ฤทธิ์ชะลอวัยของสารสกัดจากกลีบดอกอัญชันสีม่วง
Anti-aging Activity of Mauve *Clitoria Ternatea* L. Petal Extract

นิพนธ์ต้นฉบับ **Original Article**

ชนกานต์ ชีวบันเทิง[1], ชญานิศ ศรชัยธรัชวงศ์[2] และ สริน ทัดทอง[2]*

[1] นิสิตบัณฑิตศึกษา คณะเภสัชศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ องครักษ์ จ.นครนายก 26120
[2] สาขาวิชาเภสัชเคมี คณะเภสัชศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ องครักษ์ จ.นครนายก 26120

*** Corresponding author:** sarin@g.swu.ac.th

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Chanakan Cheewabanthoeng[1], Chayanid Sornchaithawatwong[2] and Sarin Tadtong[2]*

[1] Graduate Student, Facultyof Pharmacy, Srinakharinwirot University, Ongkharak, Nakhonnayok, 26120, Thailand
[2] Department of Pharmaceuical Chemistry, Facultyof Pharmacy, Srinakharinwirot University, Ongkharak, Nakhonnayok, 26120, Thailand

*** Corresponding author:** sarin@g.swu.ac.th

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ชะลอวัยของสารสกัดจากกลีบดอกอัญชันสีม่วง โดยทดสอบการยับยั้งเอนไซม์อีลาสเตส เอนไซม์คอลลาจีเนส และเอนไซม์ไฮยาลูโรนิเดส และวิเคราะห์หาปริมาณรวมของสารกลุ่มฟีนอลิก สารกลุ่มฟลาโวนอยด์ และสารกลุ่มแอนโทไซยานินในสารสกัดจากกลีบดอกอัญชันสีม่วง **วิธีการศึกษา:** เตรียมสารสกัดจากกลีบดอกอัญชันสีม่วงโดยการสกัดผงกลีบดอกอัญชันสีม่วงด้วย 80% เอทานอล และระเหยเอทานอลออกด้วยเครื่องกลั่นระเหยสารแบบหมุน ทดสอบฤทธิ์ชะลอวัย โดยรายงานผลเป็นค่าความเข้มข้นของสารที่สามารถยับยั้งการทำงานของเอนไซม์ได้ร้อยละ 50 (IC_{50}) ซึ่งทดสอบการยับยั้งเอนไซม์อีลาสเตสและเอนไซม์คอลลาจีเนสด้วยวิธี spectrophotometry โดยใช้กรดแกลลิกเป็นกลุ่มควบคุมเชิงบวก ทดสอบการยับยั้งเอนไซม์ไฮยาลูโรนิเดสด้วยวิธี colorimetry โดยใช้เควอเซทินเป็นกลุ่มควบคุมเชิงบวก วิเคราะห์หาปริมาณรวมของสารกลุ่มฟีนอลิก สารกลุ่มฟลาโวนอยด์ และสารกลุ่มแอนโทไซยานินด้วยวิธี Folin-Ciocalteu method, aluminium chloride complexation colorimetric method และ pH differential method ตามลำดับ **ผลการศึกษา:** สารสกัดกลีบดอกอัญชันสีม่วงมีค่า IC_{50} ในการยับยั้งเอนไซม์อีลาสเตส เอนไซม์คอลลาจีเนส และเอนไซม์ไฮยาลูโรนิเดสเท่ากับ 2.95, 3.37 และ 12.67 mg/ml ตามลำดับ และมีปริมาณรวมของสารกลุ่มฟีนอลิก 27.84 mg GAE, สารกลุ่มฟลาโวนอยด์ 3.47 mg QE, และสารกลุ่มแอนโทไซยานิน 0.02 mg CE ในสารสกัดกลีบดอกอัญชัน 1 กรัม **สรุป:** สารสกัดจากกลีบดอกอัญชันสีม่วงมีฤทธิ์ชะลอวัย โดยการยับยั้งเอนไซม์อีลาสเตส เอนไซม์คอลลาจีเนส และเอนไซม์ไฮยาลูโรนิเดส และพบสารกลุ่มฟีนอลิก สารกลุ่มฟลาโวนอยด์ และสารกลุ่มแอนโทไซยานินในสารสกัดจากกลีบดอกอัญชันสีม่วง ซึ่งฤทธิ์ชะลอวัยของสารสกัดจากกลีบดอกอัญชันสีม่วงน่าจะเป็นผลจากสารกลุ่มฟีนอลิกเนื่องจากพบปริมาณสารกลุ่มฟีนอลิกในสารสกัดมากที่สุด

