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Research paper

Antioxidant and anti-inflammatory activites of *Clerodendrum* leaf extracts collected in Thailand

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ABSTRACT

Introduction: *Clerodendrum* plants have been traditionally used for inflammatory related diseases in Thailand. Crude extracts of *Clerodendrum disparifolium* and *Clerodendrum laevifolium* were assessed for antioxidant and anti-inflammatory activities in order to support traditional Thai claims about the therapeutic potential of these plants.
Methods: Ethanol and hexane extracts of leaves were evaluated for the *in vitro* antioxidant activity including phenolic content assay using Folin–Ciocalteu method, flavonoid content assay using an aluminium chloride ($AlCl_3$) colorimetric method, 2, 2-Diphenyl-2-picryl-hydrazyl (DPPH) free-radical scavenging activity assay, total reductive capability assay, total antioxidant activity assay using ferric thiocyanate (FTC) and thiobarbituric (TBA) methods. In addition anti-inflammatory activity was assayed against lipoxygenase.
Results: The phenolic and flavonoid contents, relating with their antioxidant activities, of the extracts ranged from 1167.21–3344.52 GAE/g of dry extract and 9.05–59.91 mg QE/g of dry extract, respectively. Reducing power increased with increasing concentrations of extracts. The lipid peroxidation inhibition was evaluated by ferric thiocyanate (FTC) and thiobarbituric (TBA) methods, which demonstrated the maximum inhibition in ethanol *C. laevifolium* (55.49%) and *C. disparifolium*(96.17%) extracts, respectively. Furthermore the ethanol *C. laevifolium* extract exhibited highest 2, 2-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity (IC_{50} 12.70 μg/mL) and also possessed the greatest anti-inflammatory activity (IC_{50} 14.12 μg/mL).
Conclusion: This study indicated that selected *Clerodendrum* leaf extracts exhibited potential antioxidant and anti-inflammatory activities, which could be focus of further phytochemical research and may be applicable as natural medicine.

1. Introduction

Oxidative stress and inflammation underlie the pathogenesis of illnesses.Physiological and biochemical processes in the human body may produce free radicals as by products, which can cause oxidative damage to biomolecules leading to various diseases [1–5]. A number of species in the genus *Clerodendrum*(family Lamiaceae) were widely documented in indigenous systems of medicine throughout tropical and subtropical regions of the world especially Asian and African continents [6]. The presence of antioxidant and anti-inflammatory molecules in these plant species was considerably evident their beneficial actions that led researchers to investigate plant extracts targeting their activity. Antioxidant activity can be characterized with *in vitro* assays *i.e.* phenolic content assay using Folin–Ciocalteu method, flavonoid content assay using aluminium chloride ($AlCl_3$) colorimetric method. 2,2-Diphenyl-2-picryl-hydrazyl (DPPH) free-radical scavenging activity assay, total reductive capability assay, total antioxidant activity assay using ferric thiocyanate (FTC) and thiobarbituric (TBA) methods [7]. Whereas anti-inflammatory activity can be tested against lipoxygenase.In order to assess traditional claims about the therapeutic potential and to screen plants for future phytochemical research, crude leaf extracts of Thai *Clerodendrum* species with anti-inflammatory uses, *Clerodendrum disparifolium* Blume (*C. disparifolium*) and *Clerodendrumlaevifolium* (*C.laevifolium*) were assessed for their *in vitro* antioxidant activity and anti-inflammatory activity against soybean lipoxygenase enzyme.

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2. Materials and methods

2.1. Chemicals

Ammonium thiocyanate, butylatedhydroxytoluene (BHT), Folin–Ciocalteu's reagent, sodium tetraborate decahydrate, quercetin dehydrate, indomethacin, 2-thiobarbituric acid were all purchased form Sigma–Aldrich (St. Louis, MO, USA). Methanol, ethanol and hexane were purchased from J.T. Baker (NJ, USA). Iron (II) chloride ($FeCl_2$) tetrahydrate, linoleic acid and hydrochloric acid were purchased from Fluka (Buchs, Switzerland). Potassium dihydrogen orthophosphate, orthophosphoric acid, sodium hydroxide, potassium ferricyanide, sodium carbonate anhydrous and aluminium chloride ($AlCl_3$) were purchased from Ajax Finechem (NSW, Australia). Gallic acid, calcium acetate monohydrate, trichloroacetic acid, iron (III) chloride ($FeCl_3$) hexahydrate, 5-lipoxygenase (20,000 U/mL) and boric acid were purchased from Ascros organics (Geel, Belgium), POCH S.A. (Gliwice, Poland), Unilab (General Santos, Philippines), LOBA Chemie (Mumbai, India), TCI (Tokyo, Japan) and RCI Labscan (Bangkok, Thailand), respectively.

