พฤกษเคมีและฤทธิ์ในการลดเลือนริ้วรอยของสารสกัดกลีบดอกอัญชันสีน้ำเงิน
Phytochemical Study and Anti-wrinkle Activity of Blue *Clitoria ternatea* L. Petal Extract

นิพนธ์ต้นฉบับ

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Original Article

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บทคัดย่อ

วัตถุประสงค์: เพื่อวิเคราะห์ปริมาณรวมของสารกลุ่มฟีนอลิก กลุ่มฟลาโวนอยด์ และกลุ่มแอนโทไซยานินที่มีในสารสกัดกลีบดอกอัญชันสีน้ำเงิน เพื่อศึกษาฤทธิ์ลดเลือนริ้วรอยจากการยับยั้งเอนไซม์อีลาสเตส เอนไซม์คอลลาจีเนส และเอนไซม์ไฮยาลูโรนิเดสของสารสกัดจากกลีบดอกอัญชันสีน้ำเงิน **วิธีการศึกษา:** สกัดสารสกัดจากกลีบดอกอัญชันสีน้ำเงินด้วย 80% เอทานอล และระเหยตัวทำละลายออกด้วยเครื่องกลั่นระเหยแบบหมุน วิเคราะห์ปริมาณรวมของสารกลุ่มฟีนอลิกด้วยวิธี Folin-Ciocalteu วิเคราะห์ปริมาณฟลาโวนอยด์ด้วยวิธีการวัดสีของสารประกอบอลูมิเนียมคลอไรด์ วิเคราะห์ปริมาณรวมของแอนโทไซยานินด้วยวิธีการวัดในสภาวะต่าง pH และศึกษาฤทธิ์ลดเลือนริ้วรอยโดยศึกษาการยับยั้งเอนไซม์อีลาสเตสและคอลลาจีเนสด้วยวิธี spectrophotometric และศึกษาการยับยั้งเอนไซม์ไฮยาลูโรนิเดสด้วยวิธี colorimetric โดยรายงานผลเป็นความเข้มข้นของสารที่ยับยั้งการทำงานของเอนไซม์ได้ร้อยละ 50 (IC_{50}) **ผลการศึกษา:** สารสกัดกลีบดอกอัญชันสีน้ำเงิน 1 g มีปริมาณรวมของฟีนอลิก ฟลาโวนอยด์ และแอนโทไซยานินเทียบเท่ากรดแกลลิก 43.96 mg, เทียบเท่าเควอเซติน 3.33 mg และเทียบเท่าไซยานิดิน 0.026 mg ตามลำดับ การศึกษาฤทธิ์ลดเลือนริ้วรอยพบว่า สารสกัดกลีบดอกอัญชันสีน้ำเงินมีค่า IC_{50} ในการยับยั้งเอนไซม์อีลาสเตสและเอนไซม์คอลลาจีเนส เท่ากับ 4.47 mg/ml และ 3.60 mg/ml ตามลำดับ แต่มีฤทธิ์ในการยับยั้งเอนไซม์ไฮยาลูโรนิเดสมากที่สุดที่ความเข้มข้น 17 mg/ml (ร้อยละการยับยั้ง 31.38%) **สรุป:** สารสกัดกลีบดอกอัญชันสีน้ำเงินมีพฤกษเคมีกลุ่มฟีนอลิกเป็นองค์ประกอบมากที่สุด และมีฟลาโวนอยด์และแอนโทไซยานินตามลำดับ และมีฤทธิ์ลดเลือนริ้วรอยจากการยับยั้งเอนไซม์อีลาสเตสและเอนไซม์คอลลาจีเนสได้ดี แต่ไม่มีประสิทธิผลในการยับยั้งเอนไซม์ไฮยาลูโรนิเดส