คำสำคัญ: ฤทธิ์ชะลอวัย, ฤทธิ์ยับยั้งเอนไซม์อีลาสเตส, ฤทธิ์ยับยั้งเอนไซม์คอลลาจีเนส, ฤทธิ์ยับยั้งเอนไซม์ไฮยาลูโรนิเดส, สารสกัดจากกลีบดอกอัญชันสีม่วง

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Abstract

Objective: To investigate anti-aging activity of mauve *Clitoria ternatea* L. (commonly known as butterfly pea) petal extract including anti-elastase, anti-collagenase, and anti-hyaluronidase, and to determine the contents of total phenolic, total flavonoid, and total anthocyanin. **Methods:** The extract was prepared by soaking powdered mauve *C. ternatea* L. petal in 80% ethanol, then filtered and concentrated by a rotary evaporator. Anti-elastase and anti-collagenase activities were determined by spectrophotometry with gallic acid as a positive control. Anti-hyaluronidase was determined by colorimetry with quercetin as a positive control and anti-aging activity was reported as the half maximal inhibitory concentration (IC_{50}). The total phenolic content, total flavonoid content, and total anthocyanin content were determined by Folin-ciocalteu method, aluminium chloride complexation colorimetric method, and pH differential method, respectively. **Results:** Anti-elastase, anti-collagenase, and anti-hyaluronidase activities of mauve *C. ternatea* L. petal extract were exhibited with IC_{50} values of 2.95, 3.37, and 12.67 mg/ml, respectively. The total phenolic, total flavonoid, and total anthocyanin were 27.84 mg GAE/g, 3.47 mg QE/g, and 0.02 mg CE/g of the extract, respectively. **Conclusion:** Mauve *C. ternatea* L. petal extract possessed anti-aging activity by inhibiting activities of elastase, collagenase, and hyaluronidase. It contained phenolic compounds, flavonoids, and anthocyanins. The anti-aging activity of mauve *C. ternatea* L. petal extract might be the effect of phenolic compounds since it was found the highest amount in the extract.

Keywords: anti-aging activity, anti-elastase activity, anti-collagenase activity, anti-hyaluronidase activity, mauve *Clitoria ternatea* L. petal extract

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Introduction

Skin is a part of organs in the body. Its main function is to protect the internal organs from the external environment including physical, chemical, and biological agents. It is divided into three layers: the epidermis, the dermis, and subcutaneous tissue. The extracellular matrix (ECM) is the largest component of the dermis and provides a structural framework essential for the growth and elasticity of the skin. ECM is composed of elastin, collagen, fibronectin, and hyaluronic acid.[1] The skin is damaged by extrinsic factors (UV radiation, environmental pollutants, etc.) and intrinsic factors (hormones, age-dependent, oxidation, etc.). Both factors cause the increase in activity of matrix metalloproteinases (MMPs) such as elastase, collagenase and hyaluronidase which lead to degradation of ECM and skin aging. The degradation of ECM leads to loss of strength, elasticity,

smoothness, and moisture of skin which appears as wrinkles on the skin.[2]