2.2. Instrumentation

All spectra and absorbance measurements were made on UV–vis spectrophotometer Evolution 600 UV (Thermo Scientific, USA) and microplate UV–vis spectrophotometer Infinite M200 (TECAN®, USA).

2.3. Preparation of plant extract

C. disparifolium and *C. laevifolium* were taken from middle part of Thailand. The plants were identified by an expert in the Department of Pharmaceutical Botany, Mahidol University and the voucher specimen number of *C. disparifolium* and *C. laevifolium* were PBM. 5156 and PBM.05153, respectively. The specimens were deposited at Mahidol University herbarium. Then, leaves were washed, cut into small pieces, air-dried and crushed into powder. The sample powder was extracted with ethanol and hexane by maceration at room temperature for 72 h. After filtering and evaporating to dryness, the crude extracts were obtained. Stock solutions of *C. disparifolium* and*C. laevifolium* were separately prepared by dissolving 20 mg of each crude extract in 10 mL methanol (2 mg/mL).

2.4. Loss on drying

This technique was carried out using an accurate weight of about 0.5 g of each extract in a crucible and dried in an oven with the constant temperature at 105 °C for 2 h. Upon opening the oven chamber, close the crucible promptly, and allow it to come to room temperature in a desiccator before weighing. The results are expressed as mean ± SD.

2.5. Total phenolic content

Total phenolic content was determined by the Folin–Ciocalteu method [8] using gallic acid as a standard. The standard curve of gallic acid with concentration of 100–200 μg/mL was established against the absorbance. Then, 125 uL of 2 mg/mL plant extract was mixed with 2.5 mL of 2.0% sodium carbonate. After that, 125 μL of Folin–Ciocalteu reagent was added and placed at room temperature for 30 min. The absorbance was measured at 680 nm against a reagent blank. Total phenolic contents of the extracts were calculated and expressed in terms of gallic acid equivalent (GAE) mg/g of dry extract.

2.6. Total flavonoid content

Determination of the total flavonoid content in the plant extracts was carried out using $AlCl_3$ colorimetric method [9]. The standard curve of quercetin in the concentration range of 5–15 μg/mL was established. Then, 0.5 mL of 2 mg/mL plant extract, 0.5 mL of 1.2% $AlCl_3$ ethanol solution and 0.5 mL of 0.12 M calcium acetate were mixed at room temperature for 30 min. The absorbance was measured at 430 nm against a reagent blank. A yellow color indicated the presence of flavonoids. Total flavonoid contents were expressed in terms of quercetin equivalent (QE) mg/g of dry extract.

2.7. DPPH free radical scavenging activity assay

An aliquot of 100 μL of extract solution (20–1000 μg/mL) or BHT standard solution (0–100 μg/mL) was added 100 μL of 0.5 mM DPPH methanolic solution. The mixture was shaken vigorously and left to stand at room temperature for 20 min. A color change of DPPH was quantitatively measured by spectrophotometric absorbance. Measurement was conducted on a spectrophotometer at wavelength of 515 nm against a blank [10–11]. The data was derived using equation (Eq. (1)) and reported as concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period (IC_{50}). Samples were analyzed in triplicates.

$$\%\ \text{inhibition} = \left[\frac{A_c - A_s}{A_c}\right] \times 100 \quad (1)$$

where A_c = absorbance control or blank, A_s = absorbance with sample or standard

2.8. Total reductive capability

The Fe^{3+} reductive capability of all extracts were determined using Oyaizu method [12]. One mL of *C. disparifolium* and *C. laevifolium* extracts (500–1500 μg/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH = 6.6) and 2.5 mL of 30 mM potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of 0.6 M trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was transferred to mix with 2.5 mL of distilled water and 0.5 mL of 6 mM $FeCl_3$. The absorbance was measured at 700 nm against a blank.

2.9. Total antioxidant activity

2.9.1. FTC method

The FTC assay of the extracts were determined using the standard method as described by Kikuzaki and Nakatani [13]. Two mL of 1 mg/mL extracts, 2.05 mL of 80 mM linoleic acid in 99.5% ethanol, 4 mL of 50 mM phosphate buffer (pH 7.0), and 1.95 mL of water were transferred to a vial and placed in an oven at 40 °C protecting from light until use (solution 1). Then, 0.1 mL of solution 1 was aliquoted and mixed with 9.7 mL of 75% ethanol, 0.1 mL of 3.9 M ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% HCl. The absorbance measurement of the red colored-mixture, containing the extract at final concentration of 2 μg/mL, was performed at the wavelength of 500 nm within 3 min every 24 h until 1 day after the absorbance of the control reached its maximum.The percent inhibition of linoleic acid peroxidation was calculated according to the following equation:

$$\%\ \text{inhibition of linoleic acid} = 100 - \left[\frac{A_{\text{sample}}}{A_{\text{control}}}\right] \times 100 \quad (2)$$