คำสำคัญ: กลีบดอกอัญชันสีน้ำเงิน, พฤกษเคมี, ฤทธิ์ยับยั้งเอนไซม์อีลาสเตส, ฤทธิ์ยับยั้งเอนไซม์คอลลาจีเนส, ฤทธิ์ยับยั้งเอนไซม์ไฮยาลูโรนิเดส

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Abstract

Objective: To determine total phytochemical contents, including phenolic, flavonoid and anthocyanin of blue *C. ternatea* L. extract and to investigate its anti-wrinkle activity from anti-elastase, anti-collagenase, and anti-hyaluronidase activities. **Methods:** The pulverized blue *C. ternatea* L. petal was extracted by 80% ethanol and evaporated by a rotary evaporator. Total phenolic, flavonoid, and anthocyanin contents were determined by Folin-ciocalteu, $AlCl_3$ complexation colorimetric, and pH differential methods based on gallic acid, quercetin, and cyanidin standard curves, respectively. Anti-elastase and anti-collagenase activities were determined by spectrophotometric method with gallic acid as a positive control. Anti-hyaluronidase activity was determined by the colorimetric method with quercetin as a positive control. **Results:** Total phenolic, flavonoid, and anthocyanin contents of blue *C. ternatea* L. petal extract were found at 43.96 mgGAE, 3.33 mgQE, and 0.026 mgCE per 1 g of extract, respectively. The inhibition activity of blue *C. ternatea* L. petal extract on elastase and collagenase exhibited IC_{50} values of 4.47 and 3.60 mg/ml, respectively. It showed the most active hyaluronidase inhibition at 17 mg/ml (31.38% hyaluronidase inhibition). **Conclusion:** The phytochemistry including phenolic, flavonoid, and anthocyanin were found in blue *C. ternatea* L. petal extract with the highest amount of phenolic compound. The extract showed anti-wrinkle activity on anti-elastase and anti-collagenase, while it showed low anti-hyaluronidase activity.

Keywords: blue *Clitoria ternatea* L. petal, phytochemical contents, anti-elastase, anti-collagenase, anti-hyaluronidase

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Introduction

Nowadays, humans are paying attention to their skin more than in the past. As the age increases, the structure of skin degenerates. Skin aging[1-4] is characterized by wrinkling caused by both extrinsic factors (sunlight, poor nutrition, or pollution) and intrinsic factors (matrix metalloproteinase; MMPs). The effect of intrinsic factors means enzyme activation and that enzyme will affect the degradation of the skin primary structure. The enzymes involved in wrinkling, including elastase degenerated elastin protein related to the strength of the skin, collagenase degenerated collagen related to the flexibility, and hyaluronidase degenerated hyaluronic acid related to the skin moisture.

Many researchers have consistently developed processes to prevent the degradation of the skin primary structure. Natural products, another option, have been studied on wrinkling inhibition. According to the study, plants developed as anti-wrinkle products often contain phytochemicals, especially, phenolic and flavonoid. This leads to studying enzyme inhibitory effect of phenolic and flavonoid containing plants.[5]

Clitoria ternatea L. or butterfly pea is a common flowering plant in Southeast Asia that comes in a variety of colors (blue, mauve, light blue, and white). Blue flower is the most common color. *C. ternatea* L. flower contains several phytochemicals including alkaloids, anthocyanins, flavonoids, glycosides, resin, and tannins. Biological activity from different parts of blue *C. ternatea* L. was studied and found that the leaves have the effect associated with reducing wrinkles. The blue *C. ternatea* L. flower extract has been reported on antioxidant, anti-microbial, antifungal, antidiabetic, anti-inflammatory effect, and inhibition of cancer cells. In addition, several researches studied the anti-allergic effect of blue *C. ternatea* L. flower extract. However, neither of them reported its anti-wrinkle activity nor the study on a comparison of the biological activity of the variety of petal colors found in nature.