Natural skincare products may increase skin regeneration, elasticity, smoothness, and prevent the degradation of ECM and skin aging. In addition, natural skincare products cause less irritation and toxicity than synthetic skincare products.[3] There are certain phytochemicals that can inhibit MMPs. For example, phenolic compounds from *Clitoria ternatea* L. petals and leaves inhibit the production of reactive oxygen species (ROS) so they help reduce wrinkle formation.[4] Other phytochemical substances such as flavonoids (kaempferol, quercetin, and myricetin) in *Clitoria ternatea* L. petals and leaves have anti-elastase and anti-collagenase activities.[5,6] Anthocyanins stimulate the expression of ECM that increases the formation of collagen, elastin, and hyaluronic acid.[7]

Clitoria ternatea L., commonly known as butterfly pea, is grown as an ornamental plant in Thailand. There are various colors of *C. ternatea* L. flower including dark blue, light blue, mauve and white (Figure 1, mauve *C. ternatea* L. flower). *C. ternatea* L. flower contains several phytochemicals such as alkaloids, tannins, glycosides, resins, flavonoids, and anthocyanin.[8-10] Different colors of *C. ternatea* L. flowers contain different types and content of phytochemicals. The anthocyanins known as ternatins are mostly found in dark blue petals followed by light blue petals while delphinidins are only found in mauve petals. White petals do not contain anthocyanins.[11] Dark blue petal extract and an extract containing eye gel formulation have been reported for their antioxidant activity and can reduce wrinkles.[12]

Figure 1 Mauve of *C. ternatea* L. flower.

Studies on biological activities of *C. ternatea* L. flower have widely been done on dark blue petal. In addition, dark blue *C. ternatea* L. petals are mostly found in Thailand followed by mauve, light blue, and white petals, respectively. Therefore, we aimed to investigate anti- aging activity of mauve *C. ternatea* L. petal extract including anti-elastase, anti-collagenase, and anti-hyaluronidase activities, as well as to determine the content of total phenolic, total flavonoid, and total anthocyanin.

Methods

Chemicals and devices

4-(Dimethylamino)-benzaldehyde (PDMAB), collagenase type I from *Clostridium histolyticum* (6.25 units/mg solid), gallic acid, hyaluronidase from bovine testes (800 units/mg solid), N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA), N-succinyl-$(Ala)_3$-p-nitroanilide (SANA), porcine pancreatic elastase type IV (PPE, 4.5 units/mg solid), quercetin, Tricine and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis, USA). Cyanidin chloride was purchased from Fluka (China). Folin-Ciocalteu reagent was purchased from Carlo Erba Reagents (France). Glacial acetic acid was purchased from Quality Reagent Chemical (New Zealand). Hyaluronic acid was purchased from My Skin Recipes (Thailand). Hydrochloric acid 37% (HCl) was purchased from ACI Labscan (Thailand). Water for irrigation was purchased from General Drug House (GDH) (Thailand). All other chemicals used in the study were of analytical grade.

Preparation of mauve *C. ternatea* L. petal extract

Mauve *C. ternatea* L. flowers were collected in 2020 from Nonthaburi, Thailand and identified by Assoc. Prof. Dr. Sarin Tadtong. A voucher specimen number “CT_2020_001M” of this plant was deposited in herbarium at the Faculty of Pharmacy, Srinakharinwirot University, Nakhonnayok, Thailand. Mauve *C. ternatea* L. petals were separated and shade-dried under well-ventilated conditions at room temperature. The petals were dried in hot air oven at 50 °C for 4 hours and ground into powder using mechanical grinder. Powdered mauve *C. ternatea* L. petals (28 g) were macerated with 80% ethanol (300 ml) for 3 days. The extract was filtered, and the solvent was recovered using rotary evaporator (Buchi Rotavapor R-114, Switzerland) at 45 °C. The process was repeated for other four times. The combined extract was

poured into the amber air-tight glass containers and the retained solvent was removed until dry using water bath. The concentrated extract was stored at -20 °C until use.

