ฤทธิ์ยับยั้งเอนไซม์คอลลาจีเนส อีลาสเตส ไฮยาลูโรนิเดส และฤทธิ์ต้านออกซิแดนซ์ของสารสกัดดอกเข็มสีเหลือง
Collagenase, Elastase, Hyaluronidase Inhibitory and Antioxidant Activities of Yellow *Ixora coccinea* L. Flowers Extract

นิพนธ์ต้นฉบับ **Original Article**

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ยับยั้งการทำงานของเอนไซม์ไฮยาลูโรนิเดส เอนไซม์คอลลาจีเนส เอนไซม์อีลาสเตส และฤทธิ์ต้านอนุมูลอิสระ และปริมาณรวมของสารกลุ่มฟีนอลิก ฟลาโวนอยด์ แอนโทไซยานิน และแคโรทีนอยด์ในสารสกัดดอกเข็มสีเหลือง **วิธีการศึกษา:** นำดอกเข็มสีเหลืองมาบดเป็นผงและสกัดด้วยเอทานอล 80% ระเหยด้วยเครื่องระเหยสารสุญญากาศแบบหมุน ทดสอบฤทธิ์ลดเลือนริ้วรอยโดยศึกษาฤทธิ์ยับยั้ง 1) เอนไซม์ไฮยาลูโรนิเดส 2) คอลลาจีเนสและอีลาสเตส และ 3) อนุมูลอิสระ ด้วยวิธี 1) colorimetric 2) spectrophotometric และ 3) free radical scavenging assay ต่อ DPPH, ABTS, and superoxide anion ทั้งหมดใช้เควอเซตินเป็นสารควบคุมเชิงบวก (รายงานเป็นค่า IC_{50}) วิเคราะห์ปริมาณรวมของสารกลุ่มฟีนอลิก ฟลาโวนอยด์ แอนโทไซยานิน และแคโรทีนอยด์ **ผลการศึกษา:** สารสกัดดอกเข็มสีเหลืองมีค่า IC_{50} ในการยับยั้งการทำงานของเอนไซม์ไฮยาลูโรนิเดส เอนไซม์คอลลาจีเนส และเอนไซม์อีลาสเตส เท่ากับ 608.38 ± 216.71, 556.94 ± 15.75 และ 287.70 ± 17.29 µg/ml ตามลำดับ ความสามารถในการต้านอนุมูลอิสระ (IC_{50}) ด้วย free radical scavenging assay โดยใช้ DPPH, ABTS, superoxide anion เท่ากับ 73.80 ± 5.58, 27.86 ± 3.41 และ 86.55 ± 4.69 µg/ml ตามลำดับ ใน 1 g มีปริมาณฟีนอลิก ฟลาโวนอยด์ แอนโทไซยานิน และแคโรทีนอยด์เป็นค่าเทียบเท่าของกรดแกลลิก เควอเซติน ไซยานิดิน และเบต้าแคโรทีนเท่ากับ 11.49 ± 4.06 mg, 277.38 ± 5.10 mg, 63.71 ± 0.70 mg และ 74.89 ± 0.72 mg ตามลำดับ **สรุป:** สารสกัดดอกเข็มสีเหลืองมีฤทธิ์ยับยั้งเอนไซม์อีลาสเตสเอนไซม์ไฮยาลูโรนิเดส เอนไซม์คอลลาจีเนส และฤทธิ์ต้านอนุมูลอิสระ พฤกษเคมีในสารสกัดดอกเข็มสีเหลืองพบว่ามีปริมาณของสารกลุ่มฟลาโวนอยด์เป็นองค์ประกอบมากที่สุด และมีปริมาณแคโรทีนอยด์ แอนโทไซยานิน และฟีนอลิกรองลงมา

คำสำคัญ: สารสกัดดอกเข็มสีเหลือง; ฤทธิ์ยับยั้งเอนไซม์ไฮยาลูโรนิเดส; ฤทธิ์ยับยั้งเอนไซม์คอลลาจีเนส; ฤทธิ์ยับยั้งเอนไซม์อีลาสเตส; ฤทธิ์ต้านอนุมูลอิสระ; ฟีนอลิก; ฟลาโวนอยด์; แอนโทไซยานิน; แคโรทีนอยด์

