SHORT COMMUNICATION

Bioassay-guided isolation of the antioxidant constituent from *Cassia alata* L. leaves

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Abstract

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Bioassay-guided isolation of the antioxidant constituent from *Cassia alata* L. leaves
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Using DPPH radical scavenging assay to investigate the antioxidant activity of crude methanol extracts from the leaves, flowers and pods of *Cassia alata* L. found that the leaf extract exhibited a stronger antioxidant activity than the extracts from the flowers and pods. On the basis of DPPH radical scavenging assay-guided isolation, the methanol extract of *C. alata* leaves was separated by silica gel vacuum chromatography and Sephadex LH-20 gel filtration chromatography afford a light yellowish powder (CA1), which was identified as kaempferol. This compound exhibited antioxidant activity (ED_{50} 9.99 μM) that was six times stronger than that of BHT (ED_{50} 57.41 μM) and fifty eight times stronger than that of emodin (ED_{50} 578.87 μM).

Key words : *Cassia alata*, *Senna alata*, antioxidant, kaempferol, emodin

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บทคัดย่อ

ภาคภูมิ พาณิชยูปการนันท์ และ ทรงศรี แก้วสุวรรณ

การแยกสารที่มีฤทธิ์ต้านออกซิเดชั่นจากใบชุมเห็ดเทศโดยวิธี Bioassay-guided isolation

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การทดสอบฤทธิ์ต้านออกซิเดชั่นของสารสกัดจากส่วนใบ, ดอก และ ฝักของต้นชุมเห็ดเทศด้วยเมธานอล โดยวิธี DPPH radical scavenging assay พบว่าสารสกัดจากใบมีฤทธิ์ต้านออกซิเดชั่นแรงกว่าสารสกัดจากดอกและฝัก โดยหลักการของ DPPH radical scavenging assay-guided isolation ในการแยกสารสกัดเมธานอลจากใบชุมเห็ดเทศโดยวิธี silica gel vacuum chromatography และ Sephadex LH-20 gel filtration chromatography ได้สารเป็นผงสีเหลืองอ่อน (CA1) ซึ่งเมื่อทำการพิสูจน์เอกลักษณ์พบว่าเป็นสาร kaempferol สารชนิดนี้มีฤทธิ์ต้านออกซิเดชั่น (ED_{50} 9.99 μM) แรงกว่าฤทธิ์ต้านออกซิเดชั่นของ BHT (ED_{50} 57.41 μM) 6 เท่า และแรงกว่าฤทธิ์ต้านออกซิเดชั่นของ emodin (ED_{50} 578.87 μM) 58 เท่า

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Endogenous free radicals such as superoxide, nitric oxide and hydroxyl free radicals are produced in the human body everyday. In addition, oxidant by-products of normal metabolism cause extensive damage to DNA, proteins, and lipids constituting a major contribution to aging and also to degenerative diseases of aging such as cancer, cardiovascular disease, brain dysfunction and cataracts (Ames *et al*., 1993). To prevent or delay the oxidation process, addition of antioxidants to foods is the most extensively used method. Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) have been commonly used as antioxidants in foods for years, their safety has long been questioned (Branen, 1975; Ito *et at*., 1983). This has led to an increased interest in natural antioxidants (Lim *et al*., 2002; Kayano *et al*., 2002; Gyamfi and Aniya, 2002; Braca *et al*., 2002).

Strong antioxidant activity has been reported in the methanol extracts from the seed of *Cassia tora* L. and *Cassia oxidentalis* L. (Yen *et al*., 1998). In addition, while the highest active fraction of the seed extract from *C. tora* was found to contain an active compound, emodin, this compound exhibited lower activity than the fraction from which it was obtained. It was thus assumed that the other minor constituents might contribute to the strong antioxidant activity.

Cassia alata L. (Chum-Het-Thet), is a Thai medicinal plant whose chemical composition is close to that of *C. tora* (Ponglux *et al*. 1987). Our interest in *C. alata* led us to investigate and compare the antioxidant activity of each of its aerial parts. The antioxidant active compound that was purified by DPPH radical scavenging assay-guided isolation was also identified.