Determination of anti-elastase activity by spectrophotometric method

Three independent anti-elastase activity assays were performed by the method described previously using 96-well plate (Sterilin®, UK) and each experiment was run in triplicate.[13,14] Porcine pancreatic elastase (PPE) was determined by spectrophotometric method using N-succinyl-$(Ala)_3$-p-nitroanilide (SANA) as the substrate, monitoring the release of *p*-nitroaniline. PPE and SANA were dissolved in 0.2 M Tris-HCl buffer (pH 8.0). The reaction mixture contained 160 µl of different concentrations of mauve *C. ternatea* L. petal extract in 0.2 M Tris-HCl buffer (2.5, 3.75, 5, 6.25, and 7.5 mg/ml) and 20 µl of 0.01 mg/ml PPE (4.5 units/mg solid). The extract was pre-incubated with the enzyme for 10 minutes at room temperature. After incubation, 20 µl of 0.8 mM SANA was added and incubated the reaction mixture for 60 minutes at room temperature. The control used 0.2 M Tris-HCl buffer instead of the plant extract. Gallic acid was used as a positive control. The absorbance was measured at 410 nm using microplate reader (AccuReader® M965 Mate, Taiwan). The percentage of anti-elastase activity (%inhibition) was calculated as:

$$\%\ \text{inhibition} = \frac{\text{Absorbance}_{control} - \text{Absorbance}_{sample}}{\text{Absorbance}_{control}} \text{ x } 100$$

where control absorbance was the *p*-nitroaniline absorbance of control reaction, and sample absorbance was the *p*-nitroaniline absorbance of gallic acid or the plant extract reaction. Half-maximal inhibitory concentration (IC_{50}) of mauve *C. ternatea* L. petal extract was calculated from the graph of inhibition percentage versus concentration.

Determination of anti-collagenase activity by spectrophotometric method

Anti-collagenase activity assay was performed by the method described previously using 96 well plate (Sterilin®, UK) for three independent experiments which each was done in triplicate.[13,15] Collagenase type I from *Clostridium histolyticum* was determined by spectrophotometric method using N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA) as the substrate, monitoring the release of N-(3-[2-Furyl]-acryloyl)-Leu and Gly-Pro-Ala. Collagenase and FALGPA were dissolved in 0.05 M Tricine buffer (pH 7.5). The reaction mixture contained 120 µl of different concentrations of mauve *C. ternatea* L. petal extract in 0.05 M Tricine buffer (3.125, 3.75, 4.375, 5, and 5.625 mg/ml) and 15 µl of 2.5 mg/ml collagenase type I (6.25 units/mg solid). The extract was pre-incubated with the enzyme for 15 minutes at room temperature and the reaction was started with the addition of 15 µl of 0.8 mM FALGPA. In control, 0.05 M Tricine buffer was used instead of the plant extracts. Gallic acid was used as a positive control. The absorbance was measured immediately after adding FALGPA at 340 nm and continuously monitored every 2 minutes for 20 minutes using microplate reader (AccuReader® M965 Mate, Taiwan). The percentage of anti-collagenase activity (%inhibition) was calculated as:

$$\%\ \text{inhibition} = \frac{\Delta\text{Absorbance}_{control} - \Delta\text{Absorbance}_{sample}}{\Delta\text{Absorbance}_{control}} \text{ x } 100$$

where Δ control absorbance was the different absorbance initial to FALGPA final reaction of control reaction, and Δ sample absorbance was the different absorbance initial to FALGPA final reaction of gallic acid or the plant extract reaction. Half-maximal inhibitory concentration (IC_{50}) of mauve *C. ternatea* L. petal extract was calculated from the graph of the inhibition percentage versus concentration.