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Abstract

Objective: To investigate hyaluronidase, collagenase, and elastase inhibitory activities and antioxidation and the total phytochemicals contents including phenolic, flavonoid, anthocyanin, and carotenoid of yellow *Ixora coccinea* L. flower extract. **Methods:** Yellow *I. coccinea* flower was collected, dried under hot air oven at 50°C until dryness, pulverized to powder and macerated in 80% ethanol. The ethanolic extract was evaporated until dryness with a rotary evaporator. The inhibitory activity of the extract on 1) hyaluronidase, 2) collagenase and elastase, and 3) free radicals was investigated by 1) colorimetric method, 2) spectrophotometric method, and 3) free radical scavenging assay (for DPPH, ABST, and superoxide anion), respectively. Quercetin was used as a positive control. The total phenolics, flavonoids, anthocyanins and carotenoids contents were analyzed. **Results:** Yellow *I. coccinea* flower extract exhibited anti-hyaluronidase, anti-collagenase, and anti-elastase activities at IC_{50} values of 608.38 ± 216.71, 556.94 ± 15.75 and 287.70 ± 17.29 µg/ml, respectively. The antioxidant activities (IC_{50}) by free radical scavenging assay of DPPH, ABTS, and superoxide anion were 73.80 ± 5.58, 27.86 ± 3.41, and 86.55 ± 4.69 µg/ml, respectively. The total contents of phenolics, flavonoids, anthocyanins, and carotenoids in 1 g of the extract as equivalence of gallic acid, quercetin, cyanidin, and β-carotene were 11.49 ± 4.06, 277.38 ± 5.10, 63.71 ± 0.70, and 74.89 ± 0.72 mg. respectively. **Conclusion:** Yellow *I. coccinea* flower extract expressed anti-elastase, anti-hyaluronidase, anti-collagenase, and antioxidant activities. Total flavonoids content was the highest phytochemical contents found in the extract, followed by total carotenoid, total anthocyanin and total phenolic contents.

Keywords: yellow *Ixora coccinea* L. flower extract; anti-hyaluronidase; anti-collagenase; anti-elastase; antioxidant; phenolics; flavonoids; anthocyanins; carotenoids

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Introduction

Aging is a significant factor in wrinkles formation. They appear due to the decline in crucial substances in our skin, causing imbalances in the cell. Normally, human skin has an extracellular matrix (ECM) which helps the cell to remain

intact. ECM is an important part for supporting the cell, giving the cell strength and flexibility. ECM consists of proteoglycan and matrix metalloprotein (MMP). The major components of the matrix metalloprotein are collagen, elastin, and hyaluronic acid (HA). Collagen is the main structural protein important to cells, elastin maintains skin elasticity and hyaluronic acid can draw water in the cells resulting in the smooth and moisturized skin. As age increases, the function of various organs decreases, leading to the increase in the production of reactive oxygen and nitrogen species. These are free radicals that cause inflammation in different parts of the body and lead to the increase in the production of MMPs. In addition, UV radiation from sunlight can have detrimental effects on the skin. UVB radiation, in particular, can increase the activity of enzymes MMP-1, MMP-3, and MMP-9, leading to tissue degradation and skin damage.[1] This results in the decrease in the production of MMPs. The body produces fewer MMP, resulting in the decrease in the production of cell coating substances. In addition, the matrix metalloproteinase (MMPs), which includes hyaluronidase, collagenase, and elastase break down hyaluronic acid, collagen, and elastin, respectively, leading to dry and wrinkled skin. The best way to keep the skin moisturized and reduce wrinkles is to inhibit oxidation and MMPs activity, which can help prevent and reduce ECM degradation.[2,3]

