

IN VITRO EVALUATION OF ANTIFUNGAL ACTIVITY OF ANTHRAQUINONE DERIVATIVES OF *SENNA ALATA*

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ABSTRACT: *Senna alata* leaf powder was used to obtain five extracts which contain anthraquinone compounds in different forms i.e. anthraquinone aglycone extract, anthraquinone glycoside extract, anthraquinone aglycones from glycosidic fraction, crude ethanol extract, and anthraquinone aglycone from crude ethanol extract. All extracts were tested against clinical strain of dermatophytes: *Trichophyton rubrum*, *T. mentagrophytes*, *Epidermophyton floccosum*, and *Microsporum gypseum* by diffusion and broth dilution techniques to find out the active form for antifungal activity. Thin layer chromatography was developed to demonstrate the fingerprints of chemical constituents of each extract. This investigation pointed out the best *in vitro* antifungal activity of anthraquinone aglycones from glycosidic fraction qualitatively and quantitatively, compared to other extracts.

Keywords: anthraquinone, antifungal, *Cassia alata*, dermatophytes, ringworm, *Senna alata*

INTRODUCTION: *Senna alata* (Linn.) Roxb. or Chum-het-thet, former *Cassia alata* Linn., family Fabaceae (Leguminosae), is generally known as ringworm tree, Christmas candle, candlestick or candle bush. Its ethnomedicinal values have been demonstrated for centuries in worldwide records, or from collected research-based data¹. The remarkable biological effectiveness of *S. alata* was documented for the variety of medical properties from non-infectious relief i.e. constipation, anemia, antidote, dyspepsia, laxative effect, purgative and eventually inhibition of the proliferation of leukaemia cells²⁻⁷ and the treatment of several infections caused by bacteria, protozoa, fungi and virus⁸⁻¹⁸. The major active pharmacological compounds from *S. alata* have been elucidated as anthraquinone glycosides, which are responsible for laxative properties⁵, evidences showed that rhein, emodin and chrysophanol possessed antimicrobial activities^{8,14}. Because of the scientific approval of its versatile efficacies and throughout traditional usages, this plant is therefore not only accepted in primary health care programmes which have been urged by the World Health Organization, but included in Thai National List of Essential Drugs as herbal medicines according to Drug Act B.E. 2510. Products from *S. alata* are classified as herbal medicines developed for constipation³ whereas

many researches are addressed to other properties of this plant.

This study was undertaken to determine the active form of anthraquinones and prove for the antifungal activity, especially against dermatophytes, of different extracts containing different forms of anthraquinones of *S. alata* leaves. The fingerprints of those extracts are compiled by thin layer chromatography. The outcomes will scientifically support the ancient traditional use in many regions of which folklores are partially based on belief as well as evidences and, therefore, richness in diversity of native agricultures will consequently be preserved.

MATERIALS AND METHODS:

Plant materials

Leaves of *S. alata* Linn. collected from Buriram province, Thailand, in January 2000, were compared to specimens kept at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environments in Bangkok for identification while the voucher specimens were kept at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University.

Test organisms

Twenty one clinical isolates of dermatophytes from Department of Microbiology, Faculty of

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Pharmacy, Mahidol University were *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporium gypseum* and *Epidermophyton floccosum* with 3, 8, 5, and 5 isolates, respectively. These test strains, kept on Sabouraud dextrose agar, were properly sub-cultured to obtain active proficiency growth of competent organisms.

Extraction of plant materials

Leaves of *S. alata* were cleaned and air-dried before drying in a hot air oven at 50°C for 5 hours and ground until the powder readily passed through a laboratory sieve mesh of 1 mm aperture. The powder was later extracted to 5 different extracts: anthraquinone aglycone extract, anthraquinone glycoside extract, anthraquinone aglycone from glycosidic fraction, crude ethanol extract, and anthraquinone aglycone from crude ethanol extract, the extraction process was done by modification of the assay procedure of hydroxyanthracene derivatives described in the Standard of ASEAN Herbal Medicine¹⁹ and those for extraction and determination of total anthraquinones and total anthraquinone glycosides from *Senna* species.