Determination of anti-hyaluronidase activity by colorimetric method

Anti-hyaluronidase activity assay was performed by the method described previously with slight modification measuring the amount of N-acetylglucosamine (GlcNAc) fragmented from hyaluronic acid as the substrate.[1,16] Hyaluronidase from bovine testes and hyaluronic acid were dissolved in 0.1 M acetate buffer (pH 3.6). The reaction mixture contained 50 µl of different concentrations of mauve *C. ternatea* L. petal extract in 50% ethanol (10, 11, 12, 13, and 14 mg/ml), 50 µl of 9.875 mg/ml hyaluronidase (800 units/mg solid) and 250 µl of 15 mg/ml hyaluronic acid that was incubated for 20 minutes at 37 °C. After incubation, 1 ml of PDMAB solution (4 g PDMAB dissolved in 50 ml of 10N HCl and 350 ml of glacial acetic acid) was added and then 200 µl of the reaction mixture was pipetted into 96-well plate (Sterilin®, UK). The control used 0.1 M acetate buffer instead of the plant extract. Quercetin was used as a positive control. The absorbance was measured at 570 nm using microplate

reader (SpectraMax M3, Molecular Devices, USA). The assay was performed for three independent experiments and each experiment was performed in triplicate. The percentage of anti-elastase activity (% inhibition) was calculated as:

$$\%\ \text{inhibition} = \frac{\text{Absorbance}_{control} - \text{Absorbance}_{sample}}{\text{Absorbance}_{control}} \text{ x } 100$$

where control absorbance was the GlcNAc-PDMAB absorbance of control reaction, and sample absorbance was the GlcNAc-PDMAB absorbance of quercetin or the plant extract reaction. Half-maximal inhibitory concentration (IC_{50}) of mauve *C. ternatea* L. petal extract was calculated from the graph of the inhibition percentage versus concentration.

Determination of total phenolic content by Folin-Ciocalteu method

The total phenolic content was determined by the modified Folin-Ciocalteu method using gallic acid as the standard.[17,18] The reaction mixture contained 100 µl of 50 mg/ml mauve *C. ternatea* L. petal extract in 95% ethanol, 100 µl of 10% v/v Folin-Ciocalteu reagent, 100 µl of 75 mg/ml Na_2CO_3 solution and 700 µl of deionized water, then was kept in dark place for 90 minutes at room temperature. After incubation, 200 µl of the reaction mixture was pipetted into 96-well plate (Sterilin®, UK). The control used deionized water instead of the plant extract. For standard curve, the different concentrations of gallic acid (0.0313, 0.0625, 0.125, 0.25, and 0.5 mg/ml in 95% ethanol) were used instead of the plant extract. The absorbance was measured at 765 nm using microplate reader (SpectraMax M3, Molecular Devices, USA). Three independent experiments were performed, and each experiment was run in triplicate. The amount of total phenolic content was expressed as gallic acid equivalence in milligrams per gram (mg GAE/g) of extract.

Determination of total flavonoid content by aluminium chloride complexation colorimetric method

The total flavonoid content was determined by the modified aluminium chloride complexation colorimetric method using quercetin as the standard.[17,18] The reaction mixture contained 0.5 ml of 10 mg/ml mauve *C. ternatea* L. petal extract in deionized water, 0.5 ml of 8 mg/ml $AlCl_3$ in absolute ethanol and 4 ml of absolute ethanol then kept in dark for 90 minutes. After incubation, 200 µl of the reaction mixture was pipetted into 96-well plate (Sterilin®, UK). The control used absolute ethanol instead of the plant extract. For standard curve, different concentrations of quercetin (25, 50, 75, 100, and 125 µg/ml in 50% ethanol) were used instead of the plant extract. The absorbance was measured at 415 nm using microplate reader (SpectraMax M3, Molecular Devices, USA). Three independent experiments were performed, and each experiment was run in triplicate. The amount of total flavonoid content was expressed as quercetin equivalence in milligrams per gram (mg QE/g) of extract.

Determination of total anthocyanin content by pH differential method

The total anthocyanin content was determined by the modified pH differential method using cyanidin as the standard.[19] Each 10 mg of mauve *C. ternatea* L. petal extract was transferred into two microcentrifuge tubes for preparing two different pH samples; 1 ml of 35% ethanol in 0.2 M KCl buffer (pH 1.0) was added into one of them, and 1 ml of 35% ethanol in 0.4 M CH_3COONa buffer (pH 4.5) was added into the other. These samples were allowed to equilibrate for 15 minutes, not exceeding 40 minutes. 200 µl of the reaction mixture was pipetted into 96-well plate (Sterilin®, UK).

