EFFECT OF COMBINATIONS OF *EMBLICA OFFICINALIS* EXTRACT, *ACHRAS SAPOTA* EXTRACT AND SILYMARIN ON *IN VITRO* ANTI-AGING ACTIVITIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmaceutical Technology
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2014

ผลของสารผสมสารสกัดมะขามป้อม สารสกัดละมุด และซิลิมาริน ต่อฤทธิ์การชะลอวัยแบบนอกกาย

นางสาวศิรินยา เพียรทวีรัชต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	EFFECT OF COMBINATIONS OF *EMBLICA OFFICINALIS* EXTRACT, *ACHRAS SAPOTA* EXTRACT AND SILYMARIN ON *IN VITRO* ANTI-AGING ACTIVITIES
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ศิรินยา เพียรทวีรัชต์ : ผลของสารผสมสารสกัดมะขามป้อม สารสกัดละมุด และซิลิมารินต่อฤทธิ์การชะลอวัยแบบนอกกาย (EFFECT OF COMBINATIONS OF *EMBLICA OFFICINALIS* EXTRACT, *ACHRAS SAPOTA* EXTRACT AND SILYMARIN ON *IN VITRO* ANTI-AGING ACTIVITIES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภญ. ดร.วิภาพร พนาพิศาล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ภญ. ดร.อัญญพร ตันศิริคงคล , 94 หน้า.

ผิวที่ขาดความยืดหยุ่น และมีริ้วรอย เป็นสิ่งที่หลีกเลี่ยงไม่ได้ และเกิดผ่านกลไกที่ซับซ้อน มากมาย ผลิตภัณฑ์บำรุงผิวต่างๆ ที่มีส่วนผสมของสารสกัดหลายตัว เพื่อชะลอวัยและปรับเปลี่ยนสภาพผิว ได้ถูกพัฒนาขึ้นอย่างมายมายหลากหลาย อย่างไรก็ตามรายงานเกี่ยวกับผลของการผสมสารสกัดต่อฤทธิ์ทางชีวภาพของสารสกัดยังมีจำนวนจำกัด ดังนั้นการศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาผลกระทบของการผสมสารสกัดต่อฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านเอมไซม์คอนลาจิเนส และฤทธิ์ต้านเอนไซม์อิลาสเทส เปรียบเทียบกับฤทธิ์ของสารสกัดเดี่ยว สารสกัดเอทานอลของผลมะขามป้อม สารสกัดเอทานอลของผลละมุด และซิลิมาริน สารสกัดเดี่ยวแต่ละชนิดถูกนำมาทดสอบกลั่นกรองฤทธิ์ต้านสารอนุมูลอิสระ ฤทธิ์ต้านเอมไซม์คอนลาจิเนส และฤทธิ์ต้านเอนไซม์อิลาสเทส รวมถึงการหาปริมาณรวมของสารฟีนอลิคและฟลาโวนอยด์ ฤทธิ์ต้านสารอนุมูลอิสระทำการประเมินโดยวิธีวัดการเปลี่ยนแปลงสี สำหรับการประเมินฤทธิ์ต้านคอนลาจิเนสทั้ง MMP-1, MMP-2 และฤทธิ์ต้าน อีลาสเทส ทำการทดสอบโดยใช้ชุดทดสอบ EnzChek® การวิเคราะห์ปริมาณรวมของสารฟีนอลิคและฟลาโวนอยด์ ทดสอบได้ด้วยวิธีวัดการเปลี่ยนแปลงสีของการทำปฏิกิริยาระหว่างสารสกัดกับสารละลาย Folin-Ciocalteu และอลูมิเนียมคลอไรด์ ตามลำดับ สารสกัด 2 ชนิดจะถูกคัดเลือกโดยพิจารณาจากฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านเอนไซม์คอนลาจิเนส และฤทธิ์ต้านเอนไซม์อีลาสเทส และนำมาศึกษาผลกระทบจากการผสมสารสกัดทั้งสองเข้าด้วยกัน ซึ่งรายงานผลเป็นค่าดัชนีการผสมสาร หรือ CI ซึ่งถูกจัดแบ่งเป็น 3 ประเภท คือ CI < 1 การเสริมฤทธิ์, CI = 1 การรวมฤทธิ์ และ CI > 1 การต้านฤทธิ์ ค่าอ้างอิง CI คำนวณจากค่ากลางผลตอบสนอง เมื่อกล่าวถึงฤทธิ์ต้านอนุมูลอิสระ สารสกัดเอทานอลของผลมะขามป้อมแสดงฤทธิ์สูงที่สุดด้วยค่า IC_{50} เท่ากับ 1.61 ± 0.04 ไมโครกรัม / มิลลิลิตร ในขณะที่สารสกัดเอทานอลของผลละมุดแสดงฤทธิ์ต้านเอนไซม์คอนลาจิเนสและอีลาสเทสสูงที่สุด ด้วยค่า IC_{50} เท่ากับ 86.47 ± 3.04 ไมโครกรัม / มิลลิลิตรและ 35.73 ± 0.61 ไมโครกรัม / มิลลิลิตร ตามลำดับ ดังนั้นจึงนำสารสกัดมะขามป้อมและละมุดมาผสมกันที่อัตราส่วนต่างๆกัน ค่าอ้างอิง CI ที่คำนวณจากค่ากลางผลตอบสนองสำหรับฤทธิ์ต้านอนุมูลอิสระแสดงผลกระทบจากการผสมเป็นการรวมฤทธิ์ ด้วยค่า CI เท่ากับ 1.00 ± 0.02 แต่สารผสมสารสกัดมะขามป้อมและละมุดแสดงผลกระทบจากการผสมเป็นการเสริมฤทธิ์ต้านคอนลาจิเนส MMP-1 ด้วยค่า CI เท่ากับ 0.79 ± 0.02 และ MMP-2 ด้วยค่า CI เท่ากับ 0.58 ± 0.02 และฤทธิ์ต้านเอนไซม์อีลาสเทส ด้วยค่า CI เท่ากับ 0.76 ± 0.04 เป็นที่น่าแปลกใจเกี่ยวกับฤทธิ์ต้านเอนไซม์อีลาสเทสของสารผสมที่ความเข้มข้นนอกเหนือจากที่ค่ากลางผลตอบสนอง ผลกระทบจากการผสมสามารถปรากฏเป็นการต้านฤทธิ์ที่อัตราส่วนของสารผสมหนึ่งๆ สำหรับฤทธิ์ต้านเอนไซม์อีลาสเทส ผลกระทบจากการผสมอาจเป็นได้ทั้งการเสริมฤทธิ์และการต้านฤทธิ์ขึ้นอยู่กับอัตราส่วนของสารผสม โดยสรุป คือ การผสมสารสกัดหรือสารสำคัญสามารถมีอิทธิพลต่อฤทธิ์ทางชีวภาพของสารสกัด บ้างแสดงผลเป็นการรวมฤทธิ์ โดยไม่มีประโยชน์เหนือกว่าการใช้สารสกัดเดี่ยว ในขณะที่บ้างแสดงผลเป็นการเสริมฤทธิ์ซึ่งได้รับประโยชน์จากการผสม อัตราของสารผสมแสดงความสำคัญต่อฤทธิ์ต้านเอมไซม์อีลาสเทส แต่กลับไม่ปรากฏต่อฤทธิ์อื่นๆ ดังนั้น การศึกษาจำเพาะบางประเภทอาจมีความจำเป็นในระยะต้นๆของการพัฒนาผลิตภัณฑ์

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต	----------
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5576357533 : MAJOR PHARMACEUTICAL TECHNOLOGY
KEYWORDS: AMLA, SAPOTA, SILYMARIN, COMBINATION, ANTI-OXIDANT, ANTI-COLLAGENASE, ANTI-ELASTASE, 94 ANTI-AGING

SIRINYA PIENTAWEERATCH: EFFECT OF COMBINATIONS OF *EMBLICA OFFICINALIS* EXTRACT, *ACHRAS SAPOTA* EXTRACT AND SILYMARIN ON *IN VITRO* ANTI-AGING ACTIVITIES. ADVISOR: ASST. PROF. VIPAPORN PANAPISAL, Ph.D., CO-ADVISOR: ANYARPORN TANSIRIKONGKOL, Ph.D., 85 pp.

Since skin aging is an unavoidable condition involving several pathways, a combination of extracts in skincare product to alleviate the skin aging and improve skin condition becomes common. However, there are a few reports that showed the influence of combined extracts on their biological activities. This study aims to investigate an effect of combined extracts on their antioxidant, anti-collagenase and anti-elastase activity when compares with each individual extracts. Ethanolic amla (Emblica officinalis) extract, ethanolic sapota (Achras sapota) extract and silymarin were individually screened for antioxidant, anti-collagenase and anti-elastase properties as well as were quantified for their total phenolic content and total flavonoid content. The antioxidant capacity was evaluated by colorimetric methods. The effects of MMP-1, MMP-2 and elastase inhibitions were determined by using the gelatinase/collagenase and elastase assay kits (EnzChek®). The quantities of phenolic and flavonoid compounds were determined by Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively. Two extracts were chosen from their superior activities on for anti-oxidant, anti-collagenase and anti-elastase and were used in the combination study. The effect of combination was reported as combination index (CI) and categorized into three types: synergism (CI < 1), additive (CI = 1) or antagonism effects (CI > 1), respectively. The reference combination index was calculated at the median effect. Regarding to antioxidant activity, ethanolic amla extract showed the most potent activity with IC_{50} values of 1.61 ± 0.04 µg/mL. While ethanolic sapota showed the highest inhibitions on collagenase and elastase with IC_{50} of 86.47 ± 3.04 µg/mL and 35.73 ± 0.61 µg/mL, respectively. Therefore, amla and sapota were combined at different combination ratios. The combination index at median effect was revealed additive effect (CI = 1.00 ± 0.02) on anti-oxidant activity. But the amla and sapota combinations exhibited synergist effects on inhibitions of MMP-1 (CI = 0.79 ± 0.02) and MMP-2 (CI = 0.58 ± 0.02) and elastase (CI = 0.76 ± 0.04). Surprisingly, the elastase inhibitions of the combination at concentration other than the median point, antagonist effect could be observed at particular combination ratios. For the elastase inhibition, the effects could be synergist or antagonist depending on the combination ratios. In conclusion, combining extracts or actives could have an influence on the biological activities. Some activities showed additive effect presenting no advantage over usage of single extract while some showed synergist effect demonstrating some advantages. The importance of combination ratios was seen for anti-elastase activity but not for others; therefore, some special tests may be required at earlier stage of product development.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	----------
Field of Study:	Pharmaceutical Technology	Advisor's Signature	----------
Academic Year:	2014	Co-Advisor's Signature	----------

ACKNOWLEDGEMENTS

Deep appreciation and grateful thank-you are extended to the significant efforts of many people who direct and indirect contributed knowledge, time and financial support in this thesis.

This thesis would not have been possible unless the persistent help from my thesis advisor, Assistance Professor Vipaporn Panapisal, Ph.D. and co-advisor, Anyarporn Tansirikongkol, Ph.D. I would like to express my profound gratitude for their valuable guidance, financial support and incredible patience during the entire research.

I would like to special thank-you to the thesis committee and external member, Vipaporn Sareedenchai, Ph.D. for time and invaluable comments. This thesis would not be completed without your constructive suggestions.

I owe my deepest gratitude to all staff members of the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Science, Chulalongkorn University for facilitating instrument and the office area for the research and study.

I am indebted to my many colleagues who supported me with advice and encouragement. Thank you Ms. Thitita for encouraging and being my buddy. Thanks Ms. Jam for the warm help and encouraging. Mr. Sakan and Nut for the helpful technique and supplier contact. I would like to apologize if I do not write any colleague name in here but please know that I would never forget you.

Above all, I extend me everlasting gratitude to my family and relatives for love, understanding, support and encouragement that greatly inspire me to complete this thesis.

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LIST OF ABBREVIATIONS

^{o}C	degree celsius
AOs	antioxidants
DI water	distilled water
DPPH	2,2-Diphenyl-1-picrylhydrazyl
mM	millimolar
M	molar
mL	milliliter (s)
PG	propylene glycol
g	gram (s)
µL	microliter (s)
µg	microgram (s)
L	liter
GAE	gallic acid equivalent
QE	quercetin equivalent
SD	standard deviation
w/v	weight by volume
%	percentage
UV	ultraviolet

CHAPTER I

INTRODUCTION

Cutaneous aging, a complex biological phenomenon, causes skin dryness, hyperpigmentation and unwanted wrinkles and sagging on the skin. Alleviation of cutaneous aging tends to gain more interesting in the future, since the skin displays the most visible and accessible manifestations of aging. Intrinsic and extrinsic factors play a pivot role on skin aging. Intrinsic factor contributes slow chronological changes due to the time passage. On the other hand, extrinsic factors from environmental exposure considerably accelerate the visible aging of the skin. Majority of the skin aging is caused by extrinsic factors, particularly sun exposure (Assaf et al., 2010). Ultraviolet irradiation induces extensive generation of reactive oxygen species (ROS) (Demeule et al., 2000). ROS activates the mitogen-activated protein kinase signal-transduction pathway, which further induces the expression of activator protein-1 (AP-1). AP-1 induces an upregulation of matrix metalloproteinases (MMPs) such as collagenase-1 (MMP-1) and gelatinase A (MMP-2), which specifically degrade connective tissue proteins especially collagen and elastin. MMPs indirectly inhibit skin collagen synthesis, resulting in an obvious wrinkles and skin damage (Denize and Touitou, 2010). Many researchers have reported that UV irradiation alters chemicals and enzymes in the dermis. UV irradiation induced the expression of matrix metalloproteinase-1 (MMP-1), which is known to degrade collagen and other extracellular matrix (ECM) proteins in the dermis (Fisher et al., 2002). In addition, an increase in activity of elastase, elastin degrading enzyme, in mouse skin after chronic UV exposure causes the skin to be less elastic (Robert et al., 2000). Inhibition of collagenase and elastase activities together with an antioxidant property could be employed as a potential strategy against skin aging.

Matrix metalloproteinases (MMPs) are a group of calcium (Ca)-dependent zinc (Zn) containing endopeptidases, which are responsible for remodeling and degradation of ECM proteins, such as collagen and elastin. MMP-1 or collagenase-1 is in the group of collagenase that mainly cleaves collagen type I, the most abundant

collagen type in the dermis (Fujii et al., 2008). In addition, collagen represents 70 % to 80 % of the dry weight dermis, which plays a major role in skin structure. Due to the time passage and sun damage, collagen synthesis becomes slower whereas MMP-1 level becomes higher. These alterations result in the skin wrinkling and loss of elasticity. In addition to MMP-1, collagen type I is also degraded by MMP-2 or gelatinase A, which is a gelatinolytic enzyme in a member of gelatinase group. Collagen type I, IV, VII and elastin were well documented to be digested by MMP-2 (Aimes and Quigley, 1995, Lee et al., 2011). Due to its broad actions, an inhibition of MMP-2 can be, therefore, used in the screening of anti-collagenase compounds.

Another important proteolytic enzyme in a group of serene protease is elastase, which has a capability to cleave elastin, one of the vital components in the ECM. Elastin is widely distributed throughout the arteries, lung, ligaments and skin. Although elastin possesses only about 1-2 % of the dry skin weight, it is essential for maintaining the skin's elasticity and resiliency (Satardekar and Deodhar, 2010). Elastase has an ability to cleave elastin as well as collagen, fibronectin, and other ECM proteins (Thring et al., 2009, Kafienah et al., 1998). Therefore, an inhibition of elastase can be proposed as a potential way to protect against skin aging (Sokmen et al., 2012).

Cosmetic industries have vastly grown recent years. People crave for being young as long as possible. Natural source ingredients become more desirable than synthetic compounds. Natural ingredients are presumed to be safer, since they occur naturally in plants, foods, or herbs. Therefore, plant extracts have been widely investigated in the anti-aging activity. Many extracts were found to have free radical scavenging, anti-collagenase, and/or anti-elastase activities, which were key concepts to reverse or delay sign of aging. Thring et al. (2009) examined 21 plants for these three activities. White tea not only presented a high antioxidant activity, but it also highly inhibited collagenase and elastase. It contained a lot of chemical constituents such as epigallocatechin-3-gallate (EGCG), quercetin, kaempferol, gallic acid, and others. EGCG, a tea catechin, greatly inhibited elastase and moderately inhibited MMP-1 collagenase activities (Thring et al., 2009). Moreover, EGCG showed an inhibitory effect against MMP-2 gelatinase activity (Cheng et al., 2003). Quercetin and kaempferol are flavonoids, which are widely known to have antioxidant and anti-

MMP-1 properties (Sim et al., 2007). Gallic acid, a type of phenolic acid, is known to have free radical scavenging and antioxidant properties (Kaisoon et al., 2012). However, not all plant extracts contain variety of phytochemical constituents to offer various activities. Therefore, a combination of plant extracts containing these related structure components might be able to obtain several anti-aging benefits.

Emblica officinalis or *Phyllanthus emblica*, which are commonly known as Amla, was reported to have a good antioxidant activity due to rich in ascorbic acid, polyphenols and phenolic acids, such as gallic acid and ellagic acid (Kim et al., 2005, Yokozawa et al., 2007b). Fuji et al. (2008) reported that the ethanolic fruit extract significantly reduced MMP-1 collagenase production, and promoted procollagen production in human skin fibroblast with the concentration range of 10-40 μg/mL. With current literature reviews, elastase inhibition activity from the extract has not yet been reported. However, amla extracts contain a large amount of functional tannins, such as emblicanin, pedunclagin, and puniglucoin (Chaudhuri, 2002); therefore, it may exert its activities through a mechanism similar to that of EGCG (Fujii et al., 2008). Moreover, Jimenez et al. (2006) reported that phenolic acids containing in amla (i.e. ellagic acid and tannic acid) helped in reducing rate of elastin degradation in dermal fibroblast culture, and also inhibited several exogenous elastolytic enzymes including porcine pancreatic elastase. Therefore, elastase inhibiton activity of the amla extract is a promising target, and shall be further investigated.

Achras sapota or Manilkara zapota has been used as a traditional Indian medicine for decades. The sapota or sapodilla fruit is known to be a good source of antioxidants. Ma et al. (2003) found bioactive flavonoids from methanol extract of sapota fruit such as (-)-epicatechin, (+)-gallocatechin, gallic acid, quercitin, myricitrin, (+)-catechin, of which the latter three compounds were reported to inhibit enzyme collagenase and elastase activities (Sim et al., 2007, Thring et al., 2009).

Silymarin is a standardized extract of flavonolignans from the milk thistle seeds, *Silybum marianum* (L.) Gaertner. It has been used for centuries to treat liver disorder, such as cirrhosis, chronic hepatitis, liver diseases associated with alcohol consumption, and environmental toxin exposure (Kren and Walterova, 2005). The main active constituent in silymarin is silybin. Silymarin demonstrated antioxidant and anti-inflammatory properties in mouse skin which prevents skin disorders such as

photoaging or UV-related skin cancer (Katiyar et al., 2008).

In the present study, three plant extracts including Amla (*Emblica officinalis*), Sapota (*Achras sapota*), and silymarin were selected on the basis of their different phytochemical compositions. These plant extracts were proved to have antioxidant activity, but there was no data available on anti-collagenase, except amla, and anti-elastase activities. Thring (2009) and others showed unestablished correlation among these activities. Several plant extracts showing antioxidant property may not exhibit anti-collagenase or anti-elastase activities. In addition, certain plant extracts inhibited collagenase, but failed to inhibit elastase (Thring et al., 2009). Therefore, combination of extracts may help to fulfill all these properties. And in the contrary, the biological activities including antioxidant, anti-collagenase, and anti-elastase can be altered when several extracts are combined. The objective of the present study is to investigate an effect of the combined extracts for *in vitro* anti-aging activities. Both individual and combined extracts were investigated. This research would provide beneficial information, when using extracts as a combination for typical cosmetic uses.

CHAPTER II

LITERATURE REVIEW

1. Combination concept

In pharmacological study, two or several drugs combination gives the advantages of an increase of the therapeutic efficacy by affecting the same or multiple targets. In addition, the combination of drugs also provides a decrease in doses while maintaining an efficacy with less toxicity and a prevention of drug resistance development (Chou, 2006). Nowadays, the combination concepts are applied successfully in the treatment of infectious disease, depression (Wagner and Ulrich-Merzenich, 2009), and rheumatic disease (Proudman et al., 2000). When some phytochemicals such as phenolic or flavonoid combined with a chemotherapeutic agent, the combination could enhance cytotoxic effects in cancer cells (Eid et al., 2012b). The combination of flavonoid, β-carotene, with doxorubicin enhanced the cytotoxicity and reduced the effective dose of doxorubicin in Caco-2 (adenocarcinoma type) and CEM/ADR5000 (leukemia type) cancer cells. The benefits of combining drugs or active substance used to treat many diseases were widely studied and reported. Where the combinations of cosmetic actives were normally used but few studies were reported about the effect of mixing. Some synergism effect of medicinal plant mixture has been reported. The herbal mixture of alcoholic extract of aloe vera leaves, *Bacopa monniera*, *Moringa oleifera,* and rhizome of *Zingiber officinale* were tested for antioxidant (Padmanabhan and Jangle, 2012). The mixture showed synergistic effects in both DPPH scavenging activity and ferric reducing power when compared to the individual extracts at the same concentration. A synergistic effect amongst phytochemicals was responsible for overall beneficial effects derivable from constituent plants. Another report showed that combined plants of *Camellia sinensis* with other herbs also presented synergistic activity (Jain et al., 2011). The mixture of *Camellia sinensis, Vitis vinifera, Phyllanthus emblica, Punica granatum, Cinnamomum cassia,* and *ginkgo biloba* at 5:3:3:3:3:3 was found to have high contents of phenolic (250 mg GAE/g), and

flavonoid (1237 mg of catechin equivalents/g) compounds with antioxidant activity IC_{50} comparable to standard ascorbic acid.

An influence of mixing extracts still focused on antioxidant effect; therefore, for some specific activities such as anti-proteinases, it is very interesting to compare the activities between individuals and the combination. As aging causes by a lot of complex pathways, the combination of substances might affect multiple targets, and provide the overall anti-aging properties. Information gained from such study could provide some important consideration before combining the extracts to achieve a required action.

2. Causes of skin aging

Skin aging is a complex phenomenon, resulting in skin dryness, hyperpigmentation, and unwanted wrinkles and sagging of the skin. Skin aging is normally categorized into intrinsic and extrinsic agings. Intrinsic aging or true aging is age-related alterations attributable to intrinsic factors, such as the passage of time, and is mainly genetically determined. It is a chronological process accompanied by a slow progressive loss of physiological function in multiple organs. This results in thinning skin with exaggerated expression lines. Extrinsic aging is an accelerated aging that results from environmental factors such as sun exposure, smoking, excessive alcohol consumption, and poor nutrition (Baumann, 2009). This process is super-imposed upon the intrinsic factor, which creates the premature aging of skin. The majority of premature cutaneous aging comes from sun exposure, which is faster process than intrinsic aging. Ultraviolet from sunlight is categorized into three groups based on wavelengths: UVA, UVB and UVC. Shorter UVB (290-320 nm) wavelengths are able to penetrate into the epidermis while longer UVA (320-400 nm) wavelengths can get through deeper into dermis, and primarily causes photoaging. The longer UVA1 (340-400 nm) causes the most photoaging than UVA2 (320-340 nm) (McCullough and Kelly, 2006). UV irradiation from sunlight induces extensive mechanisms resulting in the decrease of ECM proteins (Figure 1) (Ainbinder and Touitou, 2010). UV induces extensive generation of ROS, which activates the mitogen-activated protein kinase (MAPK) signal-transduction pathway, which further induces the expression of activator protein-1 (AP-1) - driven genes, including

collagenases in the exposed skin. In addition, UV also induced the increased of NF-Kb transcription, which positively induces pro-inflammatory cytokine including IL-1b, TNF-α, IL-6 and IL-8 (Ainbinder and Touitou, 2010). These products act to trigger the AP-1 protein. Many researchers have reported that UV irradiation alters chemicals and enzymes in the dermis. Quan et al. (2009) reported that UV irradiation induced expression of MMP-1, which degraded collagen and other extracellular matrix (ECM) proteins. Fisher et al. (2002) also published that UV irradiation activated protein kinase-mediated signaling pathways, which finally stimulated MMP-1 production. Furthermore, Robert et al. (2000) also reported the up-regulation of elastase activity after chronic UVB irradiation.

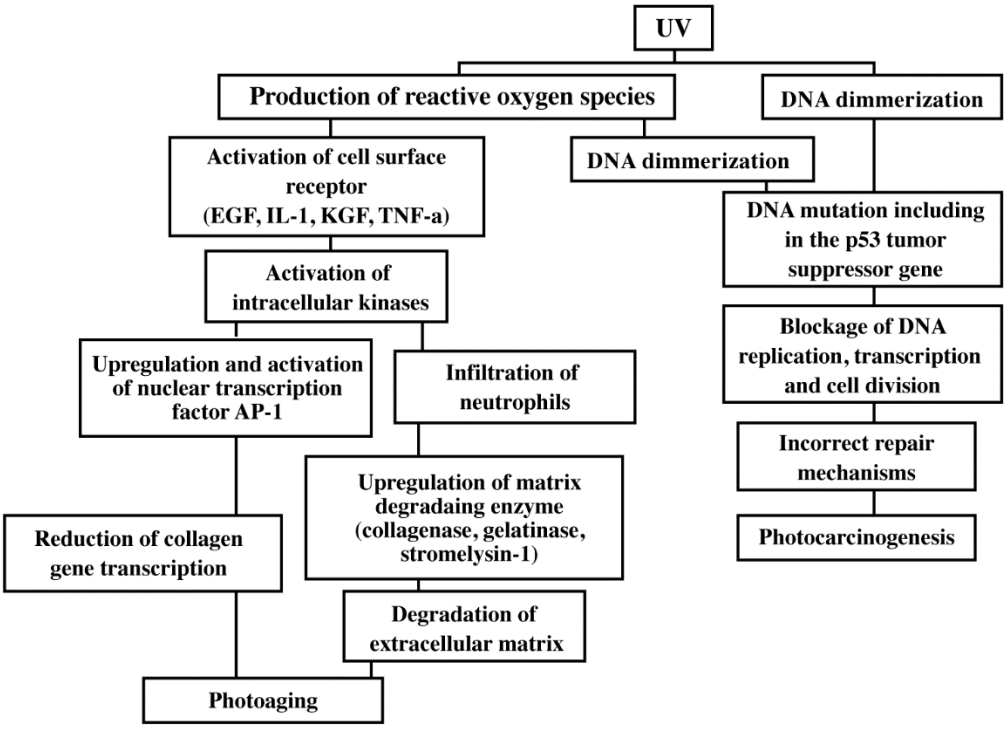


Figure 1 Mechanisms of skin photodamage

Collagen and elastin level alterations in ECM are primarily responsible for the clinical manifestations of skin aging, such as wrinkles, sagging, and laxity. According to skin aging, the atrophy of collagen and elastin fibers is influenced from the increased expression of their degradative enzymes, collagenase (MMP-1), gelatinase (MMP-2 and -9), and elastase.

