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IDENTIFICATION OF ANTHRAQUINONES FROM *CASSIA TORA* L. SEEDS AND ANTIFUNGAL ACTIVITY

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Abstract: Anthraquinones are a major group of active compounds found in Cassia plants. Cassia tora L. was a medicinal plant and its seeds and leaves have been used as laxative. C. tora seed extracts have been reported for antibacterial and antifungal activities. This study aimed to identify anthraquinones from C. tora seeds and determined antifungal activity. C. tora seeds were extracted with 80%ethanol and partial purified with column chromatography. HPLC with RP C18 column and mobile phase composition of 0.01% phosphoric acid in water and methanol were used for analysis. The antifungal activity was tested against Trichophyton mentagrophytes using broth microdilution assay. Results showed that four anthraquinone compounds were identified including aloe-emodin, emodin, chrysophanol and physcion. Total anthraquionone of crude 80% ethanolic extract was 55.60 %w/w and those of 95% ethanolic and acetone subfractions were 10.10-51.77% w/w and 54.88-70.57 %w/w, respectively. The 95%ethanolic subfraction inhibited T. mentagrophytes with a minimal concentration (MIC) of 125 and a minimal fungicidal concentration (MFC) of 1,000 μg/mL, respectively compared with ketoconazole (MIC and MFC = 20 μg/mL). Partial purified acetone fraction contained main anthraquinones including chrysophanol, physcion and emodin while 1-desmethylaurantio-obtusin and aurantio-obtusin were major compounds in ethanolic subfraction. These subfractions inhibited T. mentagrophyte about 4 to 8-folded higher than the crude extract. This extract would be useful for development of antifungal product.

Keywords: *Cassia tora* L., anthraquinones, *T. mentagrophytes*

บทคัดย่อ: สารแอนทราควิโนนเป็นกลุ่มสารสำคัญที่พบในพืชสกุล Cassia ชุมเห็ดไทยเป็นพืชสมุนไพรซึ่งเมล็ดและใบถูกนำมาใช้เป็นยาระบาย สารสกัดจากเมล็ดชุมเห็ดไทยมีรายงานว่ามีฤทธิ์ยับยั้งเชื้อแบคทีเรียและเชื้อรา การศึกษานี้มีวัตถุประสงค์เพื่อตรวจวิเคราะห์สารกลุ่มแอนทราควิโนนและตรวจสอบฤทธิ์ยับยั้งเชื้อรา เมล็ดชุมเห็ดไทยสกัดด้วย 80% เอทานอลและนำมาแยกส่วนด้วยคอลัมน์โครมาโตกราฟี เทคนิค HPLC ใช้คอลัมน์ C18 และเฟสเคลื่อนที่ประกอบด้วยเมทานอลและ 0.01%กรดฟอสฟอริกในการวิเคราะห์ การทดสอบฤทธิ์ยับยั้งเชื้อรา *Trichophyton mentagrophytes* ใช้วิธี broth microdilution ผลการศึกษาพบสารแอนทราควิโนนสี่ชนิด คือ aloe-emodin, emodin, chrysophanol และ physcion ปริมาณสารแอนทราควิโนนรวมของส่วนสกัด 80%เอทานอล มีค่าร้อยละ 55.60 โดยน้ำหนัก และของสารที่แยกโดย 95%เอทานอลและอะซิโตนมีค่าร้อยละ 10.10-51.70 และ 54.88-70.57 โดยน้ำหนักตามลำดับ สารสกัดที่แยกจาก 95%เอทานอลมีฤทธิ์ยับยั้งเชื้อรา *T. mentagrophytes* โดยมีค่าความเข้มข้นต่ำสุดในการยับยั้ง 125 μg/mL และค่าความเข้มข้นต่ำสุดในการฆ่าเชื้อ 1,000 μg/mL ตามลำดับ เปรียบเทียบกับยา ketoconazole มีค่าความเข้มข้นต่ำสุดในการยับยั้งและฆ่าเชื้อ 20 μg/mL สารสกัดที่แยกผ่านคอลัมน์ด้วยอะซิโตนยังคงพบสาร chrysophanol, physcion และ emodin ในขณะที่สารสกัดที่แยกผ่านคอลัมน์ด้วย 95% เอทานอล พบสาร 1-desmethylaurantio-obtusin และ aurantio-obtusin. สารสกัดที่ผ่านคอลัมน์และนำมาทดสอบมีฤทธิ์ยับยั้งเชื้อรา *T. mentagrophytes* เพิ่มขึ้น 4 ถึง 8 เท่าเปรียบเทียบกับส่วนสกัด 80%เอทานอล สารสกัดนี้อาจนำมาใช้ประโยชน์ในการพัฒนาผลิตภัณฑ์ต้านเชื้อรา