For the standard curve, different concentrations of cyanidin (0.125, 0.25, 0.5, 1, and 2 µg/ml) prepared under the same condition were used instead of the plant extract. The absorbance of the sample was measured at 510 (Abs_{510}) and 700 nm (Abs_{700}) using microplate reader (SpectraMax M3, Molecular Devices, USA). Three independent experiments were performed, and each experiment was run in triplicate. The absorbance of the sample was calculated as:

$$Abs_{anthocyanin} = (Abs_{510} - Abs_{700})_{pH\ 1.0} - (Abs_{510} - Abs_{700})_{pH\ 4.5}$$

The amount of total anthocyanin content was expressed as cyanidin equivalence in milligrams per gram (mg CE/g) of extract.

Results

Mauve *C. ternatea* L. petal filtrate was concentrated by a rotary evaporator to obtain 14.85 g of extract (yield 47.53 %w/w). The extract was investigated for its anti-aging activity including anti-elastase, anti-collagenase, and anti-hyaluronidase, as well as to determine the content of

phytochemicals including total phenolic, total flavonoid, and total anthocyanin.

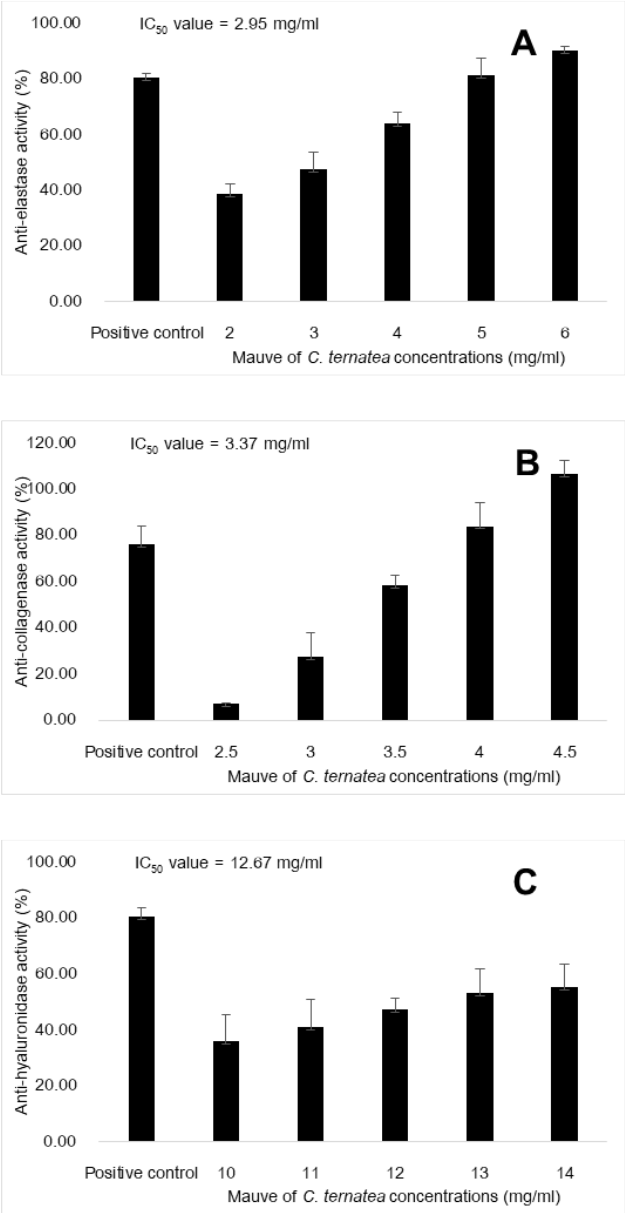

Figure 2 Anti-elastase (a), anti-collagenase (b) and anti-hyaluronidase (c) activity of mauve *C. ternatea* L. petal extract..