Currently, natural products are being increasingly used as a substitute for synthetic compounds. They are considered safer in terms of causing allergies or irritations compared to those synthetic substances. The natural products that are commonly used in anti-aging treatments contain phytochemicals such as phenolic, flavonoid, anthocyanin, and carotenoid. Phenolic compounds promote collagen synthesis and inhibit the breakdown of collagen in the skin, while flavonoids have antioxidant and anti-inflammatory effects that protect the skin from damage and reduce wrinkles. Anthocyanins enhance collagen synthesis and reduce inflammation of the skin. Carotenoids protect against UV radiation from generating free radicals, inhibit MMP activity and reduce wrinkle formation.[4-8] Antioxidants reduce wrinkles by protecting the skin from damage caused by free radicals, which are unstable molecules that can cause oxidative stress and lead to skin aging. Antioxidants neutralize free radicals and prevent them from causing further damage to the skin. They can also promote collagen synthesis, which is important for maintaining skin elasticity and reducing the appearance of wrinkles. In addition, antioxidants have anti-inflammatory effects that can help reduce redness and inflammation in the skin, further improving its overall appearance.[9-12]

Ixora coccinea L. is a plant in the Rubiaceae family, a tropical plant grown as ornamental plant in Thailand. The Ixora flower extract contains phenolics, flavonoids, anthocyanins, and carotenoids.[13] The extract also showed antioxidant activity.[14] In addition, the anti-hyaluronidase activity of flavonoid was reported.[15-18]

In our study, we investigated anti-wrinkle ability of yellow *I. coccinea* flower extract (Figure 1) by evaluating its anti-collagenase, anti-elastase, anti-hyaluronidase, and antioxidant activities. The antioxidant effect of the extract was evaluated by DPPH, ABTS, and superoxide anion assays. Moreover, we determined the quantities of phenolic, flavonoid, anthocyanin, and carotenoid in the extract to observe the relationship between the quantities of these mentioned phytochemical substances found in the extract and their anti-wrinkle effect.

Figure 1 Yellow *Ixora coccinea* L. flower.

Methods

Chemicals and equipments

All solvents used for all assays 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). Dichloromethane, ethyl Acetate, 37% hydrochloric

acid (HCl) and hexane were purchased from RCI Labscan (Thailand). Glacial acetic acid, sodium acetate (CH_3COONa), and sodium chloride (NaCl) were purchased from Quality Reagent Chemical (New Zealand). Riboflavin was purchased from Sigma (China). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma (Germany). 4-(Dimethylamino) benzaldehyde (PDMAB), Collagenase type I from *Clostridium histolyticum* (6.25 units/mg solid), gallic acid, hyaluronidase type I from bovine testes, Nitroblue tetrazolium (NBT), N-(3-[2-Furyl]-acryloyl)-Leu-Gly-ProAla (FALGPA), N-succinyl-$(Ala)_3$-p-nitroanilide (SANA), porcine pancreatic elastase type IV (PPE), quercetin, tricine, and tris HCl were purchased from Sigma (USA). β-carotene and cyanidin chloride were purchased from Fluka (China). Potassium persulfate ($K_2S_2O_8$) was purchased from Unilab (Australia). Dipotassium hydrogen phosphate (K_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), and ethylenediaminetetraacetic acid (EDTA) were purchased from Univar (Australia). Hyaluronic acid (HA) was purchased from My Skin Recipes (Thailand) and water for irrigation was purchased from A.N.B. Laboratories (Thailand). Experiment instruments included microplate readers (SpectraMax M3, Molecular Devices, USA), an incubator (Electronic Microprocessor PID Control, Memmert, Germany), a centrifuge (MX-301, Tomy, Japan) and a rotary evaporator (Rotavapor R-300, Buchi, Switzerland)