Extraction of anthraquinone aglycone and anthraquinone glycoside extracts

The extraction was done from 1 hr reflux of the aqueous part of 50 grams of *S. alata* leaf powder and 500 ml water; the aqueous extract was adjusted to pH 4 with 2M hydrochloric acid (0.1 ml), and later extracted with 5 x 200 ml of chloroform. The collected extracts from chloroform layer were evaporated to yield anthraquinone aglycone extract while the aqueous layer was added with 0.1 g of sodium bicarbonate to adjust to neutral pH. The solution was then centrifuged at 4000 rpm for 20 min. The supernatant which contained anthraquinone glycoside was evaporated to dryness to give anthraquinone glycosides extract.

Extraction of anthraquinone aglycone from glycosidic fraction

The extraction was done similar to the process of anthraquinone glycosides extract except for evaporation to dryness. Ferric chloride hexahydrate solution 10.5 % w/v (50ml) was added to the anthraquinone glycosides extract, then refluxed

for 30 min. The solution was added with concentrated HCl (15ml) and refluxed for another 30 min and filtered after cool down. The filtrate was shaken with 3 x 200 ml of chloroform in a separating funnel and separated the chloroform layer, combined and washed with 2 x 200 ml of distilled water, then evaporated to dryness to yield anthraquinone aglycone from glycosidic fraction.

Extraction of crude ethanol extract and anthraquinone aglycone from crude ethanol extract

S. alata leaf powder 450 g was extracted with 3 x 3 L of 70% ethanol using a soxhlet apparatus. The extract was evaporated to dryness to yield crude ethanol extract. A portion of the crude ethanol extract (75 g) was further extracted by adding 170 ml of water and 30 ml of 70% ethanol. After 10.5% w/v ferric chloride hexahydrate solution (50 ml) was added, the mixture was refluxed for 30 min before adding 20 ml concentrated hydrochloric acid and refluxed for another 30 min. When the mixture was cooled down, it was filtered and the filtrate was extracted with 5 x 200 ml of chloroform. The chloroform layer was combined and washed with distilled water, then evaporated to dryness to yield anthraquinone aglycone from crude ethanol extract (Scheme1).

Thin layer chromatography (TLC) analysis

Each extract was investigated for anthraquinone compound using TLC and reference standards of rhein, aloe-emodin, emodin and chrysophanic acid on silica gel plates (60 F₂₅₄) and subsequently developed by 4 different solvent systems. Those were a) Petroleum ether : Ethyl acetate : Formic acid (75 :25 : 1), b) Chloroform : Methanol (80 : 20), c) Ethyl acetate : Methanol : Water (100 : 17 : 13), and d) n-Propanol : Ethyl acetate : Water (40 : 40 : 30). The anthraquinone spots were detected by spraying with 10% methanolic KOH which showed pink-red spot.

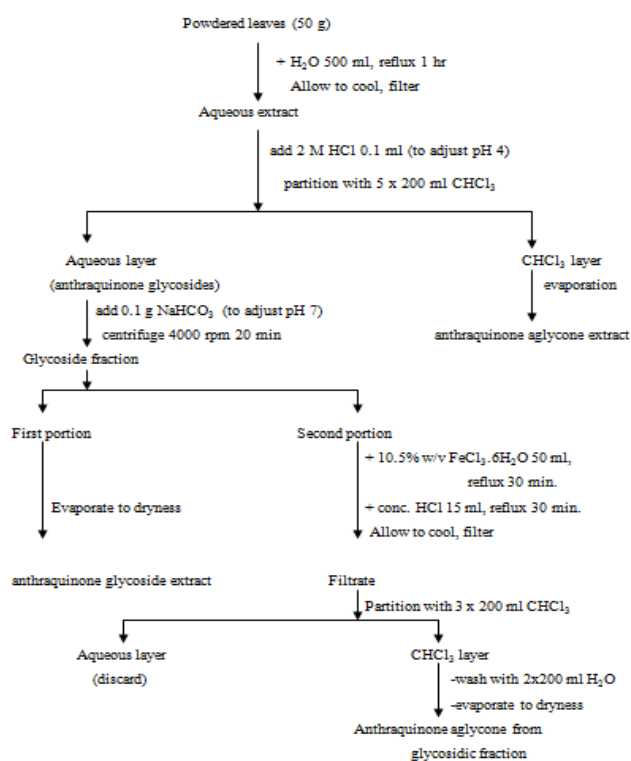
Antifungal activity test of *S. alata* leaf extracts

Antifungal activity of extracts was evaluated by agar diffusion and broth dilution method.

