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Research Article

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Fluorescence of *Senna simea* Lam. leaf extracts: A possible interference in a fluorescence-based assay

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ABSTRACT

Major depressive disorder (MDD) is a debilitating disease worldwide and occurs with a high prevalence in elderly individuals. In attempt to search for herbal medicine with antidepressant effect, each in vitro model must be properly validated. The objective of this study was to determine the effect of Senna siamea Lam. (Cassod tree) leaf extracts prepared using ethanol and water on activity of DAT, NET, SERT and total MATs in in vitro model, human neuroblastoma LAN-5 cell line using a commercial neurotransmitter transporter reuptake assay kit. After measuring the intracellular fluorescence, we calculated RFU area below curve reflecting the activity of dopamine transporter (DAT), norepinephrine transporter (NET), serotonin transporter (SERT) and all monoamine neurotransmitter transporters (MATs) in LAN-5 cells after treatment for 30 min with 25 and 100 µg/mL of Senna siamea Lam. leaf extracts in the absence or presence of combining antidepressants. We observed the fluorescence intensity below zero or minus values, thus reflecting assay interference. The inference was stronger as reflected by lesser intracellular fluorescence intensity when increasing concentrations of Senna siamea Lam. extracts. Ethanol extract resulted in a stronger interference as compared to that of water extract. To confirm that Senna siamea Lam. leaf extracts containing certain fluorescent compounds with excitation 440 nm and emission 520 nm, we measured the fluorescence of Senna siamea extracts compared with reagent blank and Mentha cordifolia Opiz ex. Frezen (Kitchen mint) leaf and Centella asiatica L (Urm.) (Gotu kola) whole plant extracts as control herbal extracts using spectrofluorometer with the excitation wavelength of 440 nm and emission wavelengths of 480-600 nm for scanning, and we found the concentration-dependent and solvent type-dependent manner in agreement with the observation in neurotransmitter transporter reuptake assay. Therefore, Senna siamea leaf extracts possessed fluorescence characteristics possibly due to certain fluorescent compounds and could be interfered in a fluorescence-based assay. It is highly recommended to know the chemical nature of each plant extract before selecting any biological assay.

Keywords: Major depressive disorder, *Senna siamea* Lam. leaf extracts, Fluorescence, monoamine Neurotransmitter transporters, LAN-5 neuroblastoma cells

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INTRODUCTION

Major depressive disorder (MDD) is a neurodegenerative disorder with profound individual and societal costs. The lifetime prevalence of MDD is around 16.2% [1] with women experiencing a disproportionately higher burden of MDD than men [2, 3]. Depressive symptoms and syndromes are very common in older persons [4, 5].

Antidepressant drugs currently available have side effects on patient such as anticholinergic, sleepy, hypotension, cardiac arrhythmia, sexual dysfunction, myoclonus, hepatic failure and renal failure [6-8]. Therefore, an alternative treatment by medicinal herbal extracts might provide an effective, safe and inexpensive therapy.

Thailand is rich of natural products, and plants are one of them that can be consumed not only as nutritional values, but as medicinal drugs as well. Our research has focused on the search for medicinal plants with advantageous properties such as neuroprotective effects and antidepressant effects using *in vitro* and *in vivo* models. Recently, our *in vitro* study demonstrated that *Rhinacanthus nasutus* extracts exhibited neuroprotective effects by preventing not only hypoxia-induced neurotoxicity [9], but also glutamate-induced and β-amyloid-induced neurotoxicity [10]. In fact, we would like to test whether these *Rhinacanthus nasutus* extracts can inhibit the activity of monoamine neurotransmitter transporters (MATs), especially serotonin transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET). However, validation of our current model with known herbal drug is required prior to testing with *Rhinacanthus nasutus* extracts.

As a known herb for folk medicine, *Senna siamea* Lam., commonly known as Cassod tree or locally called Khi Lek, is a local herb that the people use for treatment insomnia [11]. Barakol (3,4-dihydroxy-2,5-dimethyl-1,4-dioxyphenalene), a major compound in *Senna siamea* Lam. leaves, has been shown to possess the biological effects, including sedative drugs and reducing anxiety [12]. The use of barakol for therapy is currently discouraged because of its hepatotoxicity [13]. However, boiled *Senna siamea* Lam. in popular Thai curry is safe for consumption since much less barakol is left after boiling [14], thus reflecting the potential application in prevention of MDD. The human neuroblastoma cell line, LAN-5, was selected as our *in vitro* model for this current study since it has been demonstrated to express multiple neurotransmitter transporter expression [15, 16]. For the MATs activity, neurotransmitter transporter uptake assay kit was commercially available for our purpose [17].