Experimental

Materials

Cassia alata L. was collected from the field in Pattalung province, Thailand (in December 2000). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and emodin were purchased from Fluka Chemie and Sigma, respectively. All other chemicals were of reagent grade.

Preparation of the methanol extracts

The leaf, flower, and pod powders of *C. alata* were separately macerated in methanol for three days. After filtering and evaporating the filtrates to dryness *in vacuo*, the methanol extracts of the leaves, flowers and pods were obtained.

Determination of antioxidant activity

The antioxidant activity of all extracts and fractions was determined according to the DPPH radical scavenging assay (Yamazaki *et al*., 1994). Samples for testing were prepared by dissolution in absolute ethanol. A portion of sample solution (0.1 ml) was mixed with the same volume of 6×10^{-5} M DPPH in absolute ethanol. After the mixture had been allowed to stand for 30 minutes at room temperature, its absorbance was measured at 520 nm using a spectrophotometer (Hewlett Packard 8452A). All tests were run in triplicate and averaged.

Purification of the antioxidative component

The methanol extract of *C. alata* leaves (10.2 g) was subjected to silica gel vacuum chromatography eluted with chloroform/methanol solvent system. The pooled active fraction (fraction II) was further purified by Sephadex LH-20 column chromatography eluted by methanol. A yellowish powder; CA1 (22.4 mg) was obtained from the pooled active fraction (fraction VII).

Identification of CA1

Yellow powder, IR (KBr) cm^{-1} : 3340 (OH), 1660, 1615 (C=O), 1570, 1505 (C=C, Ar); ^{1}H NMR (500 MHz, in CD_3OD) δ ppm: 8.07 (2H, d, J = 9.0 Hz), 6.90 (2H, d, J = 9.0 Hz), 6.38 (1H, d, J = 1.9 Hz), 6.18 (1H, d, J = 1.9 Hz); ^{13}C NMR (125 MHz, in CD_3OD) δ ppm: 177.67, 165.57, 162.50, 160.54, 158.27, 148.07, 137.12, 130.67, 123.75, 116.31, 104.56, 99.29, 94.49.

Results and Discussion

DPPH radical scavenging assay of the methanol extracts from the leaves, flowers and pods of *C. alata* showed that the methanol extract from the leaves possessed a stronger antioxidant activity than the extract from the flowers and pods. If the ED_{50} values are taken into account, the activity of the methanol extract of the leaves was weaker than that of the synthetic antioxidant, BHT (Table 1). However, the crude extract with an ED_{50} value less than 50 µg/ml should be obtained significantly free radical scavenging active compounds (Parejo *et al*., 2003). The leaf extract was, therefore, subjected to further purification steps. The antioxidant activity of the leaf extracts, which were extracted by maceration in different organic solvents, including n-hexane, ethyl acetate and methanol, was also evaluated. It was found that the methanol and ethyl acetate extracts gave an identical antioxidant activity (ED_{50} were 28.50 ± 1.86, 27.05 ± 1.72 µg/ml, respectively) that was markedly stronger than that of the n-hexane extract activity (ED_{50} was 107.32 ± 2.16 µg/ml). Because the leaves produced a smaller amount of ethyl acetate extract (1.8 g), we were interested in studying the methanol extract (10.2 g).

Table 1. Antioxidant activity of the *C. alata* extracts from leaves, flowers and pods compared with that of BHT.

Samples	ED_{50} (µg/ml ± S.D.)
Leaf extract	28.50 ± 1.86
Flower extract	175.36 ± 2.07
Pod extract	100.18 ± 2.59
BHT	14.17 ± 1.38

Purification of the methanol extract using a silica gel vacuum chromatography gave six fractions of the isolate. Evaluation of the antioxidant activity of each fraction showed that fraction II gave an antioxidant activity that was not only stronger than the other fractions, but also stronger than BHT (Figure 1). Using Sephadex LH-20 gel filtration chromatography, we therefore further separated fraction II into eight fractions. Fraction VII gave the highest potency (Figure 2) and also the yellowish powder (CA1).