Inoculum: Pure isolated strains of test organisms were sub-cultured on SDA slants until

full growth with proper characteristic of each strain was obtained at room temperature. Spore suspension was prepared using sterile distilled water and measured density equivalent to McFarland number 1.

Diffusion method: Each extract was dissolved in corresponding solvent: chloroform for anthraquinone aglycone extract, anthraquinone aglycone from glycosidic fraction and anthraquinone aglycone from crude ethanol extract; sterile water and 70% ethanol for anthraquinone glycoside extract and crude ethanol extract. All extracts were diluted and prepared in different concentration that gave the required amount (mg/disc) when incorporated onto sterile blank disc of 6 mm diameter as the followings: anthraquinone aglycone extract at 1.00 mg/disc, anthraquinone glycoside extract at 40.0 mg/disc, anthraquinone aglycone from glycosidic fraction at 0.50 mg/disc, crude ethanol extract at 15.00 mg/disc and anthraquinone aglycone from crude ethanol extract at 0.50 mg/disc. While ketoconazole was dissolved in methanol and incorporated in sterile blank disc at 0.001 mg/disc.



Scheme 1 Preparation of anthraquinone aglycone extract, anthraquinone glycoside extract and anthraquinone aglycone from glycosidic fraction

The 100 μ L of suspended inoculum was evenly incorporated onto the surface layer of a total amount of 20 ml SDA plate. Discs containing extracts at the mentioned amount, standard agents and solvents were each dublicately placed on this agar. Culture control of each organism was made in similar way but without discs. All of the test plates were incubated at room temperature. Inhibitory zone diameters were measured at the same time of fully grown culture control and were regarded as antifungal activity of test material.

Broth dilution method: Each extract was dissolved in 2 % v/v dimethylsulfoxide (DMSO) solution. Ketoconazole, dissolved in methanol, and 2 % v/v DMSO solution in SDB (Sabouraud Dextrose broth) were used as control agent.

Twenty microliters of inocula were added to two-fold serial dilution of each sample with SDB to yield 250 to 0.49 mg/ml. The solvent, reference drug and culture control were made by similar manner. After incubated at room temperature, the concentration that gave growth inhibition was recorded.

Spot inoculation was made from serial dilution, from the first tubes with relevant growth and from the next three tubes with clear culture. Each tube was spotted at least 5 times onto the surface of SDA plate. After the growth appeared compared to the culture control plate inoculated from culture control, at least 3 from 5 spots (or 5 from 7 spots) that gave clear cut growth was regarded as positive growth. Thus, MIC (minimum inhibitory concentration) was considered as the lowest concentration of sample which inhibited the test organism; in such case, the MFC (minimum fungicidal concentration) was established.

RESULTS:

Extract yields

Anthraquinone extracts yielded from leaf powder of *S. alata* were ranging differently from 0.19% w/w of anthraquinone aglycones from glycosidic fraction up to 34.94% w/w of crude ethanol extract. While anthraquinone aglycone from crude ethanol extract and anthraquinone aglycone extract gave less than 1% w/w; while yield of anthraquinone glycoside extract showed higher production (Table 1).

TLC fingerprints of anthraquinone extracts

Investigation of TLC fingerprints of anthraquinone compounds on silica gel plate using four solvent systems and sprayed with 10% methanolic KOH, was compared to the anthraquinone standards (figure 1). Main anthraquinone compounds in all extracts were aloe-emodin and rhein except anthraquinone glycoside extract.

In vitro antifungal activity test of *Senna alata* leaf extracts

Qualitative and quantitative antifungal activity of *S. alata* leaf extracts was evidenced as average inhibitory zone diameter from diffusion test and minimum inhibition concentration from broth dilution test against dermatophyte isolates, respectively. Anthraquinone aglycone from glycosidic fraction showed the best activity for all extracts against all test strains (21.2 ± 3.8 mm) except that of crude ethanol extract for *T. rubrum* (22.8 ± 1.3 mm), whereas ketoconazole gave the strongest inhibition for *M. gypseum* (36.00 mm) (Table 2). The greatest activity of dilution test was also of anthraquinone aglycone from glycosidic fraction against all test strains (Table 3). However, because different amount of leaf powder was used to prepare the qualified extract appropriate to the test, crude ethanol extract was justified as the best activity on the basis of less amount of raw material being used (Table 4).