EXPERIMENTAL SECTION

Plant material

Senna siamea Lam. was collected from Bangkok, Thailand. The herb was authenticated by Prof. Dr. Thaweesakdi Boonkerd (Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand). The voucher specimen [013436 (BCU)] was deposited at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University.

Preparation of *Senna siamea* Lam. extracts

Leaves of *Senna siamea* Lam. were extracted using two different solvents, ethanol and water. With respect to maceration, the leaves were extracted using ethanol (Merck, Hohenbrunn, Germany) at ratio 1:10 (w/v) in shaking incubator at 120 rpm at room temperature for 48 h. Subsequent extract was filtered, and ethanol was evaporated at 60 °C. For boiling, the leaves were extracted using deionized water at ratio 1:10 (w/v) at 100 °C for 30 min. Then, resulting extract was filtered, and water was evaporated. Eventually, all crude extracts were dissolved in dimethyl sulphoxide (DMSO) (Merck) at a final concentration of 100 mg/mL (stock solution), and they were stored at -20°C in the dark. Prior to use, the extracts were filtered through a 0.2-μm pore size filter (Corning Inc., Corning, NY, USA). To serve as our controls in detection of fluorescence characteristics, leaves of *Mentha cordifolia* Opiz ex. Frezen (Kitchen mint) and whole plants of *Centella asiatica* L (Urm.) (Gotu kola) were prepared using both water and ethanol as well.

Cell culture

The human neuroblastoma cell line (LAN-5) was kindly provided by Dr. Weerah Wongkham, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. LAN-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (HyClone, Logan, UT, USA) at 37 ºC in 5% CO_2.

Cell viability assay

Effect of *Senna siamea* Lam. leaf extracts on LAN-5 viability was determined using MTT assay following the manufacturer's instruction (Merck) with slight modifications. LAN-5 cells were seeded into 96-well plates at a density of 2.5×10^5 cells per well. The cells were treated with both *Senna siamea* Lam. extracts at the concentrations of 25, 50, 100, 300, 600, 800 and 1000 μg/mL and incubated at 37 °C with 5% CO_2. After treatment for 24 h, 20 μL of MTT reagent (5 mg/mL) were pipetted into treated cells and incubated at 37 °C with 5% CO_2 for 4 h. After

incubation for 4 h, the medium was removed, and 200 µL of DMSO was added into each well to dissolve the formazan. The absorbance was measured spectrophotometrically at 550 nm and finally calculated the percentage of viable cells according to the following formula:

$$\% \text{ cell viability} = \frac{(\text{absorbance of treatment group - blank}) \times 100}{(\text{absorbance of untreated group - blank})}$$

Preparation of antidepressant drugs

Antidepressant drugs used as reference controls in this current investigation included fluoxetine hydrochloride, desipramine hydrochloride and GBR12935 dihydrochloride. They were representatives of selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs) and selective dopamine reuptake inhibitors (SDRIs), respectively. All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in deionized water at the final concentration of 10 mM. After dissolution, all drugs were filtered through a 0.2-µm pore size filter (Corning Inc.) and stored at -20 °C in the dark.

Assay of MATs activity

In this study, we investigated the MATs activity using Neurotransmitter Transporter Uptake Assay Kit (Molecular Devices, Sunnyvale, CA, USA). In principle, the kit utilizes a fluorescent substrate that mimics the biogenic amine neurotransmitters that is taken into the cell through those specific neurotransmitter transporters, resulting in increased intracellular fluorescence intensity. Since this kit detects the activity of all MATs simultaneously, use of specific neurotransmitter reuptake inhibitor (s) allows us to know transporter type (s) with activity. LAN-5 cells were seeded into black 96-well plates with transparent bottom at a density of 3.5×10^4 cells per well and were cultured at 37°C with 5% CO_2. Twenty-four hr after incubation, the medium was removed and treated cells with combining drugs as described in Table 1. Concentrations of antidepressant drugs applied in this study were similar to the report of Gang Zhao et al. [18]. After that, treated cells were incubated at 37°C with 5% CO_2 for 30 min, and the reagent was added according to the manufacturer's recommendation. The intracellular fluorescence intensity of treated cells was measured at 0, 10, 20, 30, 40, 50 and 60 min, with 440 nm excitation and 520 nm excitation. Graph of Relative Fluorescent Unit (RFU) and time (min) in Y axis and X axis, respectively were plotted, and we calculated the area below curve for analyzing MATs activity.

