and the results exhibited an effective anti-elastase and moderated anti-collagenase activities. Pientaweeratch et al. (2016) reported that the ethanolic extract of sapota fruit showed IC_{50} value of 35.73±0.61 μg/ml on anti-elastase activity which is three times more effective than standard EGCG and showed significant inhibition on collagenase activity with IC_{50} value of 86.47±3.04 μg/ml. Both effects might be contributed by polyphenols and flavonoids presenting in sapota fruit since several reports revealed that such compounds including catechin, epicatechin, gallocatechin, myricitin, quercetin and kaempferol significantly inhibited elastase and collagenase activities (Demeule et al., 2000; Kanashiro et al., 2014; Wittenauer et al., 2015).

Combination concept

In addition to nutritional purpose, plant extract has been wildly included in health care products such as medicine, supplement or cosmetic products. Complete cosmetic benefit is barely achieved by only single plant extract. Couple active substances are, thus, commonly combined in the product in order to obtain several properties. Few reports of such combination effect have been revealed. Combination of active substances may provide advantages by promoting particular each effect or enhancing overall effects. Jain and his group (2011) demonstrated an enhancement of

antioxidant property of plant extract combination. The combination of *Camellia sinensis* (green tea), *Vitis vinifera* (grape seed), *Phyllanthus emblica* (amla), *Punica granatum* (pomegranate), *Cinnamomum cassia* (cinnamon) and *Ginkgo biloba* at the ratio of 5:3:3:3:3:3 exhibited the highest antioxidant activity on DPPH, Nitric oxide and superoxide radical scavenging assays with IC_{50} of 33.5, 31.47 and 46.34 mg/ml, respectively. This result showed the highest activity compared to each individual extract and showed comparable effect to standard ascorbic acid (Jain et al., 2011). In addition, synergistic antioxidant effect of ginger and honeysuckle extracts combination and ginger and sophora extracts combination were reported by Ge and her group (2012). Both combinations revealed synergistic effect on oxygen radical absorbance capacity (ORAC) assay within the ranges of ginger concentration of 0.5-9.0 µg/ml and honeysuckle and sophora concentrations of 2.0-27.0 µg/ml. Both combinations were used as active ingredients in skin care products including liquid hand soap, antiperspirant roll-on, body wash and body lotion (Ge et al., 2012). An enhancement on antioxidant activity of combined extracts was also reported in Yang et al. (2009), *Astragalus membranaceus* (AME) in combination with *Glycyrrhiza uralensis* (GU), *Paeonia lactiflora* (PL) or *Atractylodes macrocephala* (AMA) at the ratio of 1:1 were evaluated for DPPH scavenging, hydroxyl radical and superoxide anion radical scavenging assays. Individual AME showed the weakest of all activities and it was combined with other herbs which possessed stronger effect in each assay. Interestingly, the results of combined extract exhibited synergistic effect showing higher

activity than theoretical sum of individual activity in each test. PL, GU and AMA showed potent activity than AME and they improved antioxidant activity of AME in extract combination. Furthermore, anti-inflammatory activity of combined thyme and oregano oils was reported (Bukovská et al., 2007). Thyme oil at concentrations of 0.4%, 0.2% and 0.1% were combined with oregano oil at concentrations of 0.2%, 0.1% and 0.05%, respectively, and they were investigated for anti-inflammatory activity on TNBS-induced colitis in mice. The result showed that at the medium dose of combination (0.2% thyme and 0.1% oregano oils) exhibited synergistic effect on recovery body weight gain, decreasing mortality rate, inhibition pro-inflammatory cytokines and reducing macroscopic damage in colonic tissue. Whereas at the high dose (0.4% thyme and 0.2% oregano oils) and low dose of combinations (0.1% thyme and 0.05% oregano oils) showed no significant difference in these tests. The low activities was found in high dose combination might be caused by cytotoxic effects on lymphocyte and intestinal cells due to high concentration of test compounds. Conversely, combination at low dose might be insufficient to decrease the inflammation and exert other effects (Bukovská et al., 2007). Therefore, the proper concentration of compound combination is as important.

In addition, the combination of plant extracts which enhanced overall cosmetic effects has been revealed. Individual *Astragalus membranaceus*, *Bupleurum falcatum*, *Atractylodes macrocephalae* and their combination at the ratio of 1:1:1 have been investigated. Synergistic effects on *in vitro* collagen synthesis, anti-melanogenesis,

reducing oxygenate free radical production and inhibition of lipid peroxidation were observed for the combination extract. The combination of three plant extracts was claimed for providing antioxidant effect, reducing wrinkles and fine lines, improving skin firmness and skin radiance. Moreover, this combination extract was used as active ingredients in formulations such as body cream, facial mask and body shower cream to provide various cosmetic effects (Grizaud and Mondon, 2014). These results demonstrated the superior effect of combination extract in enhancing overall cosmetic properties. Another researches of combined plant extract with enhanced overall cosmetic properties were reported. "Triple activities" including antioxidant, anti-bacterial and anti-tyrosinase were exhibited in combination of *Tridax procumbens, Lantana camara, Euphorbia hirta* and *Thevetia peruviana*. Individual *Euphorbia hirta* presented the highest effective antioxidant and anti-tyrosinase activities while individual *Tridax procumbens* possessed the most potent anti-bacterial activity. The combination extract showed synergistic effects on antioxidant and anti-bacterial activities with additive effect on tyrosinase activity. Therefore, this combination extract provided multiple effects in cosmetic and it could be used as natural preservatives instead of synthetic preservatives (Sindhuja, Prabhakaran, and Gokulshankar, 2014).

The effect of combined extracts on their activities was evaluated in many researches. However, the factor such as combination ratio which could influence the activity of combination is limited. Pientaweeratch (2014) (unpublished data) studied the effects of combined ethanolic amla and sapota extracts on *in vitro* antioxidant,

anti-collagenase and anti-elastase effects. Amla extract showed potent antioxidant activity with IC_{50} of 1.61±0.04 µg/ml comparable to standard ascorbic acid while sapota showed potent anti-collagenase and anti-elastase activities with IC_{50} of 86.47±3.04 µg/ml and 35.73±0.61 µg/ml, respectively. Amla and sapota extracts were combined at different ratios and it were prepared by 1.) fixed concentration of amla with varied concentrations of sapota extracts and 2.) fixed concentration of sapota with varied concentrations of amla extracts. The results showed additive effect on DPPH assay and synergistic effect on anti-collagenase assay for both combinations at all test ratios. Surprisingly, anti-elastase activity of different combinations presented both of synergistic and antagonistic effects depending on combination ratios. The combinations with fixed concentration of amla and varied in sapota concentrations showed synergistic effect at all ratios. However, the other combinations with fixed concentration of sapota showed antagonistic effect when the concentration of amla was more than 300 µg/ml. These results demonstrated that combination ratio could influence on biological activities and effect of combination ratio should be concerned when using as a combination. Therefore, the effect on biological activities of combination ratio of amla and sapota extracts should be investigated. The antagonistic effect might be due to high total concentration of extract which could influence the reaction where available substrate is limited or saturation is occurred. The study of Pientaweeratch considered all of concentrations or all of different ratios in dose response curve. The overall response curve of each ratio which covers the midpoint

(IC_{50}) has not been constructed. In general, only linear portion of the overall response will be used to accurately estimate inhibitory effect. Therefore, the overall response curve is necessary for chosing the right concentration and proper combination ratio.

Amla and sapota exhibited potent in different activities. In combination, by having one extract as the main constituent, the effect of combination could be either suppressive or supportive response which yet to be investigated.

The effect of combination extract might be synergism, antagonism or additive. Several methods were utilized to calculate interaction of combination such as isobolographic (Tallarida, 2002), median effect plot and combination index (CI) (Chou and Talalay, 1984). A combination index is the most popular method and it has been widely used for calculating the type of interaction of combination (e.g. additive, synergistic, antagonistic) in most scientific journals (Zhao, Wientjes, and Au, 2004). It assesses the relationship between the effect and concentration of both extracts in the combination. CI value of combined extract is calculated from dose response curve at the median effect (IC_{50}) based on the mass law action and the median effect principle (Chou and Talalay, 1984) which could be calculated by equation:

$$CI = (C_a/IC_{x,a}) + (C_b/IC_{x,b})$$

Where

C_a and C_b are concentrations of substance A and B, respectively, used in the combination.

$IC_{x,a}$ and $IC_{x,b}$ are concentrations of individual substance A and B, respectively.

When CI < 1 is synergism

CI = 1 is additive

CI > 1 is antagonism

CHAPTER III

MATERIALS AND METHODS

Materials

1. (-)-Epigallocatechin-3-gallate (EGCG) (Sigma Aldrich, USA)
2. 2,2-Diphenyl-1-pikyl-hydrazyl (DPPH) (Sigma Aldrich, USA)
3. 3,4-Dihydroxyl-L-phenylalanine (L-DOPA) >= 98% (Sigma Aldrich, USA)
4. 95% Ethanol (The Liquor Distillery Organization, Thailand)
5. Absolute ethanol (RCI Labscan, Thailand)
6. Dimethyl sulfoxide (DMSO) (Merk, Germany)
7. EnzCheck® elastase assay kit (E-12056) (Molecular probes, USA)
8. EnzCheck® gelatinase/collagenase assay kit (E-12055) (Molecular probes, USA)
9. Gallic acid (Sigma Aldrich, USA)
10. Kojic acid (Sigma Aldrich, USA)
11. L-(+)-ascorbic acid (Carlo Erba, Italy)
12. N-(1-Nephtyl) ethylenediamine dihydrochloride (Sigma Aldrich, USA)
13. 85% Ortho-phosphoric acid for analysis (Merk, Germany)
14. Potassium nitrite (Merk, Germany)
15. Propylene glycol USP (Dow Chemical ,Thailand)
16. Sodium dihydrogen phosphate dihydrate extra pure (Merk, Germany)
17. Sodium nitroprusside dihydrate (Merk, Germany)

18. Sodium phosphate dibasic (Sigma Aldrich, USA)
19. Sulfanilamide >= 99% (Sigma Aldrich, USA)
20. Tyrosinase from mushroom 2687 unit/mg solid (Sigma Aldrich, USA)

Instruments

1. Analytical balance (Mettler Toledo AX105 DeltaRange®)
2. Microplate reader (Victor3/Wallac 1420, USA)
3. Microplate reader (CLARIOStar®, Germany)
4. pH Meter (Mettler Toledo, Switzerland)
5. Pipet-Lite™ XLS™ (Mettler Toledo, Switzerland)
6. Sonicator (Elma, Germany)
7. Vortex mixer (Vortex Genie2, Scientific Industries, USA)

Methods

In this study, amla and sapota individual extracts and their combinations were prepared and evaluated for the cosmetic effects. Amla and sapota were extracted with ethanol. All test samples including amla, sapota individual extracts and combined extracts were investigated for *in vitro* antioxidant, anti-inflammatory, anti-collagenase, anti-elastase and anti-tyrosinase activities. Finally, the effects of extract combination and combination ratio were evaluated.

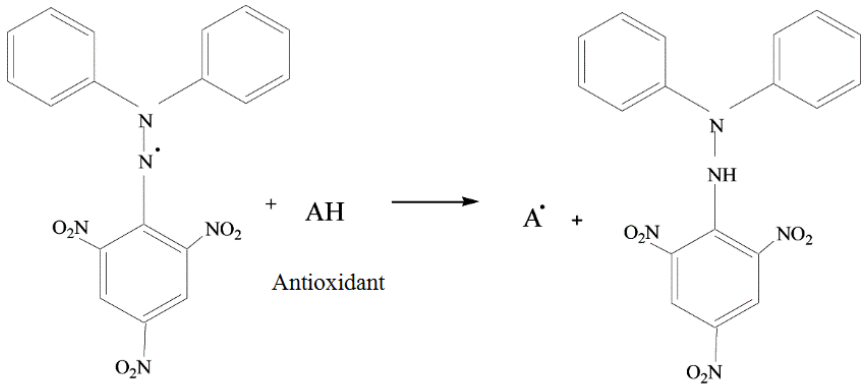
1. Preparation of plant extract

The dried amla and unripe sapota (Waan-Suk cultivar) fruits were purchased from Chao Phraya Abhaibhubejhr Hospital, Prachinburi province and Thai vegetable and fruit market in Pathum Thani province, Thailand, respectively. The dried amla fruits were ground and macerated with 95% ethanol for 24 hours. The macerated solvent was filtered and the extraction was repeated for 3 cycles. All obtained solvents were combined and evaporated by rotary evaporator at 40°C until dryness. The unripe fresh sapota fruits were sliced into small pieces and the seeds were discarded. Sapota fruits were macerated with absolute ethanol and the extraction process was followed as for amla. Both crude extracts were kept in the dark at 4°C until further used.

The combination extract were prepared by combining amla and sapota extracts at the ratios of 1:5, 1:1 and 5:1. All test samples were investigated for *in vitro* antioxidant, anti-inflammatory, anti-collagenase, anti-elastase and anti-tyrosinase activities.

2. *In vitro* antioxidant assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is commonly used for investigation antioxidant activity because this method is simple, speed analysis and not expensive. Antioxidant compounds can scavenge DPPH radical by donating hydrogen atom to DPPH radical which results in the colour change from purple to colourless (Molyneux, 2004).