On the basis of the spectroscopic data, including IR, ^{1}H NMR and ^{13}C NMR, CA1 was identified as a flavonol compound named kaempferol (Figure 3). Comparison of the antioxidant activity of kaempferol with BHT and emodin, an anthraquinone found in *C. alata*, showed that kaempferol gave an antioxidant activity that was six times

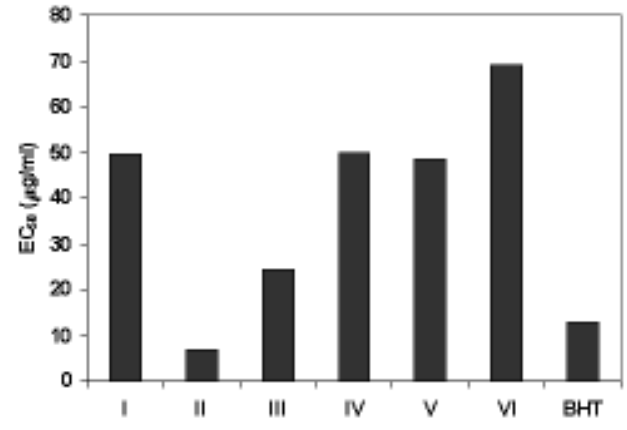

Figure 1. Antioxidation activity of the fractions, which were isolated by the silica gel vacuum chromatography, compared with that of BHT.

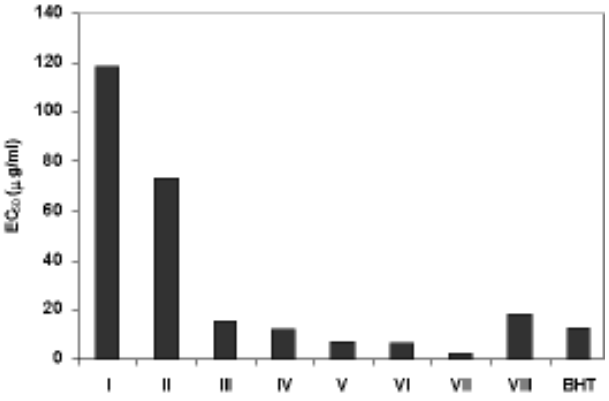

Figure 2. Antioxidation activity of the fractions, which were isolated by Sephadex LH-20 gel filtration chromatography, compared with that of BHT.

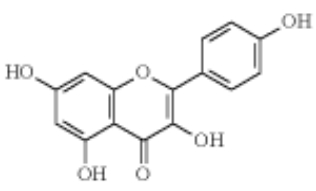

Figure 3. Structure of kaempferol

Table 2. Antioxidant activity of kaempferol, emodin and BHT.

Samples	ED_{50} (mM ± S.D.)
Kaempferol	9.99 ± 0.28
Emodin	578.87 ± 2.86
BHT	57.41 ± 0.54

stronger than that of BHT and fifty eight times stronger than that of emodin (Table 2). We therefore suggested that kaempferol is a major contributor to the antioxidant capacity of *C. alata*. Although emodin has been reported to be an active antioxidant compound in *C. tora* seeds (Yen *et al*., 1998), it showed only a very weak antioxidant potency. In addition, the active fraction that contained emodin exhibited a stronger antioxidant activity than emodin itself, implying that the component that contributed to the satisfactory antioxidant activity of *C. tora* extract was probably one of the minor components.

It has been reported that the antioxidant and the monoamine oxidase inhibiting activities of *Ginkgo biloba* extract is due primarily to the presence of kaempferol (Sloley *et al*., 2000). In addition, kaempferol showed an antioxidative activity against metal-induced lipid peroxidation (Sugihara *et al*., 1999). It also prevented protein glycosylation (Asgary *et al*. 1999). Plants that contain kaempferol can therefore be used to prevent or lower the risk of chronic diseases such as cerebrovascular disease and diabetes (Asgary *et al*.; 1999, Kayano *et al*., 2002)

It has been reported that the chronic use of anthranoid laxative has been associated with the development of pseudomelanosis coli, which might be involved in the development of colorectal carcinoma (van Gorkom *et al*., 1999; Siegers *et al*., 1993; Steer and Colin-Jones, 1975). The pigment that deposits in the intestinal wall has been identified as lipofuscin, which is a peroxidized fatty acid residue (Donnerer *et al*., 1992). Kaempferol in *C. alata* may therefore play a role in lowering the risk of pseudomelanosis coli and colorectal cancer.

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