Table 1. The combining antidepressant drugs for MATs activity test

Combining compounds	Testing for activity of
Senna siamea extract+flu+des	Only DATs
Flu+des	Control for only DATs
Senna siamea extract+flu+GBR12935	Only NETs
Flu+GBR12935	Control for only NETs
Senna siamea extract+des+GBR12935	Only SERTs
Des+GBR12935	Control for only SERTs
Senna siamea extract	All 3 MATs
1X HBSS+0.1% BSA Buffer	Control of MATs
Flu+des+GBR12935	Positive control
Notes: - Combination of 1X Hank's Balanced Salt Solution (HBSS)+0.1% bovine serum albumin (BSA) for diluting all testing compounds. *- Concentrations of herbal extracts were 25 and 100 µg/mL.* *- Concentration of Fluoxetine and Desipramine were 10 µM.* *- Concentration of GBR12935 was 0.1 µM.*	

Statistical analysis

Experiments were carried out in triplicates. The data represented the mean ± SEM of three independent experiments and were analyzed by the independent Student's *t*-test with $P < 0.05$ considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of *Senna siamea* Lam. leaf extracts on activity of DAT, NET, SERT and total MATs in LAN-5 cells

To determine effect of *Senna siamea* Lam. leaf extracts on activity of DAT, NET, SERT and total MATs in our *in vitro* model, LAN-5 cell line, we employed the commercial neurotransmitter transporter reuptake assay kit and measured the intracellular fluorescence. The calculated RFU area below curve reflecting the activity of DAT, NET, SERT and all MATs in LAN-5 cells after treatment for 30 min with 2 different concentrations of *Senna siamea*

Lam. leaf extracts prepared from 2 distinct solvents in the absence or presence of combining antidepressants as described in Table 1. In the presence of antidepressant drugs, we expected to see lesser intracellular fluorescence intensity as compared to herbal extract without drugs because specific drugs would definitively inhibit neurotransmitter transporters. With *Senna siamea* Lam. extract treatment alone, intracellular fluorescence intensity should be observed above zero as in case of 25 µg/mL of water extract in the absence of all drugs. If the water extract of *Senna siamea* Lam. leaves truly reflected the inhibitory effect of this herbal extract on activity of MATs, we should see lesser intracellular fluorescence intensity when treating LAN-5 cells with this extract at the concentration of 100 µg/mL. Our assumption might be true. However, this result considerably surprised us since intracellular fluorescence intensity was too low to be detected. Normally, the intracellular fluorescence intensity is in a range of many thousands with regard to RFU area below curve. In this study, we observed the fluorescence intensity below zero or minus values, thus reflecting assay interference. The inference was stronger as reflected by lesser intracellular fluorescence intensity when increasing concentrations of *Senna siamea* Lam. extracts. It should be mentioned that ethanol extract resulted in a stronger interference as compared to that of water extract.