DPPH (VIOLET) DPPH: H (COLOUR LESS)

The DPPH radical scavenging assay was performed based on a modified method from Marinova and Batchvarov (2011) . Briefly, 100 µl of sample in ethanol at different concentrations were mixed with 100 µl of 0.06 mM DPPH ethanolic solution in 96 well microplate. The test mixture 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:

$$\text{DPPH Inhibition (\%)} = [(A_{control}-A_{sample})/A_{control}] \times 100 \qquad \text{Equation 1}$$

Where

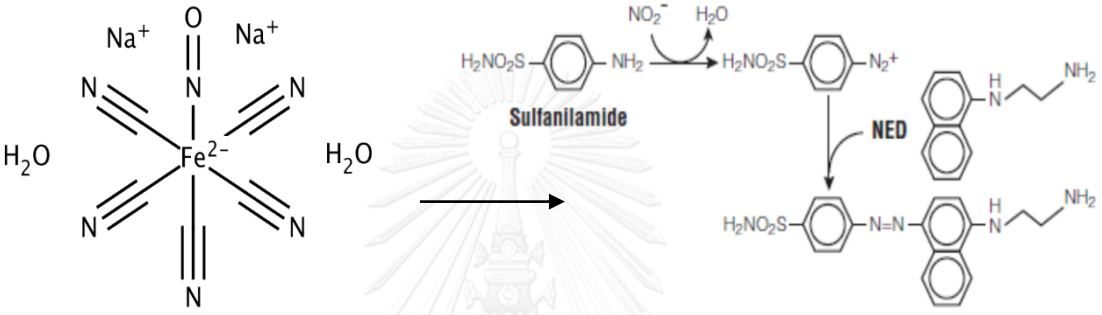
$A_{control}$ is absorbance of DPPH solution without test extract.

A_{sample} is absorbance of DPPH solution with test extract.

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

3. *In vitro* anti-inflammatory assay

Anti-inflammatory activity was determined based on nitric oxide radical scavenging assay. Nitric oxide (NO) is a free radical which interacts with oxygen to produce stable products of nitrite and nitrate. Both stable products will induce pro-inflammatory cytokines leading to inflammation.



Sodium nitroprusside
(NO donor)

Griess reagent

In vitro nitric oxide radical scavenging assay was performed based on a modified protocol from Saija et al (1999) and the resulting nitrite was detected by Griess reagent. Briefly, different concentrations of sample were mixed with 3 mM of sodium nitroprusside in 20 mM phosphate buffer, pH 7.4 and incubated for 150 minutes at room temperature. Griess reagent which was a mixture of 2% sulphanilamide, 4% H_3PO_4 and 0.2% napthylethylenediamine dihydrocholide was added and incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 544 nm by using microplate reader. Nitrite concentration was calculated by referring to the

absorbance of standard solutions of potassium nitrite. Nitric oxide inhibition was calculated by the following equation:

$$\text{Nitric oxide inhibition (\%)} = [(A_{control}-A_{sample})/A_{control}] \times 100 \quad \text{Equation 2}$$

Where

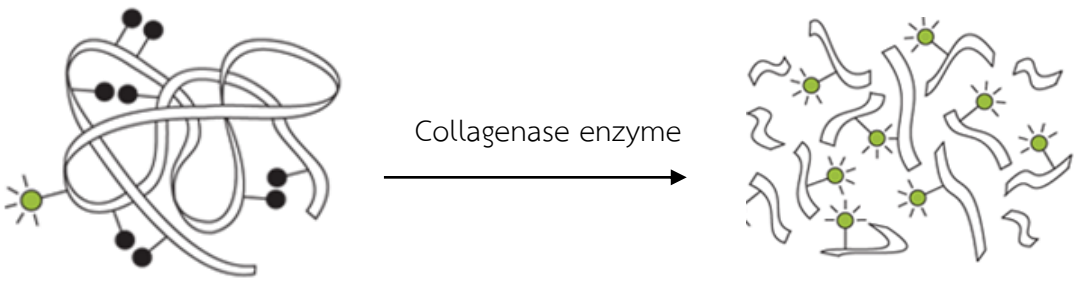
$A_{control}$ is nitrite concentration (μM) produced from nitroprusside without test extract.

A_{sample} is nitrite concentration (μM) produced from nitroprusside with test extract.

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

4. *In vitro* anti-collagenase assay

The *in vitro* anti-collagenase activity was determined by using EnzChek® E-12055 gelatinase/collagenase assay kit. The mechanism is based on the breakdown of DQ™ gelatin into multiple fragments by collagenase enzyme from *Clostridium histolyticum*. These fragments can be measured by fluorescent absorption. Once anti-collagenase substance was tested, it could inhibit collagenase enzyme as a result, the reduction in fluorescent intensity could be observed.



DQ™ gelatin substrate Multiple fragments

The *in vitro* anti-collagenase activity was determined according to the protocol from Pientaweeratch et al. (2016). 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 were measured at excitation and emission wavelengths of 485 nm and 535 nm, respectively, by microplate reader. (-)-Epigallocatechin gallate (EGCG) was used as a standard. Collagenase inhibition was calculated by equation:

$$\text{Collagenase inhibition (\%)} = [(A-B)-(C-D)]/(A-B) \times 100 \qquad \text{Equation 3}$$

Where

A is fluorescent intensity without test extract.

B is fluorescent intensity without test extract and enzyme.

C is fluorescent intensity of test extract.

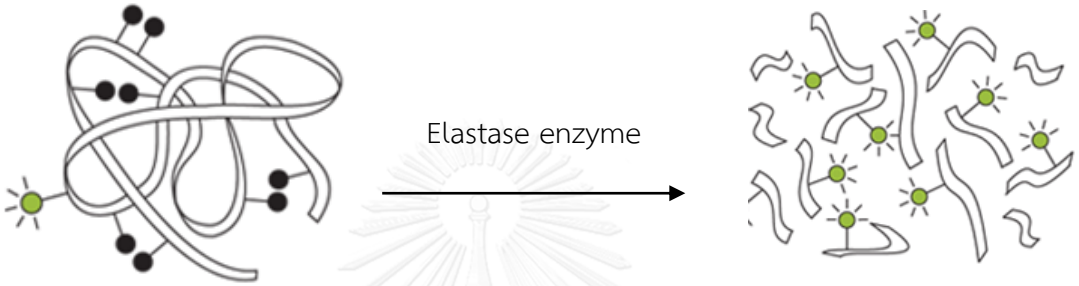
D is fluorescent intensity of test extract without enzyme.

The anti-collagenase activities of all test extracts were reported as an IC_{50} value which is concentration of the test extract providing 50% inhibition.

5. *In vitro* anti-elastase assay

The *in vitro* anti-elastase activity was determined by using EnzChek® E-12056 elastase assay kit. The mechanism is based on the breakdown of DQ™ elastin from

bovine neck ligament into multiple fragments by elastase enzyme from pig pancreas. These fragments can be measured by fluorescent absorption. Once anti-elastase substance was tested, it could inhibit elastase enzyme as a result, the reduction in fluorescent intensity could be observed.



DQ™ elastin substrate

Multiple fragments

The *in vitro* anti-elastase activity was determined according to the protocol from Pientaweeratch et al. (2016). 50 µl of sample solution was mixed with 100 µl of 0.4 U/ml elastase enzyme in 96 well microplate and pre-incubated 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 were measured at excitation and emission wavelengths 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:

Elastase inhibition (%) = [(A-B)-(C-D)]/(A-B) x 100 Equation 4

Where

A is fluorescent intensity without test extract.

B is fluorescent intensity without test extract and enzyme.

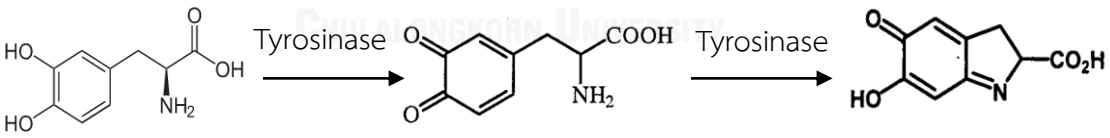
C is fluorescent intensity of test extract.

D is fluorescent intensity of test extract without enzyme.

The anti-elastase activities of all test extracts were reported as an IC_{50} value which is concentration of the test extract providing 50% inhibition.

6. *In vitro* tyrosinase inhibitory assay

In vitro anti-tyrosinase activity was evaluated by DOPA chrome method using L-DOPA as substrate. DOPA chrome is an intermediate substance in melanogenesis which is responsible for skin pigmentation.



L-DOPA DOPA quinone DOPA chrome

The method was modified from Likhitwitayawuid and Sritularak (2001). Briefly, 40 µl of sample solution was mixed with 80 µl of 20 mM phosphate buffer pH 6.8 and 40 µl of 480 unit/ml mushroom tyrosinase solution in 96 well microplate. The reaction mixture was incubated for 10 minutes at room temperature in the dark. 20 µl of 0.85

mM L-DOPA in phosphate pH 6.8 was added and incubated for 10 minutes at room temperature in the dark. The absorbance was measured at 490 nm by using microplate reader. Kojic acid was used as a standard. Tyrosinase inhibition was calculated by equation:

$$\text{Tyrosinase inhibition (\%)} = [(A-B)-(C-D)]/(A-B) \times 100 \qquad \text{Equation 5}$$

Where

A is absorbance of enzyme without test extract.

B is absorbance without test extract and enzyme.

C is absorbance of test extract with enzyme.

D is absorbance of test extract without enzyme.

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

7. Determination of combination index (CI)

A combination index (CI) has been widely used for calculating the type of combination interaction (e.g. additive, synergistic or antagonistic) in most scientific journals (Zhao et al., 2004). CI index at the median effect (IC_{50}) was calculated by equation:

$$CI = (C_a/IC_{50,a}) + (C_b/IC_{50,b}) \quad \text{Equation 6}$$

Where

C_a and C_b are concentrations of substance A and B respectively, used in the combination which provide 50% inhibition.

$IC_{50,a}$ and $IC_{50,b}$ are concentrations of individual substance A and B, respectively, which provides 50% inhibition.

(When CI<1 is synergism, CI=1 is additive, CI>1 is antagonism)

8. Statistical analysis

The data was reported as mean ± standard deviation. IC_{50} values were calculated from linear regression analysis or logarithm transformation analysis. One-way ANOVA was used to analyze the significant difference between samples and one sample t-test was used to analyze the CI_{50} values (p-value < 0.05) by SPSS version 22 software.

CHAPTER IV
RESULTS AND DISCUSSION

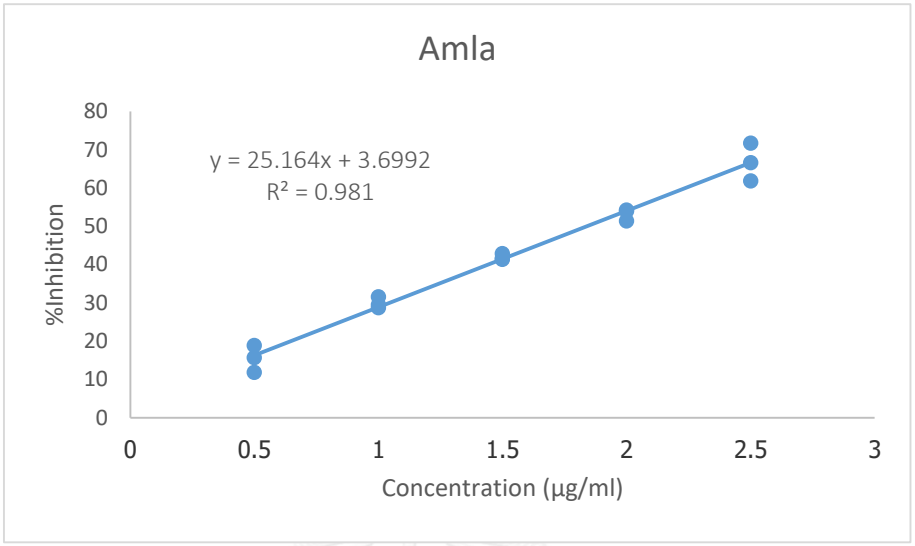
1. Preparation of plant extracts

Ethanol extract of amla and sapota fruits provided percentage yield 32.9% and 11.09% w/w, respectively. Ethanolic amla extract presented a sticky dark brown paste and soluble in 60% propylene glycol (Figure 3-A). Ethanolic sapota extract presented a viscous red brown paste and soluble in water (Figure 3-B). Ethanolic amla, sapota and their combination extracts were screened for *in vitro* antioxidant, anti-inflammatory, anti-collagenase, anti-elastase and anti-tyrosinase properties.

Figure 3 Ethanolic amla (A) and ethanolic sapota (B) extracts

2. *In vitro* antioxidant assay

The antioxidant activity was determined by DPPH radical scavenging assay. The hydrogen donation ability of all test extracts was detected by reducing the absorption of DPPH at 517 nm. The antioxidant activities of all test extracts, including individual and combination extracts, showed linear relationship between concentration and percent inhibition (Figure 4-5). The result revealed that an increase in concentration enhanced percent DPPH inhibition. All linear profiles covered the overall response curve including 50% inhibition; therefore, the regression equation which obtained by fitting all data could accurately estimate the IC_{50}.