DISCUSSION: Dermatophytoses cannot be differentiated and diagnosed for causative species on the basis of clinical appearances; and, culture method is inconvenient to be as an option in determining for drug used. However, in some cases with treatment failure or resistance to the drug of choice, susceptibility test is suggested either qualitatively or quantitatively. The perspective results of diffusion test against all test isolates revealed the best efficacy of anthraquinone aglycone from glycosidic fraction: at 21.2 ± 3.8 mm compared to 9.5 ± 2.0 to 17.7 ± 4.1 mm; except only *T. rubrum* gave better response to crude ethanol extract. However, the best efficiency of anthraquinone aglycone from glycosidic fraction was again indicated by broth dilution test at 0.27 mg/ml of MIC while the rest showed at 0.66 to 151.79 mg/ml. These interesting outcomes

emphasized the usefulness of this fraction and pave the way to strong potential possibility for *S. alata* extract using the modern technique in formulating the extract to an appropriate form to treat dermatophytoses or ringworm.

The consumption of raw material is reasonably included into consideration of the value of extract for further formulation into topical medicines. Because crude ethanol extract of this plant spent only 71.55 mg to make 1 ml of test material whereas anthraquinone aglycone from glycosidic fraction required 263.16 mg to approach the end MIC (1 : 3.67). However, although *S. alata* can be cultivated all over the country, even for specific purpose, as well as the government policy to promote the use of medicinal plant, but the

Table 1 Extract yields from *Senna alata* leaf powder

Extracts	% Yields (w/w)
Anthraquinone aglycone extract	0.57
Anthraquinone aglycone from crude ethanol extract	0.21
Anthraquinone aglycones from glycosidic fraction	0.19
Anthraquinone glycoside extract	26.20
Crude ethanol extract	34.94

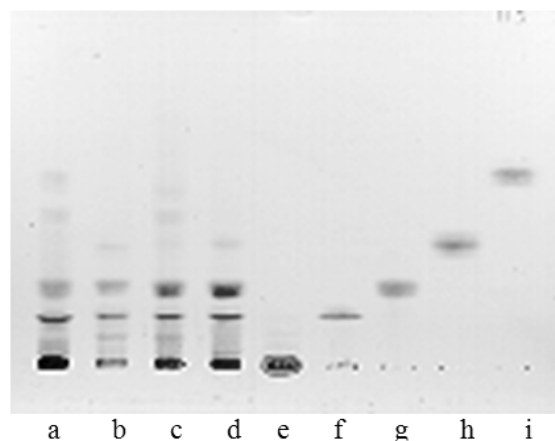


Figure 1 TLC fingerprints of *Senna alata* Linn. leaf extracts and reference standards

Solvent system: Petroleum ether : Ethyl acetate : Formic acid (75:25:1); Detection: Sprayed with 10% methanolic KOH; After spraying with 10% methanolic KOH; a = Crude ethanol extract; b = Anthraquinone aglycone from crude ethanol extract; c = Anthraquinone aglycone extract; d = Anthraquinone aglycone from glycosidic fraction; e = Anthraquinone glycoside extract; f = Rhein (hRf = 15); g = Aloe-emodin (hrf = 23.3); h = Emodin (hRf = 38.3); i = Chrysophanic acid (hRf = 58.3)

access for raw material is not appropriate.

Different methods of extraction of *S. alata* leaf were studied e.g. by maceration^{15,17}, using a soxhlet apparatus, or using sonication from pulverised leaves¹⁸, by the use of silica gel vacuum chromatographic technique where anthraquinone high-yielding *S. alata* leaf extract obtained²⁰. These extracts, no matter which sources of raw materials, ratio of solvent used, other conditional difference, and more or less, would affect the details, nature of extract and their *in vitro* efficiencies. For this instance, library of TLC

fingerprints is important information to collect, identify, differentiate, and quantify each certain extract with various qualifications from worldwide researches.

Fortunately, application of *S. alata* extract/s into modern medicinal uses is not always on the basis of certain extract that would give the greatest result on particular causative dermatophyte, thus, these extracts can be incorporated in topical medication after toxicity study demonstrates no unfavorable or disastrous effect in long term use.