MMPs family: Regulations and functions

Matrix metalloproteinases (MMPs) are a group of calcium (Ca)-dependent zinc (Zn) containing endopeptidases. They are responsible for remodeling and degradation of extracellular matrix (ECM) protein. At least 28 members have been currently identified, which are categorized into five broad groups based on substrate specificity and their structure: collagenases, gelatinases , stromelysins, matrilysins and MT-MMPs (membrane-type MMPs) (Mukherjee et al., 2013). MMPs and their substrates that involve with skin are shown in Table 1. Three predominant groups of MMPs are collagenases, gelatinases, and stromelysins (Philips et al., 2011).

Table 1 MMPs and their substrates present in the skin's extracellular matrix (Skiles et al., 2001, Mukherjee et al., 2013)

Members	Other names	Substrate	Non collageneous substrate
MMP-1	Collagenases-1; interstitial collagenase	Collagen I ,II, III, VII, VIII, X, gelatin	Proteoglycans, fibronectin, entactin, laminin, vitronectin
MMP-2	Gelatinase A, 72 kDa gelatinase, type IV collagenase, MMP-5	Gelatin, collagen I, II, III, IV, V, VII, X, XI, XIV	Elastin, laminin, fibronectin, proteoglycans
MMP-3	Stromelysin-1, pro-collagenase activator, MMP-6	Collagen II, III, IV, IX, X, XI, gelatin,	Elastin,laminin, fibronectin, proteoglycans
MMP-9	Gelatinase B; 92 kDa gelatinase, type V collagenase	Gelatin, collagen IV, V, VII, X, XIV	Elastin,laminin, fibronectin, proteoglycans

The collagenases such as MMP-1 cleave interstitial (structural) collagens. MMP-1 plays a major role in the collagen degradation (Skiles et al., 2001). Gelatinases which are primarily MMP-2 and MMP-9 cleave basement membrane collagens, collagen type I, gelatin and elastin degrade denatured structural collagens. The stromelysins such as MMP-3 cleave basement membrane collagen as well as proteoglycans and matrix glycoprotein.

MMPs are excreted by various kinds of connective tissues and pro-inflammatory cells, including fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes (Mukherjee et al., 2013). These enzymes are normally expressed as zymogens or inactive forms, which are subsequently processed by other proteolytic enzymes to generate the active forms (Jang S et al., 2012). Under normal physiological conditions, its proteolytic activity can be regulated in equilibrium by various tissue inhibitors of MMPs. However, this balance might shift to increase MMPs activity in some circumstances leading to tissue or protein degradation (Verma RP et al., 2007).

Elastase

Elastase is a proteolytic enzyme in a group of serene protease. It has a capability to cleave elastin, one of the vital components in the ECM. Elastin is widely distributed throughout the arteries, lungs, ligaments, and skin. Although elastin possesses only about 1-2 % of the dry skin weight, it is essential for maintaining the skin's elasticity and resiliency (Satardekar and Deodhar, 2010). Elastase has an ability to degrade elastin as well as collagen, fibronectin and other ECM proteins (Thring et al., 2009, Kafienah et al., 1998).

3. Prevention of aged skin

Photoaging is believed to be responsible for almost 80 % of the skin changes in the aging process (Park and Kim, 2010). Skin changes associated with extrinsic aging are widely preventable. Photoprotection is an effective approach to decrease the risks of age-related skin changes and this type of skin protection could be done at any

age including sun avoidance, wearing protective clothing, and using sunscreens. The use of sunscreens is the “gold standard” for protecting skin from UV rays. A broad-spectrum sunscreen does not only prevent sunburn, but also wrinkles and skin pigment formation. However, due to inadequate uses, incomplete spectral protection and toxicity from UV, the use of sunscreen to prevent photoaged skin might be insufficient. The addition of topical antioxidant or other anti-aging skincare shall be additionally applied for prevention of photoaging.

3.1. Antioxidant and phenolic compounds

Antioxidants (AOs) are a group of compounds or systems, which inhibits formation of reactive oxygen species or interrupts propagation of the free radical. Vitamin C, E, and natural AOs have been incorporated into many skin care products. Natural AOs have been vastly investigated against ROS. Plants normally synthesize flavonoids and phenolic compounds, which are powerful AOs to protect themselves from the sun (Pinnell, 2003). The term of phenolic compounds is included approximately 8,000 natural compounds. All of them possess one common structural feature, which is a phenol (an aromatic ring with at least one hydroxyl substituent). The major groups of human-related phenolics are phenolic acids, flavonoids, and high molecular weight polyphenols (Svobodová et al., 2003) as shown in Figure 2.

Phenolic acids that occured naturally compose of two distinguish carbon frameworks: the hydroxycinnamic and hydroxybenzoic acid structures that are caffeic acid and gallic acid, respectively. The flavonoids contain a large group of low molecular weight polyphenolic substances and a derivative of benzo-γ-pyrone structure. The high-molecular weight polyphenols are commonly known as tannin. They are divided into two groups: condensed tannin which are polymers of catechines or epicatechines and hydrolysable tannin which are polymer of gallic acid or ellagic acids.

3.2. Anti-MMPs and anti-elastase compounds

There is an emerging trend in design and synthesis of matrix metalloproteinase inhibitors and elastase inhibitor. Since MMPs and elastase are involved with collagen

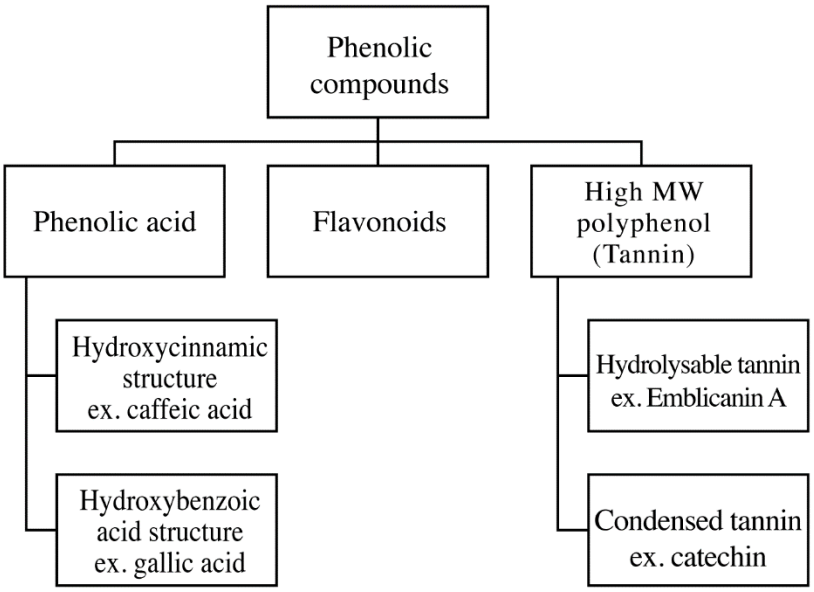


Figure 2 Scheme of phenolic compounds classification

and elastin degradations, a potential inhibitor could be further developed as anti-wrinkle skin care ingredients. Plants contain a huge variety of compounds including polyphenols, such as flavonoids, terpinoids, glycosides, vitamin E, vitamin C, phenolic acids, and tannins, which are found to provide collagenase and/or elastase inhibitory activities (Kanashiro et al., 2007, Mukherjee et al., 2013)

Twenty one plants were screened for antioxidant, anti-collagenase and anti-elastase activities (Thring et al., 2009). Some of the plant extracts had high antioxidant effect but low anti-collagenase and anti-elastase activities. Some of those showed good inhibitory activity to elastase, but poor to collagenase. However, white tea not only presented high antioxidant activity but also highly inhibited collagenase and elastase. It contained a lot of chemical constituents, such as epigallocatechin-3-gallate (EGCG), quercetin, kaempferol, gallic acid etc. EGCG, a tea catechin, greatly inhibited elastase and moderately inhibited MMP-1 collagenase activities (Thring et al., 2009). Moreover, EGCG showed an inhibitory effect against MMP-2 gelatinase activity (Cheng et al., 2003). Quercetin and kaempferol are flavonoids, which have been reported to have antioxidant and anti-MMP-1 properties (Sim et al., 2007). Gallic acid, a type of phenolic acid, is known to have free radical scavenging and antioxidant properties (Kaisoon et al., 2012). In summary, plants containing these potential substances draw an attention to be investigated as an anti-aging ingredient.

3.3. Anti-inflammatory compounds

Aging and inflammatory processes share some common signaling pathway (Maity et al., 2011). UV irradiation induced pro-inflammatory cytokine including NF-kb, IL-6 and TNF-α which further induced AP-1 production resulting in the increase of collagenase enzyme (Ainbinder and Touitou, 2010). Substances with anti-inflammatory effect might be a good candidate for anti-wrinkle agent. Silymarin was reported as NF-kb inhibitor in UV irradiation study using keratinocyte cell line (Saliou et al., 1999) and also reported as antioxidant in mouse skin (Katiyar et al., 2008). Therefore, silymarin is considered a potential anti-aging substance.

A combination of plant extracts containing previously related structure components might be able to offer several anti-aging benefits. *Emblica officinalis, Achras sapota* and silymarin were chosen based on the difference phytochemical compounds described in a next section and on their historical benefits to the skin.

4. Reviews of studied plant extracts

4.1. *Emblica officinalis* Gaertn.

Synonym: *Phyllanthus emblica* Linn.

Common name: Amla, indian gooseberry, emblic, malacca tree

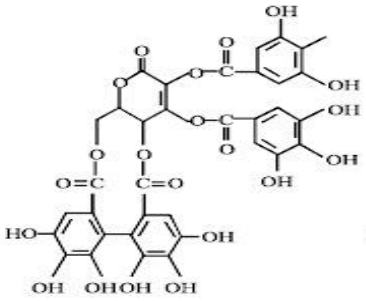
Family: Euphorbiaceae

Amla is indigenous to tropical Southeastern Asia, particularly in India, Sri Lanka, Malaysia, China, Pakistan, (Amir et al., 2011) as well as Thailand. Amla has been used in Ayurvedic medicine for over a thousand years. All parts of the plants were beneficial in treating various aliments; however, the fruits are the most common used.

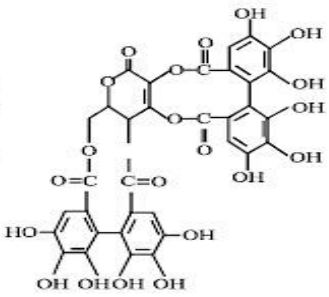
A. Phytochemistry

Amla contains extensive components, such as tannins, alkaloids, phenolic, and flavonoid compounds (Javale, 2010). The fruit contains ascorbic acid, gallic acid, ellagic acid, chebulinic acid, chebulagic acid, emblicanin A, emblicanin B, punigluconin, pedunculagin, citric acid, ellagotannin, trigallayl glucose, pectin, 1-*O*-galloyl-β-D-glucose, 3,6-di-*O*-galloyl-D-glucose, chebulagic acid, corilagin, 1,6-di-*O*-galloyl-β-D- glucose, 3 ethylgallic acid (3 ethoxy 4,5 dihydroxy benzoic acid), and

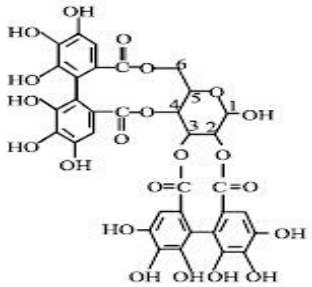
isostrictinin (Joseph et al., 2013, Zhang, 2003). Some of its phytochemical structures are illustrated in Figure 3.



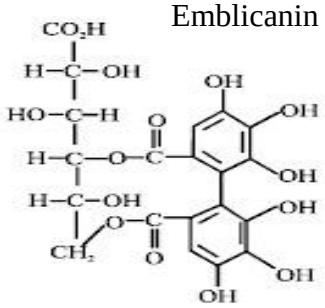
Emblicanin A



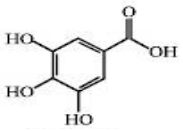
Emblicanin B



Pedunculagin



Punigluconin



Gallic acid

Figure 3 Phytochemical structures of amla

B. Ethnopharmacology

A number of researchers reported that amla extract showed a variety of pharmacological effects. The fruit extract had hypolipidemic effects (Yokozawa et al., 2007a), anti-inflammatory activities (Yokozawa et al., 2007b), chemoprevention, antitussive, antipyretic, analgesic, anti-bacterial and anti-viral (Joseph et al., 2013). Moreover, amla extract exhibited many useful skin care properties; antioxidant, anti-wrinkle, lightening, and anti-bacterial effects, which briefly summarized as following.

I. Antioxidant effect

Amla possessed a potent antioxidant property (Chaudhuri, 2002, Majeed et al., 2009). The methanolic extract of Jawarish Amla has an application towards reducing lipid peroxidation and oxidative stress. It was proved to scavenge free radicals against DPPH, nitric oxide and hydroxyl radical (Amir et al., 2011). Besides, the ethanolic extract of dried P. emblica fruit extracted by maceration showed high DPPH scavenging effect with IC_{50} of 1.22 μg/mL, and total phenolic content of 490 mg GAE/g extract (Chanpirom, 2012).

II. Lightening effect

Tyrosinase inhibition is one of the approaches to help whiten the skin. A study from Homklob (2010) showed amla extract from different sources in Thailand expressed anti-tyrosinase activity, which was detected by Dopachrome method, a spectrophotometric assay for determining tyrosinase inhibition. The ethyl acetate extract from Buriram province had superior inhibited tyrosinase activity (IC_{50} = 0.15 mg/mL) rather than ascorbic acid (IC_{50} = 0.22 mg/mL). In addition, both ellagic acid and gallic acid, which are compositions in amla fruit showed their effect in inhibiting melanogenesis (Kim, 2007, Shimogaki et al., 2000). The study in cell culture with B16 melanoma cells, when incubating mushroom-derived tyrosinase with standard ellagic acid and gallic acid, showed decreases in tyrosinase activity (Kim, 2007). Moreover, Shimogaki, et al (2000) exerted that topical applications of ellagic acid for 6 weeks in brownish guinea-pigs were effective in suppressing skin pigmentation after 2 weeks of UV-irradiation. The possibly mechanism of suppressing melanogenesis might result from the ellagic acid reacted with activated melanocytes (Shimogaki et al., 2000).

III. Anti-wrinkle effect

A cell culture study on human skin fibroblasts treated with amla decreased the production of MMP-1 (Fujii et al., 2008). Another study from Chanvorachote (2009) reported that amla also decreased the levels of collagenase activity in dose-dependent manner using Enzchek® gelatinase/collagenase kit. Recently, amla extract was demonstrated to protect the human dermal fibroblasts against the ultraviolet-B (UVB) irradiation-induced reactive oxygen species and collagen damage (Majeed et al., 2009). The authors observed that at a concentration of 0.5 mg/mL, amla extract was nearly 2.56-folds better than ascorbic acid in protecting against the UVB-induced collagen damage.

IV. Anti-bacterial effect

Drug resistance of pathogenic bacteria provoked many studies on discovery of novel anti-bacterial agent discovery. Researchers showed that chloroform extracts of amla possessed moderately antibacterial effect against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Bacellus megaterium, and Shigella boydii* (Rahman, 2009). The methanol extracts also exhibited the ability to kill *S. aureus,* which was believed to be the effect from the synergies of saponin and flavonoid in amla (Javale, 2010).

4.2. *Achras sapota*

Synonym: *Archaras sapota, Manilkara zapota, Manilkara achras*

Common name: Sapodilla, Ciku, La-mud (in Thai)

Family: Sapotaceae

Achras sapota, commonly known as Lamud in Thai, is one of the major fruits in India, Mexico, and also widely available in Thailand. It has been used as a traditional Indian medicine. Sapota fruit is known to be a good source of antioxidant.

A. Phytochemistry

Sapota contains large numbers of nutraceutical phytochemicals. Kulkarni et al. (2007) showed that its fruit juice was a good source of phytochemicals like ascorbic acid, carotenoids and phenolic compound. Bioactive phenolic and flavonoid compounds from methanol extract of sapota fruit were reported to be (-)-epicatechin, (+)-gallocatechin, gallic acid, quercitin, myricitrin, (+)-catechin (Figure 4) (Ma et al., 2003). Moreover, moderate amounts of sugar, protein and three transition metals, which were iron, copper and zinc were also found in sapota extract (Ma et al., 2003).

B. Ethnopharmacology

Phenolics provided nutraceutical value to sapota in term of free radical-scavenging potential (Kulkarni et al., 2007, Shui, 2004). Pulp and peel ethanolic extracts of sapota fruit showed DPPH, nitric oxide scavenging potential, and inhibited lipid peroxidation activity (Gomathy et al., 2013). In addition, total antioxidant capacity (TAC) and total phenolic content (TPC) of sapota fruit were changed with storage time (Shui, 2004). The TAC and TPC significantly decreased when the fruit

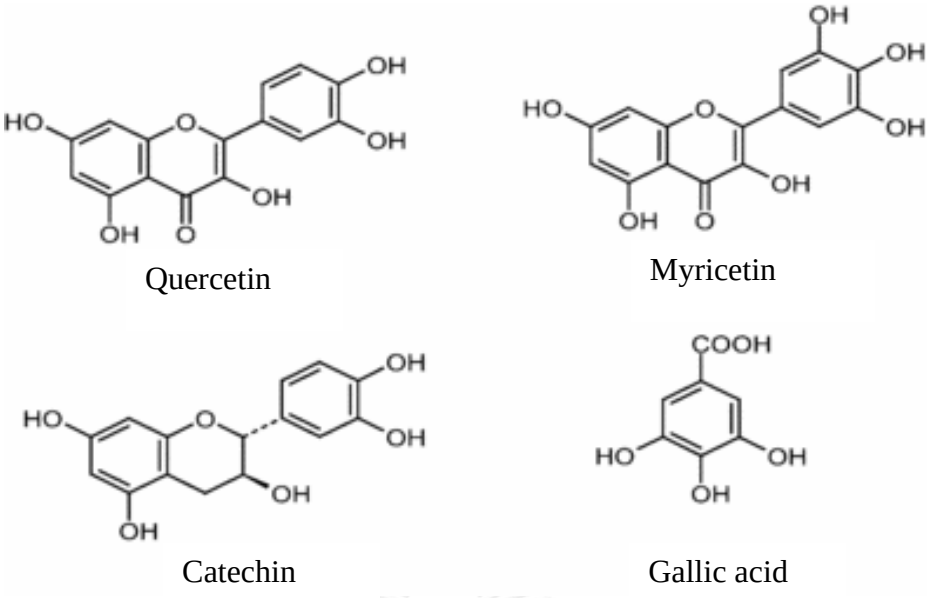


Figure 4 Phytochemical structures of sapota

gradually changed from the unripe to the overripe stage. The fresh fruit sample was suggested for the high amount of antioxidants. However, there is limited information about its phytochemicals; therefore, other skin benefits of sapota are unknown. Anti-collagenase and anti-elastase activities of sapota fruit remain unknown.

4.3. Silymarin

Synonym: *Silybum marianum* (L.) Gaertner , *Cardus marianus*

Common name: Milk thristle, Marian thistle

Family: Asteraceae

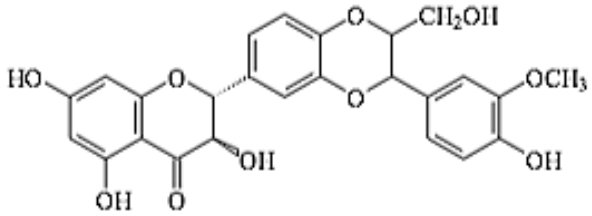
Silymarin is a standardized extract of flavonolignans from the seeds of the milk thistle, *Silybum marianum*. Silymarin has been widely used in traditional medicine for more than a century, especially for the treatment of liver diseases.

A. Phytochemistry

The main constituents of silymarin include silybin, silidianin, silychristin, and isosilybin. The principal biologically active component in silymarin is silybin (Figure 5).

B. Ethnopharmacology

A major component of silymarin, silybin, showed antioxidant (Ahmad et al., 2013) and anti-inflammatory properties, including skin cancer chemoprotective agent



Silybin

Figure 5 Phytochemical structure of silymarin

(Kren and Walterova, 2005). Treatment with silymarin prevented UV-induced infiltration of inflammatory leukocytes (Katiyar et al., 2008), which are responsible for induction of UV-induced oxidative stress in the skin. Application of silymarin to mouse skin also resulted in significant reduction in the numbers of UVB- induced hydrogen peroxide producing cells, and inducible nitric oxide synthase expressing cells (Katiyar et al., 2008). Silymarin showed a dose dependent protective effect against UV-induced damage in human keratinocytes *via* inhibition of NF-κB activation (Altaei, 2012).

5. Reviews of the test methods

5.1. Antioxidant

In vitro antioxidant testing has been reported against several oxidative species such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide etc. DPPH is a popular free radical and commonly used in antioxidant studies. DPPH assay becomes widely used to measure the ability of an interested compound to act as free radical scavenger or hydrogen donor, and to evaluate antioxidant activity of plant extracts (Prakask, 2001). Antioxidant compounds are able to donate hydrogen to DPPH radical resulting in a reduction in the color of DPPH solution from purple to pale yellow (colorless). This procedure involves the measurement of a decrease in an absorbance of DPPH at its maximum UV absorption of 517 nm. The DPPH method applies to the overall antioxidant capacity of the sample, but it is not specific to any particular antioxidant.

The structure of DPPH and its reduction product by an antioxidant are shown as Figure 6.

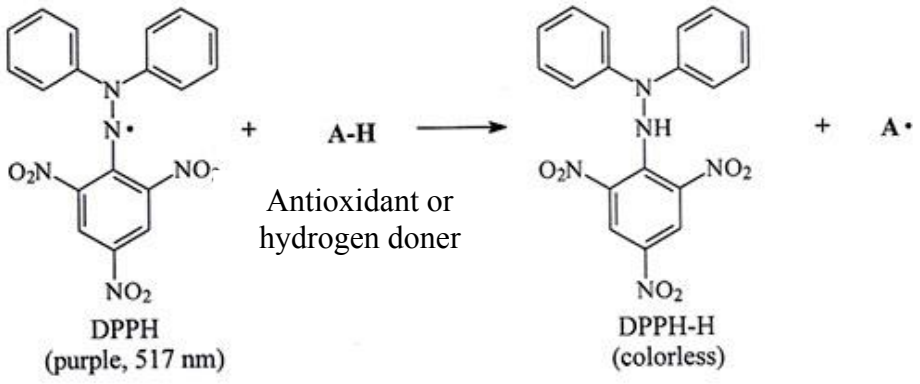


Figure 6 Structures of DPPH and its reaction with an antioxidant

5.2. Anti-collagenase

EnzChek® E-12055 gelatinase/collagenase assay kit have been used in collagenase assay (Chuadhuri, 2005, Sim et al., 2007, Chanvorachote, 2009). Uncomplicated test kit is used to detect the collagenase enzyme activity in the active form (ready form to degrade collagen). The kit contains type IV collagenase from *Clostridium histolyticum*, either DQ™ gelatin or DQ™ collagen type I as substrate, and 1,10 phenanthroline as a general metalloproteinase inhibitor. Two substrates; DQ™ gelatin and DQ™ collagen type I, are used to determine the activity of MMP-2 and MMP-1, respectively (Sim et al., 2007, Chuadhuri, 2005). Both substrates are heavily labeled with quenched fluorescein. When substrates are cleaved by collagenase enzyme, highly fluorescent peptides products are yielded. An increase in fluorescence intensity is proportional to proteolytic activity, which is monitored with a fluorescence microplate reader. Digested products from the DQ™ gelatin and DQ™ collagen type I substrates have absorption maxima at 485 $\pm$ 10 nm and fluorescence emission maxima at 535 $\pm$ 15 nm. In addition, some articles might use other substrates based on the theory that collagenase cleaves the X-glycine bond of collagen fiber or synthetic peptides (Seltzer et al., 1990). The peptides substrates should contain the sequence of Proline-X-Glycine-Proline, where X is an amino acid. Thring et al., (2009) used the synthetic substrate of N-[3-(2-furyl) acryloyl]-Leucine-Glycine-Proline-Alanine (FALGPA) and collagenase from *Clostridium histolyticum.* The kit has been used

because of high sensitivity and high throughput with a convenient method for anti-collagenase assay. A limitation of the kit is that both substrates can be digested by other proteinases (Probes, 2001).