Sample preparation

Yellow *Ixora coccinea* L. flowers were collected in 2020 from Nonthaburi, Thailand and identified by Assoc. Prof. Dr. Sarin Tadtong. A voucher specimen number "IX_2021_001" of this plant was deposited in herbarium at the Faculty of Pharmacy, Srinakharinwirot University, Nakhonnayok, Thailand. Yellow *I. coccinea* flowers were separated and dried under well-ventilated conditions at room temperature for 48 hours. The air-dried flowers were further dried in hot air oven at 50 °C for 2 hours and ground into powder using mechanical grinder. The flower powder was macerated for 3 - 5 days by using 1 g of powder per 50 ml of 80% ethanol and then filtered. The filtrate was evaporated until dryness using a rotary evaporator (Buchi Rotavapor R-114, Switzerland) at 45 °C. The process was repeated three 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.[17,19]

Biological assays

Determination of anti-hyaluronidase[20]

The inhibitory activity of hyaluronidase was analyzed by colorimetric method. The absorbance at 570 nm of the reaction between chromogen III obtained from N-acetyl-D-glucosamine (GlcNAc) reacting with hyaluronic acid and *p*-dimethylamino benzaldehyde (PDMAB) was observed by a microplate reader. Various concentrations of yellow *I. coccinea* flower extract at 50 µl was mixed with 50 µl of 7,900 units/ml bovine hyaluronidase, and 250 µl of 15 mg/ml hyaluronic acid, then incubated at 37 °C for 40 min. Then 1000 µl of PDMAB (prepared from 0.5 g PDMAB was dissolved in 6.25 mL of 10 N HCl and 43.75 mL of glacial acetic acid) was added into the previous reaction mixture. 200 µl of this reaction mixture were transferred to a 96-well plate to measure the absorbance with a microplate reader. Three independent experiments were performed and each experiment was run in triplicate. The absorbance was used to calculate the inhibition percentage from the equation below. Quercetin 600 µg/ml was used as a positive control.[21-23]

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

$Absorbance_{control}$ was the GlcNAc-PDMAB absorbance of control reaction and $Absorbance_{sample}$ was the GlcNAc-PDMAB absorbance of quercetin or the extract reaction. Half-maximal inhibitory concentration (IC_{50}) of yellow *I. coccinea* flower extract was calculated from the linear equation obtained from the graph plotted between the inhibition percentage versus concentration.

Determination of anti-collagenase[22-24]

The anti-collagenase activity was observed by spectrophotometric method. *Clostridium histolyticum* collagenase type I was used as target enzyme and N-(3-[2-Furyl]-Acryloyl)-Leu-Gly-Pro-Ala (FALGPA) was used as a substrate. If the extract can inhibit collagenase enzyme, the brown color of FALGPA will be darker. Tricine buffer pH 7.5 was prepared at a concentration of 0.05 M as a solvent and 120 µl of various concentrations of the extract was mixed with

15 µl of 6.25 collagen digestion unit (CDU) collagenase in a 96-well plate and incubated at room temperature for 15 minutes. 15 µl of 0.8 mM FALGPA solution was then added. The absorbance was measured with a microplate reader at 340 nm wavelength immediately after the reaction. Three independent experiments were performed and each experiment was run in triplicate. The absorbance was calculated for determining the percentage of inhibition by using the equation below. Quercetin 1,500 µg/ml was used as a positive control.[21-23]

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

When $Absorbance_{control}$ was the FALGPA absorbance of control reaction and $Absorbance_{sample}$ was the FALGPA absorbance of quercetin or the extract reaction. Half-maximal inhibitory concentration (IC_{50}) of yellow *I. coccinea* flower extract was calculated from the linear equation obtained from the graph plotted between the inhibition percentage versus concentration.

Determination of anti-elastase[22,23,25]

Porcine pancreatic elastase (PPE) type IV was used as target enzyme and N-Succinyl-$(Ala)_3$-p-nitroanilide (SANA) was used as a substrate in the reaction. Normally, the elastase cleaves the bonds of the precursor to yield the product as N-Succinyl-$(Ala)_3$ and *p*-nitroaniline. The absorbance of *p*-nitroaniline which was yellow was observed at wavelength of 410 nm by a microplate reader.