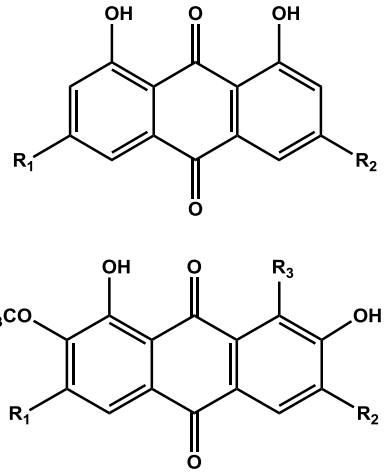
คำสำคัญ: *Cassia tora* L., anthraquinones, *T. mentagrophytes*

INTRODUCTION

Cassia tora L. (Foetid cassia) or Chumhet Thai is a plant in family Leguminosae. Its seeds and leaves have been interested as medicinal uses. *C. tora* seeds were indicated as laxative and diuretic in Thai herbal Pharmacopoeia. Methanolic extracts of seeds exhibited antioxidant activity and ethanolic extracts exhibited antihelmintic, antibacterial and antifungal activities. Leaf extract showed anti-inflammatory, antioxidant, purgative effects and

hepatoprotective activity (Choudhary *et al*., 2011). *C. tora* leaves and seeds have been used externally as germicide and antiparasite also (Jain & Patil, 2010). *C. tora* was a shrub and annual plant growing around Southeast Asia countries; i.e Thailand, India, Sri Langka, Pakistan, Bangladesh and China. *C. tora* seeds were glossy, brown and rhombohedral shape containing hull (27%), endosperm (32%) and germ (41%) (Pawar and D'mello, 2011).

C. tora seeds mainly consisted of anthraquinone, naphthopyrone and their glycosides. It also contained flavonoids, protein and mucilage. Sitosterol was found in petroleum ether extract of seeds (Pawar and D'mello, 2011). Chloroform and ethyl acetate seed extracts contained anthraquinone; chrysophanol, physcion, emodin, rubrofusarine, aurantio-obtusin, chryso-obtusin, obtusin (Dave and Ledwani, 2012). Rubrofusarin-6-β-gentiobioside and 8-hydroxy-3-methyl anthraquinone-1-β-gentiobioside were found in ethanolic extract (Choudhary *et al*., 2011). Three naphthopyrone glucosides; cassiaside, rubrofusarin-6-O-β-D-gentiobioside, toralactone-9-O-b-D-gentiobioside, were found in butanol soluble seed extract (Choudhary *et al*., 2011). Aloe-emodin and 1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone were responsible for laxative property (Pawar and D'mello, 2011). Phenolic glycosides especially torachrysone, toralactone, aloe-emodin, rhein and emodin, strongly inhibited four strains of methicillin-resistant *Staphylococcus aureus* with an MIC of 2-64 μg/mL (Hatano *et al*., 1999). Methanolic extract of *C. tora* seeds showed antimicrobial activity and emodin isolated from chloroform fraction was an active compound against *B. cereus* with the inhibition zone 20±1.4 mm (Lee *et al*., 2013). Chemical structures of main anthraquinones are showed in Figure 1.