5.3. Anti-elastase

EnzChek® E-12056 elastase kit was used in literatures for the studies of anti-elastase activity (Vallisuta et al., 2014, Li and Horwitz, 2001). The kit has a principle similar to EnzChek® gelatinase/collagenase. The elastase kit contains a substrate named DQ™ elastin, elastase enzyme from pig pancrease, and N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (a general elastase inhibitor). The principle is based on the digestion of non-fluorescent substrate (BODIPY® FL conjugated with DQ™ elastin) to yield high fluorescence fragments by elastase enzyme. The fluorescent intensity is monitored with the excitation wavelength at 485 nm, and the emission wavelength at 538 nm by using a fluorescent microplate reader. A potential elastase inhibitor shows a reduction in fluorescence intensity. The limitation is that of the same as the collagenase kit, which elastin substrate can be digested by other proteinases.

6. Combination assessment

Assessment of combination interaction (e.g. additive, synergistic or antagonistic) is complex and difficult to clearly understand. Many research groups developed a methodology to assess the combination interaction, including median effect plot (Chou and Talalay, 1984), isobologram, and combination index (CI) analyses (Chou, 2010, Eid et al., 2012a). Each method has some limitations and some require specialized computerize software to calculate. However, combination index is the most popular method which has been used in more than three thousands scientific publications until present. CI index is a value to quantify whether the relationship of combination is synergism, additive (summation), or antagonism. CI index can be calculated by following equation:

$$CI = \frac{C_a}{IC_{x,a}} + \frac{C_b}{IC_{x,b}} \qquad \text{Equation 1}$$

where C_a and C_b are the substance A and B concentrations respectively used in the combination, which produces the X % inhibition. $IC_{x,a}$ and $IC_{x,b}$ are particular substance concentrations, which show X % inhibition effect (IC_x) for each individual.

When CI < 1 ; synergism

CI = 1 ; additive

CI > 1 ; antagonism

Therefore, the dose-response curves of individual substances, or extracts together with their combination, shall be investigated and calculated the IC_x of both individual and combination. Calculation of combination index was considered at the median effect because the equation was based on the median effect principle of the mass action law (Chou and Talalay, 1984). The median effect or IC_{50} serves as the universal reference point, which indicates the 50 % effect. The dose of drug or plant extract that provides the activity or inhibition effect at 50 % is ED_{50} or IC_{50}, respectively.

The objective of the present study is to investigate the effects of the combined extracts for *in vitro* anti-aging activities. Antioxidant, anti-collagenase, and anti-elastase of both individual and combined extracts were investigated. The information gained would be taken into consideration before using the extracts as a combination in typical cosmetic uses. Moreover, some new activities of amla, sapota and silymarin that have not been reported would be discovered in this study.

CHAPTER III

MATERIALS AND METHODS

Materials

1. EnzChek® gelatinase/ collagenase assay kit (E-12055)
2. EnzChek® elastase assay kit (E-12056)
3. Dimethyl sulfoxide (DMSO) (Merck, Germany)
4. Propylene glycol (Srichand United dispensary, Thailand)
5. Transcutol® (Gatterfosse, France)
6. Absolute methanol (RCI Labscan, Thailand)
7. Absolute ethanol (RCI Labscan, Thailand)
8. Gallic acid (Sigma Aldrich, USA)
9. Folin-Ciocalteau reagent (Sigma Aldrich, USA)
10. Sodium carbonate (Ajax Finechem Pty Ltd, Australia)
11. 95 % ethanol (The Liquor Distillery Organization,Thailand)
12. Aluminium chloride (Merck, Germany)
13. Potassium acetate (May & Baker Ltd., UK)
14. Quercetin (Sigma Aldrich, USA)
15. 2,2-Diphenyl-1-pikryl-hydrazyl (DPPH) (Sigma Aldrich, USA)
16. L-(+)-ascorbic acid (Carlo Erba, Italy)

Test samples

1. Ethanolic amla extract was prepared by marceration from dried *Emblica officinalis* (amla) fruits supplied by Chao Praya Aphai Phubet Hospital (Prachinburi, Thailand).
2. *Ethanolic Achras Sapota* extract was prepared from fruits obtained from Thai market (Pathum Thani, Thailand).
3. Silymarin was gifted from Berlin Pharmaceutical Industry (Thailand), which was purchased from IVAX Pharmaceutical s.r.o. (Czech Republic).

Instruments

1. Analytical balance (Mettler Toledo, Switzerland)
2. SpectraMax® M5 multi-mode microplate readers (Molecular device, USA)
3. Micropipette (Gilson, France)
4. pH meter (Orion research, Inc., USA)
5. Rotary evaporator (Heidolph Lavorata 4011 digital, Heidolph instruments FMBH & Co, Germany)

Methods

The objective of present research was to study the *in vitro* effect of the combined extracts for their anti-aging properties. First, anti-collagenase and anti-elastase test protocols were partially verified and adapted using their general inhibitors. Second, three plant extracts (e.g. ethanolic amla extract, ethanolic sapota extract and silymarin) were screened for the potential in collagenase and elastase inhibitors. Third, the potential plant extracts were further determined for total phenolic content and total flavonoid content. Finally, each individual extract and combinations were investigated for *in vitro* anti-aging activities including antioxidant, anti-collagenase and anti-elastase.

Part 1 Partial verification of each test protocol for *in vitro* anti-aging activities

Collagenase and elastase assays were used to determine anti-aging properties of the studied extracts. Each test had different protocols and conditions as well as general inhibitors. The optimum incubation time and conditions were determined for the further experiment.

1.1. Collagenase assay

The collagenase assay was conducted using a EnzChek® E-12055 gelatinase/collagenase assay kit as a previously report (Chuadhuri, 2005). The kit contains substrate, type IV collagenase from *Clostridium histolyticum* (ChC), pH 7.4 Tris-HCL reaction buffer and a general metalloproteinase inhibitor, e.g., 1,10 phenanthroline. There are two types of substrate: DQ™ gelatin and DQ™ collagen type I. DQ™ gelatin and DQ™ collagen type I were used to determine the activity of

MMP-2 and MMP-1, respectively (Sim et al., 2007, Chuadhuri, 2005). Both substrates are heavily labeled with quenched fluorescein. Once the substrates are cleaved by enzyme, highly fluorescent peptides are yielded. An increase in fluorescence intensity is proportional to proteolytic activity, which is monitored with a fluorescence microplate reader. Digested products from the DQ^{TM} gelatin and DQ^{TM} collagen substrates have absorption maxima at 485 $\pm$ 10 nm and fluorescence emission maxima at 535 $\pm$ 15 nm. A potential inhibitor shows reduction in fluorescence intensity.

All reagents in the kit were prepared (Appendix A) and sequentially pipetted into each 96-well plate to obtain 200 μL as total final volume following the guideline from E-12055 gelatinase/collagenase product information (Probes, 2001). There were four types (A, B, C, D) of mixtures as following:

A (control) 80 μL of 1X reaction buffer
20 μL of substrate
100 μL of 0.4 U/mL collagenase, type IV solution

B (blank of A) 180 μL of 1X reaction buffer
20 μL of substrate

C (sample) 80 μL of sample solution in 1X reaction buffer
20 μL of substrate
100 μL of 0.4 U/mL collagenase, type IV solution

D (blank of C) 80 μL of sample solution in 1X reaction buffer
20 μL of substrate
100 μL of 1X reaction buffer

Firstly, both of the substrates final concentrations were used at 12.5 and 100 μg/mL to indicate the optimized substrate concentration. The sample solution was 1,10 phenanthroline at the series of final concentrations; 0.05, 0.075, 0.1 and 0.25 mM. 1X reaction buffer was used as negative control. All four types of mixtures were then incubated at 30-minute at room temperature and light protected in accordance

with the product information sheet. The fluorescent intensity was measured using fluorescent microplate reader set at excitation wavelength at 485 nm and emission wavelength at 535 nm.

The collagenase inhibition profile was plotted between % fluorescence intensity against concentrations of 1,10 phenanthroline. The lowest substrate concentration that its inhibition profile conformed to the product information sheet was chosen.

After substrate concentration was optimized, a verification of incubation period was studied by using the same inhibitor concentrations. The incubation period was varied from 30 to 120 minutes at room temperature and light protected.

The ability to inhibit against collagenase enzyme was calculated using the following equation:

$$\% \text{ Collagenase inhibition} = \left[\frac{(A-B) - (C-D)}{(A-B)} \right] \times 100 \qquad \text{Equation 2}$$

A = The fluorescent intensity without test sample (control)

B = The fluorescent intensity without test sample and collagenase enzyme (blank of A)

C = The fluorescent intensity with the test sample

D = The fluorescent intensity after incubation with the test sample without enzyme (blank of C)

The inhibition curve of 1,10 phenanthroline was plotted between % collagenase inhibitions against concentrations of 1,10 phenanthroline at the different incubation time. The shortest incubation time that provided the constant percent inhibition was chosen for further study.

1.2. Elastase assay

The elastase assay was performed by using EnzChek® E-12056 elastase kit with a similar principle to the collagenase assay (Probes, 2007). EnzChek® E-12056 elastase kit contains DQ™ elastin, elastase from pig pancrease (PE), pH 8.0 Tris-HCl

buffer and N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (CMK). Substrate, DQ^{TM} elastin, can be digested by elastase to yield high fluorescence fragments which is monitored with the excitation wavelength at 485 nm and the emission wavelength at 538 nm using a fluorescent microplate reader. A potential elastase inhibitor shows reduction in fluorescence intensity. CMK was used as a general elastase inhibitor and also used to determine the optimum incubation time and condition.

All reagents in the kit were prepared and pipetted into each 96-well plate to obtain 200 µL as total final volume following the guideline from E-12056 elastase product information (Appendix A). There were four types (A, B, C, D) of mixtures as following :

A (control) 50 µL of 1X reaction buffer
50 µL of 100 µg/mL substrate
100 µL of 0.4 U/mL elastase solution

B (blank of A) 150 of 1X reaction buffer
50 µL of 100 µg/mL substrate

C (sample) 50 µL of sample solution in 1X reaction buffer
50 µL of 100 µg/mL substrate
100 µL of 0.4 U/mL elastase solution

D (blank of C) 50 µL of sample solution in 1X reaction buffer
50 µL of 100 µg/mL substrate
100 µL of 1X reaction buffer

The sample was CMK at the series of final concentrations; 0.0001, 0.001, 0.01 and 0.1 mM. 1X reaction buffer was used as negative control. All four types of mixtures were then incubated which the incubation time was varied from 30 to 120 minutes at room temperature and light protected. The fluorescent intensity was measured using fluorescent microplate reader set at excitation wavelength at 485 nm

and emission wavelength at 535 nm. Assay of the mixture at each concentration was performed in triplicate.

The ability to inhibit against elastase enzyme was calculated using the following equation:

$$\% \text{ Elastase inhibition} = \left[\frac{(A-B) - (C-D)}{(A-B)} \right] \times 100 \qquad \text{Equation 3}$$

A = The fluorescent intensity without test sample (control)

B = The fluorescent intensity without test sample and elastase enzyme (blank of A)

C = The fluorescent intensity with test sample

D = The fluorescent intensity with test sample without enzyme (blank of C)

The inhibition curve of CMK was plotted between % elastase inhibitions against CMK concentrations at the different incubation time. The lowest incubation time that provided the constant percent inhibition was chosen for further study elastase activity of extracts.

Part 2 Screening the potential plant extracts for collagenase and elastase inhibitors

The studied plant extracts including ethanolic extract of *Emblica officinalis* (Amla), ethanolic extract of *Achras Sapota* (Sapota), silymarin were screened for their abilities to inhibit collagenase and elastase. EnzChek® E-12055 gelatinase /collagenase assay kit was used in this screening. DQ^{TM} gelatin has been used to study MMP-2 activity (Chaudhuri R., 2005). MMP-2 is capable to degrade many substrates such as collagen, elastin and gelatin (Philips et al., 2011). As a result, the substrate of MMP-2 (e.g., DQ^{TM} gelatin) was considered suitable for preliminary screenings of collagenase and elastase inhibitory activities. However, the specific substrate needs to be further investigated to confirm both activities.

Extract preparation

a). Amla extract: the dried amla fruits were groud and soaked in 95 % ethanol (1:4) for 24 hours at room temperature. The solvent was collected and the process was repeated over three cycles. All collected solvents were combined and evaporated to dryness under reduced pressure at 40 ^{o}C. The crude ethanolic amla extract was further purified by dissolving the extract in 95 % ethanol. The mixture was concentrated under reduced pressure at 40 ^{o}C.

b). Sapota extract: sapota fruits were peeled and the seeds were discarded. The fruit pulp was cut into small slices and soaked in absolute ethanol for four days at room temperature. The process was repeated over four cycles. Then, all collected solvents were combined and evaporated by rotary evaporator under reduced pressure at 40 ^{o}C.

Both extracts were kept in tight and light-resistant containers and stored in a refrigerator at 4 ^{o}C.

c). Silymarin: powder of silymarin was obtained and stored in a desiccator at room temperature.

The stock solutions of each extracts were freshly prepared at 1 mg/mL with a suitable solvent, which were 60 % propylene glycol and methanol for amla and silymarin, respectively. The stock solution of sapota was also prepared at 1 mg/mL in distilled (DI) water. Further dilution of the stock solutions may be required depending on the assay.

The preliminary screening of anti-aging activity

The method was conducted according to the developed protocol as stated in Part 1.1. All stock solutions of extracts were further diluted with 1X reaction buffer. Silymarin in methanol stock solution was poorly soluble in 1X reaction buffer, thus it was added 1 % Transcutol® as a solubilizer accordingly. 80 µL of sample solutions were added in each well to obtain 150 µg/mL as a final concentration. 20 µL of DQ™ gelatin and 100 µL of ChC were subsequently added in a 96-well plate. The final concentrations of ChC and DQ™ gelatin were 0.2 units/mL and 12.5 µg/mL, respectively. After 90-minutes of incubation, light protected at room temperature, the

fluorescence intensity was measured with the excitation wavelength at 485 nm and the emission wavelength at 538 nm using a fluorescent microplate reader. 0.1 mM of 1,10 phenanthroline, 15 μg/mL EGCG and 1X reaction buffer were used as a general metalloproteinase inhibitor (positive inhibitor), a standard inhibitor and a negative control, respectively. All determinations were performed in triplicate. The ability to inhibit against gelatinase (MMP-2) / collagenase (MMP-1) was expressed as percentage of inhibition calculating by the Equation 2.

The plant extracts that reach 50 % inhibition were selected to determine total phenolic content, total flavonoid content and the biological activities of each individual extract for anti-aging properties.

Part 3 Determination of total phenolic content, total flavonoid content and the anti-aging activities of individual extracts

The selected plants were individually determined both total phenolic and total flavonoid contents. Anti-aging activities of the selected plants were investigated including antioxidant, anti-collagenase and anti-elastase.

3.1. Total phenolic content

Total phenolic content of all samples was assessed by using a modified Folin-Ciocalteau method (Miliauskas et al., 2004). Folin-Ciocalteau (FC) reagent is a mixture of phosphomolydate and phosphotungstate, which oxidizes phenolates and reduces the heteropoly acids to a blue complex.

20 μL of diluted sample stock solution in 95 % ethanol was mixed with 100 μL of the 10 % FC reagent and 80 μL of 75 g/L sodium carbonate solution in a 96 well plate. DI water was used as blank. After a 60-minute incubation period at room temperature and light-protected, the absorbance of the reaction mixture was measured at 765 nm with a microplate reader. All samples were performed in triplicate. A standard curve of gallic acid at the concentration range between 2.5-40 μg/mL was prepared for quantifying the phenolic content of the extract. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract (mg GAE/g).

3.2. Total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay with slight modification (Chang et al., 2002).

A volume of 20 μL of diluted samples solution were mixed with 60 μL of 95 % ethanol, 4 μL of the 10 % aluminium chloride solution, 4 μL of 1 M potassium acetate (MW 98.15) and 112 μL DI water in a 96-well plate. Ten percent aluminum chloride was substituted by DI water as a blank. After 30-minutes of incubation at room temperature and light protected, the absorbance of the reaction mixtures were measured at 415 nm with a microplate reader. The standard curve of quercetin at the concentration range between 1-16 μg/mL was prepared for quantifying the flavonoid content of the extract. All samples were performed in triplicate. The results were expressed as milligrams of quercetin equivalent (QE) per gram of the extract (mg QE/g).

3.3. DPPH free radical scavenging activity

Antioxidant activities of individual extracts were determined based on a modified protocol from Marinova and Batchvarov (2011).

A volume of 0.06 mM DPPH in ethanol was mixed with different concentrations of samples or reagents as following. The final volume of each well was 200 μL.

A (control) = 100 μL DPPH solution + 100 μL absolute ethanol

B (blank of control) = 200 μL absolute ethanol

C (sample) = 100 μL diluted sample + 100 μL DPPH solution

D (blank of sample) = 100 μL diluted sample + 100 μL absolute ethanol

After a 30-minute incubation period with light protected, the absorbance was measured at 517 nm by using a microplate reader. Each absorbance was corrected with its background, which was the sample without DPPH solution (D). Ascorbic acid and ethanol was employed as a standard and a blank, respectively. All samples were performed in triplicate. The antioxidant of extracts was calculated using the following equation:

$$\text{DPPH inhibition (\%)} = \left[\frac{(A\text{-}B) - (C\text{-}D)}{(A\text{-}B)} \right] \times 100 \qquad \text{Equation 4}$$

Where A = the absorbance of DPPH solution and absolute ethanol

B = the absorbance of pure absolute ethanol

C = the absorbance of sample with DPPH solution

D = the absorbance of sample and absolute ethanol

The antioxidant activities of all extracts were finally expressed as an IC_{50} value that is defining as the concentration (μg/mL) of extract showing 50 % inhibition.

3.4. Collagenase assay

The method was conducted following the developed protocol from Part 1.1. Volumes of 80 μL of diluted extract, 20 μL of DQ™ gelatin and 100 μL of ChC were mixed in a 96-well plate. The final concentrations of ChC and gelatin substrate were 0.2 units/mL and 12.5 μg/mL, respectively. After 90 minutes of incubation, light-protected at room temperature, the fluorescence intensity was measured with the excitation wavelength at 485 nm and the emission wavelength at 538 nm using a fluorescent microplate reader. EGCG and 0.1 mM 1,10-phenanthroline were used as a standard and a general inhibitior of metalloproteinase. The ability to inhibit against gelatinase (MMP-2)/collagenase (MMP-1) enzyme was calculated using the Equation 2. All samples were performed in triplicate. The collagenase inhibitions of all individual extracts were finally expressed as an IC_{50} value. Lower IC_{50} value indicated higher collagenase inhibition activity.

3.5. Elastase assay

The assay was employed based on the developed protocol from Part 1.2. A volume of 50 μL of diluted extract was preincubated with 100 μL PE for 15 min. Then, 50 μL of DQ™ elastin was added into the mixture. The final 200 μL reaction mixture contained the diluted sample, 0.2 U/mL PE and 25 μg/mL DQ™ elastin substrate. After a 30-minute incubation period, light-protected at room temperature, the fluorescence intensity was measured with the excitation wavelength at 485 nm and

the emission wavelength at 538 nm using a fluorescent microplate reader. EGCG and CMK were used as a standard and a general elastase inhibitor, respectively. 1X reaction buffer was performed as negative control. The ability to inhibit against elastase was calculated using the Equation 3. All samples were performed in triplicate. The elastase inhibitions of all extracts were finally expressed as an IC_{50} value. Lower IC_{50} value indicated higher elastase inhibition activity.

The extracts that provided the highest DPPH radical scavenging activity, collagenase and/or elastase inhibition activities were selected for further study as a combination.

Part 4 Effects of combining extracts on their biological activities

Plant extracts that showed the lowest IC_{50} of each assay in Part 3 were selected in order to study the effect of combined extracts on their biological activities. Each extract might possess different activities. According to the cosmetic application, these extracts might be mixed together to enhance anti-aging effect. Therefore, it was interesting to study the effect on their biological activities when extracts were combined. The result of a combination might be overall synergism or antagonism or additive.

The studied concentrations of selected extracts that gave % inhibition within the linear range in previous experiment were chosen for a combination study. Anti-aging activities of combinations were determined by the addition of extract A at the fixed concentration to extract B with various concentrations and vice versa.

Three anti-aging activities and total phenolic content of combined extracts were performed similar to the individual extract experiments. Regarding to collagenase assay, DQ™ collagen type I substrate was an additional substrate to determine the activity against MMP-1. In conclusion, there are two substrates: DQ™ gelatin and DQ™ collagen type I were used to determine the activities of MMP-2 and MMP-1, respectively (Sim et al., 2007, Chuadhuri, 2005). The method was the same as using DQ™ gelatin as the substrate. All samples were performed in triplicate.

A combination index (CI index) method was used to assess the nature of the interaction (synergist, addition or antagonist) between extracts as a function of their

concentrations and percent inhibition (IC_{50}) (Chou, 2010, Eid et al., 2012a). CI value is calculated by:

$$CI = \frac{C_a}{IC_{50,a}} + \frac{C_b}{IC_{50,b}} \qquad \text{Equation 5}$$

where C_a and C_b are the extract A and extract B concentrations respectively used in the combination which produces the median effect (50 % inhibition). $IC_{50,a}$ and $IC_{50,b}$ are particular extract concentrations which show median effect value (IC_{50}) for each individual. CI value is used to quantitatively describe synergism (CI < 1), additive effect (CI = 1) and antagonism (CI > 1).

Table 2 Example of planned combinations along with the result scheme

Combinations of		4.1.Antioxidant	4.2. Anti-collagenase		4.3. Anti-elastase
Extract A* (μg/mL)	Extract B* (μg/mL)	CI value DPPH	CI value		CI value Elastase
			MMP-1	MMP-2	
30	20 40 60 80 100				
20 40 60 80 100	30				

* The concentration of extract A and B are changed based on the individual results.

CHAPTER IV

RESULT AND DISCUSSION

Part 1 Partial verification of each test protocol for in vitro anti-aging activities

1.1 Collagenase assay

EnzChek® gelatinase/collagenase assay kit was used to determine anti-collagenase activity of extracts (Chuadhuri, 2005). Limited information is provided in the test kit product E-12055 information sheet. Therefore, the test procedure should be developed and specified prior to the study. 1,10 phenanthroline is a strong metal chelater (Bigg et al., 1994) served as a general inhibitor of collagenase enzyme. It was used to determine the optimized substrate concentration and sample's incubation time.

Per product information sheet, DQ™ gelatin substrate is suggested to be used in the experiment at concentration range of 12.5-100 µg/mL. To verify the optimized substrate concentration used in the present experiment, substrate concentration at 12.5 µg/mL and 100 µg/mL, the minimum and maximum concentration suggested by the test kit, were selected. Firstly, the final concentration of either 12.5 µg/mL or 100 µg/mL DQ™ gelatin substrate and 0.2 U/mL collagenase were incubated for 30 minutes with an increase of 1,10 phenanthroline concentration. The reaction sample was measured the fluorescent intensity at excitation 485 nm and emission 535 nm. The decrease of fluorescent intensity represents the enzyme inhibition. 1,10 phenanthroline inhibited the activity of collagenase as showed the reduction of fluorescent intensity with both concentrations of the substrate (Figure 7A). At 100 µg/mL substrate, the inhibition results were similar to one shown in the product information sheet (Figure 7B) at the same concentration of substrate and enzyme which suggested that the inhibitory of collagenase enzyme could be reproduced. The inhibitory profile at 12.5 µg/mL substrate showed slightly lower than those of 100 µg/mL, which may be a result of lower substrate concentration. However, % fluorescent intensity of both concentrations decreased with the same extends. For

example, the same 10 % reduction in fluorescent intensity was obtained when increased 1,10 phenanthroline concentration from 0.075 to 0.1 mM at both substrate concentration (e.g., 12.5 µg/mL and 100 µg/mL). Therefore, the substrate concentration at 12.5 µg/mL was chosen for further studies.

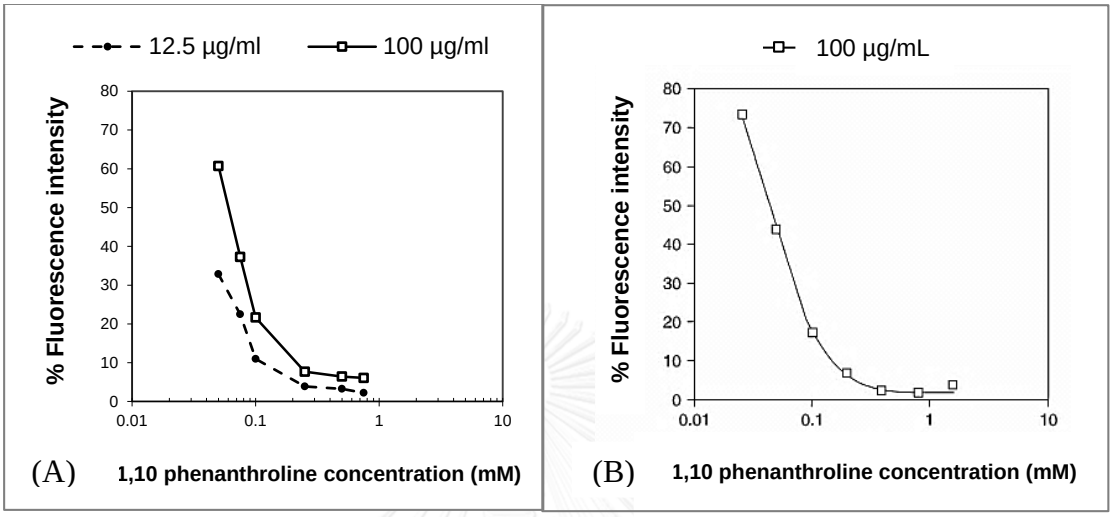


Figure 7 Collagenase inhibitory profiles of 1,10 phenanthroline with 12.5 µg/mL and 100 µg/mL DQ™ gelatin substrate concentration in the current study (A) and the product information sheet E-12055 (B)

The incubation times were varied from 30 to 120 minutes. The MMP-1 and MMP-2 inhibitions were indicated by using the specific substrates of DQ™ collagen type I and DQ™ gelatin, respectively. The 1,10 phenanthroline inhibited MMP-1 and MMP-2 in a dose dependent manner (Figure 8). Figure 8A and Figure 8B illustrate percentages of MMP-1 and MMP-2 inhibitions of 1,10 phenanthroline at different incubation times. The results differently suggested the optimum incubation time between MMP-1 and MMP-2 inhibitions. Percentages of MMP-2 inhibition at all 1,10 phenanthroline concentrations were relatively constant during 30-120 minutes of incubation times. On the other hand, percentages of MMP-1 inhibition showed constant during 90-120 minutes, where at 30-60 minute incubation times showed some fluctuation which might be due to different structures of the substrates. DQ™ collagen type I is more complex structure relative to DQ™ gelatin (Probes, 2001); therefore, the longer incubation period of DQ™ collagen type I was required. However, in order to use the same incubation period for both MMP-1 and MMP-2

inhibition studies, the optimum incubation time were chosen at 90 minutes. This result was consonant with the product information that the appropriated incubation time for the reaction is suggested at approximately 1-2 hours.