0.2 M Tris-HCl buffer pH 8.0 was used as a solvent and 160 µl of various concentrations of yellow *I. coccinea* flower extract was mixed with 20 µl of 0.01 mg/ml of PPE type IV (4.5 unit/mg solid) in a 96-well plate and incubated at room temperature for 10 minutes then 0.9 mM N-succinyl-$(Ala)_3$-*p*-nitroanilide 20 µl was added. Incubated the reaction plate at room temperature for 60 min, after that the absorbance was measured immediately. Three independent experiments were performed and each experiment was run in triplicate. The absorbance was calculated for determining the percentage of inhibition by using the equation below. Quercetin 300 µg/ml was used as a positive control.[21-23]

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

$Absorbance_{control}$ was the *p*-nitroaniline absorbance of control reaction and $Absorbance_{sample}$ was the *p*-nitroaniline absorbance of quercetin or the extract reaction. Half-maximal inhibitory concentration (IC_{50}) of the extract was calculated from the linear equation obtained from the graph plotted between the inhibition percentage versus concentration.

Determination of antioxidant

DPPH radical scavenging assay[26]

DPPH is a purple highly stable free radical. If the plant extract had antioxidant activity, hydrogen atoms were transferred to DPPH, which would change from purple to yellow (DPPH-H). 1 mg/ml Quercetin was used as a positive control.[1-3] 100 µl of 500 µM DPPH was mixed with 100 µl of various concentrations of the extract in a 96-well plate and incubated at room temperature for 30 min. After that, the absorbance was measured at wavelength of 517 nm by a microplate reader. Three independent experiments were performed and each experiment was run in triplicate. The absorbance was calculated for determining the percentage of inhibition by using the equation below.

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

$Absorbance_{control}$ was the DPPH absorbance of control reaction and $Absorbance_{sample}$ was the DPPH absorbance of quercetin or the extract reaction. Half-maximal inhibitory concentration (IC_{50}) of the extract was calculated from the linear equation obtained from the graph plotted between the inhibition percentage versus concentration.

ABTS radical scavenging assay[27]

7 mM ABTS was oxidized with 2.45 mM potassium persulfate ($K_2S_2O_8$) to form $ABTS^{+\bullet}$ (radical form) which was bluish-green solution. When $ABTS^{+\bullet}$reacts with plant extract, it was reduced back to ABTS, a colorless clear solution. 1mg/ml Quercetin was used as a positive control. To perform the assay, 180 µl of $ABTS^{+\bullet}$ radical solution (absorbance 0.8±0.1) was mixed with 20 µl of various concentrations of the extract in a 96-well plate and incubated at room temperature for 6 min. Then measured the absorbance by a microplate reader at a wavelength of 735 nm. Three independent experiments were performed and each experiment was run in

triplicate. The absorbance was calculated for determining the percentage of inhibition by using the equation below.

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

$Absorbance_{control}$ was the ABTS absorbance of control reaction and $Absorbance_{sample}$ was the ABTS absorbance of quercetin or the extract reaction. Half-maximal inhibitory concentration (IC_{50}) of the extract was calculated from the linear equation obtained from the graph plotted between the inhibition percentage versus concentration.

Superoxide anion scavenging activity assay[26]

The reaction occurred when riboflavin was photoactivated to form a free radical. Then this free radical oxidized oxygen into superoxide anions. Superoxide anion then reacted with nitro-blue tetrazolium chloride (NBT), turning NBT into the blue formazan. If the tested substance has antioxidant activity, it can inhibit the reaction and prevent the formation of blue formazan. 1 mg/ml Quercetin was a positive control. 50 mM Potassium phosphate buffer (pH 8.0) 20 µl was mixed with 40 µl of various concentrations of the extract in a 96-well plate and 20 µl each of 1mM EDTA and 750 µM were added. 100 µl of 266 µM riboflavin were added to the mixture, then incubated under fluorescent light at room temperature for 10 minutes. Absorbance with a microplate reader at a wavelength of 570 nm was measured. Three independent experiments were performed and each experiment was run in triplicate. The absorbance was calculated for determining the percentage of inhibition by using the equation below.