In the search for a diverse resources of natural anti-wrinkle and anti-aging agents, there is a need to study the phytochemicals and anti-wrinkle activity of other parts, such as blue petal extract which is the most common color and the most studied on its biological activity.[6-8] This study aimed to determine the content of phytochemical of blue *C. ternatea* L. including phenolic, flavonoid, and anthocyanin and investigates its anti-wrinkle activity on anti-elastase, anti-collagenase, and anti-hyaluronidase activities.

Methods

Materials and Instruments

Plant materials were collected from natural habitats, obtained from Ladkrabang district, Bangkok, Thailand and identified by Assoc.Prof.Dr.Sarin Tadtong. A voucher specimen number "CT_2020_002B" of this plant was deposited in a herbarium at the Faculty of Pharmacy, Srinakharinwirot University, Nakhonnayok, Thailand. All solvents used were of analytical grade. 95% Ethanol was purchased from Samchai Chemical Co., LTD. (Bangkok, Thailand). Absolute ethanol and calcium chloride ($CaCl_2$) were purchased from Merck (Germany). Folin-ciocalteu reagent was purchased from Carlo Erba Reagents (France). Potassium chloride (KCl), Sodium carbonate (Na_2CO_3) anhydrous, and sodium hydroxide (NaOH) were purchased from Ajax Finechem (New Zealand). Aluminium chloride ($AlCl_3$) was purchased from Kemaus (Australia). 37% Hydrochloric acid (HCl) was purchased from ACI Labscan (Thailand). Glacial acetic acid, Sodium acetate (CH_3COONa), and Sodium chloride (NaCl) were purchased from Quality Reagent Chemical (New Zealand). 4-(Dimethylamino) benzaldehyde (PDMAB), Collagenase type I from *Clostridium histolyticum*, Gallic acid, Hyaluronidase Type I from bovine testes, N-(3-[2-Furyl]-acryloyl)-Leu-Gly-Pro-Ala (FALGPA), N-succinyl-$(Ala)_3$-p-nitroanilide (SANA), Porcine pancreatic elastase type IV (PPE), Quercetin, Tricine, and Tris-HCl were purchased from Sigma (USA). Cyanidin chloride was purchased from Fluka (China). Hyaluronic acid (HA) was purchased from My Skin Recipes (Thailand) and water for irrigation was purchased from A.N.B. Laboratorues (Thailand). Experiment instruments included microplate readers (SpectraMax M3, Molecular Devices, USA and Accu reader® M965 Mate, Taiwan), an incubator (Electronic Microprocessor PID Control, Memmert, Germany), a centrifuge (MX-301, Tomy, Japan, a rotary evaporator (Rotavapor R-300, Buchi, Switzerland).

Sample preparation[9-12]

Blue *Clitoria ternatea L.* petals were separated and dried in a hot air oven at 50°C for 2 hours. All dried blue petals were ground in a normal grinder. The petal powder was extracted for 3 days by using 1 g of powder per 50 ml of 80% ethanol and then filtered. The filtrate was evaporated by using a rotary evaporator and dried the extract on the water bath.

Total phenolic content (TPC) determination[13,14]

The phenolic content was determined by using the spectrophotometric method. Three independent experiments were performed. 100 µl of 50 mg/ml sample extract in 95% ethanol was incubated with 100 µl of 10% v/v Folin-ciocalteu reagent, 100 µl of 75 mg/ml Na_2CO_3 solution, and 700 µl deionized water for 90 minutes at room temperature in a microcentrifuge tube. After incubated, 200 µl of the reaction mixture was transferred to a 96-well plate and the absorbance was measured at 765 nm using a SpectraMax M3 microplate reader. Each experiment was done in triplicate. Quantification of TPC was done using standard curve of gallic acid (0.0313 - 0.5 mg/ml). The amount of total phenolic content was present in gallic acid equivalent (GAE) in milligrams per g (mg/g) sample extract.