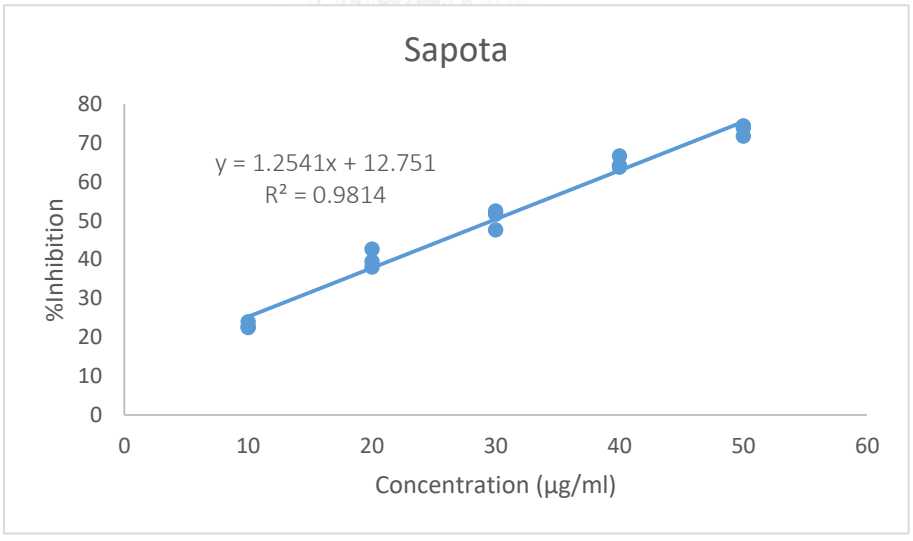
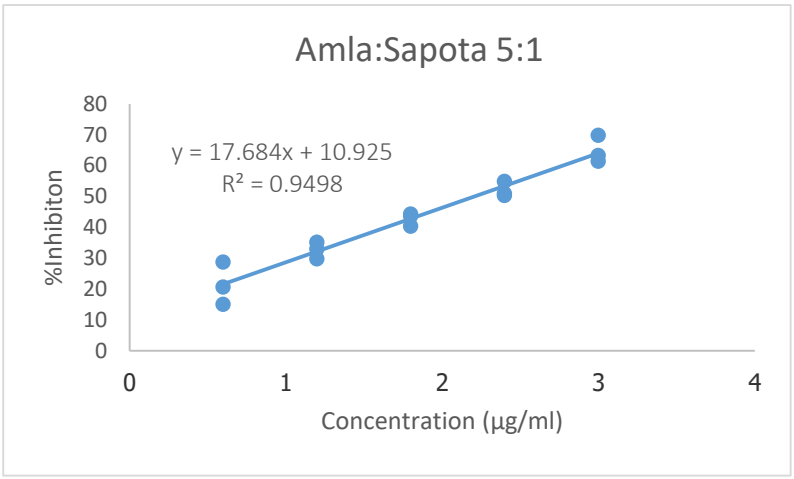
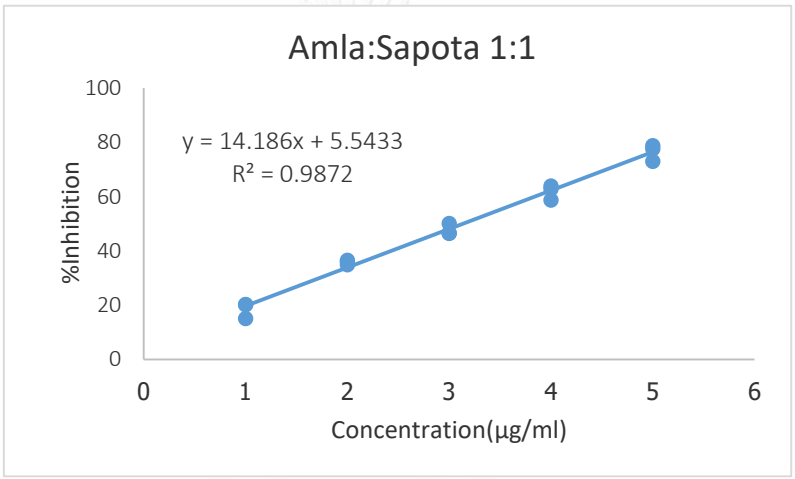


Figure 4 DPPH inhibition activities of ethanolic amla extract and ethanolic sapota extract





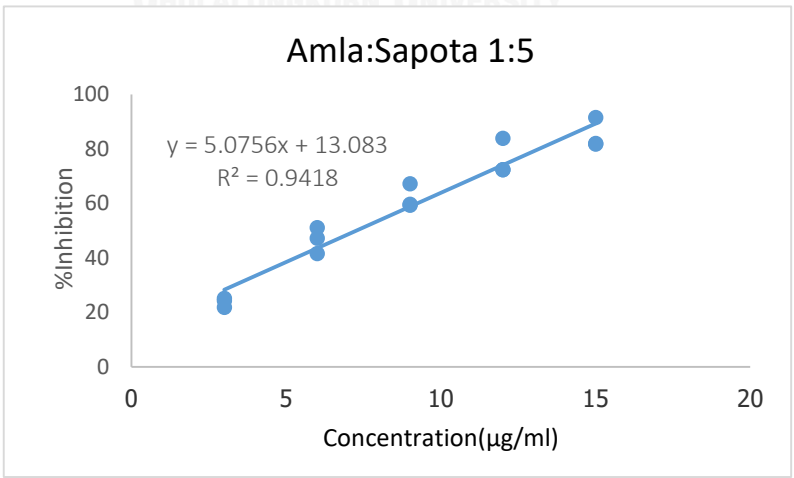


Figure 5 DPPH inhibition activities of combination amla and sapota extracts at the ratios of 5:1, 1:1 and 1:5.

The IC_{50} of individual extracts, combination extracts and standard ascorbic acid are presented in Table 1. Amla showed potent DPPH scavenging activity with approximately 16 folds stronger than that of sapota extract. Individual amla extract exhibited IC_{50} of 1.84±0.1 µg/ml comparable to standard ascorbic acid. This result was in accordance with previous study of Pientaweeratch in which the same extraction procedure was followed and their result revealed the IC_{50} of 1.70±0.06 µg/ml (Pientaweeratch et al, 2016). Amla was reported to contain various phytochemicals especially gallic acid and ellagic acid which showed potent antioxidant activity (Liu, Cui, et al., 2008; Luo et al., 2011; Luo et al., 2009). Therefore, its antioxidant effect might be related to the presence of gallic acid and ellagic acid in the test extract. Individual sapota extract exhibited weaker activity with IC_{50} of 29.70±1.29 µg/ml (Table 1). The antioxidant activity may be related to phenolic content presenting in plant extract. Pientaweeratch et al. (2016) revealed that antioxidant activities of amla and sapota showed strong correlation with total phenolic content (TPC). Amla extract contained higher TPC with 362.43±11.22 mg GAE/g extract and presented higher antioxidant activity compared to sapota extract which showed TPC at 38.56±1.98 mg GAE/g extract resulting in the lower antioxidant effect. Although, sapota extract showed lower antioxidant activity than amla extract, however, it exhibited much more potent activity than other fruits such as strawberry, plum, mango, orange, lemon or apple (Leong and Shui, 2002). Catechin compounds such as catechin, epigallocatechin and epigallocatechin gallate presenting as a major compounds in sapota fruit showed

potent inhibition DPPH radical activity with SC_{50} of 2.4, 1.8 and 1.2 μM, respectively (Nanjo et al., 1996).

Table 1 DPPH radical scavenging activities of individual and combination extracts. The result expressed as IC_{50} (mean ± SD, n=3)

	Sample / Standard	IC_{50} (μg/ml)
Individual extract	Amla	1.84 ± 0.10
	Sapota	29.70 ± 1.29*
Combination extract Amla : Sapota	5:1	2.21 ± 0.15
	1:1	3.13 ± 0.06*
	1:5	7.30 ± 0.73*
Standard	Ascorbic acid	1.33 ± 0.06

* Significant difference from the standard (p-value < 0.05)

The combination of amla and sapota at the ratio of 5:1 exhibited the IC_{50} of 2.21±0.15 μg/ml comparable to standard ascorbic acid and combination ratio at 1:1 which provided IC_{50} of 1.33±0.06 and 3.13±0.06 μg/ml, respectively. Combination at the ratio of 5:1 was insignificantly different compared to individual amla extract. Amla showed a predominant in antioxidant activity. Therefore, the highest activity of combination 5:1 revealed effective effect as expected because this combination contains amla as a main constituent. Combination extract at the ratio of 1:5 showed IC_{50} of 7.30±0.73 μg/ml. Although the majority of this combination was sapota, its antioxidant activity of this combination was 4 folds more potent than individual sapota.

Amla was a powerful antioxidant and, hence, it could improve antioxidant effect of sapota in combination. The result suggested that all three combinations were considered effective antioxidants where combination extract at ratios of 5:1 and 1:1 provide potent antioxidant property.

The combination extracts of all ratios revealed either additive or synergistic effect. The combination index at the median effect (CI_{50}) demonstrated additive effect of amla and sapota combination at ratios of 5:1 and 1:5 and showed synergistic effect at ratio of 1:1 (Table 2).

Table 2 Interactions of combination extracts on DPPH radical scavenging activity (mean ± SD, n=3).

	Sample / Standard	CI_{50} index	Type of Interaction
Combination extract Amla : Sapota	5:1	1.01 ± 0.07	Additive
	1:1	0.9 ± 0.02*	Synergism
	1:5	0.87 ± 0.09	Additive

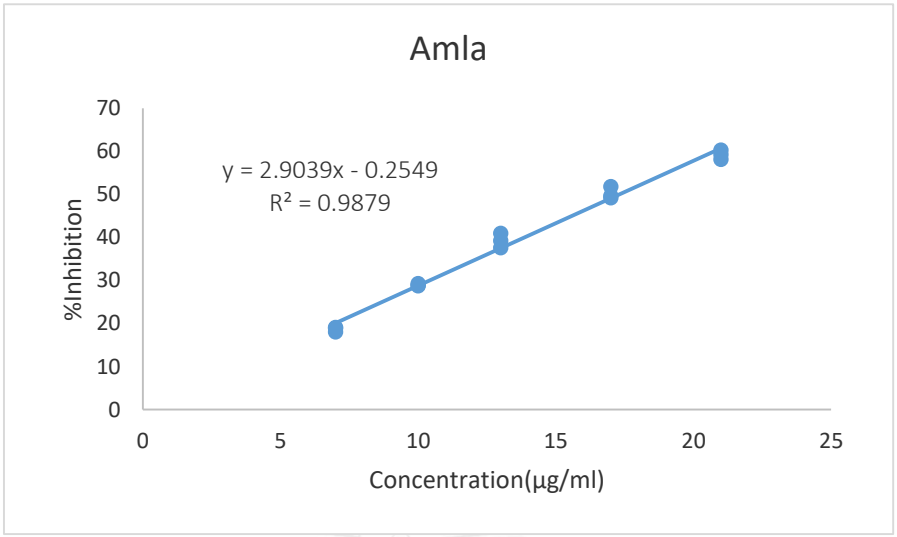
*Significant difference from 1 (CI < 1, p-value < 0.05)

Synergistic or additive effects of combinations might be due to various types of polyphenolic compounds presenting in both extracts. Antioxidant activity of amla is mainly due to the presence of gallic acid, ellagic acid and ascorbic acid in the extract while such activity of sapota comes from catechin compounds, quercetin and myricetin. Difference in active components of each test extract might, therefore,

contribute to synergistic or additive effects of combination extracts. The result of present study is in accordance with previous studies of different research groups about synergistic and/or additive effect of combined extracts. Liao and Yin (2000) reported the synergistic effect in antioxidant activity by inhibiting Fe^{2+} induced lipid oxidation assay of ascorbic acid combined with catechin or epicatechin. Moreover, combination of ascorbic acid with other polyphenol including myricetin, quercetin, gallic acid also showed synergistic effect of all combinations. Ascorbic acid was reported to have ability in reducing anion radical from phenoxide oxidation of EGCG in green tea and in recycling phenoxyl radical to regenerate EGCG (Dai, Chen, and Zhou, 2008). However, combination of quercetin and ellagic acid showed additive effect and combination of catechin and ellagic acid exhibited antagonistic effect on antioxidant activity evaluated by inhibition of LDL oxidation assay (Meyer, Heinonen, and Frankel, 1998). The antagonistic effect may be because of hydrogen-bonding interaction between carbonyl group on lactone ring of ellagic acid and dihydroxyphenols of catechin leading to blocking the hydrogen donation of catechin (Meyer et al., 1998). The effect of combined purified polyphenol from several researches revealed all interaction typed i.e., synergistic, additive and antagonistic effects. Therefore, combination of several polyphenolic compounds may exert either synergistic, additive or antagonistic effects depending on interaction between compounds that present in the test extracts.

3. *In vitro* anti-inflammatory assay

Nitric oxide radical scavenging assay was used to determine anti-inflammatory activity. Sodium nitroprusside solution generates nitric oxide radical which reacts with oxygen to produce nitrite product. The nitrite production can be detected by Griess reagent. The anti-inflammatory activities of each test extract are presented in Figure 6. Linear relationship between concentration and percent inhibition was exhibited. Increase in extract concentration linearly enhanced inhibition of nitric oxide. Similarly, increase in percent inhibition with increasing combination extract concentration was detected. However, linear relationship was not exhibited (Figure 7). Logarithm transformation was, therefore, used to fit dose response curve since it showed better fit than linear regression.