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

$Absorbance_{control}$ was the superoxide (blue formazan) absorbance of control reaction exposed to fluorescent light subtract with absorbance of control reaction keep out of the fluorescent light. $Absorbance_{sample}$ was the superoxide (blue formazan) absorbance of quercetin or the extract reaction exposed to fluorescent light subtract with absorbance of quercetin or the extract reaction keep out of the fluorescent light. Half-maximal inhibitory concentration (IC_{50}) of the extract was calculated from the linear equation obtained from the graph plotted between the inhibition percentage versus concentration.

Total phenolics content determination[22,23]

Determination of total phenolic compounds by the Folin-Ciocalteu method, is based on the absorbance principle by a UV-Vis spectrophotometer. The independent experiment was repeated 3 times with 3 replicates and each time gallic acid was used as the standard. 10%v/v Folin-Ciocalteu reagent 100 µl was mixed with 5 mg/ml extract 100 µl, 75 mg/ml sodium carbonate (Na_2CO_3) 100 µl, and deionized 700 µl of water in a 2 ml microcentrifuge tube, then incubated in the dark at room temperature for 90 minutes. The solution was added into a 96-well plate, 200 µl/well. Measured the absorbance using a microplate reader at a wavelength of 765 nm. The relationship between the absorbance and the gallic acid standard solution concentration was plotted to make a standard curve. The total phenolics content was calculated from the linear equation obtained from the standard curve and reported as milligrams of gallic acid equivalent (GAE) per gram of the extract (mg/g).

Total flavonoids content determination[22,23]

Total flavonoids content was determined by the aluminum chloride complex colorimetric method using quercetin as a standard. Three independent experiments were done and each experiment was run in triplicate. Initially, 1 ml of 8 mg/mL aluminum chloride ($AlCl_3$) was mixed with 1000 µl of the extract 10 mg/ml in a centrifuge tube, then ethanol was added to make a final volume of 10 ml and incubated in the dark at room temperature for 40 min. The solution was added into a 96-well plate, 200 µl/well. The absorbance was measured with a microplate reader at a wavelength of 415 nm. The relationship between the absorbance and quercetin standard solution concentration was plotted to make a standard curve. The total flavonoids content was calculated from the linear equation obtained from the standard curve and reported as milligrams of quercetin equivalent (QE) per gram the extract (mg/g).

Total anthocyanins content determination[22,23]

The anthocyanin content was determined by the pH differential method, which used two solutions, KCl buffer and CH_3COONa, at pH 1.0 and 4.5, respectively. Cyanidin as a standard was dissolved in two solvents (1000 µl of KCl pH 1.0 and CH_3COONa pH 4.5) and stored in the dark for 15 to 40 min. Standard solutions were added into a 96-well plate, 200 µl/well, the absorbance was measured using a microplate

reader at wavelengths of 510 and 700 nm. Three independent experiments were done and each experiment was run in triplicate. The absorbances of cyanidin and 10 mg/ml extract were calculated as the equation below. The relationship between the calculated cyanidin absorbance and cyanidin standard solution concentration was plotted to make a standard curve. Total anthocyanins content was calculated from the linear equation obtained from the standard curve and reported as milligrams of cyanidin equivalence (CE) per gram of the extract (mg/g).

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

Total carotenoids content determination[28]

Determination of total carotenoids by spectrophotometry used ethyl acetate (EA), dichloromethane (DCM) and hexane (Hex) at a ratio of 20:4:1 as solvent. β-carotene was used as standard. 1000 µl of the extract 10 mg/ml was mixed with 1000 µl of EA:DCM:Hex in a centrifuge tube and 8000 µl of ethanol was added, then incubated in the dark at room temperature for 10 minutes. 200 µl of the mixture was transferred into 96-well plates and measured absorbance with a microplate reader at a wavelength of 450 nm. Three independent experiments were performed and each experiment was run in triplicate. The relationship between the absorbance and β-carotene standard solution concentration was plotted to make a standard curve. The total carotenoids content was calculated from the linear equation obtained from the standard curve and reported as milligrams of β-carotene equivalent (BCE) per grams of the extract (mg/g).