Compounds	R_1	R_2
Rhein (R)	H	COOH
Aloe emodin	H	CH_2OH
Emodin	OH	CH_3
Chrysophanol	H	CH_3
Physcion	OCH_3	CH_3

Compounds	R_1	R_2	R_3
Aurantio-obtusin	OH	CH_3	OCH_3
1-desmethylaurantio-obtusin	OH	CH_3	OH

Figure 1. Chemical structures of anthraquinones in *C. tora* seeds

Anthraquinone found in family Liliaceae, Polygonaceae, Rubiaceae also. Anthraquinones (rhein, physcion, aloe-emdin, chrysophanol) isolated from *Rheum emodi* rhizome exhibited antifungal activity against *Trichophyton mentagrophytes* with MIC of 25-50 μg/mL compared with methanolic extract 250 μg/mL and ketoconazole (MIC = 2.5 μg/mL) (Agarwal *et al*., 2000). *Senna alata* leaf ethanolic extracts showed antifungal activity against *E. floccosum*, *M. gypseum*, *T. rubrum* and *T. mentagrophytes* with MIC of 3.75, 10.42, 18.75 and 19.64 mg/mL, respectively. In addition, anthraquinone aglycone from crude ethanol extract (MIC = 0.55-0.88 mg/mL) and from glycosidic fraction (0.13-0.34 mg/mL) showed higher antifungal activity against *E. floccosum*, *M. gypseum*, *T. rubrum* and *T. mentagrophytes* compared with crude ethanol extract and glycoside extract, MIC = 3.75-19.64 and 62.5-218.75 mg/mL, respectively (Wuthi-udomlert *et al*., 2010). In similar, another study reported anthraquinone high yield extract showed strong antifungal activity against *T. rubrum*, *T. mentagrophytes* and *M. gypseum* with MIC of 15.62-250 μg/mL, respectively. Aloe-

emodin inhibited *T. rubrum* (MIC = 0.98 μg/mL) while rhein inhibited *T. mentagrophytes* (MIC = 62.5 μg/mL) (Sakunpak, 2009). Dealcoholized *C. tora* leaf extract showed 88.20-95.30 percent spore germation inhibition of *C.albicans*, *A.niger*, *S.cerevisiae* and *T. mentagrophytes* at 300 μg/mL compared with 83.95-97.70% of griseofulvin at 1,000 μg/mL (Mukherjee, *et.al*., 1996). In opposite, methanolic leaf extracts of *C. tora* showed 40-46% hyphal growth inhibition of *T. rubrum*, *M. gypseum* and *P. marneffei* at the concentration of 1 mg/mL (Pongpaichit *et al*., 2004). Moreover, one study reported isolation of chrysophanic acid-9-anthrone was the active compound against *T. rubrum*, *T. mentagrophytes*, *M. canis* and *M. gypseum* (Acharya & Chatterjee, 1975). Because cassia or senna was in the same family which shared similar anthraquinones active components, this study focused on isolation of some anthraquinones from *C.tora* seed extracts.

Trichophyton mentagrophytes and *M. gypseum* were species of fungi which was a communicable pathogen caused skin disease in humans and animals. *T. mentagrophytes* was characterized by flat-suede-like colonies with a white cream color while *M. gypseum* was a dermatophyte that was cottony or powdery with color range from white, pink to red or yellow. The color on the underside of *T.mentagrophytes* colonies was a yellow to reddish brown color. The powdery appearance of *M. gypseum* colonies was due to the production of macroconidia on the older mycelium. *T. mentagrophytes* was zoonotic and can be spread from animals to humans through direct contact. It caused tinea infections in humans affecting feet, face and other parts of body; for example between toes and under arm. The appearance of infected areas showed peeling, wrinkling and breaking of skin. The appearance can be distinguished by pustule, blister and vesicle formation. *T. mentagrophytes* caused ringworm in animals and manifested as an inflamed ring with flakey or crusting skin with or without hair loss. *M. gypseum* caused infection on the upper dead skin of mammals especially Tinea capitis and Tinea corporis. It usually only occurred in rural areas and clinical manifestations appeared as impetigo, scleroderma or psoriasis. This study interested to investigate the antifungal activity of *C. tora* seed extracts against *T. mentagrophytes* and *M. gypseum.*