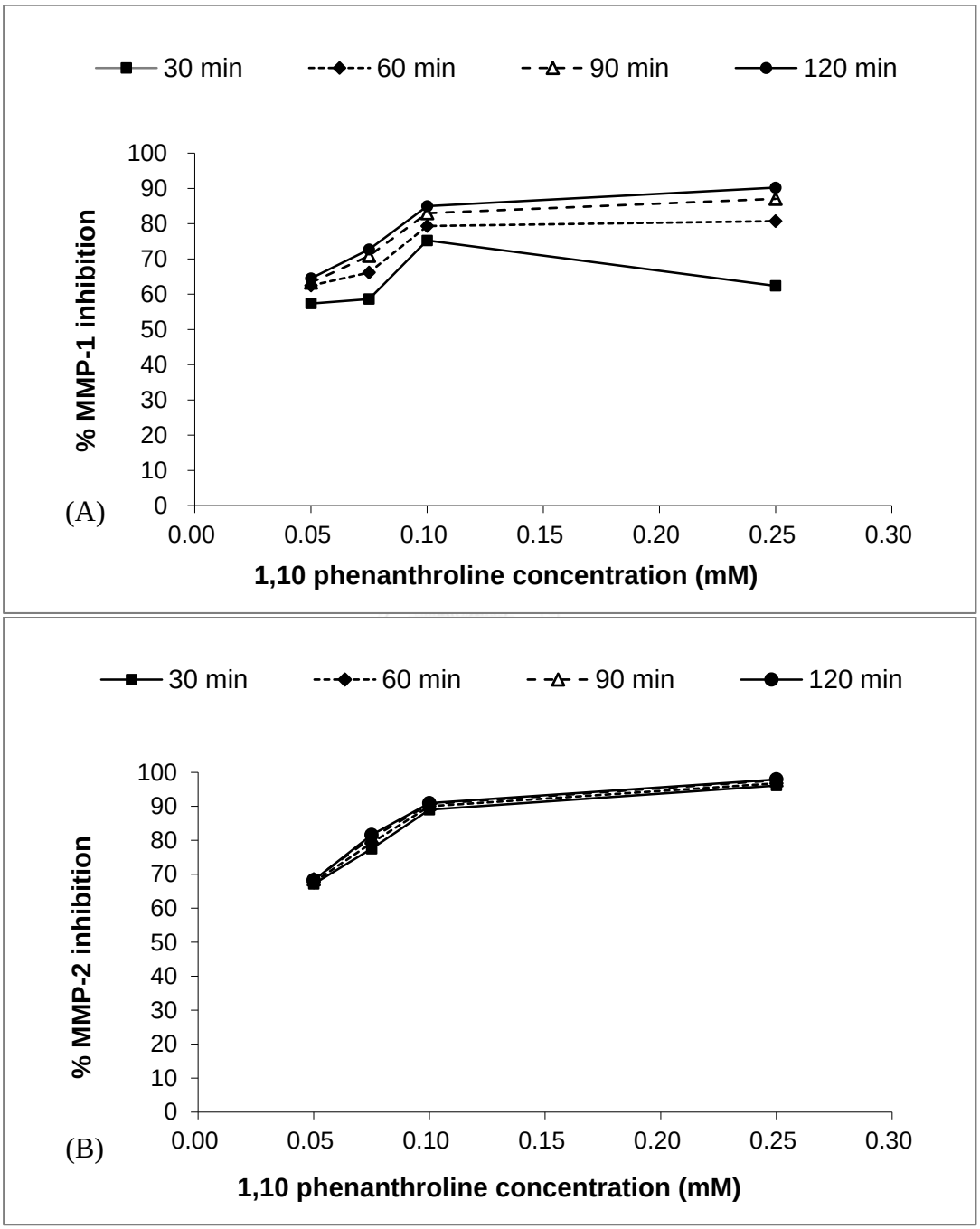


Figure 8 MMP-1 (A) and MMP-2 (B) inhibitions of 1,10 phenanthroline at different incubation times

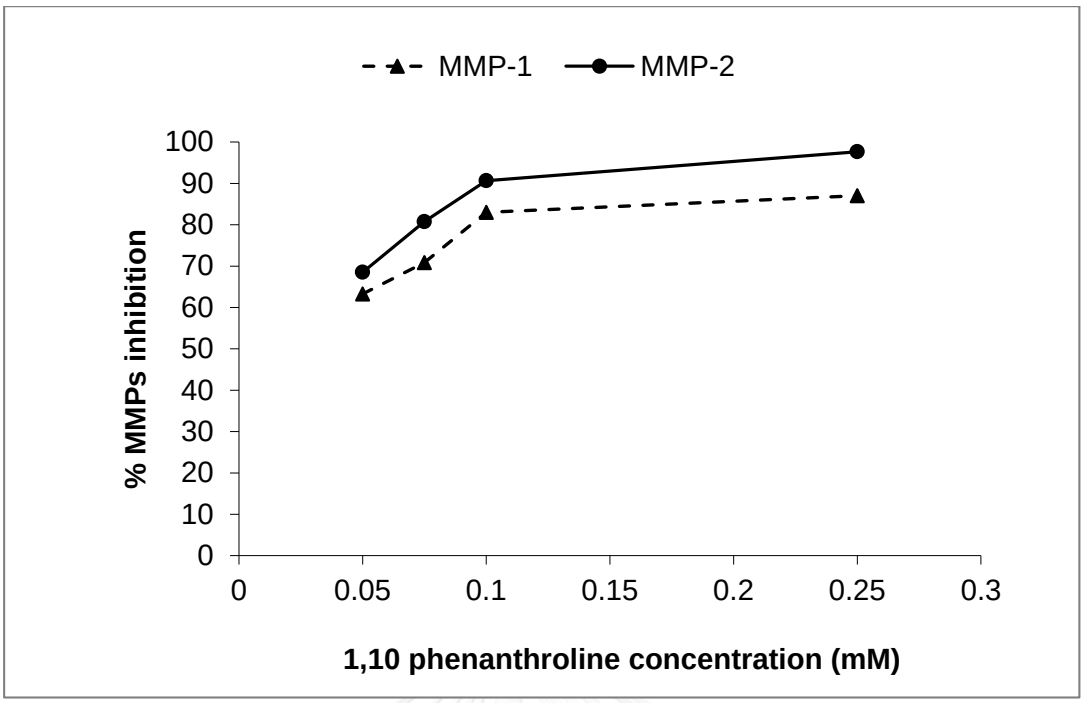


Figure 9 Comparative inhibitory profiles between MMP-1 and MMP-2 by 1,10 phenanthroline at 90-minute incubation time

Although 1,10 phenanthroline showed slightly higher affinity with MMP-2 rather than MMP-1 (Figure 9), similar inhibitory profiles showed between MMP-1 and MMP-2 within the concentration range. As a result, MMP-2 could be primarily used for studying the collagenase inhibition activity of test extract.

In conclusion, the optimized anti-collagenase protocol was to use both substrates at the same concentration of 12.5 μg/mL and type IV collagenase enzyme at the concentration of 0.2 U/mL with the 90-minute incubation time and protected from light.

1.2. Elastase assay

EnzChek® E-12056 elastase assay kit was used to study anti-elastase activity using the same principle as EnzChek® gelatinase/collagenase assay kit. The elastase inhibitor, *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (CMK) was chosen to optimize the incubation time for anti-elastase assay.

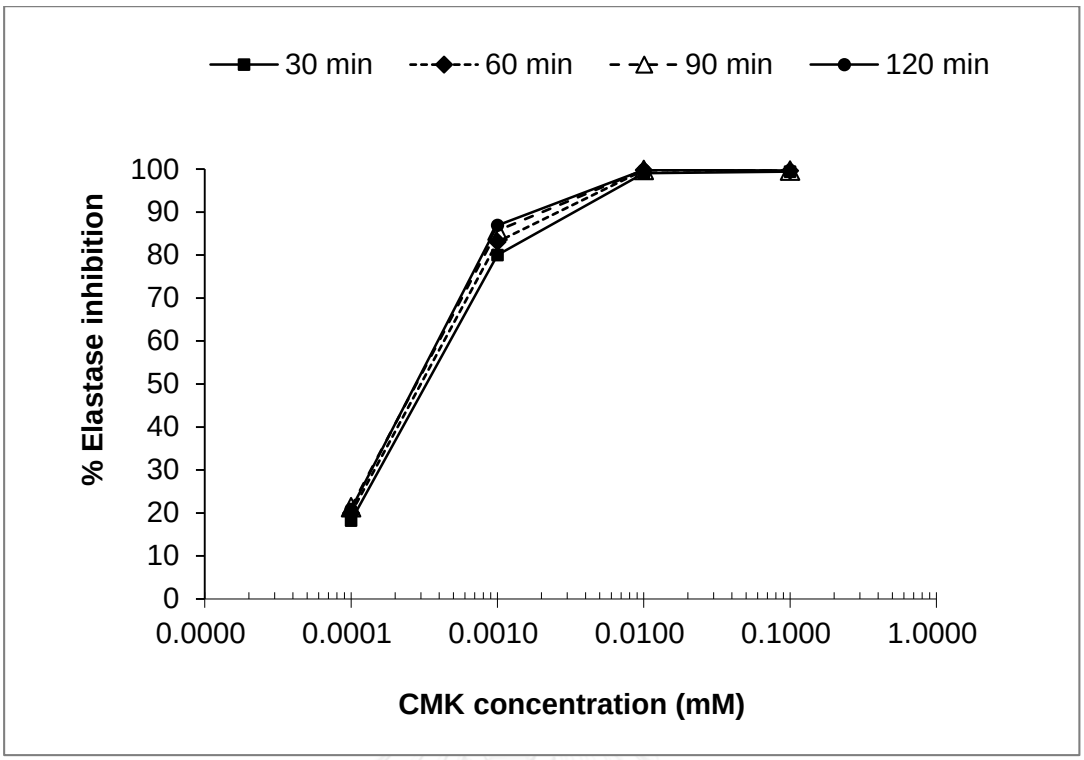


Figure 10 Elastase inhibitions of *N*-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (CMK) at different incubation times

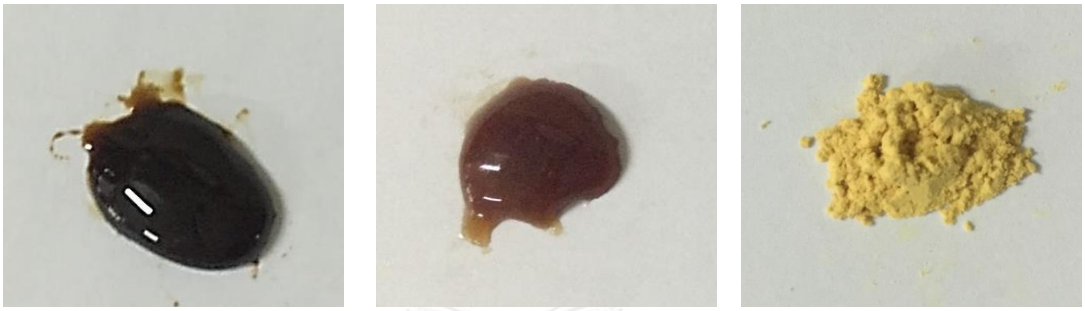
CMK is a general elastase inhibitor. Elastase enzyme was inhibited in a dose dependent manner in accordance to E-12056 product information data. The percentage of elastase inhibition at each CMK concentration was considered constant when using the incubation time range from 30 to 90 minutes as shown in Figure 10. Therefore, the optimum incubation time for further experiment was chosen at the shortest time.

In conclusion, the optimized anti-elastase protocol was to use the substrate at 25 μg/mL, porcine elastase at 0.2 U/mL with the 30-minute incubation time and protected from light.

Part 2 Screening the potential plant extracts for *in vitro* anti-aging activity

Ethanolic amla extract, ethanolic sapota extract, silymarin were screened for anti-aging activity. All extracts possess different phytochemicals, which structures are

related to the inhibitions of collagenase and elastase. The screening of anti-aging activity was performed based on collagenase assay because MMP-2 could degrade fibers including collagen, gelatin and elastin. 1,10 phenanthroline, EGCG and 1X reaction buffer were a positive inhibitor, a standard inhibitor and a negative control, respectively.



A. B. C.

Figure 11 Ethanolic amla extract (A), ethanolic sapota extract (B) and silymarin (C)

Extract preparation

Ethanolic amla extract and ethanolic sapota extract was prepared as stated in methodology Part 2 and yielded 17.59 % w/w and 11.09 % w/w, respectively. Appearances of all extracts are illustrated in Figure 11 (A, B and C). Amla extract was a viscous dark brown paste, which was soluble in 60 % propylene glycol but sparingly soluble in water. Sapota extract was a slightly red brown paste and water soluble. Silymarin was a yellowish powder, and freely soluble in methanol. Silymarin was water insoluble; however, with the use of surfactant named Transcutol® could enhance the solubilty of silymarin in water (Woo et al., 2007). Ethanolic amla extract, ethanolic sapota, and silymarin were screened for the potential anti-aging agents. MMP-2 was able to degrade both collagen and elastin (Philips et al., 2011). Thus, EnzChek® gelatinase/collagenase assay kit with DQ™ gelatin substrate was used for this preliminary screening.

The preliminary screening of anti-aging activity

Transcutol® was required to solubilize silymarin. Fluorescent intensities of 1X reaction buffer and the buffer with 1% Transcutol® were insignificantly different (p-

value > 0.05). Studied extracts showed insignificant fluorescence quenching (p-value > 0.05) under the test condition regarding to similar fluorescence intensities between substrate solution and substrate solution with the extract.

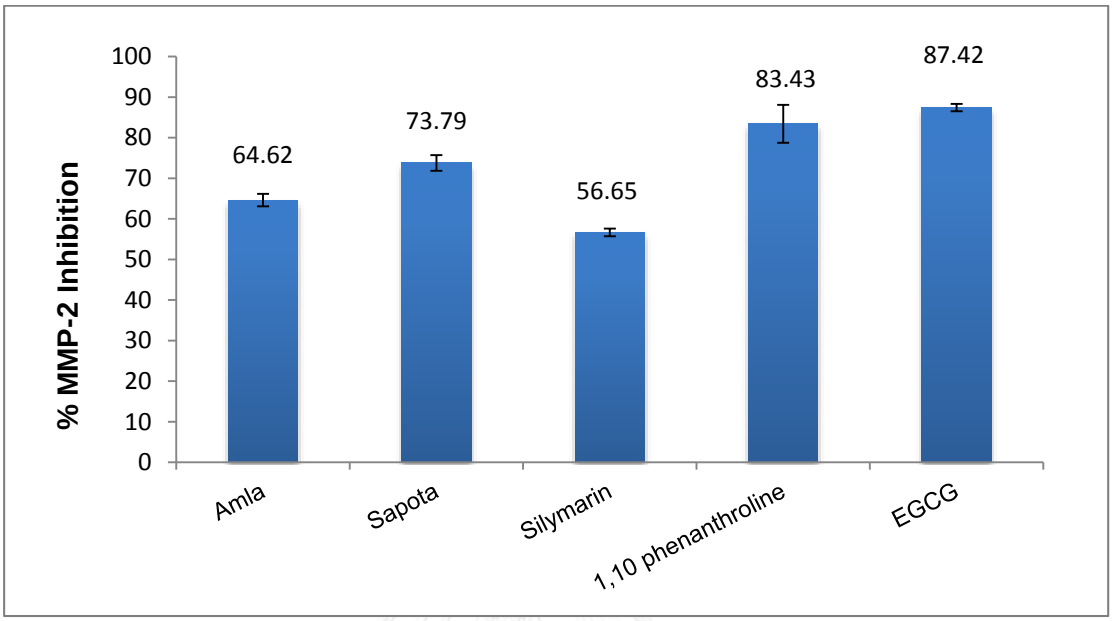


Figure 12 % MMP-2 inhibitions of extracts at fixed concentration (150 µg/mL), 0.1 mM 1,10 phenanthroline, and EGCG (15 µg/mL) presented as mean ± SD, n=3

Chuadhuri (2005) showed that 150 µg/mL of amla extract significantly inhibited collagenase enzyme with the inhibition above 50 %. If an extract at this concentration could inhibit the enzyme, decrease in fluorescent intensity could also be accurately detected. Therefore, the concentrations of all tested extracts were fixed at 150 µg/mL in order to compare the activity amongst the extracts. According to the Figure 12, sapota exhibited the highest MMP-2 inhibition (73.79 ± 1.93 %) following by amla (64.62 ± 1.54%) and silymarin (56.65 ± 0.94 %). All three extracts showed above 50 % inhibition against MMP-2 at 150 µg/mL concentration which was considered as potential inhibitors. The inhibition activity of 0.1 mM 1,10 phenanthroline and 15 µg/mL EGCG were 83.43 ± 4.67 % and 87.42 ± 1.13 %, respectively. The inhibition activity of the 1,10 phenanthroline was comparable to the previous result.

Ethanolic amla, sapota and silymarin, which showed MMP-2 inhibition higher than 50 % at each of 150 µg/mL, were further studied for the total phenolic content,

total flavonoid content and other *in vitro* anti-aging activities of each individual extract.

Part 3 Determination of total phenolic content, total flavonoid content and the anti-aging activities of individual extracts

In vitro anti-aging activities including DPPH antioxidant, anti-collagenase, and anti-elastase, also total phenolic content (TPC) and total flavonoid content (TFC) were studied for selected extracts.

3.1. Total phenolic content

The total phenolic contents of all selected extracts were examined using Folin Ciocalteu's method. The results expressed in term of mg gallic acid equivalent (GAE) per gram of the extract calculating from gallic acid calibration curve (Appendix B). Phenolic compounds in the test sample reduced the phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids which present in the FC reagent to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) under alkaline condition in the presence of sodium carbonate (Santhanam, 2013). The existence of blue oxides represents the quantity of phenolic compounds.

According to Table 3, amla extract showed the highest TPC at 362 $\pm$ 11.22 mg GAE/g extract followed by silymarin and sapota extract of 233.37 $\pm$ 7.53 and 38.56 $\pm$ 1.98 mg GAE/g extract, respectively. Amla extract expressed higher phenolic content which might be due to the fact that major components of amla was reported to be gallic and ellagic acids (Amir et al., 2011, Majeed et al., 2009, Yokozawa et al., 2007b).

3.2. Total flavonoid content

The flavonoid content (TFC) was determined based on aluminium-flavonoid complexes. Quercetin was used as the standard to generate the standard calibration curve (Appendix B). Silymarin exhibited the highest total flavonoid content (21.04 $\pm$

Table 3 Total phenolic content (TPC) and total flavonoid content (TFC) of the three extracts

Plant / extract	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QE/g extract)
Amla	**362.43 ± 11.22**	**6.40 ± 0.88**
Sapota	**38.56 ± 1.98**	**UD**
Silymarin	**233.37 ± 7.53**	**21.04 ± 0.67**

UD : undetectable , GAE : gallic acid equivalent , QE : quercetin equivalent

0.67 mg QE/g extract), whereas amla extract contained lower amounts of flavonoid (6.40 ± 0.88 μg/mL). Total flavonoid content was undetectable in sapota extract.

Silymarin unsurprisingly showed the highest total flavonoid content since it is clearly known as a flavonolignan, one of the flavonoid compounds. Its carbonyl and hydroxyl groups on C ring would preferably form a complex with aluminium chloride that could be detected at 405-419 nm (Spiridon et al., 2011).

Total flavonoid content of the tested sapota extract was undetectable, which was different to those of a previously article (Gomathy et al., 2013). Ethanolic sapota pulp extract was reported to present total flavonoid content at 5.05 ± 0.17 mg rutin equivalent per gram fresh pulp. Different results may be caused by different sources of fruits as well as different test method and standard.

3.3. DPPH free radical scavenging activity

The antioxidant capacities of the tested extracts were determined by DPPH free radical scavenging assay. This method is common and widely used in the plant, and food research for screening antioxidant activity (Floegel et al., 2011, Jain et al., 2011, Karadag et al., 2009). Antioxidant compounds donate hydrogen to DPPH radical resulting in a reduction of purple solution to yellow solution, which is detected by measuring the absorbance at 517 nm. Ascorbic acid was performed as a standard.

Table 4 Antioxidant activities of the extracts tested by DPPH scavenging assay. The results expressed as IC_{50} (mean ± SD)

Sample / Standard	IC_{50} (µg/mL)
Amla	**1.70 ± 0.06**
Sapota	**37.63 ± 1.18**
Silymarin	**27.85 ± 0.98**
Ascorbic acid	**1.38 ± 0.05**

The results of antioxidant among three plant extracts are shown in Table 4. The study revealed that all extracts inhibited DPPH free radical in a dose dependent manner (Figure 13). Amla showed the most potent ability to scavenge DPPH free radical among the extracts at IC_{50} of 1.70 ± 0.06 µg/mL, which was comparable to L-(+)-Ascorbic acid, a standard, at IC_{50} of 1.38 ± 0.05 µg/mL. The DPPH inhibition activities of silymarin and sapota were much lower than amla with IC_{50} of 27.85 ± 0.98 and 37.63 ± 1.18 µg/mL, respectively.

Total phenolic content (TPC) in plants showed strong correlation with their antioxidant activity as shown in terms of % inhibition of single plant (Figure 14A) and IC_{50} among three plants (Figure 14B) with pearson correlation coefficients of 0.99 and 0.93, respectively. Test extract presenting high antioxidant activity contained higher amount of TPC. Correlation between TPC and DPPH free radical activity may be because of the similar test principle that is electron transfer mechanism and both could be detected with a change in color. Folic-Ciocalteu reagent (FC reagent) contains phosphomolybdic / phosphotungstic acid complexes. After the phenolic compounds transfer electron to FC reagent, the mixture becomes blue complex which can be detectable (Karadag et al., 2009). Regard to DPPH free radical scavenging activity, the purple DPPH radical is reduced by antioxidant or reducing agents to yellow color.