Results

Sample preparation

The amount of yellow *I. coccinea* flower extract was 47.39%w/w. The extract was stored in an amber bottle at -20 °C prior to use.

Biological assays

Anti-collagenase, anti-elastase, and anti-hyaluronidase assays

The inhibitory effect of yellow *I. coccinea* flower extract against various enzymes involving in wrinkle formation (hyaluronidase, collagenase, and elastase) were reported as the half minimal concentration effect (IC_{50}). The ant-wrinkle effect of yellow *Ixora* sp. flower extract depended on the concentration of the extract (dose-dependent) by inhibiting the hyaluronidase, collagenase, and elastase at IC_{50} of 608.38 ± 216.71, 556.94 ± 15.75, and 287.70 ± 17.29 µg/ml, respectively. While a positive control, quercetin showed anti-hyaluronidase, anti-collagenase, and anti-elastase at IC_{50} of 561.49 ± 43.31, 1,420.17 ± 212.98, and 275.04 ± 120.82 µg/ml, respectively (Figure 2). When comparing it was found that the inhibitory activity of hyaluronidase and elastase of the extract was similar to quercetin (P-value > 0.05), while the anti-collagenase activity of the extract was better than that of quercetin (P-value < 0.05).

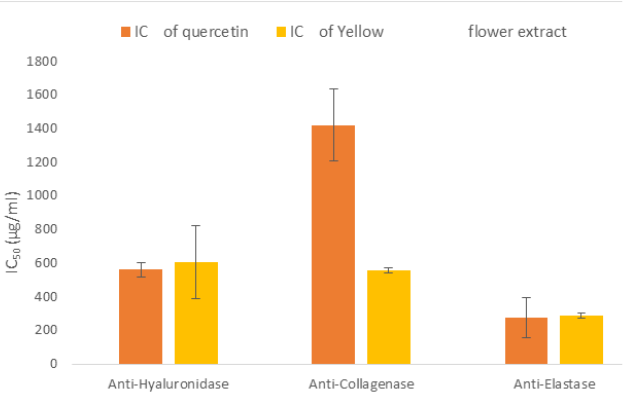

Figure 2 The inhibitory effect of yellow Ixora coccinea L. flower extract against various enzymes involving in wrinkle formation (hyaluronidase, collagenase, and elastase) at IC_{50}. *P-value < 0.05 when compared with quercetin.

Antioxidant activity of yellow *I. coccinea* flower extract

Antioxidant activity of yellow *I. coccinea* flower extract from DPPH radical scavenging, ABTS free radical scavenging, and superoxide anion radical scavenging assays were compared with those of quercetin and Trolox. It was found that the extract showed antioxidant activity by DPPH, ABTS, and superoxide anion assays at IC_{50} of 73.80 ± 5.58, 27.86 ± 3.41, and 86.55 ± 4.69 µg/ml respectively. Quercetin exhibited antioxidant activity at IC_{50} of 7.25 ± 0.15, 4.01 ± 0.85, and 19.37 ± 2.25 µg/ml respectively, while Trolox had antioxidant activity at IC_{50} of 12.86 ± 0.26, 8.83 ± 1.00, and 292.48 ± 8.04 µg/ml, respectively (Figure 3). Our results showed that the antioxidant activity by DPPH, ABTS and superoxide anion of the extract was less than that of quercetin (P-value < 0.05).

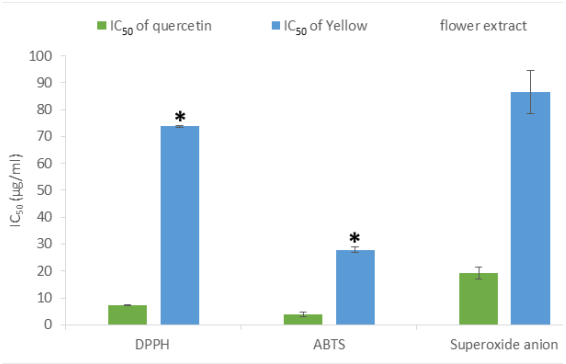

Figure 3 Antioxidant activity at IC_{50} from DPPH, ABTS and superoxide anion assays. *P-value < 0.05 when compared with quercetin.