The contents of anthraquinones and anthraquinone glycosides were determined using UV-visible spectrophotometry, TLC densitometry and HPLC. Total anthraquinone glycoside contents calculated as rhein and aloe-emodin of *Cassia fistula* L pods were determined using UV-visible spectrophotometer (Sakulpanich and Gritsanapan, 2008). TLC fingerprint of *Cassia fistula* leaf extracts was performed on a precoated aluminium plate of silica gel 60 F254 using ethyl acetate : methanol : water (100 : 17 : 13) as the mobile phase (Sakulpanich & Kritsanapan, 2009). Another study reported TLC fingerprint of *Senna alata* L. Leaf extracts using silica gel60 F254 and petroleum ether : ethyl acetate : formic acid (75:25:1) as the mobile phase (Wuthi-udomlert *et al*., 2010). RP-HPLC both isocratic and gradient elution was used for determined anthraquinones. Simultaneously rhein, aloe-emodin, emodin, chrysophanol in *Senna alata* leaves were analyzed with ODS column and the mixture of methanol and 2%aqueous acetic acid as the mobile phase (Panichayupakranant *et al*., 2009). Another HPLC method using phenyl-hexyl column and the mixture of methanol and 0.01% phosphoric acid with gradient analyzed anthraquinones from rhizomes of *Rhei*, *Coptidis* and *Radix scutellaiae* (Jia-Li *et al*., 2008). This study attempted to extract main anthraquinones using less toxic solvent and evaluated their antifungal activity. The content of anthraquinones was analyzed by RP-HPLC with photo diode array detector. The objectives of the study were to determine anthraquinones from *Cassia tora* L. seed extract and to evaluate antifungal activity.

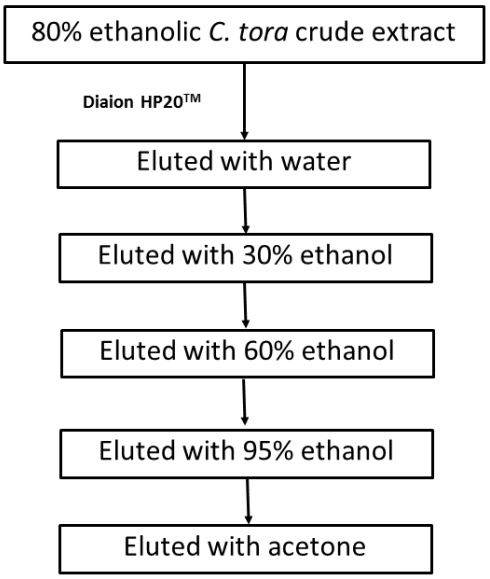
MATERIALS AND METHODS

Ripe *C. tora* seeds were bought from Charoensuk Herbal Manufacturing. Aloe-emodin and physcion were bought from Biopurify Phytochemicals Ltd., China. Chrysophanol, emodin and rhein were obtained from Sigma Aldrich, US. Commercial grade ethanol was obtained from Samchai company. Analytical grade methanol and ethanol were obtained from Avantor Performance Materials, Inc., US. Dimethyl sulfoxide (DMSO) and HPLC grade methanol were obtained from Carlo Erba and B&J Scientific.