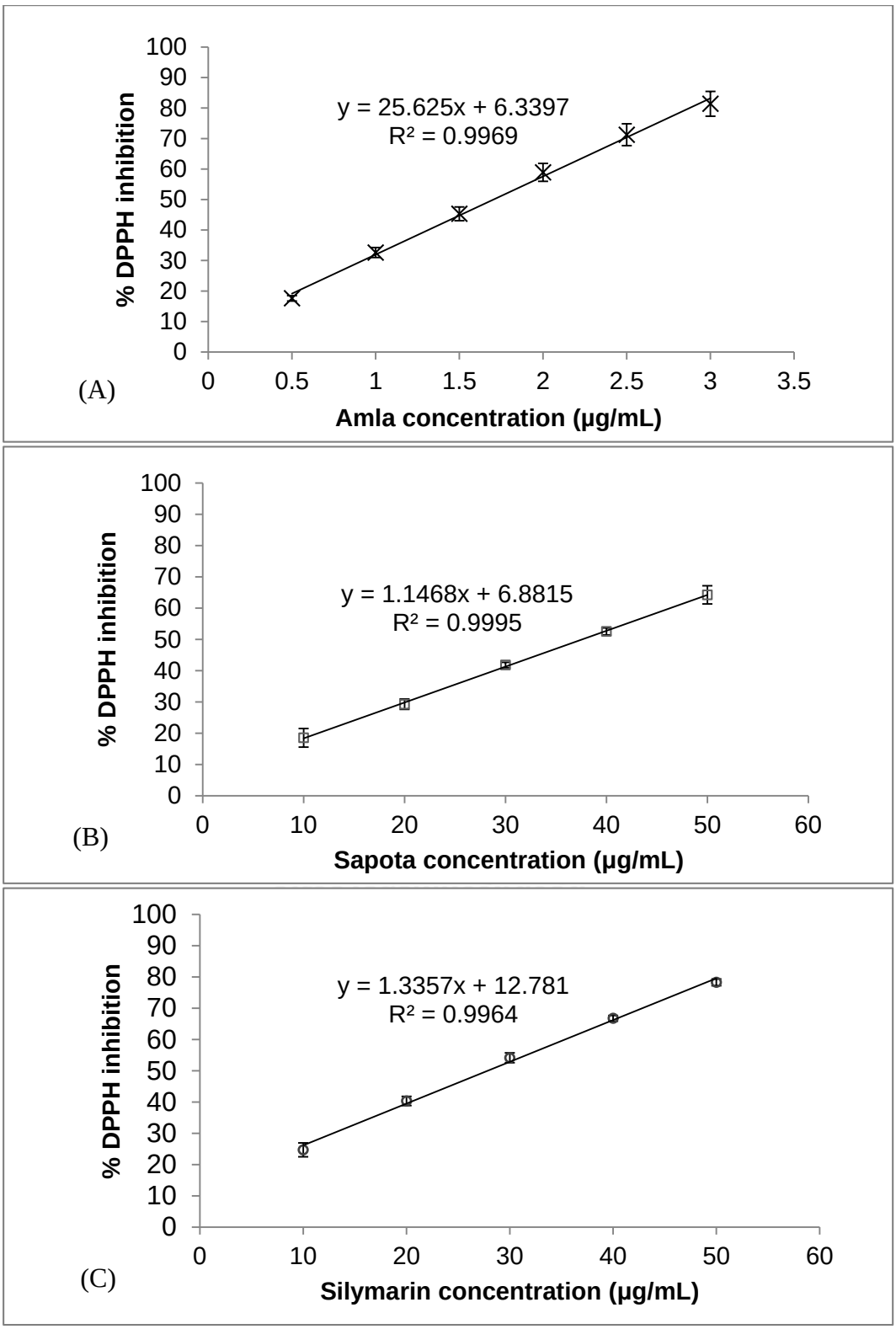


Figure 13 Effect of ethanolic amla extract (A), ethanolic sapota extracts (B) and silymarin (C) on DPPH inhibition activity

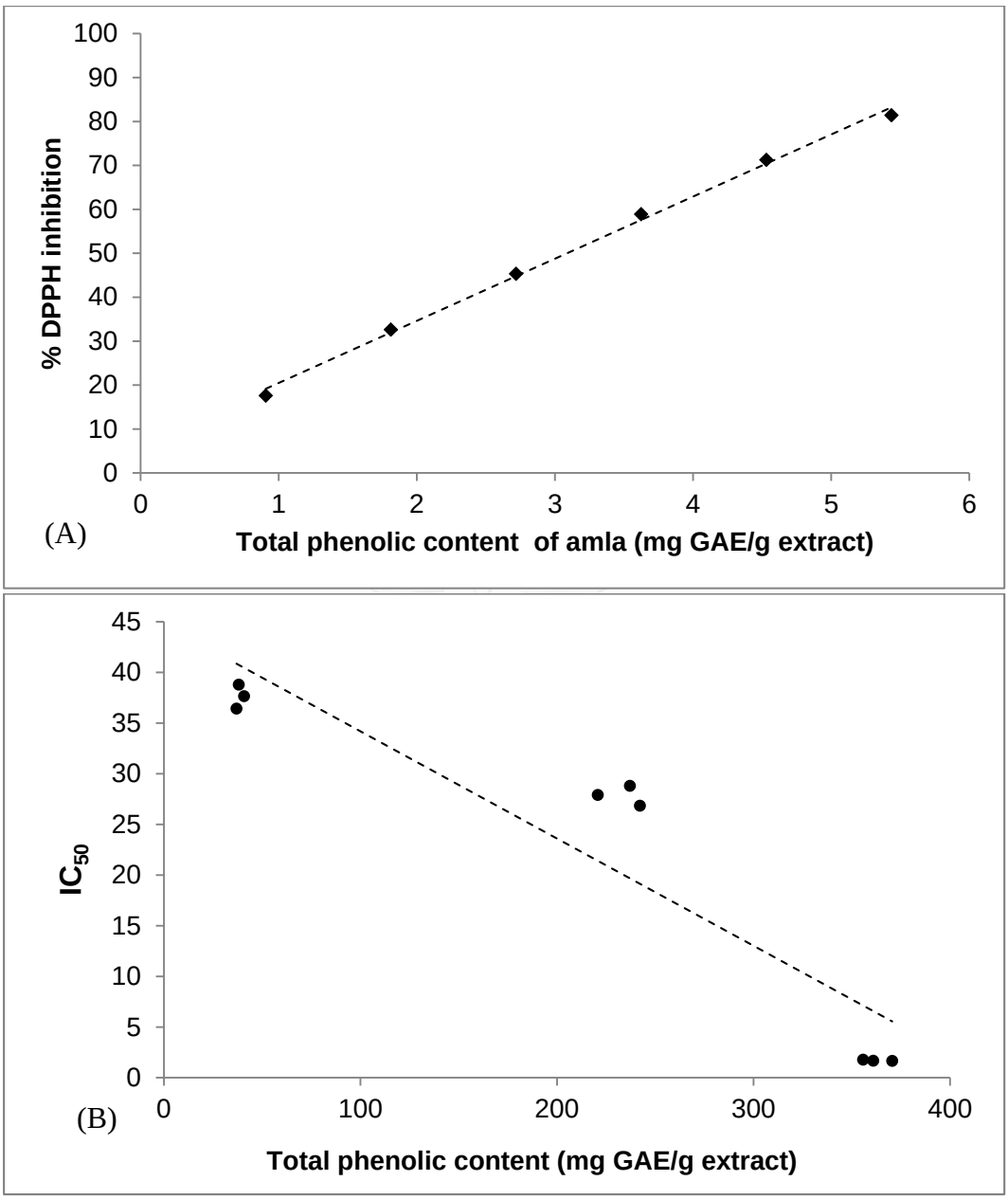


Figure 14 Correlation - between total phenolic content of amla (mg GAE/g extract) and % DPPH inhibition (A); between total phenolic content of three extracts and IC_{50} of DPPH scavenging assay (B)

The plants containing flavonoid were reported to be sources of antioxidant (Chatatikun and Chiabchalard, 2013, Floegel et al., 2011, Pallab et al., 2013). In this study, silymarin and amla presented some amounts of TFC with the antioxidant effect. Wherever, TFC was undetectable for sapota but sapota still showed the antioxidant effect. On the other hand, the antioxidant activity showed no correlation to TFC,

which this finding also agreed with Floegel et al. (2011). Antioxidant activity showed stronger correlation with TPC than TFC. Total phenolic and total flavonoid content may only be screening parameters for the antioxidant activity of the test substance. However, the degree of antioxidant property depends not only on concentration of phenolic or flavonoid content but also on the type of chemical entity as well as its presenting structure in the substance. Therefore, a compound with a low total phenolic and flavonoid content might also possess antioxidant effect due to the presence of other bioactive compounds like sapota.

Skin aging is a complex process. Antioxidant property is the simplest screening for anti-aging substances. Other specific targets such as anti-collagenase and anti-elastase might be required to get more guarantee anti-aging effect. Therefore, anti-collagenase and anti-elastase were further investigated.

3.4. Collagenase assay

Collagenase inhibitory effects were performed using the test kits and the results are shown in Table 5. DQ™ gelatin was used as a substrate to illustrate the activity of MMP-2. MMP-2 was primarily used for studying the inhibitory effect of individual test extracts.

The test extracts inhibited MMP-2 in a dose-dependent manner (Figure 15). Among the test extracts, amla (IC_{50} = 89.41 $\pm$ 3.26 μg/mL) and sapota (IC_{50} = 86.47 $\pm$ 3.03 μg/mL) showed the most effective in MMP-2 inhibition at almost one fold higher than silymarin (IC_{50} = 133.69 $\pm$ 1.16 μg/mL). However, these effects were much lower compared to that of a standard catechin, EGCG, (IC_{50} = 8.19 $\pm$ 0.4 μg/mL).

MMPs are a group of zinc-containing proteinase. This group of enzymes includes collagenase and gelatinase which are responsible for breakdown of collagen, gelatin and elastin. Depletion of the structural fibers in skin results in the lack of skin integrity and elasticity contributing to wrinkle formation and aging skin. Amla extract at 0.5 mg/mL was previously reported to inhibit MMP-2 activity with inhibition of 72.11 $\pm$ 5.95 % determined by using EnzChek gelatinase/collagenase assay kit (Chanvorachote, 2009) with the use of DQ™ gelatin substrate.

Table 5 Anti-MMP-2 activities of the extracts tested by EnzChek® E-12055 gelatinase/collagenase test kit. The result expressed as IC_{50} (mean ± SD)

Sample / Standard	IC_{50} (µg/mL)
Amla	**89.41 ± 3.26**
Sapota	**86.47 ± 3.03**
Silymarin	**133.69 ± 1.16**
EGCG	**8.19 ± 0.4**

Amla extract of a current study showed more potent activity than that of a previous study (Chanvorachote, 2009). It significantly inhibited collagenase with activity comparable to that of sapota. The inhibition effect of amla extract might involve several mechanisms. Hydroxyl groups of polyphenol could interact with the backbone or other functional group side chain of collagenase. In addition, hydrophobic interaction between the benzene ring of polyphenol and collagenase could also result in the conformational changes leading to unfunctioned enzyme (Madhan et al., 2007). Another mechanism involves Zn ion active site on collagenase. The Zn ion active site plays a major role in facilitating interaction with an inhibitor (Bigg et al., 1994). Since a collagenase from *C. histolyticum*, widely used in the study of collagenase activity (Chuadhuri, 2005, Thring et al., 2009), contains a structural Zn ion at its active site (Jung et al., 1999), the polyphenol might bind to a Zn ion active site and prevent the substrate from enzyme digestion (Mcdonald et al., 1996) supported by the current experiment. *E. officinalis* fruit contains a high amount of polyphenol compounds including gallic acid and hydrolysable tannin which are known to be metal chelators. In accordance with the literature, gallic acid showed an inhibitory property to collagenase in a dose dependent manner (Wittenauer et al., 2015).

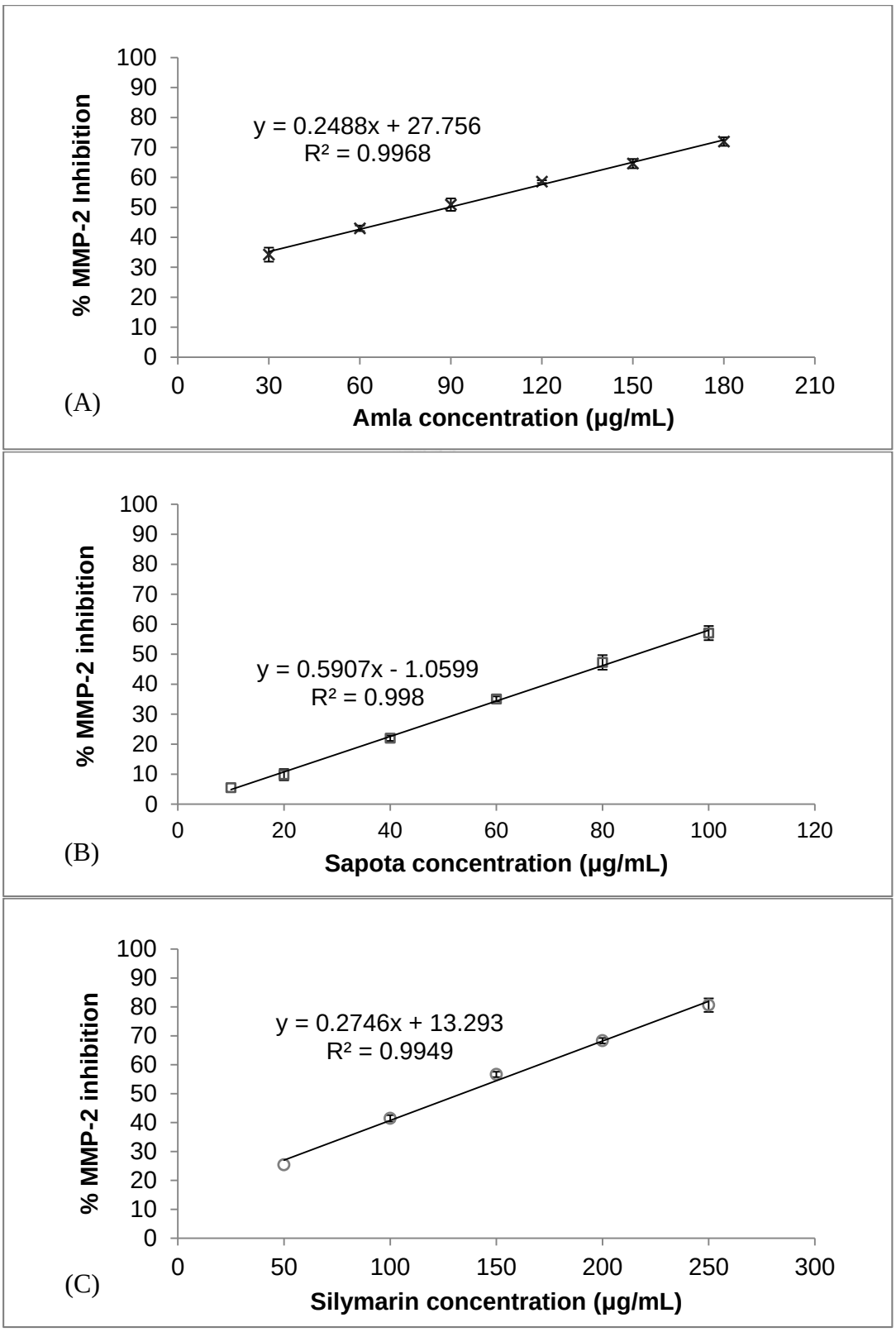


Figure 15 Effect of ethanolic amla extract (A), ethanolic sapota extract (B) and silymarin (C) on MMP-2 activity

In addition to the polyphenol, flavonoids also chelated Zn metal by its 3-hydroxyflavon structure (Malesev and Kuntic, 2007). Silymarin could inhibit collagenase by interaction between its flavonoids and Zn active binding site of the enzyme. Their effects were, however, lower than EGCG; a known major component of green tea exhibiting antioxidant and anti-collagenase activities. Several studies have reported that EGCG contains additional galloyl and hydroxyl groups (Madhan et al., 2007) compared to polyphenols found in amla. With these two functional groups, EGCG could better interact with collagenase and resulting in effectively inhibiting the enzyme.

Sapota showed significantly anti-collagenase activity which was comparable to amla but lower than EGCG. Sapota contained some total phenolic content which may include some polyphenols such as catechin, gallocatechin and epicatechin as well as gallic acid as reported earlier in Ma et al. (2003) work. Some of these phytochemicals were previously reported to inhibit collagenase. For example, catechin was reported to inhibit collagenase from *C. histolyticum* (Madhan et al., 2007). The hydroxyl groups of catechin could form hydrogen bond with the amide backbone and other side chain functional groups of collagenase enzyme and resulting in an effective collagenase inhibition. Another example, gallic acid, major component found in amla, also showed collagenase inhibition (Wittenauer et al., 2015) which may due to its chelating property. In summary, some phytochemicals which were reported to be found in sapota extract could possess an anti-collagenase activity even though all those may not be detected as total phenolic and total flavonoid content.

3.5. Elastase assay

Elastase inhibitory effects were also determined using the elastase test kit. Regards to Table 6, sapota and silymarin exhibited significant inhibition in elastase activity with IC_{50} of 35.73 $\pm$ 0.61 and 38.57 $\pm$ 0.04 μg/mL, respectively. They showed a potent anti-elastase property that was nearly three-folds superior to EGCG (93.99 $\pm$ 3.44 μg/mL), while amla extract exhibited weak elastase inhibition with IC_{50} of 520.83 $\pm$ 18.71 μg/mL. All test extracts inhibited elastase in a dose-dependent manner as in Figure 16.

Table 6 Anti-elastase activities of the extracts tested by EnzChek® E-12056 elastase test kit. The result expressed as IC_{50} (mean ± SD)

Sample / Standard	IC_{50} (µg/mL)
Amla	**520.83 ± 18.71**
Sapota	**35.73 ± 0.61**
Silymarin	**38.57 ± 0.04**
EGCG	**93.99 ± 3.39**

The data was consistent with previous reports from several groups where certain phenolic compounds and flavonoids possessed anti-elastase activity in dose dependency (Hrenn et al., 2006, Jimenez et al., 2006). Phenols, such as epicatechin, catechin, resveratrol and procyanidin B2 (Wittenauer et al., 2015, Hrenn et al., 2006), significantly inhibited elastase activity. In agreement with Witternauer et al. (2015), gallic acid as major component of amla extract possessed poor inhibitory property toward elastase enzyme. Surprisingly, sapota showed significant collagenase inhibition comparable to amla, and showed significant higher elastase inhibition though it contained lower total phenolic and undetectable flavonoid content. Sapota, thus, might contain different types of phenolic compounds or other bioactive components, which perform other mechanism of inhibition. Inhibition of porcine elastase of sapota extract might be due to catechol (Gomathy et al., 2013), since plant extracts containing catechol structure showed the strong inhibitory effect of human neutrophil elastase (Melzig et al., 2001, Alasbahi, 2008). Skin aging involves in many complex pathways. This study suggested that TPC and/or TFC might refer to the antioxidant property in some extent, but the correlations between neither anti-collagenase nor anti-elastase and TPC/TFC were not corelated in this study (data are not shown).

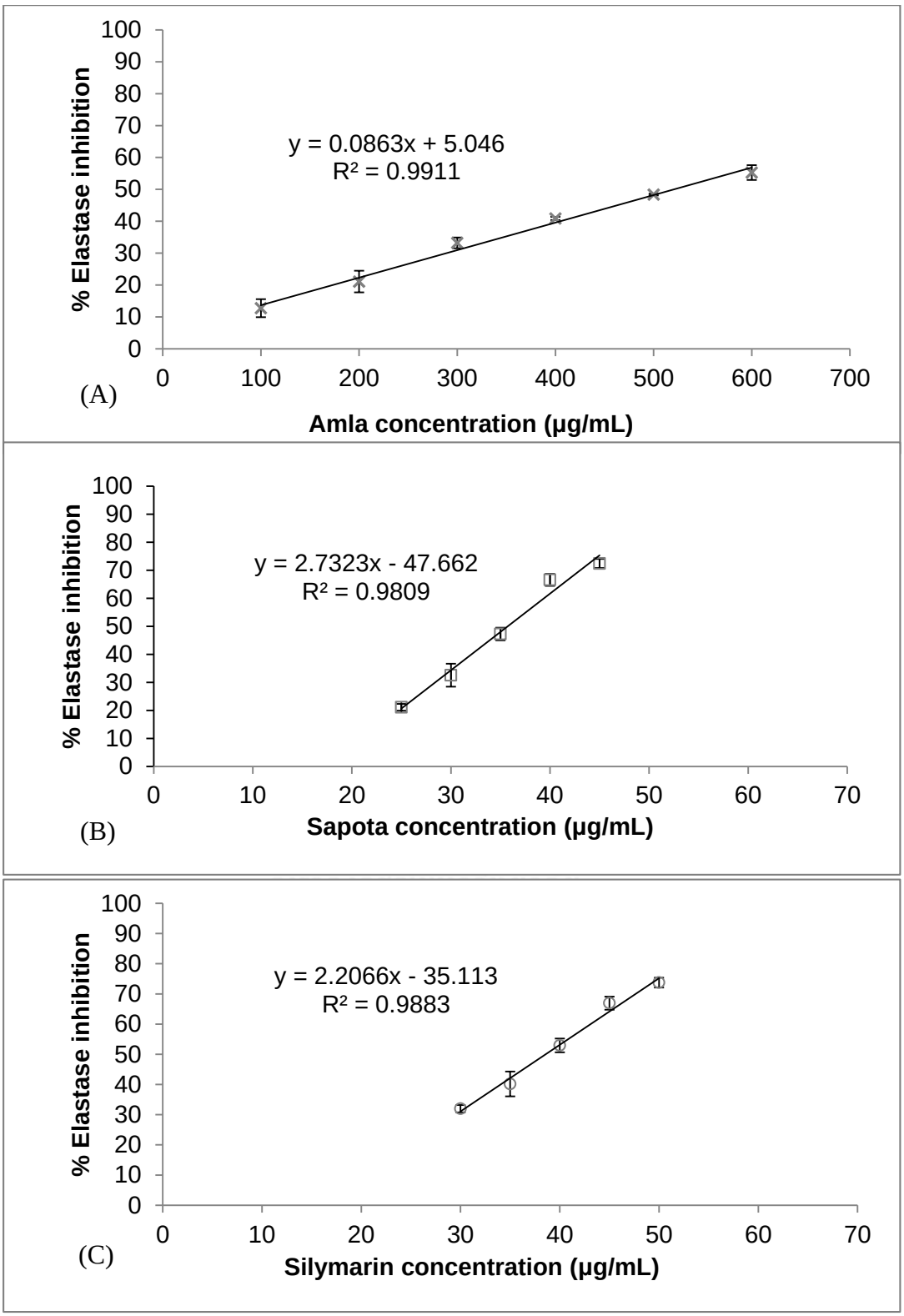


Figure 16 Effect of ethanolic amla extract (A), ethanolic sapota extract (B) and silymarin (C) on elastase activity

Fewer test parameters of TPC, TFC, and DPPH free radical scavenging assay were frequently used for the screening process property, which might not provide enough information to identify the potential anti-aging agent. For example, amla showed strong antioxidant but weak elastase inhibitor. In contrary, sapota showed the least antioxidant among three plants and TFC was not detected but sapota provided the excellent in both collagenase and elastase inhibitions. Oftentimes extracts might be ignored when the result comes out with the poor antioxidant activity. However, more specific activities could be presented by those plants, sapota was a good example. Thus, some target activities such as anti-collagenase and anti-elastase, etc shall be considered for cosmetic active screening.

In conclusion, different plant extracts had different activities for anti-aging. Among the test extracts, ethanolic amla extract contained high phenolic content and showed the most potent antioxidant with moderate collagenase and poor elastase inhibitions. Sapota showed the highest collagenase and elastase inhibition with slightly antioxidant effect. Silymarin showed high flavonoid content, and inhibited the elastase comparable to sapota, but was poor in anti-collagenase activity compared to others. There is hardly an ‘all in one’ component that exhibited total anti-aging effects on each of the skin aging-pathway. Thus, extracts might be mixed to provide overall anti-aging effects in cosmetic products. And also, the biological activities including antioxidant, anti-collagenase, and anti-elastase can be altered when extracts are combined.

Amla and sapota extracts were selected to study the effect of combination extracts on anti-aging activities. Amla exhibited the most potent on DPPH scavenging activity, whereas sapota possessed the highest inhibitory properties on elastase and collagenase. In cosmetic industry, the potential extracts are normally combined and aimed for the overall effects of the mixture. The influence of combined extracts on their biological activities is, therefore, very interesting to be investigated compared with the individuals.

Part 4 Effects of combining extracts on their biological activities

Amla had the lowest IC_{50} on antioxidant assay among three extracts, whereas sapota showed lowest IC_{50} on MMP-2 and elastase assay. Due to an imperfect anti-aging activity of single extract, combining of the extracts might become a practical way in cosmetic industry in order to fulfill overall anti-aging effect. The biological activities could be changed in positive or negative direction or even unchanged when two or more extracts are combined. Therefore, amla and sapota extracts were combined in order to determine the effect on anti-aging activities. Antioxidant, anti-collagenase, anti-elastase properties and total phenolic content were again investigated and compared the IC_{50} between combination mixtures and the individual extracts. Study was designed to obtain different ratios of amla-sapota combination by adding a fixed concentration of one extract into another extract which was varied in concentrations. Since each extract showed superior in one activity to another, both extracts was alternatively fixed and added to another. Fixed concentrations of amla and sapota were selected from the linear range of their dose-response curves at which provided 10-35 % inhibition because higher % inhibition was expected when combining extracts so the total effect would not exceed the linear range. The fixed concentration was chosen based on type of extract and assay carried in order to obtain proper results. Therefore, different fixed concentrations were used in each experiment.

Combination index (CI) was used to categorize an interaction of the combination where CI <1, CI =1 and CI>1 indicate synergism, additive effect and antagonism, respectively. This method has been widely used in pharmacology to interpret drug interaction without the requirement of mechanism of action (Rodea-Palomares et al., 2010). CI was globally cited in more than three thousands of scientific articles and the equation of CI was derived from median effect (50 %) equation (Chou, 2010). CI value was calculated by:

$$CI = \frac{C_a}{IC_{50,a}} + \frac{C_b}{IC_{50,b}} \qquad \text{Equation 5}$$

where C_a and C_b are the extract A and extract B concentrations respectively used in the combination which produces the median effect (50 % inhibition). $IC_{50,a}$ and $IC_{50,b}$ are particular extract concentrations which show median effect value (IC_{50}) for each individual.

4.1. DPPH free radicle activity

Amla and sapota were combined and CI value at 50 % DPPH inhibition of extract combination was determined. The DPPH inhibition of amla was more potent than that of sapota with approximately 20 folds. In order to get good estimation of median effect (or IC_{50}), fixed amla and sapota concentrations were different.

In combination A, sapota concentration was fixed at 10 µg/mL which provided about 13.52 % inhibition, when sapota at fixed concentration was combined with various concentrations of amla, a linear dose response curve covering 50 % inhibition would be expected. Fixed 10 µg/mL sapota was mixed with various concentrations of amla ranging from 0.5-2.5 µg/mL, which provided total concentrations of the combinations ranging from 10.5-12.5 µg/mL (Figure 17). The IC_{50} of combination A was 11.21 $\pm$ 0.14 µg/mL (Table 7), which contained amla and sapota approximately at 1.21 and 10 µg/mL, respectively.