Phytochemical of yellow *I. coccinea* flower extract

Total phenolics, total flavonoids, total anthocyanins, and total carotenoid contents found in yellow *I. coccinea* were 11.49 ± 4.06 mg GAE/g extract (11.49 ± 0.41 % w/w dry extract), 277.38 ± 5.10 mg QE/g extract (27.74 ± 0.50 % w/w dry extract), 63.71 ± 0.70 mg CE/g extract (6.37 ± 0.07 % w/w dry extract), and 74.89 ± 0.72 mg BCE/g extract (7.49 ± 0.07 %w/w dry extract) as shown in Table 1.

Table 1 Phytochemical determination of yellow *Ixora coccinea* L. flower extract.

Phytochemicals	Amount of phytochemicals found in yellow *Ixora coccinea L.* flower extract
Phenolic	11.49 ± 4.06 mg GAE/g extract
Flavonoid	277.38 ± 5.10 mg QE/g extract
Anthocyanin	63.71 ± 0.70 mg CE/g extract
Carotenoid	74.89 ± 0.72 mg BCE/g extract

Discussions and Conclusion

Yellow *I. coccinea* flower extract possessed enzymes involving in wrinkle formation inhibitory ability (collagenase, elastase, and hyaluronidase), and antioxidant property. Our result showed antioxidant activity related to that of *Ixora coccinea* L. mentioned by Chen et al (2016).[14] Interestingly, the extract could inhibit elastase and hyaluronidase activities almost to that of quercetin, the positive control. However, its anti-collagenase is less than quercetin. Evaluation of the antioxidant activity of the extract using various methods demonstrated that ABTS assay displayed more sensitivity in antioxidant assay, whereas DPPH and superoxide anion assays presented less sensitivity. The results also suggested that the extract had antioxidant property by DPPH radical was reduced by accepting an electron from the antioxidant, resulting in a change in its color from purple to yellow. In ABTS assay, ABTS was oxidized to be a blue-green colored radical and was reduced by antioxidant donating an electron, resulting in a change in its color from blue-green to colorless. Finally, in the superoxide anion assay, the antioxidant reduces the superoxide anion by donating an electron, resulting in a change in its color of nitro-blue tetrazolium chloride (NBT) from blue to colorless-reduced,[29-31] representing that the yellow *I. coccinea* flower extract is a multi-targeting anti-wrinkle agent.

The phytochemicals study in yellow *I. coccinea* flower extract found in our experiments was consistent with other studies.[11,30] This study also discovered that the highest amount of phytochemicals found in yellow *I. coccinea* flower extract was flavonoids, followed by carotenoids, anthocyanins and phenolics. The variation in the quantity of phytochemicals found in different yellow *I. coccinea* flower samples was attributed to the diverse cultivation locations, soil quality, and varying cultivation practices.[32-35] In addition, the extraction method was another factor that affected the amount of phytochemicals obtained from the yellow *I. coccinea* flower.

Studies reported that flavonoid such as quercetin, apigenin, kaempferol, myricetin, and luteolin possessed anti-elastase, anti-hyaluronidase, anti-collagenase, and antioxidant activities.[36-39] Wen et al (2012) showed that *Ixora parviflora* extract (IPE) significantly suppressed the overexpression of MMP-1, MMP-3, MMP-9 activity, and elastase activity, in a dose-dependent manner.[1] Therefore, we suggested that the anti-elastase, anti-hyaluronidase, anti-collagenase, and antioxidant activities were due to flavonoids found in the yellow *I. coccinea* flower extract. In addition, we also suggested that yellow *I. coccinea* flower extract can be used as anti-wrinkle agent in cosmetics.

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