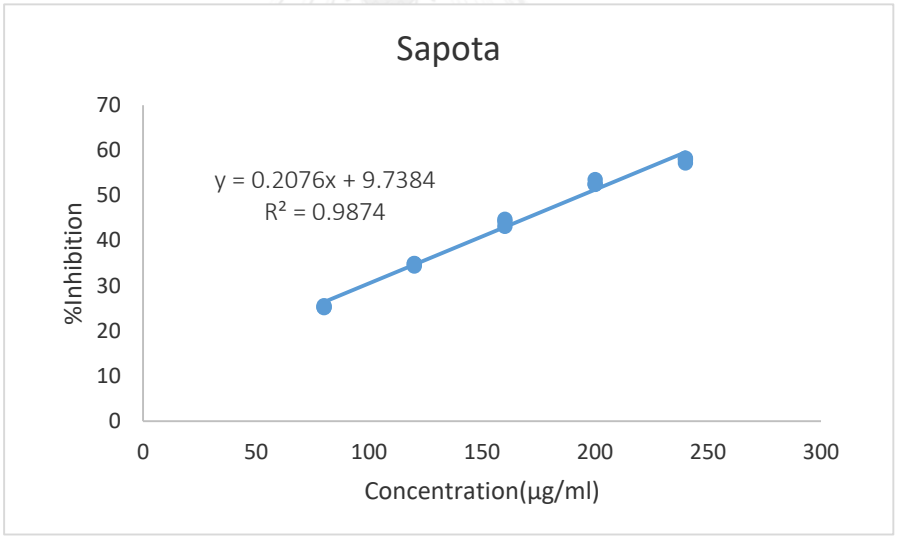
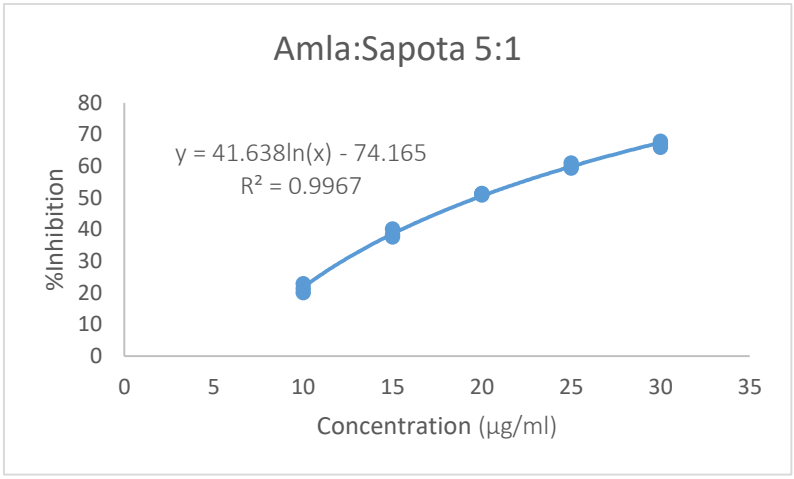
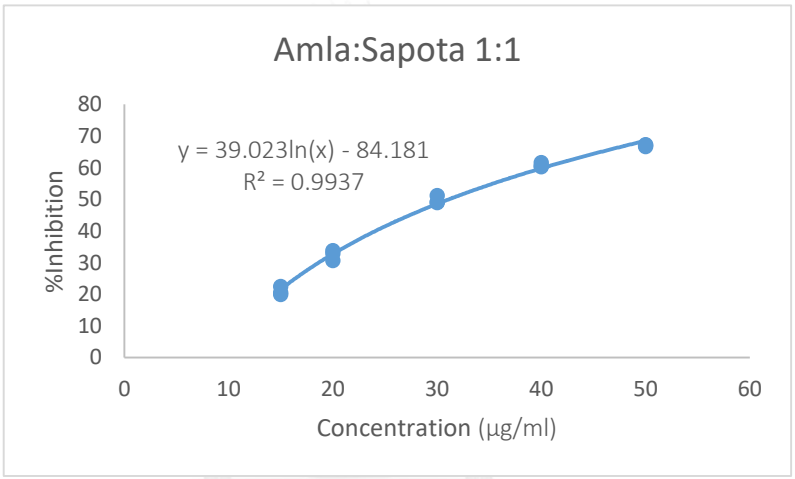


Figure 6 Nitric oxide inhibition activities of ethanolic amla extract and ethanolic sapota extract





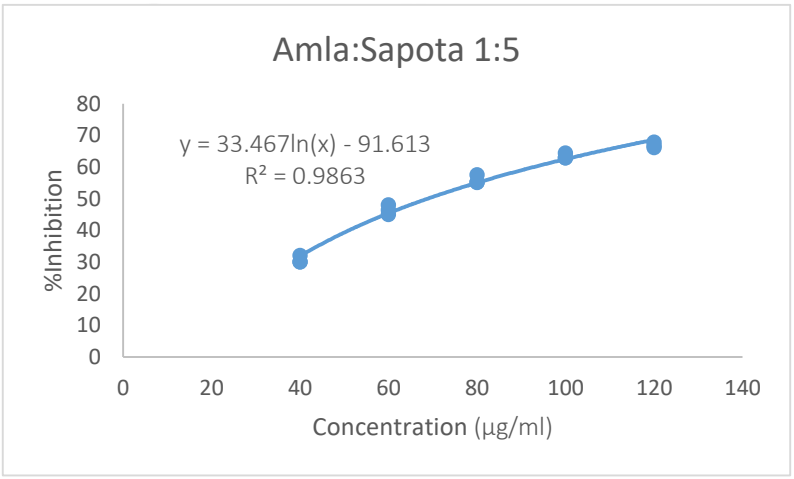


Figure 7 Nitric oxide inhibition activities of combination amla and sapota extracts at the ratios of 5:1, 1:1 and 1:5

The IC_{50} values of individual extracts, combination extracts and standard gallic acid were calculated from linear regression equations or equations from logarithm transformation. All are presented in Table 3. Amla showed effective activity with IC_{50} of 17.31±0.37 µg/ml, however, its activity was less than standard gallic acid which possessed IC_{50} of 4.72 ± 0.19 µg/ml.

However, this result was different from previous study of Kumara and Karunakaran (2006) who investigated on methanolic amla extract partitioned with ethyl acetate. Nitric oxide radical scavenging activity of ethanolic amla in the present study was two folds superior than that of their test extract which exhibited IC_{50} of 39.27±1.27 µg/ml (Kumaran and Karunakaran, 2006). The different result might be due to the difference in sources of material or difference in extraction solvents affecting the compound being extracted. Nitric oxide radical scavenging and antioxidant activities showed correlation with total phenolic content within the test extract (Ebrahimzadeh et al., 2010). Since ethanolic amla extract exhibited higher total phenolic content (TPC) and antioxidant activity with activity of 362.43±11.2 mg GAE/g extract and IC_{50} of 1.70±0.06 µg/ml, respectively (Pientaweeratch et al., 2016) than methanolic amla extract which provided TPC of 120.9±2.1 mg GAE/g extract and antioxidant activity with IC_{50} of 11.23±0.9 µg/ml (Liu, Zhao, et al., 2008). Ethanolic amla extract might, therefore, offer the superior effect. Conversely, sapota extract, showed weaker activity with approximately 11 folds lower than amla. Its IC_{50} was 193.99±2.66 µg/ml. This result

was in similar order as of DPPH radical scavenging assay where amla showed more effective activity than sapota.

The effective activity depended on the type of phytochemicals component in the extract. 2,3 double bond at C ring, 5,7-dihydroxyl group at A ring and 4'-hydroxyl group at B ring of flavonoid structure provided strong inhibition on nitric oxide production (Matsuda et al., 2003; J. Wang and Mazza, 2002). These structures were presented in gallic acid, quercetin, myricetin and kaempferol which were found in amla. In addition, gallic acid revealed a strong anti-inflammatory activity. Gallic acid at 10 μM showed 90% nitrite reduction in activated macrophages followed by epigallocatechin (EGC), epigallocatechin gallatee (EGCG) and epicatechin (EC), respectively (Lin and Lin, 1997). Since these compounds acid was found as a major compound in amla fruit extract, anti-inflammatory activity of amla extract might be contributed by the presence of such compounds.

Table 3 Nitric oxide radical scavenging activities of individual and combination extracts. The result expressed as IC_{50} (mean ± SD, n=3).

	Sample / Standard	IC_{50} (μg/ml)
Individual extract	Amla	17.31 ± 0.37*
	Sapota	193.99 ± 2.66*
Combination extract Amla : Sapota	5:1	19.72 ± 0.35*
	1:1	31.14 ± 0.49*
	1:5	68.80 ± 1.35*
Standard	Gallic acid	4.72 ± 0.19

*Significant difference from standard gallic acid (p-value < 0.05)

The IC_{50} data of combination extracts are shown in Table 3. The combination amla and sapota at the ratio of 5:1 exhibited the highest activity compared with other combinations and possessed the IC_{50} of 19.72±0.35 µg/ml. Nitric oxide scavenging activity decreased as increasing the ratio of sapota extract. The IC_{50} of the combination ratio 1:1 nd 1:5 were 31.14±0.49 µg/ml and 68.80±1.35 µg/ml, respectively. Since amla showed a predominant in NO scavenging activity, the combination extract at the ratio of 5:1 containing amla as a main constituent exhibited effective effect as expected. Although the majority of combination extract at the ratio of 1:5 was sapota, its anti-inflammatory activity of this combination was 3 folds more effective than that of individual sapota. Amla was a potent anti-inflammatory effect and, hence, it could improve such effect of sapota in combination.

Table 4 Interactions of combination extracts on nitric oxide radical scavenging (mean ± SD, n=3).

	Sample / Standard	CI_{50}	Type of interaction
Combination extract Amla : Sapota	5:1	0.97 ± 0.02*	Synergism
	1:1	0.98 ± 0.02	Additive
	1:5	0.96 ± 0.02*	Synergism

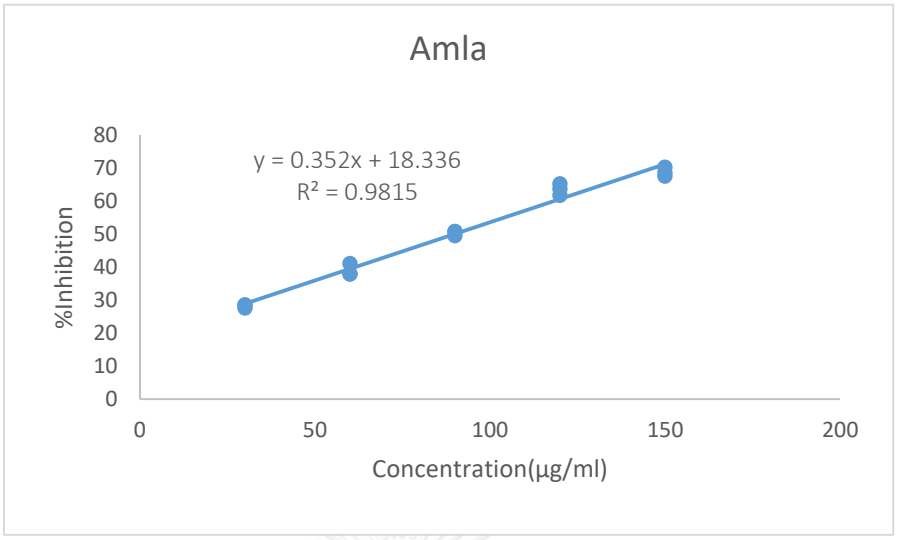
*Significant difference from 1 (CI < 1, p-value < 0.05)

Combination indexes at the median effect (CI_{50}) of all combinations were calculated and are presented in Table 4. Combination amla and sapota extract at the

ratios of 5:1 and 1:5 revealed synergistic effect and combination at the ratio of 1:1 showed additive effect. Effect of combination might result from the presence of various types of polyphenolic compounds within both extracts. Few studies reported the effect of polyphenolic combination on anti-inflammatory activity. The decreasing in nitric oxide derivatives was related with reduction in tumor growth and cancer cells proliferation (Thomsen and Miles, 1998). Combination of quercetin and kaempferol and of ascorbic acid and EGCG showed synergistic effect on anti-proliferative effect in human cancer cell lines (Ackland, VAN De Waarsenburg, and Jones, 2005) and on human lung adenocarcinoma inhibition activity (Li, Wu, and Tu, 2010), respectively. These combinations, thus, possessed anti-inflammatory property. Quercetin and kaempferol were known as effective compounds to inhibit NO production while ascorbic acid could enhance inhibitory ability of EGCG by protection oxidation of EGCG. Although amla and sapota extracts were reported to contain quercetin, kaempferol, ascorbic acid, and EGCG, these compounds were not major components in the test extracts. Therefore, the additive effect might be, instead, exhibited.

4. *In vitro* anti-collagenase assay

Anti-collagenase effect was determined by using EnzChek® E-12055 gelatinase/collagenase assay kit. Substrate DQ™ gelatin was breakdown by collagenase enzyme and provide fluorescence absorption. The decreasing of fluorescence absorption by test extracts was measured for collagenase inhibition activity. The anti-collagenase activity of all test extracts, including individual extracts and combination extracts, showed linear relationship between concentration and percent inhibition (Figure 8-9). The result demonstrated that an increase in extract concentration enhanced percent collagenase inhibition. All the linear profiles covered the range including 50% inhibition which was used to calculate the IC_{50} value.