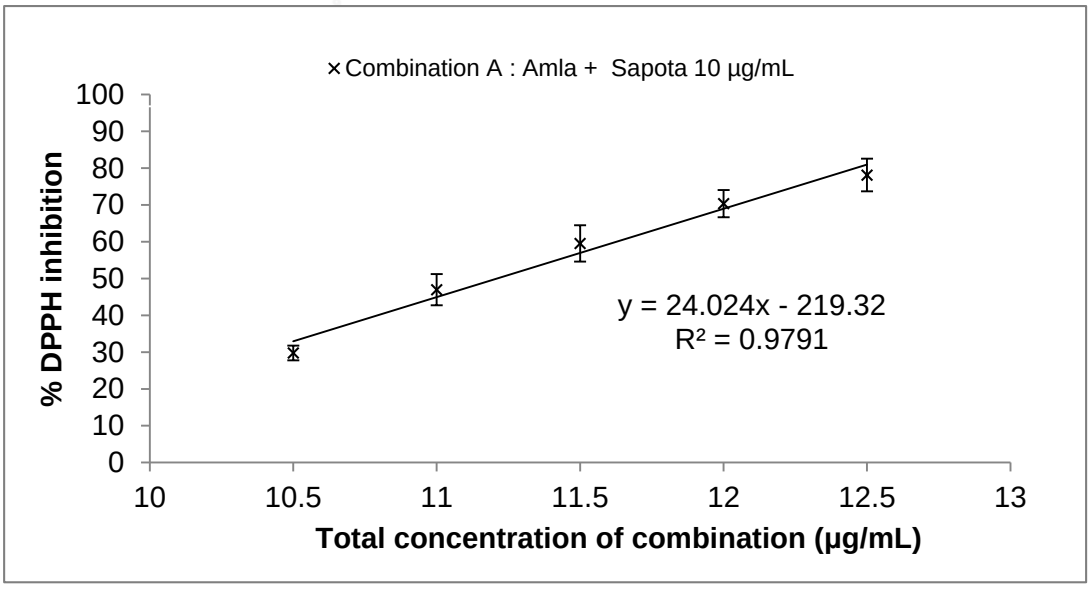


Figure 17 Effect of combination A on DPPH scavenging activity

In combination B, amla was fixed at 0.5 µg/mL providing about 17.92 % inhibition. It was mixed with various concentrations of sapota ranging from 10-50 µg/mL, which provided total concentration of the combination ranging from 10.5-50.5 µg/mL (Figure 18). The IC_{50} of combination B was 25.97 ± 0.92 µg/mL (Table 7), which contained amla and sapota approximately at 0.5 and 25.47 µg/mL, respectively.

Table 7 Combination indexes (CIs) of the combination A and B including the IC50 of individual and combination extracts for DPPH scavenging activity (Mean ± SD)

Combination	Extract	IC_{50} of Individual (µg/mL)	IC_{50} of combination (µg/mL)	CI index at IC_{50} of combination	Type of interaction
A	Amla	1.61 ± 0.04	11.21 ± 0.14*	1.03 ± 0.08	addition
	Sapota	36.89 ± 0.54			
B	Amla	1.61 ± 0.04	25.97 ± 0.92**	1.00 ± 0.02	addition
	Sapota	36.89 ± 0.54			

*** contained amla and sapota extracts at 1.21± 0.14 and 10 µg/mL, respectively**

**** contained amla and sapota extracts at 0.5 and 25.47 ± 0.92 µg/mL, respectively**

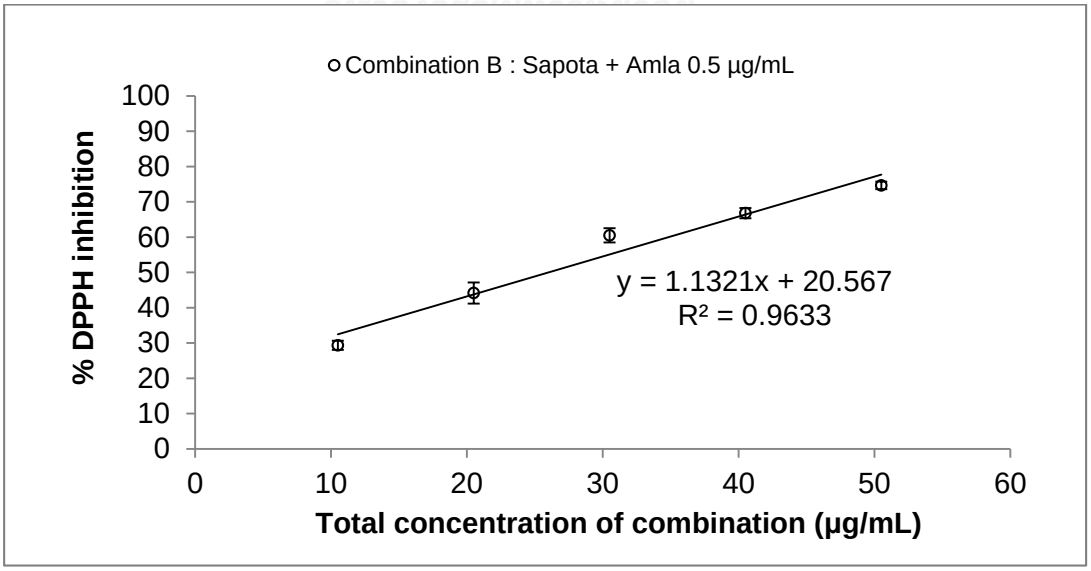


Figure 18 Effect of combination B on DPPH scavenging activity

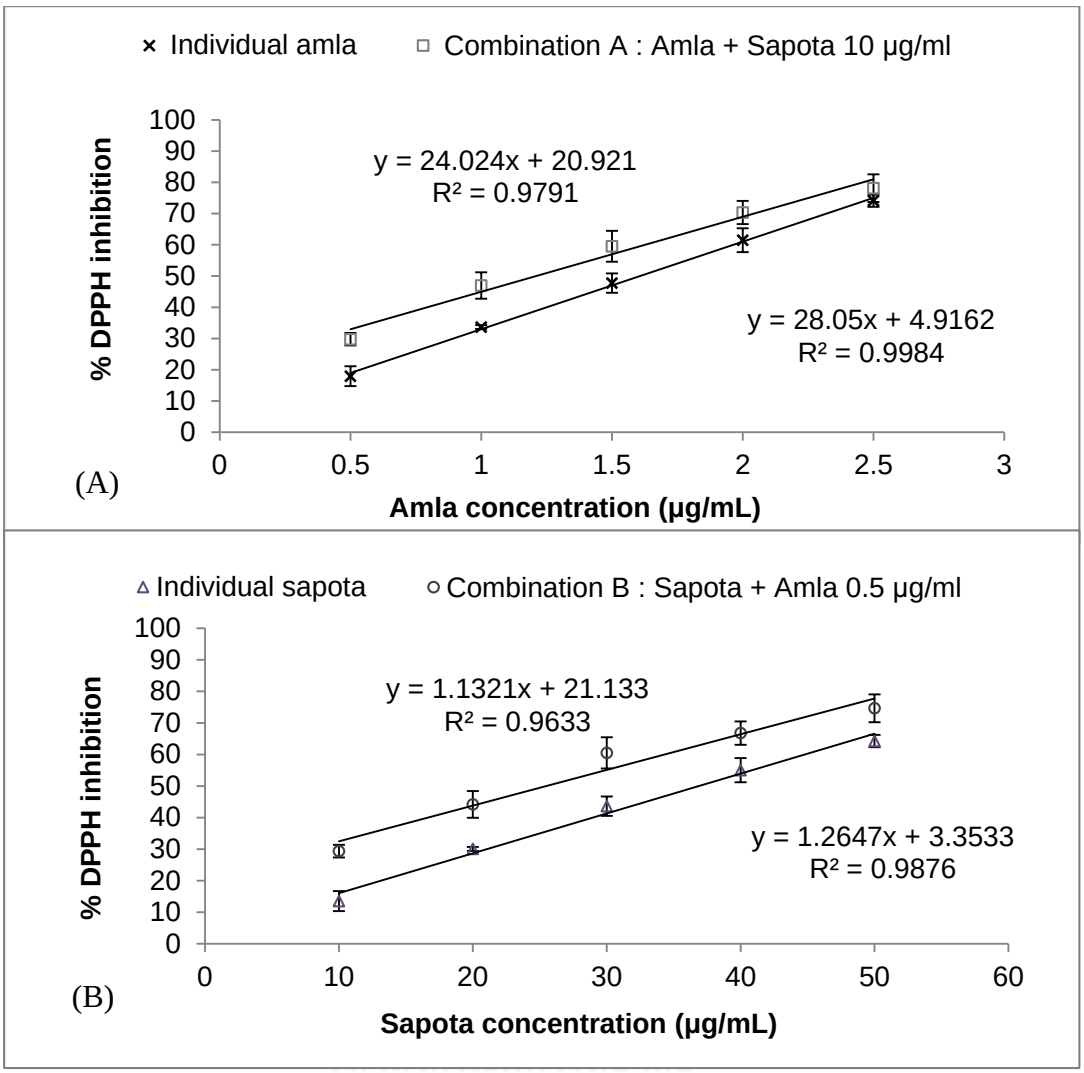


Figure 19 Effect of individual extracts and combination A (A) or combination B (B) on DPPH scavenging activity

Both combinations A and B increased antioxidant activity with similar extent along the studied concentration range and could be observed from the parallel lines between one extract and combined extract (Figure 19). The median effect CIs showed the values of 1.03 ± 0.08 and 1.00 ± 0.02 for combination A and B, respectively (Table 7).

The CIs of both combinations A and B at all ratios or at other % inhibitions ranged from 0.92 to 1.13 (Figure 20, Appendix B, Table 22-23). Most combination ratios revealed as addition effect (CI = 1) even though some data showed slightly more or less than 1 but statistically insignificant (p-value > 0.05). In summary, most

combination ratios showed no advantage of combining two extracts when considered DPPH scavenging activity or showed no adverse effect when the extracts were combined.

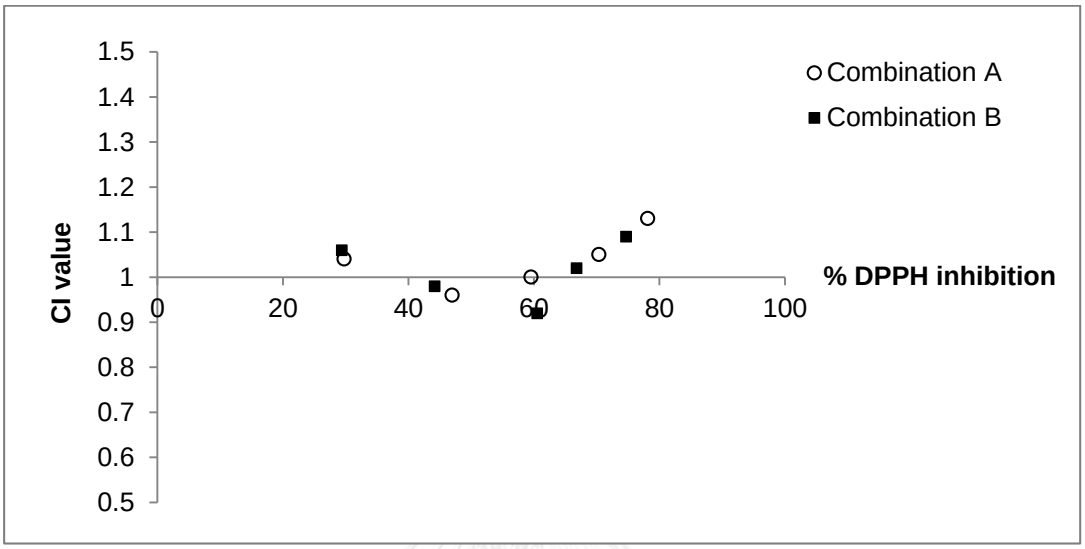


Figure 20 DPPH inhibition - CI indexes for all combination ratios of combination A and B

4.2 Collagenase assay

In addition to MMP-2 inhibition effect, MMP-1 inhibition using DQ^{TM} collagenase type I was additionally included in order to study a specific and complete influence of combined extracts on collagen type I, IV, and VII breakdowns by collagenase enzyme. All experiments were conducted similar to previous experiment. In order to specify the reaction, the collagenase inhibition experiments were performed with specific substrates; DQ^{TM} gelatin indicated MMP-2 and DQ^{TM} collagenase type I indicated MMP-1. All experiment methods were similar to previous experiment.

Similar to DPPH study, different fixed amla and sapota concentrations were chosen as well as the curve fitting used to estimate IC_{50} were different. For combination A, instead of typical untransformed X-axis, ln-transformed X-axis was required to have a better fit to the data and also a good estimate of IC_{50}. For example, MMP-1 inhibitory profile of combination A showed better curve fitting with ln-

transformation of X-axis (R^2=0.9669) while curve fitting with linear regression showed lower R^2 (R^2=0.9061).

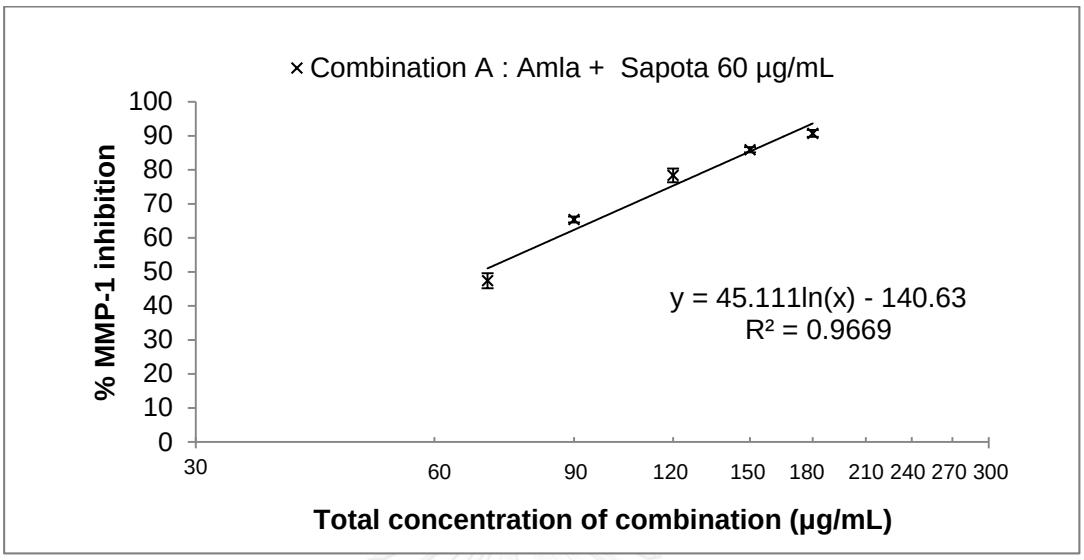


Figure 21 Effect of combination A on MMP-1 inhibition

Regards to MMP-1 study, combination A was a mixture of sapota at fixed concentration of 60 µg/mL, which provided about 30.99 % inhibition, and amla at various concentrations ranging from 10-120 µg/mL. Total concentrations of the combination A were between 70-180 µg/mL (Figure 21). The IC_{50} of combination A was 71.90 ± 1.42 µg/mL (Table 8), which contained amla and sapota extracts approximately at 11.90 and 60 µg/mL, respectively.

Table 8 Combination indexes (CIs) of the combination A and B including the IC_{50} of individual and combination extracts for anti-MMP-1 activity (Mean ± SD)

Combination	Extract	IC_{50} of Individual (µg/mL)	IC_{50} of combination (µg/mL)	CI index at IC_{50} of combination	Type of interaction
A	Amla	95.97 ± 3.27	71.90 ± 1.42*	0.79 ± 0.02	synergism
	Sapota	89.61 ± 0.96			
B	Amla	95.97 ± 3.27	74.00 ± 1.08*	0.80 ± 0.02	synergism
	Sapota	89.61 ± 0.96			

*** contained amla and sapota extracts at 11.90 ± 1.42 and 60 µg/mL, respectively**

**** contained amla and sapota extracts at 30 and 44.00 ± 1.08 µg/mL, respectively**

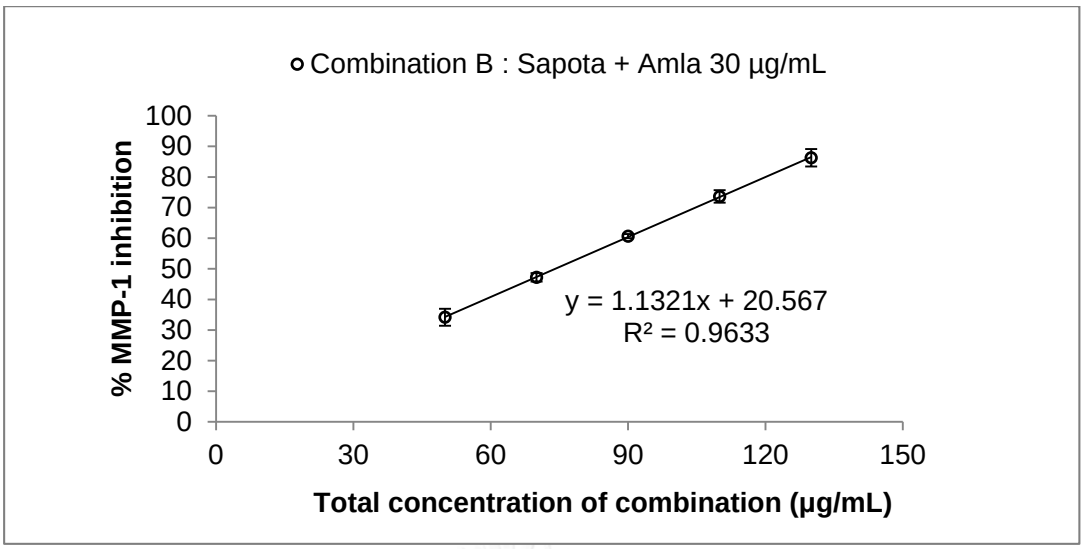
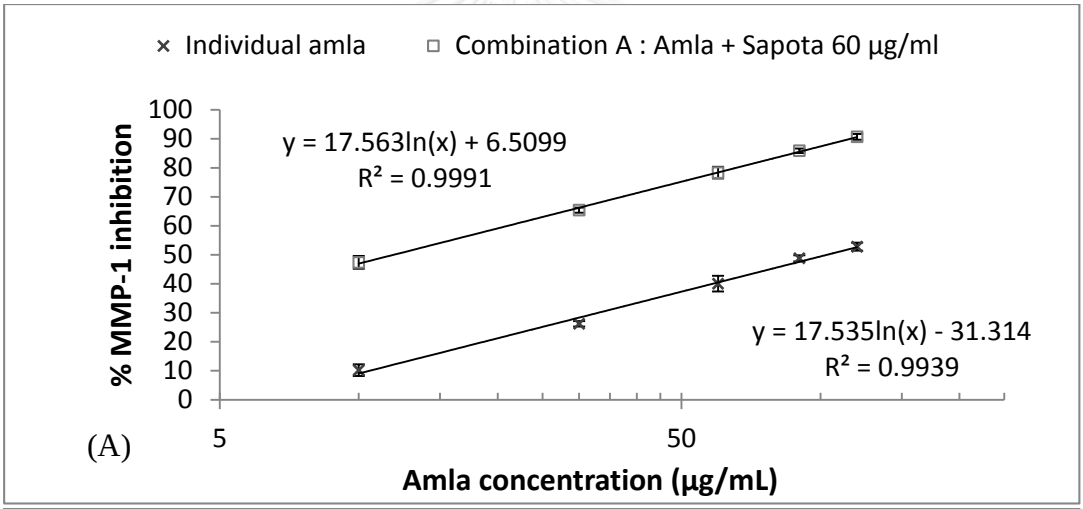


Figure 22 Effect of combination B on MMP-1 inhibition



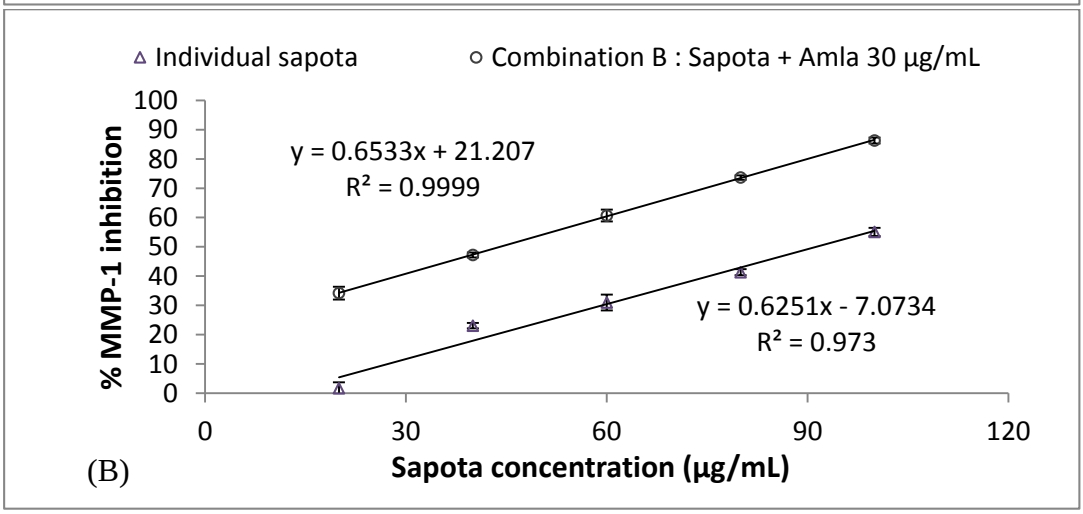


Figure 23 Effect of individual extracts and combination A (A) or combination B (B) on anti-MMP-1 activity

In combination B, amla was fixed at 30 µg/mL providing about 26.21 % inhibition. It was mixed with various concentrations of sapota ranging from 20-120 µg/mL, which provided total concentrations of the combination ranging from 50-150 µg/mL (Figure 22). The IC_{50} of combination B was 74.00 $\pm$ 1.08 µg/mL (Table 8), which contained amla and sapota approximately at 30 and 44.00 µg/mL, respectively.

Both combinations A and B increased MMP-1 activity with similar extent along the studied concentration range and could be observed from the parallel lines between single extract and combined extracts (Figure 23). With the CI calculation at IC_{50} of combination (median effect), CI of the combination A was 0.79 $\pm$ 0.02 while CI of the combinations B was 0.80 $\pm$ 0.02 (Table 8). The CIs at all combination ratios of both combinations A and B were in the range of 0.53 to 0.96 (Figure 24, Appendix B, Table 24-25). Both combinations' CI values were significantly categorized as synergist effect (CI < 1) for MMP-1 activity at all combination ratios (p-value < 0.05). If we separately used the extracts at their IC_{50}, the summation of % inhibitions from individual extract in combination A was approximately 40 % but when combined both extracts at the given concentrations could achieve 50 % inhibition which clearly showed an advantage of combination.

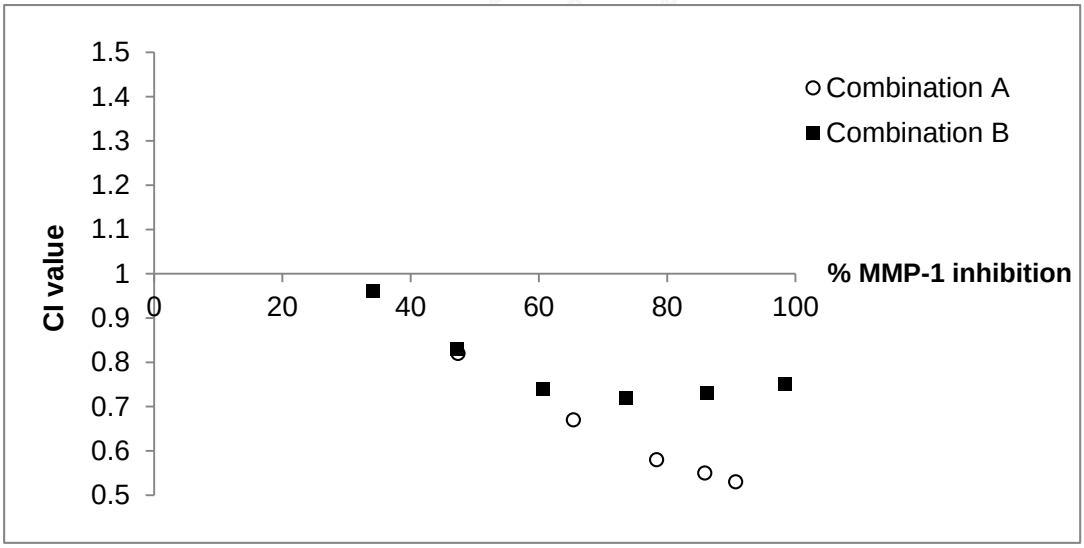


Figure 24 MMP-1 inhibition - CI indexes for all combination ratios of combination A and B

With regards to MMP-2, the combination C was a mixture of sapota at fixed concentration of 20 μg/mL, which provided about 9.83 % inhibition, and amla at various concentrations ranging from 15-120 μg/mL. Total concentrations of the combination C were between 35-140 μg/mL (Figure 25). The IC_{50} of the combination C was 49.31 ± 0.61 μg/mL (Table 9) which contained amla and sapota extracts approximately at 29.31 and 20 μg/mL, respectively.

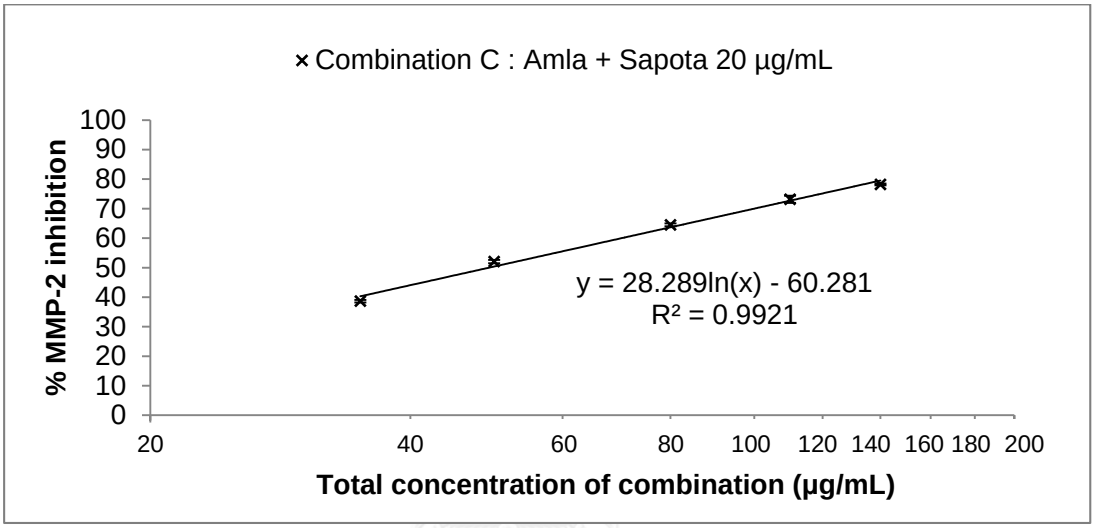


Figure 25 Effect of combination C on MMP-2 inhibition

Table 9 Combination indexes (CIs) of the combination C and D including the IC_{50} of individual and combination extracts for anti-MMP-2 activity (Mean ± SD)

Combination	Extract	IC_{50} of Individual (μg/mL)	IC_{50} of combination (μg/mL)	CI index at IC_{50} of combination	Type of interaction
C	Amla	89.40 ± 3.26	49.31 ± 0.61*	0.58 ± 0.02	synergism
	Sapota	86.48 ± 3.04			
D	Amla	89.40 ± 3.26	47.27 ± 1.01**	0.55 ± 0.02	synergism
	Sapota	86.48 ± 3.04			

*** contained amla and sapota extracts at 29.31 ± 0.61 and 20 μg/mL, respectively**

**** contained amla and sapota extracts at 30 and 17.27 ± 1.01 μg/mL, respectively**

In combination D, amla was fixed at 30 µg/mL providing about 34.22 % inhibition. It was mixed with various concentrations of sapota ranging from 20-100 µg/mL, which provided total concentrations of the combination ranging from 50-130 µg/mL (Figure 26). The IC_{50} of the combination D was at 47.29 ± 1.01 µg/mL which contained amla and sapota approximately at 30 and 17.27 µg/mL (Table 9), respectively.