Anti-aging activity

The inhibitory activity of mauve *C. ternatea* L. petal extract on elastase, collagenase and hyaluronidase was expressed as the mean ± standard error and IC_{50} value (Figure 2). Mauve *C. ternatea* L. petal extract inhibited the activities of elastase, collagenase, and hyaluronidase in a dose-dependent manner. Anti-elastase, anti-collagenase, and anti-hyaluronidase activities of mauve *C. ternatea* L. petal extract exhibited IC_{50} values of 2.95, 3.37, and 12.67 mg/ml, respectively.

Phytochemical characterization

The total phenolic content exhibited as gallic acid equivalent value of 27.84 mg GAE/g of the extract. Flavonoids are the phytochemical members of phenolic compounds as well as anthocyanins which are subgroup phytochemicals of flavonoids. The total flavonoid content exhibited as quercetin equivalent value of 3.47 mg QE/g of the extract, and the total anthocyanin content exhibited as cyanidin equivalent value of only 0.02 mg CE/g of the extract.

Discussions and Conclusion

Skin aging is influenced by the ultraviolet exposure, oxidation and MMPs (elastase, collagenase, and hyaluronidase) activity. Many precedent studies have shown that *C. ternatea* L. petal and leaf extracts contained phenolic compounds which were reported for their antioxidant and wrinkle reducing activities.[4,12] In addition, *C. ternatea* L. leaf extract has been reported for anti-elastase, anti-collagenase, and anti-hyaluronidase activities.[20] Therefore, preventing skin aging is not only the responsibility of antioxidation but also the inhibition of MMPs activity. The previous studies have shown that *C. ternatea* L. leaf extract contained phenolics and flavonoids (kaempferol, quercetin, and myricetin) similar to those found in *C. ternatea* L. petal extract.[8] Flavonoids such as kaempferol, quercetin, and myricetin have been reported to exhibit anti-elastase and anti-collagenase activities.[5,6] Also, anthocyanins are phytochemicals subgroup of flavonoids stimulating the expression of ECM. The expression of ECM increases the formation of collagen, elastin, and hyaluronic acid in the skin leads to the protection of wrinkle formation and skin aging.[7]

Our present study showed that mauve *C. ternatea* L. petal extract exerted anti-aging activity by inhibiting the activities of elastase, collagenase, and hyaluronidase. Our study is the first report on the anti-elastase, anti-collagenase, and anti-hyaluronidase of the mauve *C. ternatea* L. Only the blue line was reported for its antioxidant activity.[12] อะไรคือ blue line Mauve *C. ternatea* L. petal extract expressed better anti-elastase and anti-collagenase activities than anti-hyaluronidase activity which was contrasted to those of *C. ternatea* L. leaf extract. The previous studies revealed *C. ternatea* L. leaf extract present better anti-collagenase and anti-hyaluronidase activities than anti-elastase activity.[20] Although, *C. ternatea* L. petal extract contains phytochemicals

similar to those found in *C. ternatea* L. leaf extract, but in different amount of phytochemicals due to the different parts of *C. ternatea* L.[9,10] Moreover, the previous studies have shown the lack of correlation between polyphenols amount and hyaluronidase (*r* values 0.24)[21] that might cause mauve *C. ternatea* L. petal extract had less hyaluronidase inhibitory effect. In addition, our study found that mauve *C. ternatea* L. petal extract contained phenolic compounds, flavonoids, and anthocyanins. Total phenolic content was found in highest amount among them. Thus, it can be deduced that the anti-aging activity of mauve *C. ternatea* L. petal extract should be the effect of phenolic compounds contained in the extract. Finally, anti-skin aging or anti-wrinkle formulation containing mauve *C. ternatea* L. petal extract should be developed and studied for its activity in the future.

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