Total flavonoid content determination[15,16]

The flavonoid content was determined by using the spectrophotometric method. Three independent assays were performed. 1 ml of 10 mg/ml sample extract in deionized water was mixed with 1 ml of 8 mg/ml $AlCl_3$ and 8 ml of absolute ethanol, incubated the mixture in the dark place at room temperature for 40 minutes in a microcentrifuge tube. After incubated, 200 µl of the reaction mixture was transferred to a 96-well plate and the absorbance was measured at 415 nm

using a SpectraMax M3 microplate reader. Each experiment was done in triplicate. Quantification of flavonoid content was done using standard curve of quercetin (2.5 - 12.5 µg/ml). The amount of total flavonoids content was present in quercetin equivalent in milligrams per g (mg/g) sample extract.

Total anthocyanin content determination[17]

The anthocyanin content was determined by using the pH differential colorimetric method. Three independent assays were performed. Two of the 10 mg of sample extracts were separately mixed with 1 ml of 35% ethanol in 0.2 M KCl buffer (pH 1.0) and 35% ethanol in 0.4 M CH_3COONa buffer (pH 4.5), respectively in microcentrifuge tubes. After 15 minutes, not more than 40 minutes, 200 µl of the reaction mixture were transferred to a 96-well plate and the absorbance of each mixture solution was measured at 510 nm (Abs_{510}) and 700 nm (Abs_{700}) using a SpectraMax M3 microplate reader. The absorbance of anthocyanin content was calculated by the following equation:

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

Each experiment was done in triplicate. Quantification of anthocyanin content was done using a standard curve of cyanidin (0.125 - 4.0 µg/ml). The amount of total anthocyanins content was present in cyanidin equivalent in milligrams per g (mg/g) sample extract.

Determination of anti-elastase activity[18-20]

Anti-elastase activity was determined by using spectrophotometric method slightly modified from Wongsukkasem et al. Three independent assays were performed. The 0.2 M Tris-HCl buffer (pH 8.0) was used as solvent. The reaction mixture contained 160 µl different concentrations of sample extract in buffer was pre-incubated with 20 µl of 0.01 mg/ml (4.5 unit/mg) porcine pancreatic elastase (PPE) in a 96-well plate for 10 minutes at room temperature. After 10 minutes, 20 µl of 0.8 mM N-succinyl-(Ala)3-p-nitroanilide (SANA) was added as the substrate and incubated the reaction mixture for 60 minutes at room temperature. Gallic acid was used as a positive control. The absorbance was measured at 410 nm using an Accu Reader® M965 Mate. Each experiment was run in triplicate. Anti-elastase activity of the sample extracts was calculated as the following equation:

$$\%inhibition = \frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}} \times 100$$

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

Determination of anti-collagenase[20-22]

Three independent experiments of anti-collagenase activity assay were determined by using spectrophotometric method slightly modified from Wongsukkasem et al. Three independent assays were performed. The 0.05 M Tricine buffer (pH 7.5) was used as solvent. The reaction mixture contained 120 µl different concentrations of sample extract in buffer was pre-incubated with 15 µl of 2.5 mg/ml (6.25 collagen digestion unit) collagenase type I in 96-well plate for 15 minutes at room temperature. After 15 minutes, 15 µl of 0.8 mM FALGPA was added as the substrate. The absorbance was measured at 340 nm using an Accu Reader® M965 Mate immediately and continuously measured every 2 minutes for 20 minutes after mixed. Each experiment was done in triplicate. Gallic acid was used as a positive control. Anti-collagenase activity of the sample extracts was calculated as the following equation:

$$\%inhibition = \frac{\Delta Absorbance_{control} - \Delta Absorbance_{sample}}{\Delta Absorbance_{control}} \times 100$$

Where $\Delta Absorbance_{control}$ was the different absorbance initial to FALGPA final reaction of control reaction and $\Delta Absorbance_{sample}$ optical density was the different absorbance initial to FALGPA final reaction of gallic acid or the plant extract reaction. Half-maximal inhibitory concentration (IC_{50}) of *C. ternatea* L. petal extract was calculated from the graph of the inhibition percentage versus concentration.