Preparation of Cassia tora seed extract

Seeds were ground to powder and sieved (No.20). Seeds (150 g) were macerated in 80% ethanol (1,500 mL) in ultrasonic bath for 30 minutes and repeated until exhausted (5 times), monitored by Bontrager's test. The ethanolic extract was removed solvent using rotary evaporator.

Crude 80% ethanolic extract was separated on Diaion HP20^{TM} column chromatography (2.5 x 32 cm). Crude extract (1.41 g) was dissolve in 95%ethanol, divided to 2 portions and loaded to the column. The separations were performed 2 times; the first time obtained subfraction 1 (SF1) and repeated the same procedure to obtain subfraction 2 (SF2). Water, 30%, 60%, 95% ethanol and acetone (each 200 mL) were eluted at flow rate of 2-3 mL/min (Scheme 1). The subfractions were collected and solvent was evaporated and dried in lyophilizer. Then these subfractions (SF1, SF2) were analyzed with HPLC and tested against *T. mentagrophytes* and *M. gypseum*.



Scheme 1 Procedure for column chromatography

HPLC Determination of anthraquinones

The dried extracts was determines anthraquinone using RP-HPLC [Agilent 1260] with OpenLab software. Standard anthraquinones; aloe-emodin (AE), rhein (R), emodine (E), chrysophanol (C) and physcion (P), were prepared at the concentration of 1 mg/mL in methanol. Then standard solutions were further diluted in the range of 1.25 – 40 μg/mL. The extracts and subfractions were soluble in 80%ethanol (10 mg/mL). Anthraquinones were separated on an ACE C18 (4.6 x 250 mm, 5 μm). Mobile phase was a composition of (A) 0.01% phosphoric acid in water (pH 2.89) and (B) methanol with gradient elution:0-4 min,70

%B 4-5 min; 70-75%B; 5-10 min 75%B; 10-15 min 75-90%B; 15-22 min 90%B; 22-25 min, 90-70%B (Jia-Li *et al*., 2008). The detection was observed at 435 nm. Samples were prepared at the concentration of 1 mg/mL and filtered through nylon 0.45 μm. The injection volume was 10 μL(n=2). Each anthraquinone was calculated the content from their peak areas and standard curves. Total anthraquinone was calculated from the relative area percent of four anthraquinones (AE, E, C, P).

Evaluation of antifungal activity

The extracts were evaluated against *T. mentagrophyte* (SWU strain) and *M. gypseum* (RSU strain). *T. mentagrophyte* and *M. gypseum* were grown in sabouraud dextrose agar broth for 14 days. The fungal colonies were precultured and isolated for the tests. Sample solutions were prepared as a stock solution in DMSO (100 mg/mL) and two-fold serial dilution of tested extracts were prepared at the concentrations of 1,000, 500, 250 and 125 μg/mL. Ketoconazol (0.5-20 μg/mL) was a positive control and DMSO (1% v/v) was a negative control. Antifungal activity was tested using broth microdilution assay. Each 20 μL of sample and control solution (n=3) were added into 96-well plate. Then 80 μL of media was added and 100 μL of fungal colonies (final concentration 10^5 CFU/well) was added. The plates were incubated at 25-30 ^oC for 4 days and determined MIC. The clear solution was further determined for MFC values.

RESULTS AND DISCUSSION

Four anthraquinones were identified in crude *C. tora* seed extracts; aloe-emodin, emodin, chrysophanol and physcion. Since rhein was rarely detected in all samples, it was not included in the calculation of total anthraquinones in this study. The contents of anthraquinones identified by RP-HPLC are shown in Table 1. The highest anthraquinone content was chrysophanol in 80%ethanolic crude extract and all subfractions. Acetone subfraction eluted from the first column chromatography (SF1) showed the highest percent yield and highly increased in total anthraquinones compared with crude extract and other subfractions. Although total anthraquinone of the same solvent subfractions were relatively different, the elution profile of each subfraction showed similar separation (Figure 3-4). Since the yields from SF1 95% ethanol and SF2 acetone subfractions were relatively small amount, they were not tested for anti-fungal activity against *T. mentagrophytes*. Acetone and 95% ethanolic subfractions showed 4-8 folded increased in antifungal activity against *T. mentagrophytes* compared with crude *C. tora* seed extract. The antifungal activity was approximately 6-12 folded lower potency than ketoconazole. In similar, ethanolic subfractions inhibited *M. gypseum* 4-8 folded higher than crude extract. Acetone subfractions increased 2-4 folded *M. gypseum* inhibition compared with crude extract although the antifungal activity was 12-50 fold lower potency than ketoconazole. MIC and MFC values from broth microdilution assay are shown in Table 2.