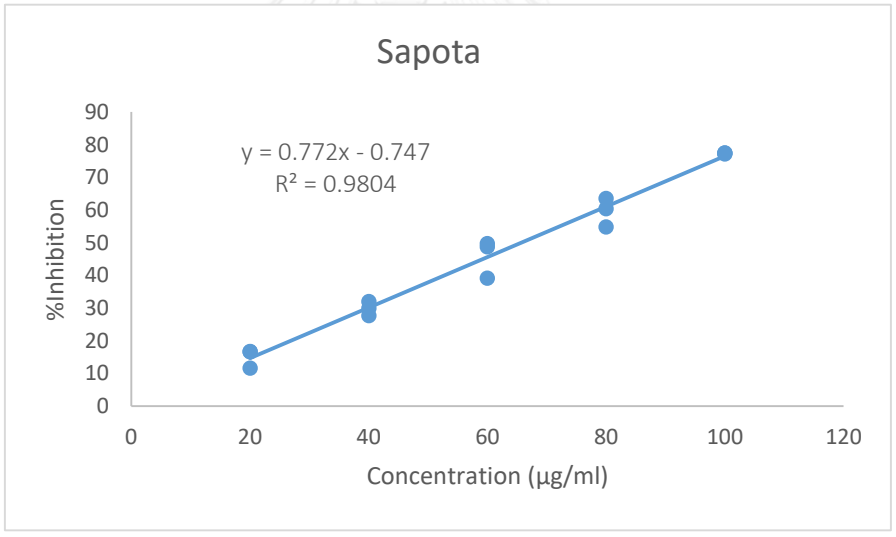


Figure 8 Collagenase inhibition activities of ethanolic amla extract and ethanolic sapota extract

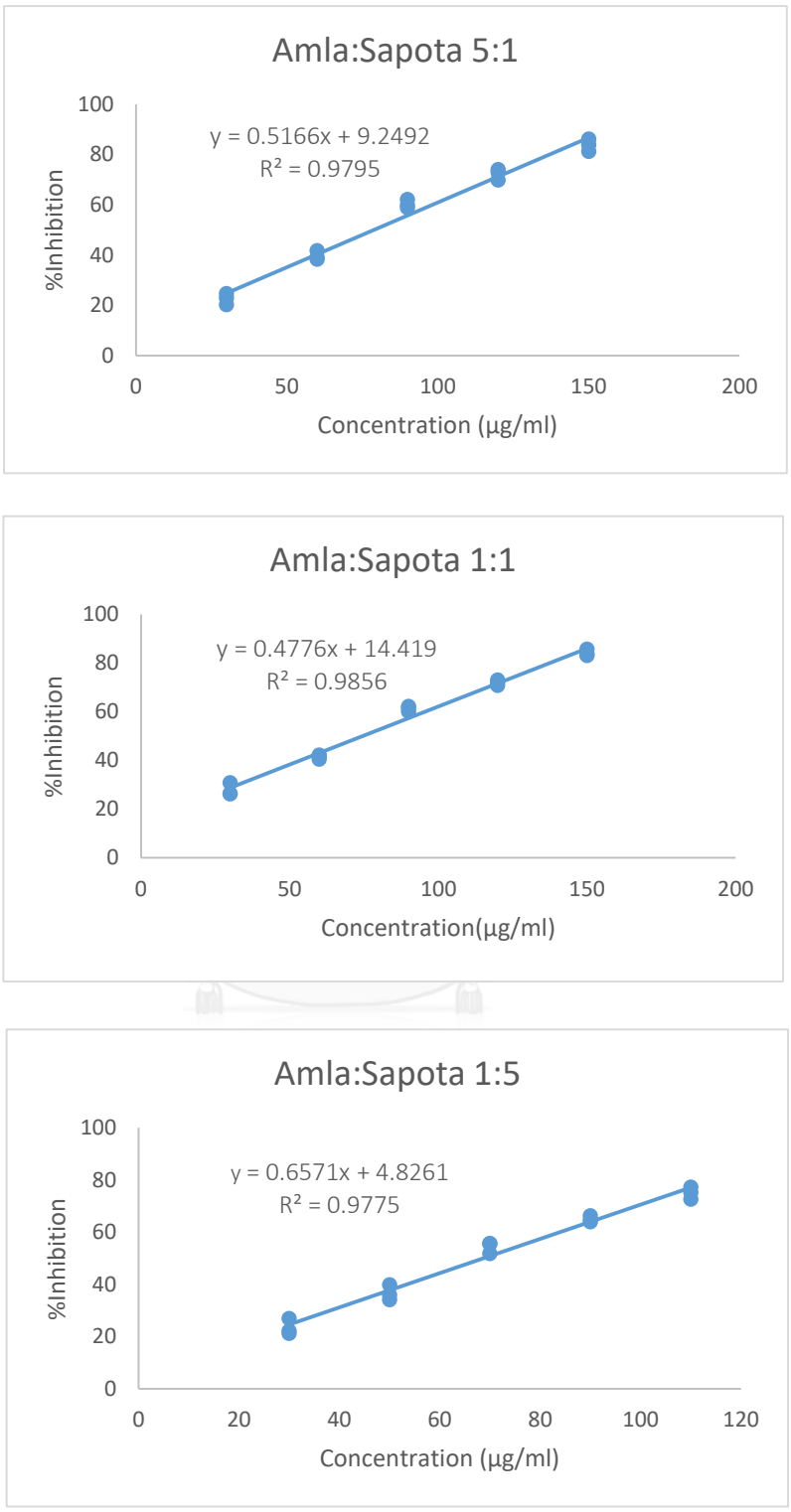


Figure 9 Collagenase inhibition activities of combination amla and sapota extracts at the ratios of 5:1, 1:1 and 1:5

The IC_{50} of individual extracts, combination extracts and standard EGCG are presented in Table 5. Sapota showed higher collagenase inhibition activity than amla with IC_{50} of 65.68±3.63 and 89.96±0.79 μg/ml, respectively. However, the activities of both extracts were much lower than that of standard EGCG. The result of present study was in agreement with Pientaweeratch (2016). The activity of ethanolic amla extract in this study showed comparable effect to ethanolic amla in their study which possessed IC_{50} of 89.41±3.26 μg/ml. However, sapota extract in the present study exhibited slightly more effective than theirs although the extraction procedures were identical (Pientaweeratch et al., 2016).

Collagenase enzyme is a matrix-metalloproteinase which relates with Zn ion responsible for collagen degradation (Mukherjee et al., 2013). Catechin compounds in green tea especially epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) were reported to potently inhibit collagenase enzyme (Demeule et al., 2000). Epigallocatechin gallate (EGCG) showed effective inhibition on MMP-2 with IC_{50} of 15 μM (Sartor et al., 2002). It was due to the presence of three hydroxyl groups and galloyl moiety on benzene ring. The structures could bind Zn ion which inhibit enzyme activity leading to inhibition of substrate digestion (Sartor et al., 2002). Sapota possessed anti-collagenase activity might be because it contain catechins as a major compound (H. Wang et al., 2012) while Amla contained various polyphenols such as gallic acid, ellagic acid, myricetin, quercetin and kaempferol which significantly inhibited collagenase enzyme activity and possessed a metal chelating property

leading to collagenase enzyme disfunction (Devipriya et al., 2007; Rafat, Cillard, and Cillard, 1987; Sim et al., 2007; Wittenauer et al., 2015).

Table 5 Anti-collagenase activities of individual and combination extracts. The result expressed as IC_{50} (mean ± SD, n=3).

	Sample / Standard	IC_{50} (µg/ml)
Individual extract	Amla	89.96 ± 0.79*
	Sapota	65.68 ± 3.63*
Combination extract Amla : Sapota	5:1	78.90 ± 3.42*
	1:1	74.48 ± 1.61*
	1:5	68.68 ± 2.69*
Standard	EGCG	4.47 ± 0.18

*Significant difference from standard (p-value < 0.05)

The combination of amla and sapota at ratio of 1:5 showed the highest activity with IC_{50} of 68.68±2.69 µg/ml, followed by the combination at the ratio of 1:1 and 5:1 which provided IC_{50} of 74.48±1.61 and 78.90±3.42 µg/ml, respectively (Table 5). Combination extract at 1:5 was insignificantly different compared to individual sapota extract. Sapota revealed potent activity approximately 1.5 folds more than amla and therefore, combination which contained sapota as a main constituent showed higher activity than other combinations as expected.

Table 6 Interactions of combination extracts on anti-collagenase activity (mean ± SD, n=3).

	Sample / Standard	CI_{50}	Type of interaction
Combination extract Amla : Sapota	5:1	0.93 ± 0.04*	Synergism
	1:1	0.98 ± 0.02	Additive
	1:5	0.99 ± 0.04	Additive

*Significant difference from 1 (C < 1, p-value < 0.05)

Interaction at median effect (CI_{50}) of combination amla and sapota extract at the ratios of 1:1 and 1:5 demonstrated as an additive effect and combination at the ratio of 5:1 revealed synergistic effect (Table 6). From previous study of Pientaweeratch (2014), combination amla and sapota extracts at the ratios of 1:1.3, 1:2, 1:3.3, 1.5:1, 3:1, 4.5:1 and 6:1 exhibited combination index as synergistic effect. However, their combinations were prepared by fixed concentration of one extract with varied concentrations of another resulting in the difference in combination ratio and in extract concentration. Consequently, the interaction effects of combinations were evaluated at different point on dose response curve **(**Pientaweeratch, 2014). In the present study, amla and sapota extracts were combined by fixed ratio at 5:1, 1:1, and 1:5 and their combination indexes were calculated at the median effect to obtain more accurately result.

5. *In vitro* anti-elastase assay

Anti-elastase effect was determined by using EnzChek® E-12056 elastase assay kit. Substrate DQ™ elastin was breakdown by elastase enzyme from porcine pancreas and provide fluorescence absorption. The decreased of fluorescence absorption by test extracts was measured and interpreted for elastase inhibition activity. The anti-elastase activity of individual and combination test extracts are presented in Figure 10-11. Linear relationship between concentration and percent inhibition was exhibited in individual test extracts. Increase in test extract concentration enhanced inhibition of elastase enzyme. Similarly, increase in percent inhibition with increasing concentration of combination extract was also detected. However, plateau activity was observed at high concentration resulting in non-linear relationship all combination ratios. Therefore, logarithm transformation was used to fit dose response curve since it showed better fit than linear regression.

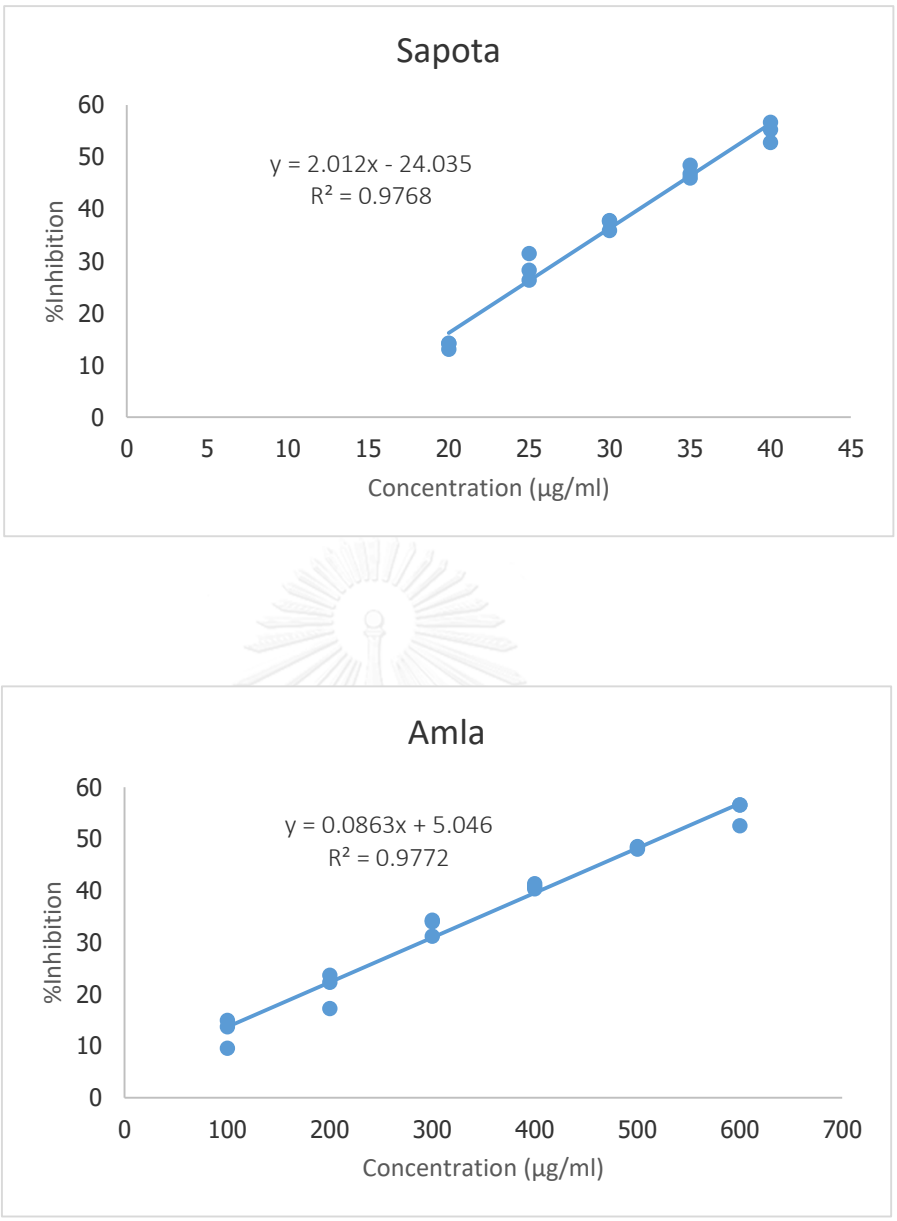


Figure 10 Elastase inhibition activities of ethanolic amla extract and ethanolic sapota extract