Determination of anti-hyaluronidase[23,24]

Anti-hyaluronidase activity was determined by using colorimetric method. Three independent assays were performed in 0.1 M acetate buffer (pH 3.6). The reaction mixture contained 50 µl different concentrations of sample extract in 50% ethanol was incubated with 50 µl of 7900 units/ml hyaluronidase from bovine testes and 250 µl of 15 mg/ml hyaluronic acid in microcentrifuge tube for 20 minutes at 37 °C. After 20 minutes 1 ml of PDMAB solution (4 g of 4-(Dimethylamino)benzaldehyde dissolved in 50 ml of 10 N HCl and 350 ml of glacial acetic acid) was added and 200 µl of the reaction mixture was transferred to 96-well plate. The absorbance of mixture solution was measured

at 410 nm using a SpectraMax M3 microplate reader immediately. Each reaction was replicated for 3 times. Quercetin was used as a positive control. Anti-hyaluronidase activity of the sample extracts was calculated as the following equation:

$$\%inhibition = \frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}} \times 100$$

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

Results

Sample preparation

481.46 g of blue *Clitoria ternatea* L. petal was macerated with 80% Ethanol for 5 times, filtered and concentrated to obtain 274.75 g extract (yield 57.07%w/w). The extract was stored in an amber bottle at -20 °C. Phytochemical study and anti-wrinkle activity of the extract were performed by the methods mentioned above.

Phytochemical of blue *C. ternatea* petal extract

The amount of total phenolic content in blue *C. ternatea* L. petal extract was based on the absorbance of the reaction mixture with Folin-ciocalteu reagent. The standard curve of gallic acid was plotted to quantify the phenolic content in sample extract. The amount of total phenolic content of blue *C. ternatea* L. petal extract was found at 43.96 mg/g of extract or 4.40%w/w of the extract equivalent to gallic acid.

The amount of total flavonoid content in blue *C. ternatea* L. petal extract was based on the absorbance of the reaction mixture with $AlCl_3$. The standard curve of quercetin was plotted to quantify the flavonoid content in sample extract. The amount of total flavonoid content of blue *C. ternatea* L. petal extract was found at 3.33 mg/g of extract or 0.33%w/w of the extract equivalent to quercetin.

The amount of total flavonoid content in blue *C. ternatea* L. petal extract was based on the absorbance of anthocyanin content in different pH mixture. The standard curve of cyanidin was plotted to quantify the anthocyanin content in sample extract. The amount of total anthocyanin content of blue *C. ternatea* L. petal extract was found at 0.026 mg/g of extract or 0.003%w/w of the extract equivalent to cyanidin.

Anti-wrinkle activity of blue *C. ternatea* L. petal extract

The inhibition activity of blue *C. ternatea* L. petal extract on elastase, collagenase, and hyaluronidase were reported as the half minimal inhibitory effect (IC_{50}) value. Blue *C. ternatea* petal extract showed the activities of elastase and collagenase inhibition in a dose-dependent manner, as shown in Figure.1, and exhibited IC_{50} values of 4.47 and 3.60 mg/ml, respectively. Unfortunately, the extract exhibited inhibitory activity on hyaluronidase less than 50% at the concentration of 17 mg/ml (%inhibition = 31.38%) which was the maximum concentration of the extract being used in our experiment. Hence, we can conclude that blue *C. ternatea* L. petal extract possessed no hyaluronidase inhibitory activity.