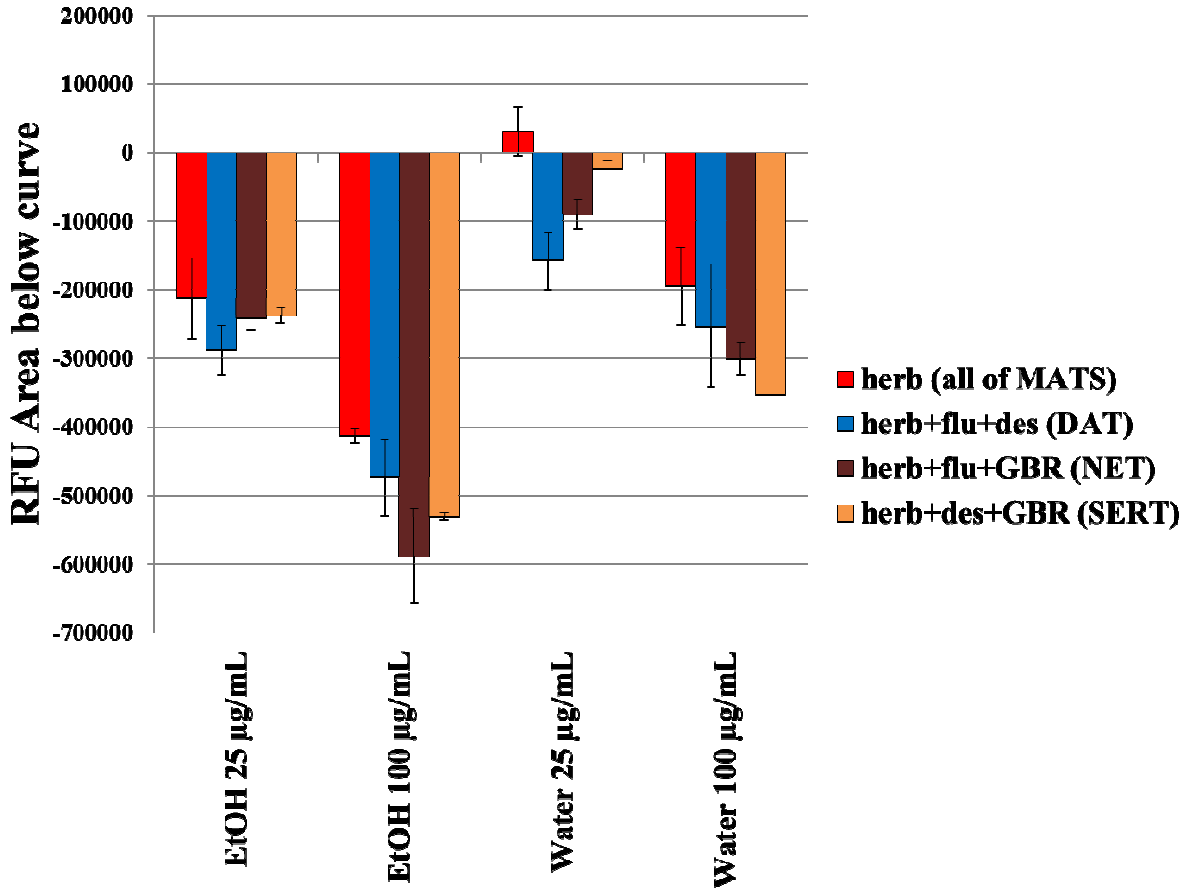


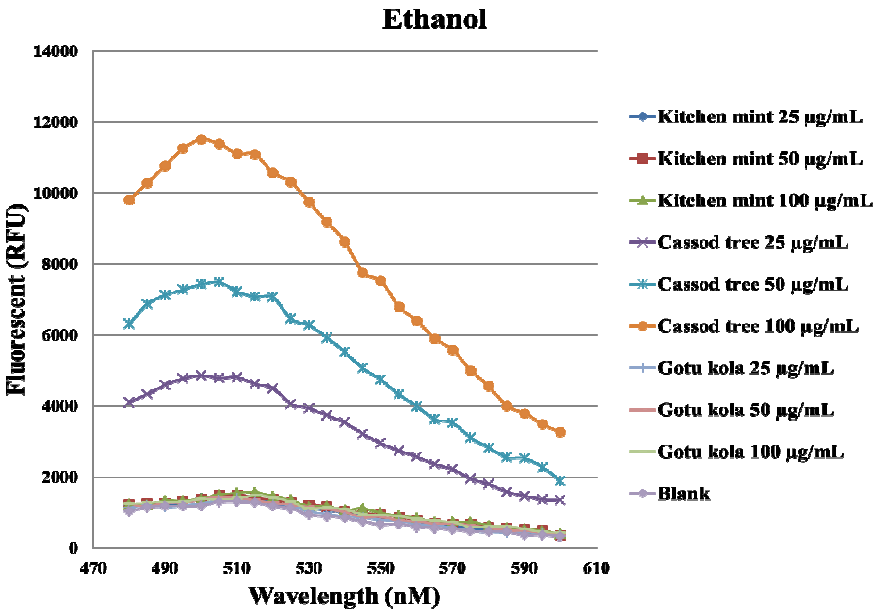
Figure 1. Effect of *Senna siamea* Lam. leaf extracts on the activity of DAT, NET, SERT and all MATs in LAN-5 cells. The measurement was carried out after LAN-5 cells were treated with *Senna siamea* Lam. leaf extract for 30 min in the absence or presence of combining compounds described in Table 1. With regard to the concentrations, 25 and 100 µg/mL of the *Senna siamea* extract, 10 µM of fluoxetine and desipramine and 0.1 µM of GBR12935 were applied

The data are expressed as mean ± SEM from three independent experiments.