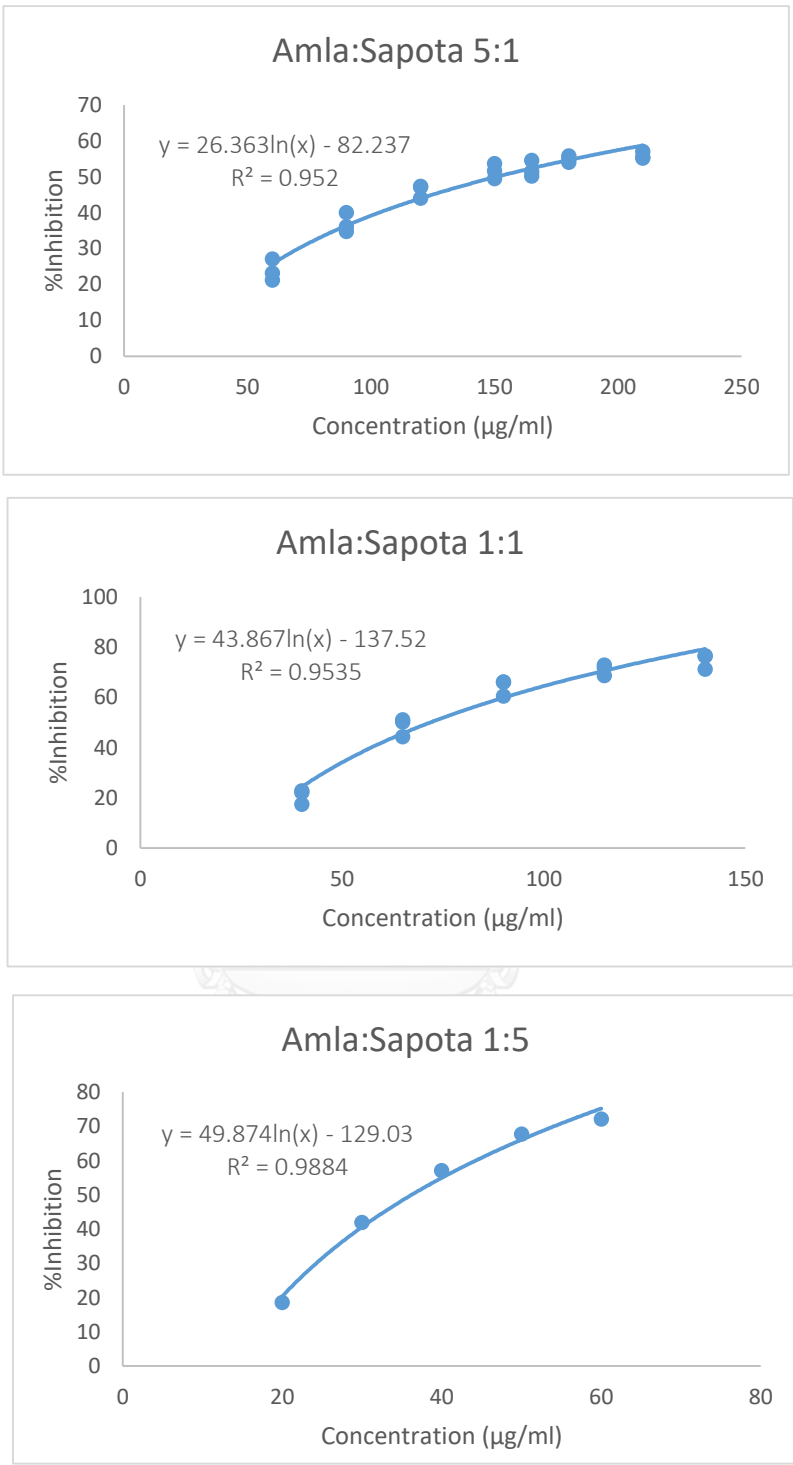


Figure 11 Elastase inhibition activities of combination amla and sapota extracts at the ratios of 5:1, 1:1 and 1:5

All the dose response curve profiles covered the range including 50% inhibition which was used to calculate the $IC_{50.}$ The IC_{50} of individual extract, combination extract and standard EGCG are presented in Table 7. Sapota exhibited effective anti-elastase activity with IC_{50} of 36.82±0.72 µg/ml. Its activity was higher than that of standard EGCG and 14 folds superior than amla whose IC_{50} was 520.83±18.72 µg/ml. This result was in agreement with previous study where sapota extract presented much higher activity than amla extract **(**Pientaweeratch, 2014). Anti-elastase activity of sapota might be caused by the presence of several effective polyphenolic compounds. Catechin compounds which were found in sapota has metal chelating ability (Le Nest et al., 2004; Torreggiani et al., 2008) and it might possess anti-elastase property since elastase enzyme could be inhibited by chelating agent (Homsy et al., 1988; Vallee and Falchuk, 1993). In addition, larger molecular weight of polyphenolic offer more ability in decreasing elastase activity than lower molecular weight. Large molecule could fit into elastase active site and numerous hydroxyl groups exhibited strong hydrogen interaction with amino acids on elastase structure leading to enzyme inactivation (Brás et al., 2010; Hrenn et al., 2006). Large molecular weight of oligomeric proanthocyanidins of catechin, epicatechin gallate (ECG) or epigallocatechin gallate (EGCG) which were found as a major compound in sapota (H. Wang et al., 2012) significantly inhibited elastase enzyme activity (Brás et al., 2010; Demeule et al., 2000; Wittenauer et al., 2015) and it might contribute to strong anti-elastase activity of sapota extract. Conversely, gallic acid which was a major compound in amla showed weak

inhibition on elastase enzyme activity (Isenburg et al., 2006; Ko et al., 2011; Wittenauer et al., 2015). Although, gallic acid is known as metal chelator, it is a small molecule which might not fit well into elastase active site. It might, thus, lead to poor anti-elastase activity of amla.

Table 7 Anti-elastase activities of individual and combination extracts. The result expressed as IC_{50} (mean ± SD, n=3).

	Sample / Standard	IC_{50} (µg/ml)
Individual extract	Amla	520.83 ± 18.72*
	Sapota	36.82 ± 0.72*
Combination extract Amla : Sapota	5:1	150.72 ± 7.90*
	1:1	71.90 ± 5.06*
	1:5	36.22 ± 0.99*
Standard	EGCG	93.99 ± 3.39

*Significant difference from standard (p-value < 0.05)

The IC_{50} data of combination extracts were shown in Table 7. The combination of amla and sapota at the ratio of 1:5 showed the highest activity with IC_{50} of 36.22±0.99 µg/ml, followed by combination at the ratio of 1:1 which provided IC_{50} of 71.90±5.06 µg/ml. Both combinations exhibited higher activity than standard EGCG. Unsurprisingly, the activity of combination extract at the ratio of 1:5 showed potent property comparable to that of individual sapota while combination at ratio of 5:1 containing amla as a main constituent provided lower activity with IC_{50} of 150.72±7.90

µg/ml. Its activity was much lower than standard EGCG. Sapota exhibited a predominant effect in anti-elastase activity. The highest activity of combination 1:5 revealed effective effect as expected since this combination contained sapota as a main constituent and it could improve activity of amla.

Table 8 Interactions of combination extracts on anti-elastase activity (mean ± SD, n=3).

	Sample / Standard	CI index	Type of interaction
Combination extract Amla : Sapota	5:1	0.92 ± 0.04	additive
	1:1	1.05 ± 0.07	additive
	1:5	0.83 ± 0.02*	synergism

*Significant difference from 1 (CI < 1, p-value < 0.05)

In the present study, combination amla and sapota extract was prepared at fixed ratio and the anti-elastase property was determined by varied concentration in dose response curve. Combination index (CI) was analyzed at the median effect to accurately estimate the effect. Combination of amla and sapota extract at the ratio of 1:5 offered combination index at the median effect (CI_{50}) as synergistic effect while combination with more concentration of amla including ratios 1:1 and 5:1 revealed as additive effect (Table 8).

Regards to study of Pientaweeratch and her group, combination with fixed concentration of sapota and varied concentration of amla extracts at ratio of 5:1 (total concentration 120 µg/ml) showed synergistic effect on anti-elastase activity. However,

increased in amla concentration to ratio of 15:1 (total concentration 320 µg/ml), 20:1 (total concentration 420 µg/ml) and 25:1 (total concentration 520 µg/ml) tend to enhance antagonistic effect (Pientaweeratch, 2014). Combination of amla and sapota extracts in previous study were varied both in combination ratios and concentrations in dose response curve. Therefore, antagonistic effect at high total concentration of extract might be caused by the limitation of substrate in the reaction.

6. *In vitro* anti-tyrosinase assay

Anti-tyrosinase activity of all test samples were determined by DOPA chrome method using L-DOPA as substrate (Likhitwitayawuid and Sritularak, 2001). DOPA chrome is an intermediate substance in melanogenesis which is responsible for skin pigmentation. Tyrosinase enzyme oxidize substrate L-DOPA to DOPA quinone and convert to DOPA chrome by auto-oxidation. Anti-tyrosinase effect of all test extracts were detected by the decrease of DOPA chrome absorbance at 490 nm.

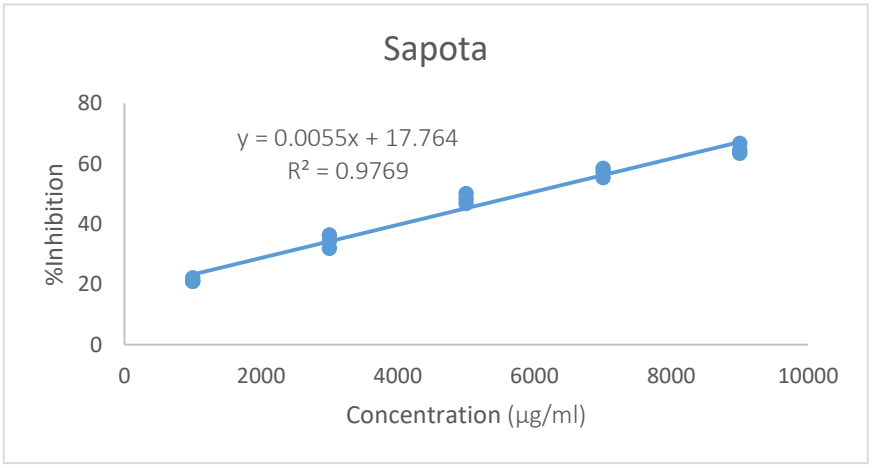


Figure 12 Tyrosinase inhibition activity of ethanolic sapota extract

Tyrosinase inhibition activity of ethanolic sapota extract are presented in Table 9. Sapota extract exhibited weak tyrosinase inhibition activity with a dose dependent manner (Figure 12). The linear profile was observed and it was used to calculate the IC_{50} value which is concentration of the test extract providing 50% inhibition. Sapota extract revealed IC_{50} of 5862.52±169.50 µg/ml (Table 9). Its activity showed more than 100 times inferior to standard kojic acid which exhibited IC_{50} of 42.14±2.08 µg/ml. Flavan-3-ol with 3-galloyl moiety at D ring on structure such as epicatechin gallate (ECG), gallocatechin gallate (GCG) and epigallocatechin gallate (EGCG) plays an important role for tyrosinase inhibition activity. It significantly decreased mushroom tyrosinase activity with IC_{50} of 15.74, 7.95 and 15.63 µg/ml for ECG, GCG and EGCG, respectively (No et al., 1999). Although, these compounds were found in sapota fruit, however, it might not be enough for exhibiting anti-tyrosinase activity.

Table 9 Anti-tyrosinase activities of individual extracts and standard kojic acid. The result expressed as IC_{50} (mean ± SD, n=3).

	Sample / Standard	IC_{50} (µg/ml)
Individual extract	Amla	UD
	Sapota	5862.52 ± 169.50*
Standard	Kojic acid	42.14 ± 2.08

*Significant difference from standard (p-value < 0.05), UD=Undetectale

Surprisingly, anti-tyrosinase property of amla extract could not be detected. Generally, DOPA chrome is a product from interaction between tyrosinase enzyme and L-DOPA substrate. Decrease in the absorbance of DOPA chrome at 490 nm demonstrates the anti-tyrosinase effect of the test extract. However, the present study revealed that an increase in concentration of amla extract resulted in the negative effect where the absorbance of DOPA chrome was enhanced. This result was in contrast to the several previous studies in which the amla extract showed significant inhibition on tyrosinase activity (Homklob, 2010; Jithavech, 2005; Sripanidkulchai and Fangkrathok, 2014; Sungthong and Phadungkit, 2015). The negative effect of amla extract in this experiment was, therefore, investigated.

Amla extract was directly incubated with only tyrosinase enzyme. Increase in absorbance unit was observed similarly to that of interaction system between L-DOPA substrate and tyrosinase enzyme. This result demonstrated that amla extract might behave as an alternate substrate and might interact with enzyme leading to an increase in the absorbance at the same wavelength of DOPA chrome. This interfering absorbance might cause the negative effect in the anti-tyrosinase assay of amla extract. Kubo et al. (2000) reported that gallic acid and its short alkyl chain ester could act as alternate substrate for mushroom tyrosinase and they were oxidized by the enzyme if amount of oxygen was presented in the systems. The resulting product enhanced the absorbance leading to decrease in anti-tyrosinase activity.

Disagreement of tyrosinase inhibition activity of amla extract in this study compared to others might be due to several conditions such as the difference in extracting condition, harvesting season or harvesting place of material. However, amla might inhibit hyper-pigmentation by other mechanisms which shall be further evaluated.

Table 10 In vitro biological properties of ethanolic amla extract, ethanolic sapota extract and their combination at the ratios of 5:1, 1:1 and 1:5

Activity	**Individual extract/ Combination Amla:Sapota extract**	**IC_{50} (µg/ml)**	**CI_{50}**	**Interaction**
Antioxidant	Amla	1.84 ± 0.10	-	-
	Sapota	29.70 ± 1.29	-	-
	5:1	2.21 ± 0.15	1.01 ± 0.07	Additive
	1:1	3.13 ± 0.06	0.90 ± 0.02*	Synergism
	1:5	7.30 ± 0.73	0.87 ± 0.09	Additive
Anti-Inflammation	Amla	17.31 ± 0.37	-	-
	Sapota	193.99 ± 2.66	-	-
	5:1	19.72 ± 0.35	0.97 ± 0.02*	Synergism
	1:1	31.14 ± 0.49	0.98 ± 0.02	Additive
	1:5	68.80 ± 1.35	0.96 ± 0.02*	Synergism
Anti-collagenase	Amla	89.96 ± 0.79	-	-
	Sapota	65.68 ± 3.63	-	-
	5:1	78.90 ± 3.42	0.93 ± 0.04*	Synergism
	1:1	74.48 ± 1.61	0.98 ± 0.02	Additive
	1:5	68.68 ± 2.69	0.99 ± 0.04	Additive
Anti-elastase	Amla	520.83 ± 18.72	-	-
	Sapota	36.82 ± 0.72	-	-
	5:1	150.72 ± 7.90	0.92 ± 0.04	Additive
	1:1	71.90 ± 5.06	1.05 ± 0.07	Additive
	1:5	36.22 ± 0.99	0.83 ± 0.02*	Synergism
Anti-tyrosinase	Amla	UD	-	-
	Sapota	5862.52 ± 169.50	-	-

*CI significant difference from 1 (CI < 1, p-value 0.05), UD=Undetectable

CHAPTER V
CONCLUSION

The changes in skin conditions involve several pathways. Plant extracts have been widely used as active ingredient in cosmetic product, however, a plant extract with complete all of cosmetic properties is barely achieved. Combination of plant extract might, therefore, enhance overall effects. In the present study, ethanolic amla and sapota extracts and their combinations at the ratios of 5:1, 1:1 and 1:5 were investigated for *in vitro* antioxidant, anti-inflammatory, anti-collagenase, anti-elastase and anti-tyrosinase activities. The result revealed that amla extract showed a predominant in antioxidant and anti-inflammatory activities with IC_{50} of 1.84±0.10 and 17.31±0.37 µg/ml, respectively while sapota extract showed a predominant in anti-collagenase and anti-elastase activities with IC_{50} of 36.82±0.72 and 65.68±3.63 µg/ml, respectively. The combination of amla and sapota extracts at the ratio of 5:1 exhibited the highest antioxidant and anti-inflammatory activities with IC_{50} of 2.21±0.15 and 19.72±0.35 µg/ml, respectively. However, all combination ratios presented the potent antioxidant and anti-inflammatory properties. The highest anti-collagenase and anti-elastase activities were observed in combination of amla and sapota extracts at the ratio of 1:5 with IC_{50} of 68.68±2.69 and 36.22±0.99 µg/ml, respectively. Since combination extract at the ratio of 1:5 provided potent antioxidant and

anti-inflammatory activities and exhibited the highest anti-collagenase and anti-elastase effects, thus, this combination might be used as an active ingredient for further formulation study.