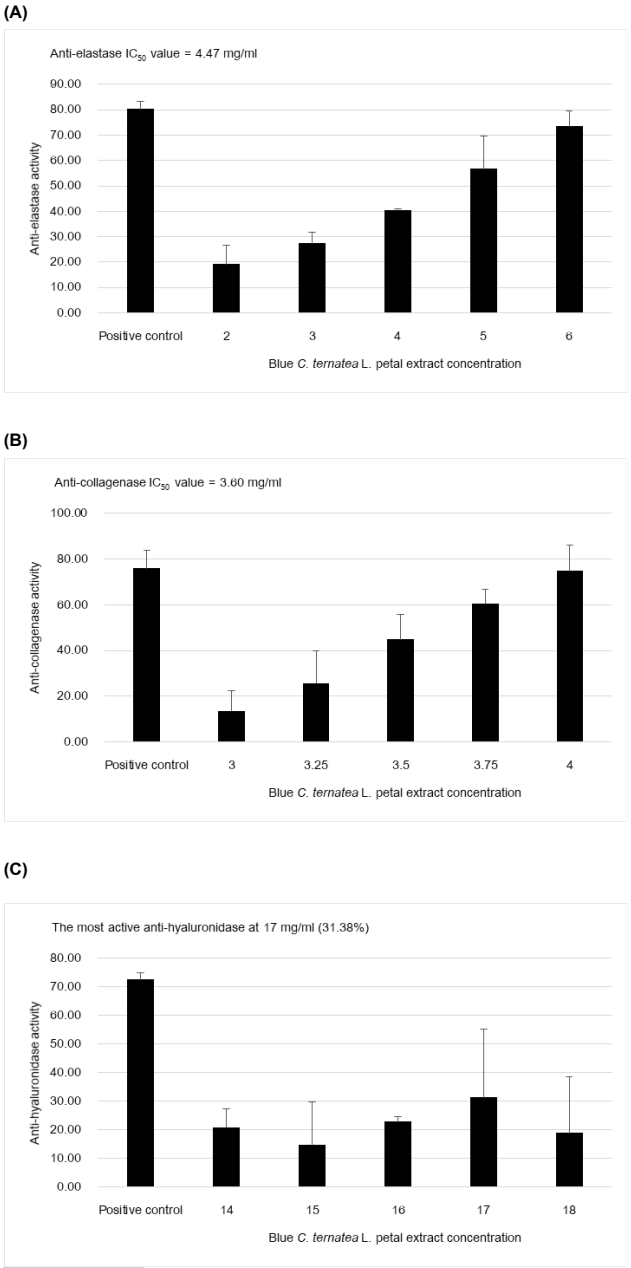

Figure 1 Anti-elastase (A), anti- collagenase (B), and anti-hyaluronidase (C) activity of blue *C. ternatea* L. petal extract.

Discussions and Conclusion

It was found that blue *C. ternatea* L. petal extract contained phenolics, flavonoids and anthocyanins with the highest amount of phenolic compound. The flavonoids found in blue *C. ternatea* L. petal extract were kaempferol, quercetin, and myricetin. Moreover, anthocyanin which could be found in *C. ternatea* L. flowers with various colors were delphinidins and ternatins. However, the amounts of the various phytochemical compounds found in the *C. ternatea* L. petal extract may differ in the plant samples from different cultivation sites. Therefore, further studies should be conducted to compare amounts of these compounds in samples collected from each growing area.[6-9]

In anti-wrinkle activity test, we found blue *C. ternatea* L. petal extract could effectively inhibit elastase and collagenase with the better ability to inhibit the collagenase than elastase. Its IC_{50} value on anti-collagenase assay was lower than that on anti-elastase assay. For anti-hyaluronidase activity, the percentage of inhibition of hyaluronidase activity was very low with a 31.38% hyaluronidase inhibition at 17 mg/ml which was the concentration that possessed the highest inhibition in this assay. Therefore, it can be concluded that blue *C. ternatea* L. petal extract showed no anti-hyaluronidase activity.

In addition, in Thailand, *C. ternatea* L. found in nature are blue, mauve, light blue and white. As one of the promising abundant natural sources of beneficial phytochemical coumpounds, the comparisons of these compounds in *C. ternatea* L. with various colors should be conducted. Specific biological activity of each of these phytochemical components should also be further investigated.

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