Table 2 Activity of *Senna alata* extracts against dermatophyte isolates exhibited as average zone diameters

Dermatophytes	Average zone diameter (mm)					Ketoconazole (10 µg/disc)
	A (1 mg/disc)	B (40 mg/disc)	C (0.5 mg/disc)	D (15 mg/disc)	E (0.5 mg/disc)	
<i>T. mentagrophytes</i>	9.4 ± 1.5 (n=5)	12.8 ± 2.5 (n=8)	21.1 ± 4.9 (n=8)	14.3 ± 1.2 (n=8)	18.2 ± 4.9 (n=8)	31.7 ± 1.9 (n=5)
<i>T. rubrum</i>	13 (n=1)	16.0 ± 3.8 (n=3)	21.3 ± 1.5 (n=3)	22.8 ± 1.3 (n=3)	20.3 ± 2.3 (n=3)	34.0 ± 0.7 (n=3)
<i>M. gypseum</i>	8.7 ± 2.1 (n=3)	11.0 ± 2.2 (n=3)	19.2 ± 4.3 (n=3)	16.3 ± 5.5 (n=3)	15.3 ± 3.8 (n=3)	36 (n=1)
<i>E. floccosum</i>	No applicable	15.1 ± 1.4 (n=4)	22.4 ± 2.7 (n=5)	14.4 ± 2.1 (n=4)	17.6 ± 3.6 (n=5)	No applicable
Average	9.5 ± 2.0 (n=9)	13.5 ± 2.9 (n=18)	21.2 ± 3.8 (n=19)	16.1 ± 3.9 (n=18)	17.7 ± 4.1 (n=19)	32.8 ± 2.2 (n=8)

A = anthraquinone aglycone extract, B = anthraquinone glycoside extract, C = anthraquinone aglycone from glycosidic fraction, D = crude ethanol extract, E = anthraquinone aglycone from crude ethanol extract

Table 3 Activity of *Senna alata* extracts against dermatophyte isolates exhibited as MICs

Dermatophytes	The average minimum inhibitory concentrations (MIC) (mg/ml)				
	A	B	C	D	E
<i>T. mentagrophytes</i>	2.19 (n=4)	218.75 (n=8)	0.34 (n=8)	19.64 (n=7)	0.88 (n=8)
<i>T. rubrum</i>	0.63 (n=4)	62.5 (n=2)	0.19 (n=2)	18.75 (n=2)	0.75 (n=2)
<i>M. gypseum</i>	1.88 (n=4)	166.67 (n=6)	0.34 (n=4)	10.42 (n=6)	0.60 (n=5)
<i>E. floccosum</i>	0.47 (n=2)	62.5 (n=5)	0.13 (n=5)	3.75 (n=5)	0.35 (n=5)
Average	1.45 (n=14)	151.79 (n=21)	0.27 (n=19)	11.56 (n=20)	0.66 (n=20)

A = anthraquinone aglycone extract, B = anthraquinone glycoside extract, C = anthraquinone aglycone from glycosidic fraction, D = crude ethanol extract, E = anthraquinone aglycone from crude ethanol extract

Table 4 Amount of *Senna alata* leaf powder used for preparations at each MICs

EXTRACT	MICs (mg/ml)	Yield (% w/w)	Raw powder used (mg/ml)
1. Anthraquinone aglycones extract	2.5	0.57 %	438.60
2. Anthraquinone glycoside extract	250.00	26.20 %	954.20
3. Anthraquinone aglycones from glycosidic fraction	0.50	0.19 %	263.16
4. Crude ethanol extract	25.00	34.94 %	71.55
5. Anthraquinone aglycone from crude ethanol extract	1.00	0.21 %	476.19

CONCLUSION: Anthraquinone aglycone from glycosidic fraction extracted from *Senna alata* leaves exhibited the superior *in vitro* activity, both qualitatively and quantitatively against clinical strain of dermatophytes in comparison with anthraquinone aglycones extract, anthraquinone glycosides extract, anthraquinone aglycones from crude ethanol extract, and crude ethanol extract. Among these extracts, crude ethanol extract, which contained both anthraquinone aglycones, anthraquinone glycosides and other chemical polar compounds, acquired less leaf ground powder to prepare for test material in order to obtain the effective anti-dermatophytes MICs. Several extracts prepared from various sources of plant, solvents, method of extraction, different condition used can be library collected and compared by TLC fingerprints.