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APPENDIX

Appendix A

Reagent preparation

1. DPPH radical scavenging assay

a) Preparation of 0.06 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution

Freshly prepared DPPH stock solution by weighing 1.18 mg (MW 394.3) and diluting in 50 ml absolute ethanol.

b) Preparation of test extract

Each test extract was dissolved in absolute ethanol. It was diluted in absolute ethanol to obtain final concentration in well plate.

Test extract		Final concentration (µg/ml)
Individual	Amla	0.5, 1, 1.5, 2, 2.5
	Sapota	10, 20, 30, 40, 50
Combianation Amla : Sapota	5:1	0.6, 1.2, 1.8, 2.4, 3
	1:1	1, 2, 3, 4, 5
	1:5	3, 6, 9, 12, 15

c) Preparation of standard ascorbic acid

10 mg ascorbic acid was dissolved in 10 ml absolute ethanol. It was diluted in absolute ethanol to obtain final concentrations at 0.5, 1, 1.5, 2, 2.5 µg/ml.

2. Nitric oxide radical scavenging assay

a) Preparation of 20 mM phosphate buffer solution pH 7.4

296.5 mg $NaH_2PO_4.2H_2O$ and 1150.6 mg Na_2HPO_4 were dissolved in 500 ml deionized water to obtain 20 mM phosphate buffer solution pH 7.4

b) Preparation of sodium nitroprusside (SNP) solution

3 mM working solution was prepared by weighing SNP 8.94 mg and dissolving in 10 ml 7.4 phosphate buffer solution.

c) Preparation of Griess reagent

Phase A: 2% w/v sulfanilaminde was prepared by weighing 1000 mg and dissolving in 50 ml deionized water. Then, 4% w/v phosphoric acid was added by adding 1.17 ml phosphoric acid into sulfanilamined solution.

Phase B: 0.2% w/v nepthylethylenediamine dihydrochloride was prepared by weighing 100 mg and dissolving in 50 ml deionized water.

Phase A and B were mixed at the ratio of 1:1 immediately before assay.

d) Preparation of standard reference

Standard curve KNO_2 was prepared by dissolving KNO_2 in phosphate buffer solution. It was diluted in phosphate buffer to preparing working stock solution. It was diluted in well plate to obtain final concentrations at 3.125, 6.25, 12.5, 25, 50, 100 µg/ml.

e) Preparation of test extract

5 mg amla and 5 mg sapota extracts were dissolved in 5 ml 60% Propylene glycol (PG) and 5 ml deionized water, respectively. Each test extract was diluted in phosphate buffer to preparing working stock solution. Then, it was diluted in well plate to obtain final concentrations.

Test extract		Final concentration (µg/ml)
Individual	Amla	7, 10, 13, 17, 21
	Sapota	80, 120, 160, 200, 240
Combianation Amla : Sapota	5:1	10, 15, 20, 25, 30
	1:1	15, 20, 30, 40, 50
	1:5	40, 60, 80, 100, 120

f) Preparation of standard gallic acid

10 mg gallic acid was dissolved in 10 ml deionized water. It was diluted in phosphate buffer to obtain final concentrations at 1, 3, 5, 7, 9 µg/ml.

3. Anti-collagenase assay (Enzchek® gelatinase/collagenase assay kit E-12055)

a) Preparation of 1X reaction buffer, pH 7.6

20 ml of 10X reaction buffer was diluted in 180 ml deionized water to obtain 200 ml 1X reaction buffer.

b) Preparation of DQ™ gelatin solution

1 ml deionized water was added in DQ™ gelatin vial (1 mg). Then, 125 µg/ml DQ™ gelatin solution was prepared by adding into 7 ml of 1X reaction buffer. 20 µl of this stock solution was used in 200 µl well to obtain final concentration at 12.5 µg/ml.

c) Preparation of *Clostridium histolyticum* collagenase solution

0.5 ml of deionized water was added into 500 U *Clostridium histolyticum* enzyme vial to obtain 1000 U/ml stock solution. Then, 20 µl of this stock solution was added into 50 ml of 1X reaction buffer to provide 0.4 U/ml. 100 µl of 0.4 U/ml collagenase enzyme was used in 200 µl well to obtain final concentration at 0.2 U/ml.

d) Preparation of general metalloproteinase inhibitor 1,10-phenanthroline

9.9 mg 1,10-phenanthroline was dissolved in 25 µl ethanol. Then, 10 µl of this solution was added in to 5 ml 1X reaction buffer to provide 10 mM stock solution. 0.25 µg/ml working solution was prepared by adding 16 µl of 10 mM stock solution into 624 µl 1X reation buffer. 80 µl of 0.25 µg/ml working solution was used in 200 well to obtain final concentration at 0.1 µg/ml.

e) Preparation of test extract

Amla and sapota were separately dissolved in 60% Propylene glycol (PG) and deionized water, respectively. Each test extract was diluted

in 1X reaction buffer to preparing working stock solution. Then, it was diluted in well plate to obtain final concentrations.

Test extract		Final concentration (µg/ml)
Individual	Amla	30, 60, 90, 120, 150
	Sapota	20, 40, 60, 80, 100
Combianation Amla : Sapota	5:1	30, 60, 90, 120, 150
	1:1	30, 60, 90, 120, 150
	1:5	30, 50, 70, 90, 110

f) Preparation of standard -(-)epigallocatechin gallate (EGCG)

5 mg EGCG was dissolved in deionized water and it was diluted with 1X reaction buffer to preparing working stock solution. Then, it was diluted in well plate to obtain final concentrations at 5, 10, 15, 20 µg/ml.

4. Anti-elastase assay (Enzchek®elastase assay kit E-12056)

a) Preparation of 1X reaction buffer, pH 8

18 ml of 10X reaction buffer was diluted in 162 ml deionized water to obtain 180 ml 1X reaction buffer.

b) Preparation of DQ™ elastin solution

1 ml deionized water was added in DQ™ elastin vial (1 mg). Then, 100 µg/ml DQ™ elastin solution was prepared by adding into 9 ml of 1X reaction buffer. 50 µl of this stock solution was used in 200 µl well to obtain final concentration at 25 µg/ml.

c) Preparation of elastase enzyme solution.

0.5 ml of deionized water was added into 50 U elastase enzyme vial to obtain 100 U/ml stock solution. Then, 100 µl of this stock solution was added into 25 ml of 1X reaction buffer to provide 0.4 U/ml. 100 µl of 0.4 U/ml collagenase enzyme was used in 200 µl well to obtain final concentration at 0.2 U/ml.

d) Preparation of standard inhibitor 1 mM *N-methoxysuccinyl-alanyl-alanyl-prolyl-valine*-chloromethyl *ketone* (CMK) solution

50 µl DMSO was added into 500 µg CMK vial. 1 mM stock solution was prepared by adding 20 µl of this solution in to 380 µl 1X reaction buffer. Then, this stock solution was further diluted to provide 0.04 mM working solution. 50 µl of working solution was used in 200 well to obtain final concentration at 0.001 µM.

e) Preparation of test extract

Amla and sapota were separately dissolved in 60% Propylene glycol (PG) and deionized water, respectively. Each test extract was diluted in

1X reaction buffer to preparing working stock solution. Then, it was diluted in well plate to obtain final concentrations.

Test extract		Final concentration (µg/ml)
Individual	Amla	100, 200, 300, 400, 500
	Sapota	20, 25, 30, 35, 40
Combianation Amla : Sapota	5:1	60, 90, 120, 150, 165, 180, 210
	1:1	40, 65, 90, 115, 140
	1:5	20, 30, 40, 50, 60

f) Preparation of standard -(-)epigallocatechin gallate (EGCG)

5 mg EGCG was dissolved in deionized water and it was diluted with 1X reaction buffer to preparing working stock solution. Then, it was diluted in well plate to obtain final concentrations at 20, 40, 60, 80, 120 µg/ml.

5. Anti-tyrosinase assay

a) Preparation of 20 mM phosphate buffer solution pH 6.8

312 mg $NaH_2PO_4.2H_2O$ and 284 mg Na_2HPO_4 was separately dissolved in 100 ml deionized water. Then, 51 ml of $NaH_2PO_4.2H_2O$ solution and 49 ml Na_2HPO_4 solution were mixed to obtain 20 mM phosphate buffer solution pH 6.8.

b) Preparation of 0.85 mM 3,4-Dihydroxyl-L-phenylalanine (L-DOPA) solution

0.8 mg L-DOPA was dissolved in 10 ml phosphate buffer solution pH 6.8 to obtain concentration at 0.85 mM.

c) Preparation of 480 unit/ml mushroom tyrosinase solution

1.786 mg tyrosinase enzyme (labeled 2687 U/mg) was dissolved in 10 ml phosphate buffer solution to obtain concentration at 480 U/mg.

d) Preparation of test extract

Amla and sapota extracts were dissolved in 60% Propylene glycol (PG) and deionized water, respectively. Each test extract was diluted in phosphate buffer solution to preparing working stock solution. Then, it was diluted in well plate to obtain final concentration.

Test extract		Final concentration (µg/ml)
Individual	Amla	100, 200, 300, 400, 500
	Sapota	20, 25, 30, 35, 40

e) Preparation of standard kojic acid

10 mg kojic acid was dissolved in phosphate buffer and it was diluted with phosphate buffer solution to preparing working stock solution. Then, it was diluted in well plate to obtain final concentrations at 20, 30, 40, 50, 60 µg/ml.

Appendix B

Standard curve

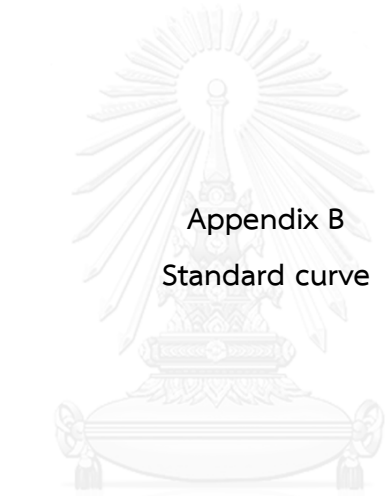

Table 11 Absorbance of standard potassium nitrite (KNO_2) for determination of anti-inflammatory activity using nitric oxide radical scavenging assay

Concentration (µg/ml)	Absorbance at 544 nm				
	N1	N2	N3	Average	SD
3.13	0.082	0.081	0.083	0.082	0.001
6.25	0.162	0.161	0.165	0.163	0.002
12.50	0.323	0.319	0.322	0.321	0.002
25	0.629	0.625	0.633	0.629	0.004
50	1.207	1.207	1.210	1.208	0.002
100	2.232	2.215	2.248	2.232	0.017

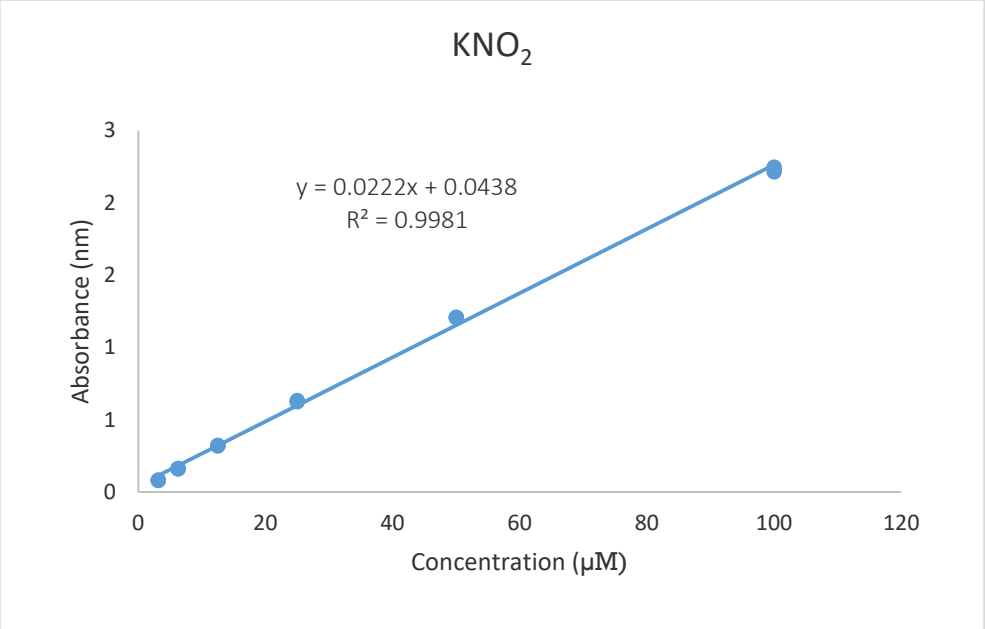

Figure 13 Potassium nitrite (KNO_2) standard curve

Appendix C
Light setting and concentration screening of sodium nitroprusside

Table 12 Absorbance of 3 mM sodium nitroprusside incubation under the light in 96 well plate (2 plates)