The result in Figure 1 actually suggested that the *Senna siamea* Lam. leaf extracts might contain certain fluorescent compounds with excitation 440 nm and emission 520 nm, the spectra monitored for this assay. Barakol and barakol derivatives were found to possess fluorescence characteristics [19, 20]. In *Senna siamea* Lam. leaves, other fluorescent compounds may also be presented and contributed to this assay. To test this hypothesis, we measured the fluorescence of *Senna siamea* extracts compared with reagent blank and *Mentha cordifolia* Opiz ex. Frezen (Kitchen mint) and *Centella asiatica* L (Urm.) (Gotu kola) as control herbal extracts using spectrofluorometer with the

excitation wavelength of 440 nm and emission wavelengths of 480-600 nm for scanning. As in Figure 2, we found that all concentrations of *Senna siamea* Lam. leaf extracts from both ethanol and water possessed fluorescence characteristics in a concentration-dependent fashion. Ethanol extract of *Senna siamea* leaves contained greater fluorescent intensity as compared to that of water extract. The concentration-dependent and solvent type-dependent manner was in agreement with the observation in Figure 1. Blank and other 2 herbal extracts as our controls had a fluorescence in lower levels than those of *Senna siamea* extracts.

A



B

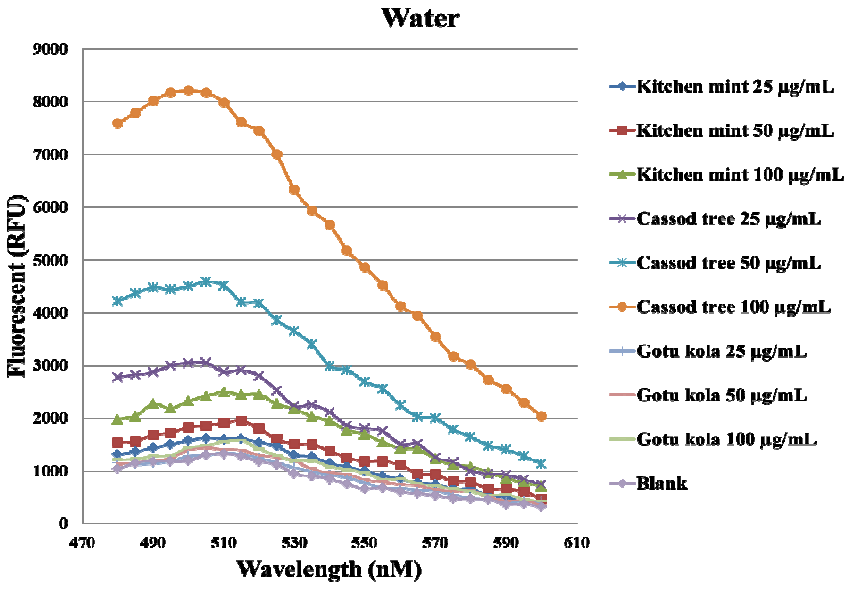


Figure 2. Fluorescence spectra of *Senna siamea* Lam. leaf (Cassod tree) extracts at the excitation wavelength of 440 nm and the emission wavelengths of 480-600 nm compared to *Mentha cordifolia* Opiz ex. Frezen (Kitchen mint) leaf and *Centella asiatica* L (Urm.) (Gotu kola) whole plant extracts and reagent blank. All herbs were extracted in either ethanol (A) or water (B)
The data are expressed as mean ± SEM from three independent experiments.

Recently, phytochemical properties of five species of *Cassia*, including *Cassia spectabilis*, *Cassia siamea*, *Cassia fistula*, *Cassia biflora* and *Cassia hirsute* were elucidated, thus discerning certain constituents [21]. Likewise, it is of great importance to explore the phytochemical properties of *Senna* species. So, we may gain more information regarding structures and functions of herbal compounds based on a comparative study. It is feasible that other species of *Senna* may also possess fluorescence features. In addition, research-based knowledge may open the door to useful applications. For instance, *Senna uniflora* was employed to remove copper ions from aqueous solutions, thus suggesting the potential application in solving copper-contaminated water found in industrial effluents [22].

CONCLUSION

We found in this study that *Senna siamea* leaf extracts possessed fluorescence characteristics possibly due to certain fluorescent compounds and could be interfered in a fluorescence-based assay. It is highly recommended to know the chemical nature of each plant extract before selecting any biological assay.

Acknowledgments
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