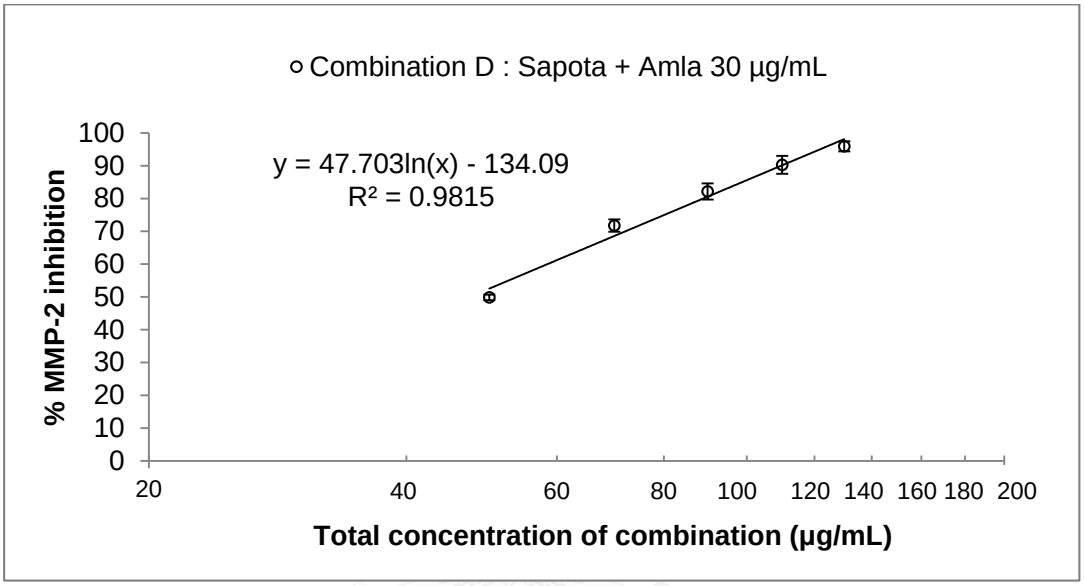


Figure 26 Effect of combination D on MMP-2 inhibition

MMP-2 activity of both combinations C and D increased with similar extent along the studied concentration range and could be observed from the parallel lines between one extract and combined extract (Figure 27A-B), which showed the same results to those of MMP-1. MMP-2 inhibitory profile of combination D showed better curve fitting with ln-transformation of X-axis (R^2=0.9979) (Figure 27C) while curve fitting with linear regression showed lower R^2 (R^2=0.9276) (Figure 27B).

With the CI calculation, CIs of the combination C was 0.58 ± 0.02 while CIs of the combinations D was 0.55 ± 0.02 (Table 9). The CIs at all combination ratios of both combinations C and D were in the range of 0.52 to 0.75 (Figure 28, Appendix B, Table 26-27). Similar findings as MMP-1 study, both combinations C and D at all ratios were categorized as synergist effect (CI < 1) for MMP-2 activity.

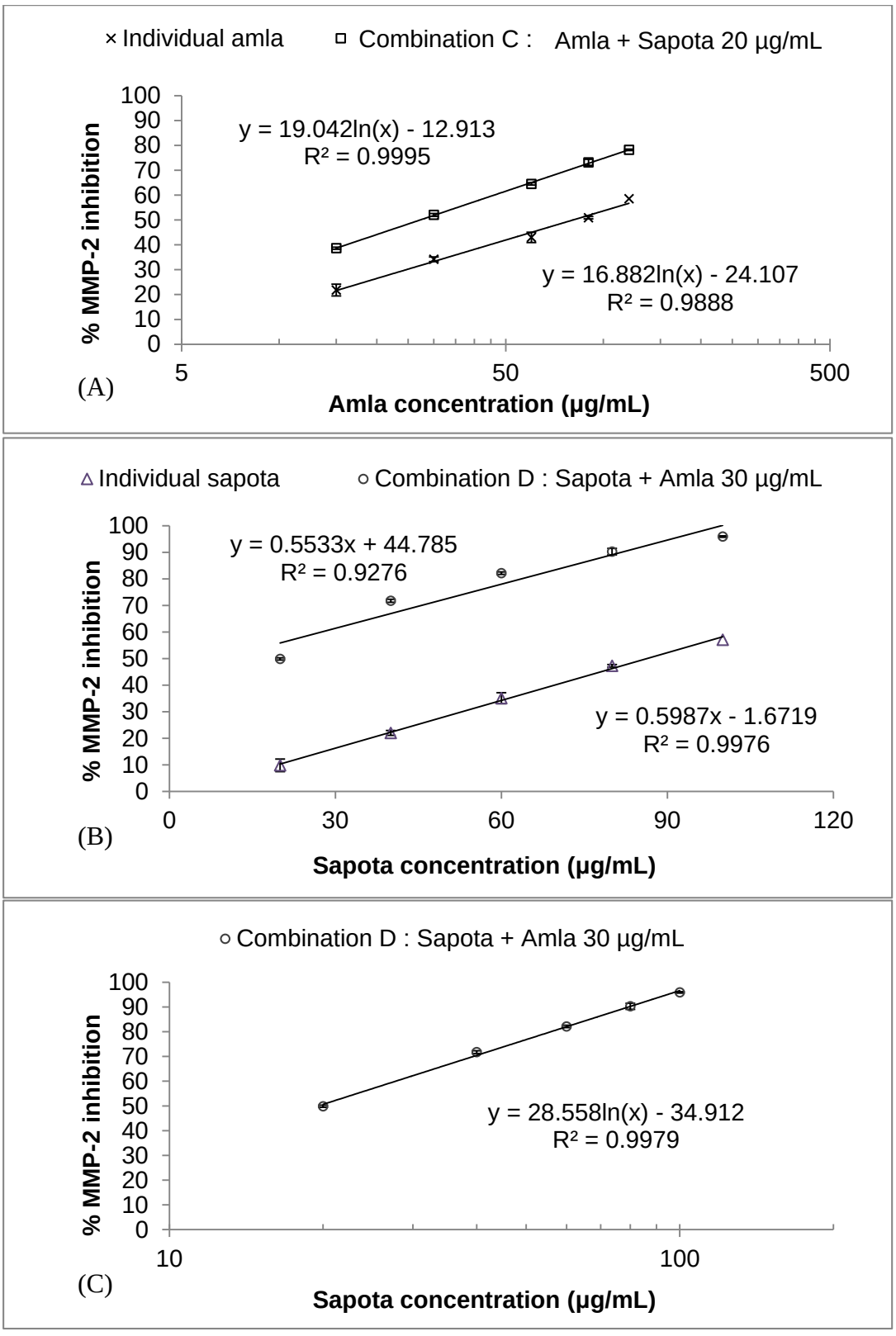


Figure 27 Effect of individual extracts and combination on anti-MMP-2 activity; combination C (A) and combination D in linear scale (B) and log scale (C)

If we separately used the extracts at IC_{50}, the summation of % inhibitions from individual extract in combination C was approximately 43 % but when combined both extracts at the given concentrations could achieve 50 % inhibition which clearly showed an advantage of combination.

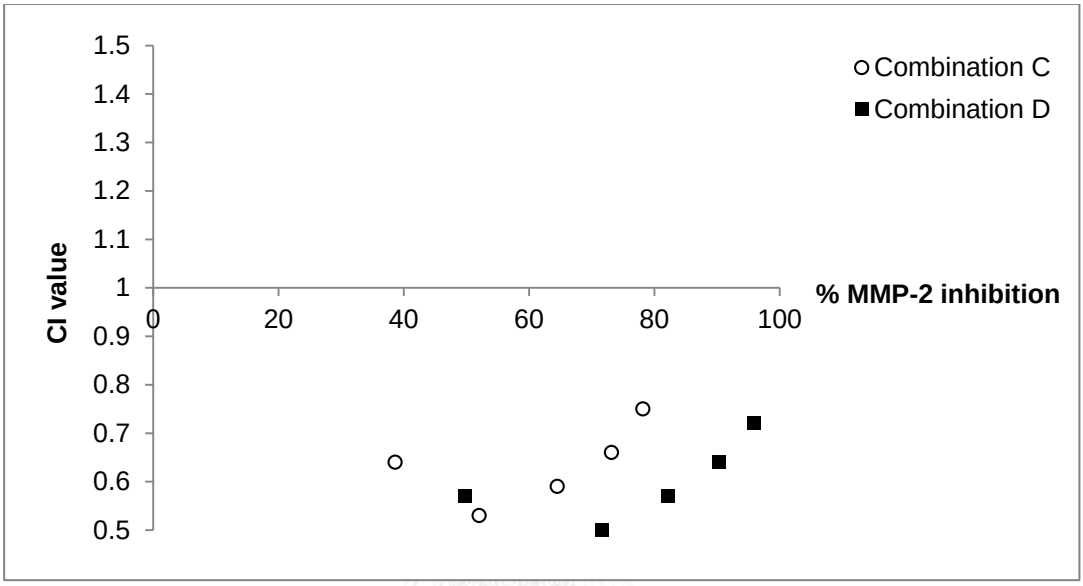


Figure 28 MMP-2 inhibition - CI indexes for all combination ratios of combination C and D

In agreement with 1,10 phenanthroline results, the MMP-2 inhibition by the combined extracts seemed easier than the MMP-1 inhibition regarding to lower concentration was required to achieve the same % inhibition (seen in Table 8 and 9). These findings may also support that more complex structure of MMP-1 could hinder the interaction with the test sample when compared with MMP-2.

In summary, both combinations' CI values were categorized as synergist effect for anti-MMP-1 and anti-MMP-2 at all combination ratios. There was an advantage of combining two extracts when considered anti-collagenase activity. On the other hand, positive effect could also be assumed when combining the extracts.

4.3 Elastase assay

In combination A, sapota concentration was fixed at 20 μg/mL provided about 18.74 % elastase inhibition. Fixed 20 μg/mL sapota was mixed with various

concentrations of amla ranging from 100-500 µg/mL. Total concentrations of the combination A were between 120-520 µg/mL (Figure 29). The IC_{50} of the combination A was 131.32 ± 24.89 µg/mL (Table 10) which contained amla and sapota extracts approximately at 111.32 and 20 µg/mL, respectively.

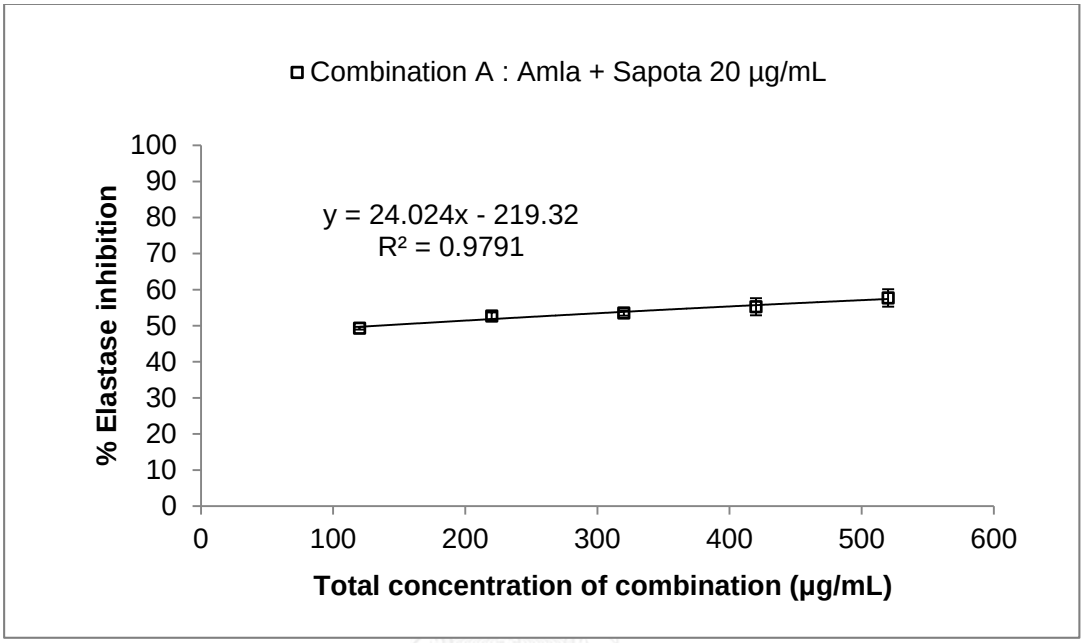


Figure 29 Effect of combination A on elastase inhibition

Table 10 Combination indexes (CIs) of the combination including the IC_{50} of individual and combination extracts on anti-elastase activity (Mean ± SD)

Combination	Extract	IC_{50} of Individual (µg/mL)	IC_{50} of combination (µg/mL)	CI index at IC_{50} of combination	Type of interaction
A	Amla	520.83 ± 18.71	131.32 ± 24.89*	0.76 ± 0.04	synergism
	Sapota	36.40 ± 0.45			
B	Amla	520.83 ± 18.71	120.04 ± 0.33**	0.74 ± 0.01	synergism
	Sapota	36.40 ± 0.45			

*** contained amla and sapota extracts at 111.32 + 24.89 and 20 µg/mL, respectively**

**** contained amla and sapota extracts at 100 and 20.04 ± 0.33 µg/mL, respectively**

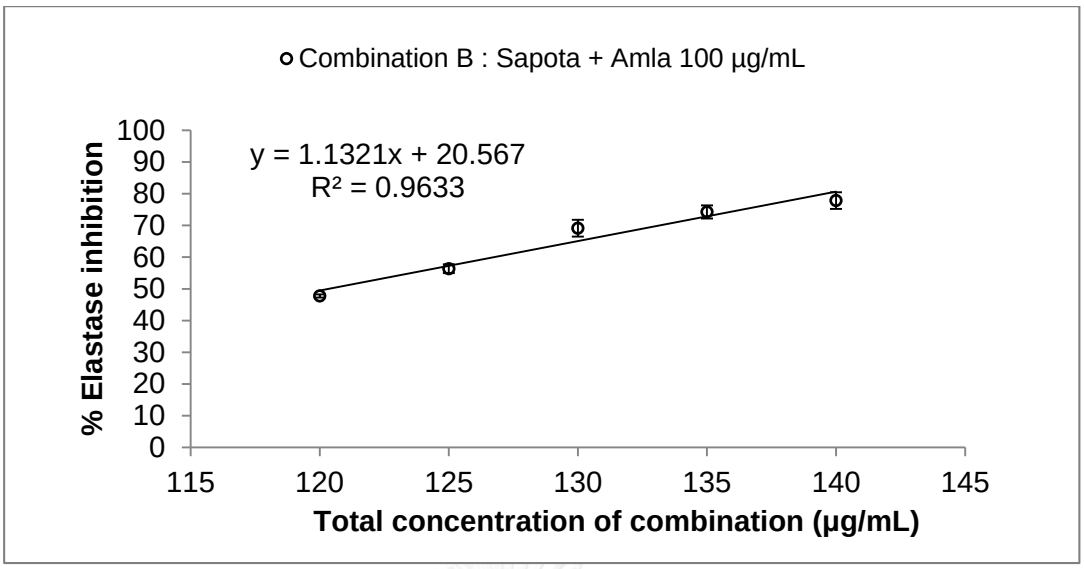


Figure 30 Effect of combination B on elastase inhibition

In combination B, amla was fixed at 100 µg/mL providing about 12.74 % inhibition. It was mixed with various concentrations of sapota range from 20-45 µg/mL, which provided total concentrations of the combination ranging from 120-145 µg/mL (Figure 30). The IC_{50} of combination B was at 120.04 $\pm$ 0.33 µg/mL (Table 10) which contained amla and sapota approximately at 100 and 20.04 $\pm$ 0.33 µg/mL, respectively.

According to Figure 31, unparalleled-lines showed that elastase activities of combination A were changed with different extents along the studied concentration range. The % elastase inhibitions of combination A slightly increased and reached a plateau whereas % inhibitions of combination B increased with similar extent along the studied combination ratio range. The median effect CI calculated at 50 % inhibition showed 0.76 ± 0.04 for combination A and showed 0.74 ± 0.01 for combinations B (Table 10). These could imply that at the median effect, both combinations A and B showed synergist effects. On the other word, if we separately used the extracts at IC_{50}, the summation of % inhibitions from individual extracts approximately 33 % inhibition for combination A and 32 % inhibition for combination B but when combined both extracts at the given concentrations of combinations A and B could achieve 50 % inhibition which clearly showed an advantage of combination. Regards to other ratios of combination A (Table 11 and Figure 32), CI values became more than 1 or presenting antagonist effect when the

concentration of amla exceeded 300 µg/mL, increasing amla interfered the anti-elastase activity of sapota which considered unwanted effect when combining extracts. In contrast, the combination B at all combination ratios (Figure 32) significantly revealed average CI indexes less than 1 or synergist effect (p-value < 0.05) so increasing sapota concentration still maintained the advantages of mixing. In conclusion, the combination ratios played a significant effect on the anti-elastase of mixing effects and should be taken into the consideration when formulating a product.

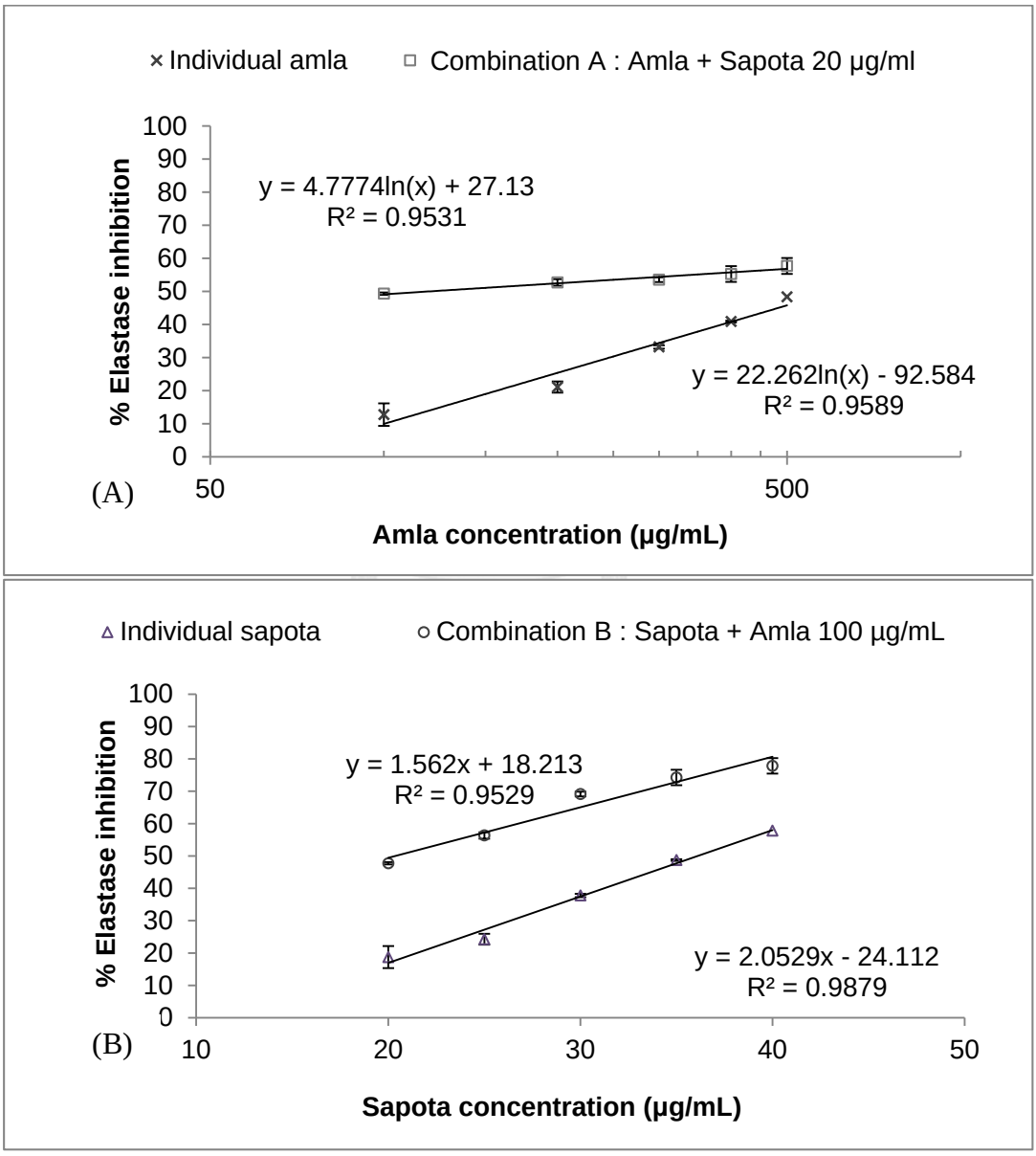


Figure 31 Effect of individual extracts and combination A (A) or combination B (B) on anti-elastase activity

Table 11 Combination indexes (CIs) of the combination including the IC_{50} of individual and combination extracts on anti-elastase activity (Mean ± SD)

Concentration (µg/mL)			Average %inhibition	SD	Average CI index	Type of interaction
Total	Amla	Sapota				
Combination A						
120	100	20	49.31	0.37	0.75*	synergism
220	200	20	52.71	1.00	0.89*	synergism
320	300	20	53.55	0.73	1.06*	antagonism
420	400	20	55.26	2.38	1.20*	antagonism
520	500	20	57.70	2.41	1.32*	antagonism
Combination B						
120	100	20	47.76	0.51	0.77*	synergism
125	100	25	56.36	1.38	0.80*	synergism
130	100	30	69.14	2.65	0.79*	synergism
135	100	35	74.26	2.09	0.85*	synergism
140	100	40	77.85	2.64	0.92*	synergism

*significantly different from CI = 1, p-value < 0.05

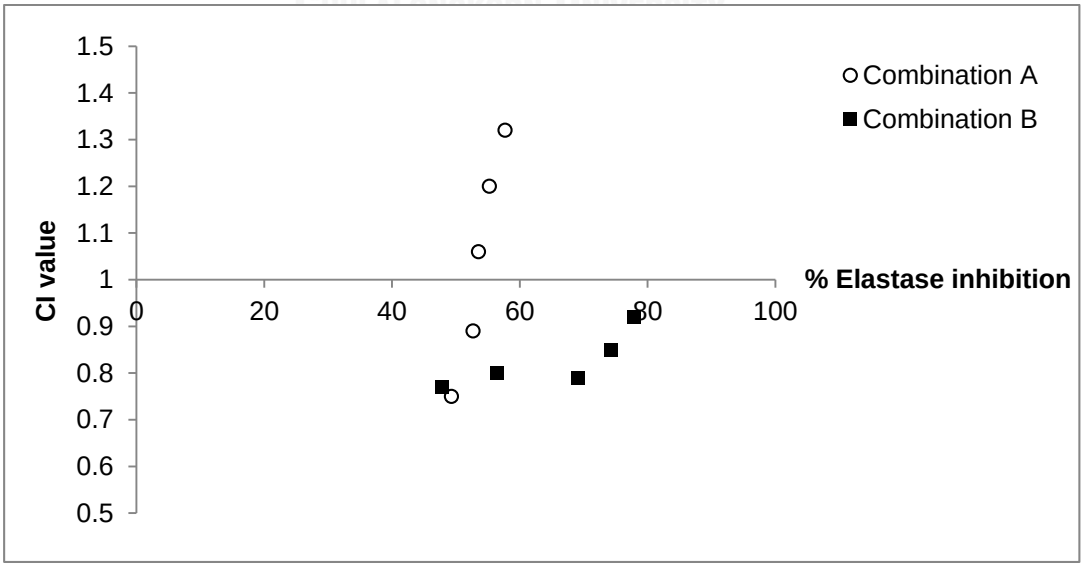


Figure 32 Elastase inhibition - CI indexes for all combination ratios of combination A and B

4.4 Total phenolic content of the combination A and B

The total phenolic of each combination ratio at median effect (IC_{50}) were determined. The content of amla in the combination provided strong correlation to TPC which higher amla content tended to show higher TPC value (Figure 33). For example, amla: sapota ratios at 5:1 and 1:5 contained TPC of 325 and 107 mg GAE/g (Table 12), respectively. TPC did not correlate with the potential of collagenase or elastase inhibition. For instance, difference TPC values showed the same % inhibition effect (50 % inhibition) on MMP-1. Therefore, TPC and DPPH free radical results were not sufficient to conclude anything about anti-collagenase or anti-elastase activities.