0.869	0.875	0.873	0.866	0.861	0.853	0.843	0.845	0.849	0.851	0.860	0.870
0.886	0.905	0.897	0.887	0.888	0.888	0.891	0.871	0.882	0.892	0.898	0.902
0.917	0.916	0.904	0.886	0.885	0.895	0.884	0.864	0.894	0.897	0.905	0.921
0.910	0.919	0.907	0.898	0.895	0.904	0.887	0.882	0.889	0.896	0.912	0.920
0.913	0.934	0.912	0.907	0.907	0.886	0.891	0.890	0.894	0.906	0.921	0.925
0.921	0.931	0.918	0.914	0.906	0.911	0.894	0.888	0.890	0.897	0.914	0.924
0.917	0.932	0.921	0.907	0.912	0.902	0.898	0.900	0.892	0.902	0.911	0.881
0.904	0.904	0.893	0.893	0.888	0.879	0.868	0.879	0.871	0.872	0.887	0.902
Average					0.895						
SD					0.012						
%CV					2.225						

0.892	0.887	0.877	0.817	0.873	0.863	0.811	0.861	0.863	0.848	0.849	0.876
0.893	0.925	0.916	0.903	0.890	0.876	0.887	0.887	0.886	0.885	0.886	0.844
0.935	0.940	0.911	0.894	0.899	0.891	0.883	0.886	0.886	0.883	0.893	0.873
0.939	0.926	0.921	0.904	0.898	0.887	0.885	0.891	0.891	0.888	0.899	0.808
0.936	0.931	0.911	0.907	0.900	0.899	0.891	0.884	0.889	0.881	0.897	0.833
0.943	0.931	0.912	0.901	0.894	0.889	0.885	0.864	0.889	0.894	0.884	0.876
0.853	0.935	0.906	0.895	0.893	0.887	0.887	0.893	0.887	0.877	0.871	0.873
0.903	0.883	0.848	0.869	0.866	0.849	0.850	0.854	0.849	0.848	0.813	0.881
Average					0.885						
SD					0.027						
%CV					3.143						

Appendix D

Raw data

Table 13 DPPH radical scavenging activities of ethanolic amla extract, ethanolic sapota extract and standard ascorbic acid

Sample/ Standard	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
Amla	0.5	11.87	15.67	18.83	15.46	3.49
	1	29.44	28.72	31.54	29.90	1.46
	1.5	41.33	41.62	42.89	41.95	0.83
	2	51.38	54.27	53.95	53.20	1.58
	2.5	61.82	66.62	71.72	66.72	4.95
	IC_{50}	1.94	1.84	1.74	1.840	0.1
Sapota	10	22.68	24.00	22.46	23.05	0.83
	20	39.44	42.63	38.07	40.05	2.34
	30	51.62	52.50	47.57	50.56	2.63
	40	64.17	66.65	63.79	64.87	1.55
	50	74.38	73.87	71.78	73.34	1.38
	IC_{50}	29.64	28.44	31.02	29.70	1.29
Ascorbic acid	0.5	20.74	15.97	15.71	17.47	2.83
	1	42.46	35.60	35.41	37.82	4.02
	1.5	63.07	58.18	59.10	60.12	2.60
	2	75.52	79.07	77.50	77.36	1.78
	2.5	88.80	87.04	86.26	87.37	1.30
	IC_{50}	1.26	1.36	1.37	1.33	0.06

Table 14 DPPH radical scavenging activities of combination amla and sapota extract at the ratios of 5:1, 1:1 and 1:5

Combination Amla:Sapota	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
5:1	0.6	15.02	28.77	20.68	21.49	6.91
	1.2	29.76	35.13	33.01	32.64	2.71
	1.8	40.37	43.65	44.30	42.77	2.11
	2.4	50.34	50.89	54.91	52.05	2.49
	3	61.42	63.28	69.81	64.84	4.41
	IC_{50}	2.36	2.20	2.07	2.21	0.15
1:1	1	15.10	20.34	20.23	18.55	3.00
	2	34.98	35.99	36.63	35.87	0.83
	3	50.13	46.48	46.66	47.75	2.06
	4	63.97	62.84	58.71	61.84	2.77
	5	78.85	77.61	73.04	76.50	3.06
	IC_{50}	3.09	3.10	3.20	3.13	0.06
1:5	3	25.20	24.29	21.82	23.77	1.75
	6	51.06	47.28	41.59	46.64	4.77
	9	67.16	59.69	59.36	62.07	4.41
	12	83.87	72.40	72.39	76.22	6.63
	15	91.56	81.98	81.81	85.11	5.58
	IC_{50}	6.50	7.47	7.93	7.30	0.73

Table 15 Nitric oxide radical scavenging activities of ethanolic amla extract, ethanolic sapota extract and standard gallic acid

Sample/ Standard	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
Amla	7	18.91	19.04	18.04	18.66	0.55
	10	28.87	29.23	28.69	28.93	0.28
	13	40.95	39.19	37.54	39.23	1.70
	17	51.74	49.17	49.65	50.19	1.36
	21	60.28	58.08	59.20	59.19	1.10
	IC_{50}	16.89	17.58	17.47	17.31	0.37
Sapota	80	25.30	25.14	25.59	25.34	0.23
	120	34.85	34.38	34.97	34.73	0.31
	160	44.21	43.15	44.75	44.04	0.81
	200	52.55	52.49	53.51	52.85	0.57
	240	57.92	57.19	58.31	57.81	0.56
	IC_{50}	193.97	196.66	191.33	191.33	191.33
Gallic acid	1	18.05	18.12	19.99	18.72	1.10
	3	37.01	36.42	38.53	37.32	1.09
	5	58.01	56.38	59.57	57.99	1.60
	7	69.70	67.44	72.37	69.83	2.47
	9	75.78	76.03	77.66	76.49	1.02
	IC_{50}	4.77	4.88	4.51	4.72	0.19

Table 16 Nitric oxide radical scavenging activities of combination amla and sapota extract at the ratios of 5:1, 1:1 and 1:5

Combination Amla:Sapota	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
5:1	10	20.02	22.82	21.36	21.40	1.40
	15	37.65	40.18	38.33	38.72	1.31
	20	50.82	51.21	51.38	51.14	0.28
	25	59.35	59.69	61.00	60.02	0.87
	30	65.93	67.01	67.84	66.93	0.96
	IC_{50}	20.13	19.50	19.54	19.72	0.35
1:1	15	20.55	22.50	19.98	21.01	1.32
	20	32.86	33.83	30.63	32.44	1.64
	30	51.14	48.94	49.14	49.74	1.22
	40	61.64	60.56	60.28	60.83	0.72
	50	67.27	66.68	67.05	67.00	0.30
	IC_{50}	30.70	31.03	31.67	31.14	0.49
1:5	40	30.00	32.08	30.05	30.71	1.18
	60	46.41	47.98	44.95	46.45	1.51
	80	57.55	55.08	55.19	55.94	1.39
	100	64.44	62.88	62.95	63.42	0.88
	120	67.90	66.12	66.71	66.91	0.91
	IC_{50}	67.96	68.08	70.36	68.80	1.35

Table 17 Anti-collagenase activities of ethanolic amla extract, ethanolic sapota extract

Sample/ Standard	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
Amla	30	28.56	27.68	28.62	28.29	0.53
	60	37.93	38.04	41.11	39.02	1.81
	90	49.57	50.82	50.81	50.40	0.72
	120	65.27	63.67	61.73	63.56	1.77
	150	70.30	68.61	67.56	68.83	1.38
	IC_{50}	89.11	90.66	90.11	89.96	0.79
Sapota	20	16.72	16.67	11.69	15.03	2.89
	40	32.06	30.01	27.75	29.94	2.15
	60	49.76	48.76	39.14	45.88	5.87
	80	60.42	63.60	54.86	59.63	4.42
	100	77.52	77.20	77.43	77.38	0.16
	IC_{50}	63.60	63.56	69.87	65.68	3.63
EGCG	5	48.60	49.33	48.75	48.90	0.39
	10	69.28	70.57	70.85	70.23	0.84
	15	84.19	84.92	83.90	84.34	0.53
	20	95.42	95.74	95.51	95.55	0.17
	IC_{50}	4.66	4.31	4.43	4.47	0.18

Table 18 Anti-collagenase activities of combination amla and sapota extract at the ratios of 5:1, 1:1 and 1:5

Combination Amla:Sapota	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
5:1	30	22.90	20.22	24.61	22.58	2.21
	60	41.80	38.28	39.14	39.74	1.84
	90	59.98	58.90	62.14	60.34	1.65
	120	72.94	69.81	74.07	72.27	2.20
	150	83.92	81.30	86.20	83.81	2.45
	IC_{50}	77.65	82.77	76.29	78.90	3.42
1:1	30	30.91	26.30	26.37	27.86	2.64
	60	41.68	42.31	40.59	41.53	0.87
	90	60.09	62.21	61.64	61.32	1.10
	120	72.22	73.12	70.84	72.06	1.14
	150	83.13	85.79	83.76	84.23	1.39
	IC_{50}	73.10	74.08	76.25	74.48	1.61
1:5	30	26.90	21.14	22.14	23.39	3.08
	50	39.82	35.88	34.09	36.60	2.93
	70	55.48	51.71	55.64	54.28	2.22
	90	64.56	63.96	66.22	64.91	1.17
	110	75.15	72.55	77.15	74.95	2.31
	IC_{50}	66.07	71.45	68.53	68.68	2.69

Table 19 Anti-elastase activities of ethanolic amla extract, ethanolic sapota extract and standard EGCG

Sample/ Standard	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
Amla	100	13.70	9.56	14.97	12.74	2.83
	200	23.71	17.23	22.31	21.08	3.41
	300	34.35	34.00	31.26	33.20	1.69
	400	41.41	40.85	40.39	40.88	0.51
	500	48.45	48.56	48.05	48.35	0.27
	600	56.61	56.58	52.60	55.26	2.31
	IC_{50}	508.36	511.78	542.35	520.83	18.72
Sapota	20	14.17	13.07	14.21	13.82	0.65
	25	26.38	28.24	31.45	28.69	2.56
	30	35.90	37.69	37.78	37.12	1.06
	35	46.79	48.44	45.98	47.07	1.25
	40	52.82	56.69	55.27	54.92	1.96
	IC_{50}	37.57	36.13	36.76	36.82	0.72
EGCG	20	20.69	20.60	19.43	20.24	0.70
	40	30.17	31.95	25.37	29.16	3.40
	80	49.53	43.21	43.77	45.50	3.50
	120	58.65	57.65	62.70	59.67	2.67
	IC_{50}	91.44	97.83	92.69	93.99	3.39

Table 20 Anti-elastase activities of combination amla and sapota extract at the ratios of 5:1, 1:1 and 1:5

Combination Amla:Sapota	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
5:1	60	23.19	27.10	21.25	23.85	2.98
	90	36.13	40.07	34.79	37.00	2.74
	120	47.34	47.14	44.04	46.17	1.85
	150	53.70	51.63	49.51	51.61	2.09
	165	54.56	51.48	50.21	52.08	2.24
	180	55.28	55.78	54.02	55.03	0.91
	210	55.55	57.03	55.24	55.94	0.96
	IC_{50}	146.60	145.73	159.82	150.72	7.90
1:1	40	17.49	22.81	22.22	20.84	2.92
	65	44.32	50.18	51.15	48.55	3.70
	90	60.56	66.23	66.11	64.30	3.24
	115	68.71	72.91	71.87	71.16	2.19
	140	71.25	76.61	76.50	74.78	3.06
	IC_{50}	77.80	68.92	69.14	71.95	5.06
1:5	20	18.27	20.75	16.56	18.53	2.10
	30	43.44	41.63	40.64	41.90	1.42
	40	58.82	57.22	55.04	57.03	1.90
	50	68.80	68.00	66.29	67.70	1.28
	60	73.02	72.15	70.93	72.03	1.05
	IC_{50}	35.51	35.80	37.36	36.22	0.99

Table 21 Anti-tyrosinase activities of ethanolic amla extract, ethanolic sapota extract and standard kojic acid

Sample/ Standard	Concentration (µg/ml)	%Inhibition			Average	SD
		N1	N2	N3		
Amla	500	-73.80	-73.35	-68.66	-71.94	2.84
Sapota	1000	21.03	21.06	22.20	21.43	0.67
	3000	31.94	36.43	34.35	34.24	2.25
	5000	46.83	50.09	48.39	48.44	1.64
	7000	58.53	57.31	55.41	57.08	1.57
	9000	63.49	66.79	64.52	64.93	1.69
	IC_{50}	5984.29	5668.93	5934.34	5862.52	169.50
Kojic acid	20	32.00	32.72	34.03	32.92	1.03
	30	44.80	45.55	45.29	45.21	0.38
	40.	52.00	54.19	51.05	52.41	1.61
	50	60.00	60.47	58.38	59.62	1.10
	60	67.47	67.80	66.23	67.17	0.83
	IC_{50}	41.25	40.65	44.52	42.14	2.08

Table 22 Screening anti-tyrosinase activity of ethanolic amla extract at different concentrations

Sample/ Standard	Concentration (µg/ml)	%Inhibition
Amla	100	-48.38
	500	-73.57
	1000	-78.30
Kojic acid	50	55.61

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