REFERENCES:

- Girón LM, Freire V, Alonzo A, Cáceres A. 1991. Ethnobotanical survey of the medicinal flora used by the Caribs of Guatemala. *J Ethnopharmacol* 34(2-3): 173-87.
- Ajibesin KK, Ekpo BA, Bala DN, Essien EE, Adesanya SA. 2008. Ethnobotanical survey of Akwa Ibom State of Nigeria. *J Ethnopharmacol* 115(3): 387-408.
- Chokevivat V, Chuthaputti A. 2005. The role of Thai traditional medicine in health promotion. The 6th Global Conference on Health Promotion Bangkok, Thailand; 2005. August 7-11;p. 9.
- Clement YN, Morton-Gittens J, Basdeo L, Blades A, Francis MJ, Gomes N, Janjua M, Singh A. 2007. Perceived efficacy of herbal remedies by users accessing primary healthcare in Trinidad. *BMC Complement Altern Med* 7: 4-12.
- Elujoba AA, Ajulo OO, Iweibo GO. 1989. Chemical and biological analyses of Nigerian *Cassia* species for laxative activity. *J Pharm Biomed Anal* 7(12): 1453-7.
- Pieme CA, Penlap VN, Ngogang J, Kuete V, Catros V, Moulinoux JP. 2009. *In vitro* effects of extract of *Senna alata* (Ceasalpiniaceae) on the polyamines produced by Leukaemia cells (L1210). *Phcog Mag* 5(17): 8-13.
- Ssegawa P, Kasenene JM. 2007. Medicinal plant diversity and uses in the Sango bay area, Southern Uganda. *J Ethnopharmacol* 113(3): 521-40.
- Agarwal SK, Singh SS, Verma S, Kumar S. 2000. Antifungal activity of anthraquinone derivatives from *Rheum emodi*. *J Ethnopharmacol* 72(1-2): 43-6.
- Asase A, Akwetey GA, Achel DG. 2010. Ethnopharmacological use of herbal remedies for the treatment of malaria in the Dangme West District of Ghana. *J Ethnopharmacol* 129(3): 367-76.
- Benjamin TV, Lamikanra A. 1981. Investigation of *Senna alata*, a Plant Used in Nigeria in the Treatment of Skin Diseases. *Pharm Biol* 19(2-3): 93-6.
- Bone K, Mills S. 2000. Principles and Practice of Phytotherapy: Modern Herbal Medicine. In: Principles of Herbal Pharmacology. London: Churchill Livingstone; p. 49.
- Longuefosse JL, Nossin E. 1996. Medical ethnobotany survey in Martinique. *J Ethnopharmacol* 53(3): 117-42.
- Magassouba FB, Diallo A, Kouyaté M, Mara F, Mara O, Bangoura O, et al. 2007. Ethnobotanical survey and antibacterial activity of some plants used in Guinean traditional medicine. *J Ethnopharmacol* 114(3): 44-53.
- Manojlovic NT, Solujic S, Sukdolak S. 2002. Antimicrobial activity of extract and anthraquinones from *Caloplaca schaeferi*. *Lichenologist* 34: 83-5.
- Owoyale JA, Olatunji GA, Oguntoye SO. 2005. Antibacterial Activities of an Alcoholic Extract of *Senna alata* Leaves. *J Appl Sci Environ Mgt* 9(3): 105-7.
- Pesewu GA, Cutler RR, Humber DP. 2008. Antibacterial activity of plants used in traditional medicines of Ghana with particular reference to MRSA. *J Ethnopharmacol* 116(1): 102-11.
- Phongpaichit S, Pujenjob N, Rukachaisirikul V, Ongsakul M. 2004. Antifungal activity from leaf extracts of *Cassia alata* L., *Cassia fistula* L. and *Cassia tora* L. *Songklanakarin J Sci Technol* 26: 741-8.
- Wuthi-udomlert M, Prathanturug S, Soonthornchareonnon N. 2001. Antifungal activities of *Senna alata* extracts using different methods of extraction. *World Conference of Medicinal and Aromatic Plants*; 2001 Jul 8-11; Budapest, Hungary. Abstract; p 304.
- ASEAN countries. 1993. Standard of ASEAN Herbal medicines vol.1. Jakarta: Aksara Buana.
- Sakunpak A, Sirikatitham A, Panichayupakaranant P. 2009. Preparation of anthraquinone high-yielding *Senna alata* extract and its stability. *Pharm Biol* 47(3): 236-41.