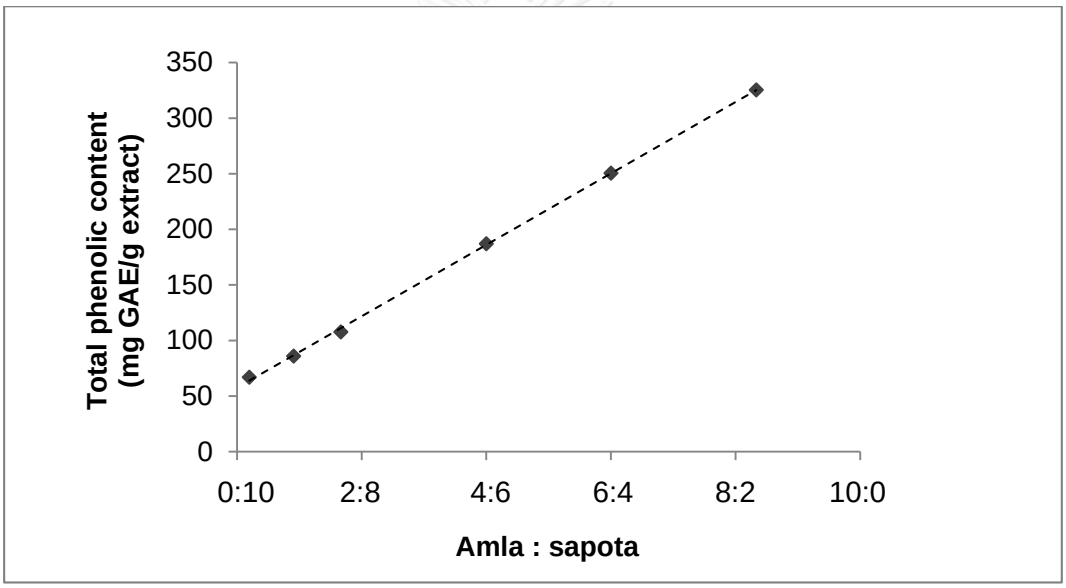


Figure 33 The correlation of total phenolic content and ratio of amla : sapota content in the combination

In conclusion, combining extracts showed influences on antioxidant, anti-collagenase, and anti-elastase activities. Some activity was the summation of each extract activities like DPPH, where some activity like anti-collagenase showed synergist at all combination ratios. On the other hand, anti-elastase activity also affected by the combination but depending on the combination ratios. Some ratios showed synergist but some showed opposite. With the knowledge gained from the present study could change mindset about simply mixing two extracts together to

achieve cosmetics development goals. More specific tests may be required to assure the success.

Table 12 Total phenolic content of the combination at median effect (mean ± SD)

Assay	Median ratio of combination (Amla : sapota)	TPC (mg GAE/g extract)
DPPH	A (1:10)	85.92 ± 2.92
	B (1:50)	66.89 ± 3.43
MMP-1	A (1:5)	107.61 ± 0.80
	B (2:3)	186.97 ± 3.65
MMP-2	C (3:2)	250.49 ± 4.02
	D (3:2)	250.49 ± 4.02
Elastase	A (5:1)	325.36 ± 8.52
	B (5:1)	325.36 ± 8.52

CHAPTER IV

CONCLUSIONS

Anti-aging effect of skin becomes more interested in the future. Wrinkle and sagging are caused by the depletion of collagen and elastin which collagenase and elastase play a pivot role in the degradation. Tons of natural extracts have been screened for anti-aging properties. There is ‘no all in one’ plant that complete all wanted effects. Amla showed potential antioxidant and anti-collagenase while sapota showed the best anti-collagenase and elastase among three extracts. Silymarin also showed antioxidant and anti-elastase but lower than amla and sapota, respectively. Therefore, the overall anti-aging effect could be obtained by combining extracts. The mixture of amla and sapota at proper ratio provided advantages over the use of single extract since the combination showed comparable antioxidant activity and the synergist effects on anti-collagenase and anti-elastase activities. The mechanism of this synergism remains unknown and the determination of phytochemical is required to explain the interaction of the extracts.

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Appendix A
Test kits information

I. EnzChek® Gelatinase/ Collagenase Assay kit (E-12055) contains

a) 10X Reaction buffer consist of 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6

b) DQ™ gelatin from pig skin, a fluorescein conjugated substrate (MMP-2 substrate)

c) DQ™ collagen type I (MMP-1 substrate)

d) Collagenase type IV from *Clostridium histolyticum*, pH 7.5

e) 1,10-Phenanthroline, monohydrate (MW = 198.2), a general metalloproteinase inhibitor

Reagent preparation

1. Preparation of 1X reaction buffer

1X reaction buffer was prepared by diluting 20 mL of 10X reaction buffer in 180 mL deionized water.

2. Preparation of DQ™ gelatin and collagen type I substrate

A DQ™ gelatin vial (1 mg) was directly added 1 mL deionized water. 125 μg/mL DQ™ gelatin was prepared by adding 1 mg/mL substrate into 7 mL of 1X reaction buffer. 20 μL of this substrate solution was used in each 200 μL well to obtain 12.5 μg/mL as a final concentration. Reconstituted DQ™ gelatin was stored in dark at $4^{O}C$ (do not freeze). DQ™ collagen type I was prepared at the same concentration and method as DQ™ gelatin.

3. Preparation of *Clostridium* collagenase solution

A 1000 U/mL stock solution was prepared by dissolving the contents of enzyme vial (500 Unit) in 0.5 mL deionized water. Then, 0.4 U/mL of working solution was obtained by adding 20 μL of this stock solution into 50 mL of 1X

reaction buffer. 100 µL of this solution was used in an assay to obtain 0.2 U/mL as a final concentration in each well.

4. Preparation of 1,10 phenanthroline as a general metalloproteinase inhibitor

9.9 mg of 1,10 phenanthroline was weighted and dissolved with 25 µL ethanol. A 10 mM working solution was prepared by adding 10 µL of this solution to 2 mL of 1X reaction buffer. This inhibitor solution was further diluted with 1X reaction buffer to obtain final concentration at 0.05, 0.075, 0.1, 0.25 mM in a well plate.

II. EnzChek® elastase assay kit (E-12056) contains

a) 10X Reaction buffer consist of 1 M Tris-HCl, pH 8, and 2 mM sodium azide

b) DQ™ elastin from bovine neck ligament, a fluorescein conjugated substrate

c) Elastase from pig pancreas, pH 7.5

d) N-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MW = 503), an inhibitor of elastase

Reagent preparation

1. Preparation of 1X reaction buffer

1X reaction buffer was prepared by diluting 20 mL of 10X reaction buffer in 180 mL deionized water.

2. Preparation of DQ™ elastin substrate solution

A DQ™ elastin vial (1 mg) was directly added 1 mL deionized water. 100 µg/mL DQ™ elastin was prepared by adding 1 mg/mL substrate into 9 mL of 1X reaction buffer. 50 µL of this substrate solution was used in each well to obtain 25 µg/mL as a final concentration. Reconstituted DQ™ elastin was stored in dark at 4 °C (do not freeze).

3. Preparation of elastase solution

A 100 U/mL stock solution was prepared by dissolving the contents of enzyme vial (50 unit) in 0.5 mL deionized water. Then, 0.4 U/mL of working solution was obtained by adding 100 µL of this stock solution into 25 mL of 1X reaction buffer. 100 µL of this solution was used in each well to obtain 0.2 U/mL as a final concentration.

4. Preparation of 1 mM *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone as an elastase inhibitor

50 µL of DMSO was added into 500 µg of the inhibitor vial. (Reconstituted inhibitor can be kept in -20 ^{o}C for three month.) 1 mM working solution was obtained by mixing 20 µL of this solution to 380 µL of 1X reaction buffer. Then, this inhibitor solution was further diluted with 1X reaction buffer to 0.0004, 0.004, 0.04 mM in order to evaluate the optimum incubation time.

Appendix B

Standard curve

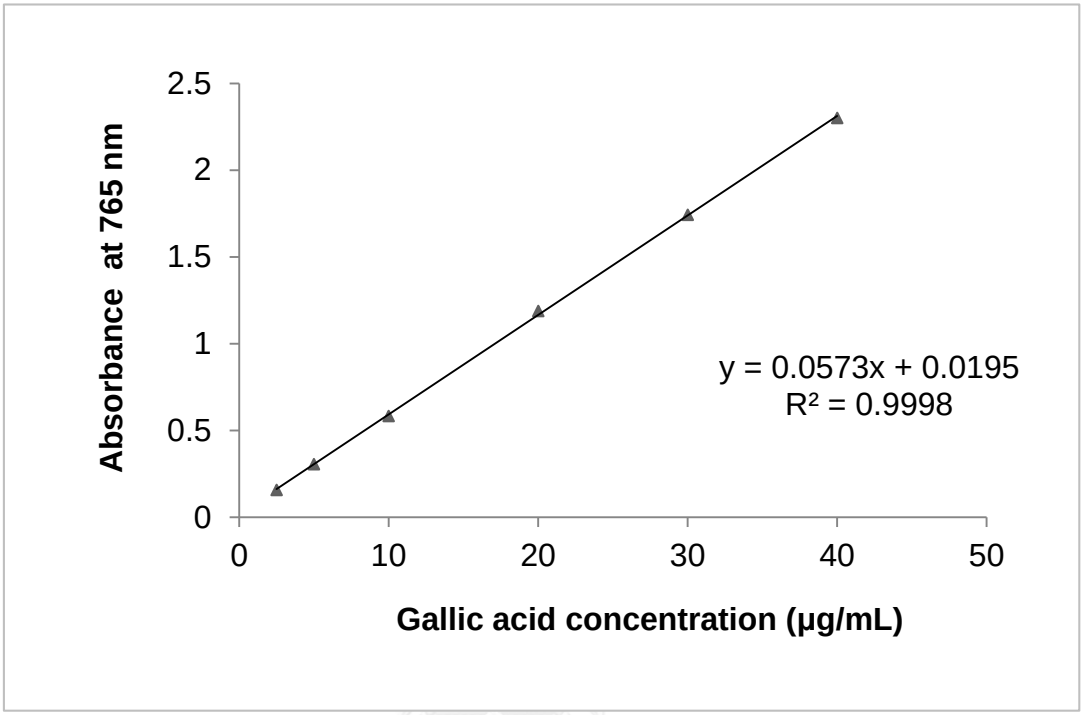

Figure 34 Gallic acid standard curve

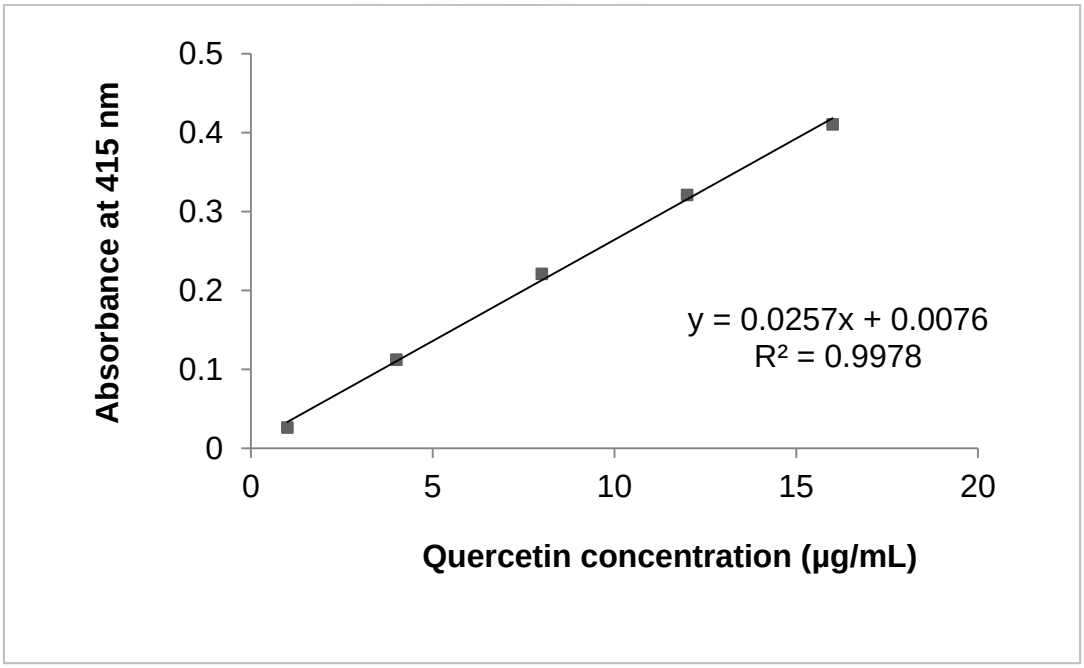

Figure 35 Quercetin standard curve

Appendix C

Raw data

Table 13 Collagenase inhibitory profiles of 1,10 phenanthroline with 12.5 µg/mL and 100 µg/mL DQTM gelatin substrate concentration

1,10 phenanthroline	% Fluorescent intensity, DQ gelatin at	
Concentration	100 µg/mL	12.5 µg/mL
0.05	60.72	32.85
0.075	37.27	22.48
0.1	21.66	10.97
0.25	7.68	3.88
0.5	6.43	3.27
0.75	6.07	2.24

Table 14 MMP-1 (A) and MMP-2 (B) inhibitions of 1,10 phenanthroline at different incubation times

1,10 phenanthroline concentration (mM)	% MMP-1 inhibition at different incubation times				% MMP-2 inhibition at different incubation times			
	30 min	60 min	90 min	120 min	30 min	60 min	90 min	120 min
0.05	57.35	57.35	62.47	63.26	67.14	67.66	68.52	68.25
0.075	58.62	58.62	66.13	70.86	77.52	79.25	80.76	81.56
0.1	75.23	75.23	79.33	82.98	89.02	90.04	90.66	90.95
0.25	62.36	62.36	80.72	87.04	96.12	96.78	97.66	97.91

Table 15 Comparative inhibitory profiles between MMP-1 and MMP-2 by 1,10 phenanthroline at 90-minute incubation time

1,10 phenanthroline concentration (mM)	% MMP-1 inhibition at 90-minute incubation time	% MMP-2 inhibition at 90-minute incubation time
0.05	62.47	68.52
0.075	66.13	80.76
0.1	79.33	90.66
0.25	80.72	97.66

Table 16 Elastase inhibitions of *N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl* ketone (CMK) at different incubation times

CMK concentration (mM)	% Elastase inhibition at different incubation times			
	30 min	60 min	90 min	120 min
0.0001	18.20	19.92	21.33	20.86
0.001	80.01	83.07	85.68	86.91
0.01	99.06	99.78	99.80	99.78
0.1	99.41	99.61	99.64	99.69

Table 17 % MMP-2 inhibitions of extracts at fixed concentration (150 µg/mL), 0.1 mM 1,10 phenanthroline, and EGCG (15 µg/mL) presented as mean + SD, n=3

Concentration	% MMP-2 inhibition				
	150 µg/mL			0.1 mM	15 µg/ml
	Amla	Sapota	Silymarin	1,10 phenanthroline	EGCG
N1	64.02	72.03	55.89	87.26	88.45
N2	63.46	75.85	57.69	78.23	86.84
N3	66.37	73.49	56.36	84.79	86.97
Average	64.62	73.79	56.65	83.43	87.42
SD	1.54	1.93	0.94	4.67	0.89

Table 18 Correlation - between total phenolic content of amla (mg GAE/g extract) and % DPPH inhibition (A); between total phenolic content of three extracts and IC_{50} of DPPH scavenging assay (B)

	Extracts	Concentration (μg/mL)	Total phenolic content (mg GAE/g extract)	% DPPH inhibition
A	Amla	0.5	0.91	17.62
		1	1.81	32.61
		1.5	2.72	45.32
		2	3.62	58.89
		2.5	4.53	71.26
		3	5.44	81.40

	Extracts	N	Total phenolic	IC_{50} DPPH
B	Sapota	N1	40.74	37.66
		N2	38.06	38.80
		N3	36.89	36.43
	Silymarin	N1	220.74	27.91
		N2	242.22	26.85
		N3	237.14	28.80
	Amla	N1	355.76	1.78
		N2	360.93	1.67
		N3	370.60	1.66

Table 19 Effect of ethanolic amla extract (A), ethanolic sapota extracts (B) and silymarin (C) on DPPH inhibition activity

Concentration (µg/mL)		% DPPH inhibition			Average	SD
		N1	N2	N3		
Amla	0.5	18.13	17.92	16.80	17.62	0.71
	1	30.46	32.81	34.56	32.61	2.06
	1.5	42.38	45.05	48.52	45.32	3.08
	2	56.22	61.02	59.44	58.89	2.45
	2.5	68.89	72.77	72.12	71.26	2.08
	3	79.86	82.45	81.89	81.40	1.37
	IC_{50}	1.78	1.67	1.66	1.70	0.06
Sapota	10	18.91	15.39	21.31	18.54	2.98
	20	30.60	27.40	29.85	29.28	1.67
	30	42.24	40.82	42.38	41.81	0.86
	40	51.26	52.99	53.37	52.54	1.13
	50	60.94	65.33	66.48	64.25	2.92
	IC_{50}	38.80	37.66	36.43	37.63	1.18
Silymarin	10	23.16	27.23	23.73	24.71	2.21
	20	38.83	40.47	41.74	40.35	1.46
	30	52.66	55.77	54.04	54.16	1.56
	40	66.12	67.81	66.41	66.78	0.90
	50	77.34	79.45	78.03	78.28	1.08
	IC_{50}	28.80	26.85	27.91	27.85	0.98
Ascorbic acid	0.5	20.95	16.81	18.08	18.61	2.12
	1	37.61	35.29	38.24	37.05	1.55
	1.5	58.70	56.15	56.34	57.06	1.42
	2	73.34	69.93	72.78	72.01	1.83
	2.5	89.31	84.86	84.19	86.12	2.78
	IC_{50}	1.33	1.42	1.38	1.38	0.05

Table 20 Effect of ethanolic amla extract (A), ethanolic sapota extract (B) and silymarin (C) on MMP-2 activity

Concentration (µg/mL)		% MMP-2 inhibition			Average	SD
		N1	N2	N3		
Amla	30	36.27	31.65	34.73	34.22	2.35
	60	43.28	41.96	43.76	43.00	0.93
	90	48.85	50.89	53.00	50.91	2.08
	120	59.05	58.53	58.12	58.57	0.47
	150	64.02	63.46	66.37	64.62	1.54
	180	70.49	73.35	72.06	71.97	1.43
	IC_{50}	89.24	92.63	86.10	89.41	3.26
Sapota	10	7.60	3.94	4.94	5.49	1.89
	20	10.63	9.95	8.90	9.83	0.87
	40	22.11	21.12	22.77	22.00	0.83
	60	37.85	33.33	34.08	35.09	2.43
	80	49.55	47.49	44.83	47.29	2.37
	100	59.08	55.04	57.04	57.05	2.02
	IC_{50}	82.98	87.99	88.45	86.43	3.03
Silymarin	50	24.14	26.31	25.72	25.39	1.12
	100	41.49	42.38	40.48	41.45	0.95
	150	55.89	57.69	56.36	56.65	0.94
	200	70.72	66.05	68.14	68.30	2.34
	250	82.77	79.50	79.55	80.61	1.88
	IC_{50}	132.91	133.14	135.02	133.69	1.16
EGCG	5	32.50	29.22	30.23	30.65	1.67
	10	62.76	60.72	61.59	61.69	1.02
	15	88.45	86.84	87.43	87.57	0.81
	20	98.47	98.32	98.51	98.43	0.10
	IC_{50}	7.90	8.48	8.11	8.19	0.40

Table 21 Effect of ethanolic amla extract (A), ethanolic sapota extract (B) and silymarin (C) on elastase activity

Concentration (µg/mL)		% Elastase inhibition			Average	SD
		N1	N2	N3		
Amla	100	13.7	9.56	14.97	12.74	2.83
	200	23.71	17.23	22.31	21.08	3.41
	300	34.35	34	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	52.6	55.26	2.31
	IC_{50}	508.36	511.78	542.35	520.83	18.71
Sapota	25	19.88	21.12	22.3	21.1	1.21
	30	28.82	36.94	31.88	32.55	4.09
	35	46.08	49.86	45.79	47.25	2.26
	40	64.06	67.77	67.84	66.56	2.16
	45	71.57	71.36	74.28	72.4	1.62
	IC_{50}	36.41	35.23	35.56	35.73	0.61
Silymarin	30	31.09	30.84	33.96	31.96	1.734
	35	41.81	39.80	38.91	40.17	1.488
	40	52.37	53.51	52.92	52.93	0.57
	45	67.17	68.82	64.83	66.94	2.00
	50	73.05	74.05	74.14	73.74	0.67
	IC_{50}	38.58	38.52	38.61	38.57	0.04
EGCG	20	20.69	20.60	19.43	20.24	0.71
	40	30.17	31.95	25.37	29.16	3.40
	80	49.53	43.21	43.77	45.51	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 22 Effect of combination A on DPPH scavenging activity

Concentration (µg/mL)			% DPPH inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination A								
10.5	0.5	10	32.04	28.28	28.99	29.77	2.00	1.04
11	1	10	49.31	42.08	49.54	46.98	4.24	0.96
11.5	1.5	10	64.67	54.83	59.11	59.54	4.93	1.00
12	2	10	73.79	66.43	70.87	70.36	3.71	1.05
12.5	2.5	10	82.93	74.21	77.27	78.14	4.42	1.13

Table 23 Effect of combination B on DPPH scavenging activity

Concentration (µg/mL)			% DPPH inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination B								
10.5	0.5	10	30.81	28.97	28.29	29.36	1.30	1.06
20.5	0.5	20	47.30	43.88	41.32	44.17	3.00	0.98
30.5	0.5	30	59.56	59.14	62.85	60.52	2.03	0.92
40.5	0.5	40	68.4	65.63	66.34	66.79	1.44	1.02
50.5	0.5	50	73.88	74.22	75.85	74.65	1.05	1.09

Table 24 Effect of combination A on MMP-1 inhibition

Concentration (µg/mL)			% MMP-1 inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination A								
70	10	60	44.92	48.15	49.11	47.39	44.92	0.82*
90	30	60	64.50	65.47	66.23	65.40	64.50	0.67*
120	60	60	76.05	79.27	79.75	78.36	76.05	0.58*
150	90	60	85.61	86.76	85.28	85.88	85.61	0.55*
180	120	60	90.39	91.81	89.88	90.70	90.39	0.53*

* significantly different from CI = 1, p-value < 0.05

Table 25 Effect of combination B on MMP-1 inhibition

Concentration (µg/mL)			% MMP-1 inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination B								
50	30	20	32.91	32.30	37.37	34.19	2.77	0.96
70	30	40	48.66	45.87	47.10	47.21	1.40	0.83*
90	30	60	61.09	61.01	59.89	60.67	0.67	0.74*
110	30	80	75.35	74.18	71.41	73.65	2.02	0.72*
130	30	100	85.65	89.42	83.84	86.30	2.85	0.73*
150	30	120	98.59	98.37	98.07	98.35	0.26	0.75*

* significantly different from CI = 1, p-value < 0.05

Table 26 Effect of combination C on MMP-2 inhibition

Concentration (µg/mL)			% MMP-2 inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination C								
35	15	20	39.07	38.22	38.64	38.65	0.42	0.64*
50	30	20	51.49	52.02	52.65	52.05	0.58	0.53*
80	60	20	64.85	63.96	64.71	64.51	0.48	0.59*
110	90	20	73.86	71.77	73.90	73.18	1.22	0.66*
140	120	20	78.09	78.52	77.97	78.19	0.29	0.75*

* significantly different from CI = 1, p-value < 0.05

Table 27 Effect of combination D on MMP-2 inhibition

Concentration (µg/mL)			% MMP-2 inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination D								
50	30	20	50.73	49.04	49.79	49.85	0.85	0.57*
70	30	40	72.47	69.61	73.14	71.74	1.88	0.50*
90	30	60	81.33	80.17	84.91	82.14	2.47	0.57*
110	30	80	91.28	87.20	92.35	90.28	2.71	0.64*
130	30	100	96.58	94.16	97.03	95.92	1.54	0.72*

*significantly different from CI = 1, p-value < 0.05

Table 28 Effect of combination A on elastase inhibition

Concentration (µg/mL)			% Elastase inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination A								
120	100	20	49.28	48.96	49.7	49.31	0.37	0.75*
220	200	20	51.59	53.02	53.51	52.71	1.00	0.89*
320	300	20	53.1	54.39	53.15	53.55	0.73	1.06*
420	400	20	53.25	54.98	52.55	55.26	2.38	1.20*
520	500	20	58.4	55.68	55.02	57.70	2.41	1.32*

*significantly different from CI = 1, p-value < 0.05

Table 29 Effect of combination B on elastase inhibition

Concentration (µg/mL)			% Elastase inhibition			Average	SD	Average CI index
Total	Amla	Sapota	N1	N2	N3			
Combination B								
120	100	20	47.62	48.32	47.33	47.76	0.51	0.77*
125	100	25	56.71	54.83	57.53	56.36	1.38	0.80*
130	100	30	67.75	72.19	67.47	69.14	2.65	0.79*
135	100	35	74.05	76.45	72.29	74.26	2.09	0.85*
140	100	40	80.28	78.23	75.05	77.85	2.64	0.92*

*significantly different from CI = 1, p-value < 0.05

Table 30 The correlation of total phenolic content and ratio of amla : sapota content in the combination

Ratio of amla : sapota	Total phenolic content of the combination (mg GAE/g extract)				
	N1	N2	N3	Average	SD
1:10	88.68	82.86	86.24	85.93	2.92
1:50	69.35	68.35	62.97	66.89	3.43
1:5	107.27	108.52	107.03	107.61	0.80
2:3	189.56	182.79	188.55	186.97	3.65
3:2	252.28	245.88	253.32	250.49	4.03
5:1	326.01	333.53	316.54	325.36	8.52

VITA

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