Table 1. Anthraquinone contents

Samples	%Yield	mg/g extract (dried weight)				Total anthraquinone (%PA)
		AE	E	C	P	
C. tora crude extract	17.0	0.44	0.39	8.43	1.81	55.60
SF1 95%ethanol	2.84	1.33	ND	ND	ND	10.10
SF1 acetone	21.27	ND	0.70	18.76	5.06	70.57
SF2 95%ethanol	4.26	5.02	12.34	59.02	16.66	51.77
SF2 acetone	4.26	1.47	4.57	23.40	6.52	54.88

Total anthraquinone were calculated from %PA of 4 anthraquinones (AE, E, C, P)
ND = not detected or lower than 1.25 μg/mL

Table 2. MIC and MFC values of *C. tora* seed extracts and subfractions

Samples	*T. mentagrophytes*		*M. gypseums*	
	MIC (μg/mL)	MFC (μg/mL)	MIC (μg/mL)	MFC (μg/mL)
C. tora crude extract	500-1,000	> 4,000	1,000	1,000
SF1 95%ethanol	Not tested	Not tested	125	125
SF1 acetone	250	500	250	500
SF2 95%ethanol	125	1,000	250	500
SF2 acetone	Not tested	Not tested	500	> 500
Ketoconazole	20	20	10	> 40

HPLC chromatograms of five standard anthraquinones, *C. tora* crude extracts and subfractions are shown in Figures 2-4. From the retention times, aloe-emodin (RT 9.02 min), rhein (RT 11.64 min), emodin (RT 17.70 min), chrysophanol (RT 19.69 min) and physcion (RT 22.90 min) were eluted in order in the mixture of standards. These compounds were also observed in *C. tora* crude extracts and subfractions in corresponding retention time. Anthraquionones were not observed in water subfraction and other ethanolic subfractions (30%, 60%), their chromatogram were not shown here.

In this study major anthraquinoes identified from *C. tora* seeds were chrysophanol and physcion similar to the results reported by Sakunpheuk *et al*., 2012 that mature *C. tora* seeds showed high contents of emodin, chrysophanol, and physcion (2.62, 13.64, 14.27 mg/g dried weight) respectively. In contrast, five anthraquinones including aurantio-obtusin, 1-desmethylaurantio-obtusin, chryso-obtusin, obtusin and 1-desmethylchryso-obtusin were isolated and purified by high-speed counter-current chromatography (Zhu, *et al*., 2008). In comparison, aloe-emodin and rhein presented in high levels in *C. alata* pods and leaves (Sakulpanich and Gritsanapan, 2008; Sakulpanich and Gritsanapan, 2009). Anthraquinone high-yielding extract used silica gel column chromatography and eluted with hexane-ethyl actetate showed increasing in total anthraquinone content (16.70 & 1.13%w/w) compared with crude extract especially aloe-emodin and emodin (Sakunpak, 2009). The other used liquid-liquid partition with chloroform yielding anthraquinone aglycone from crude ethanol extract (Wuthi-udomlert *et al*., 2010). This study was partial purified through one column separation using Diaion HP20^{TM} as a stationary phase. Although the purified compound was not obtained, it needed further separation either another column chromatography or preparative chromatography.

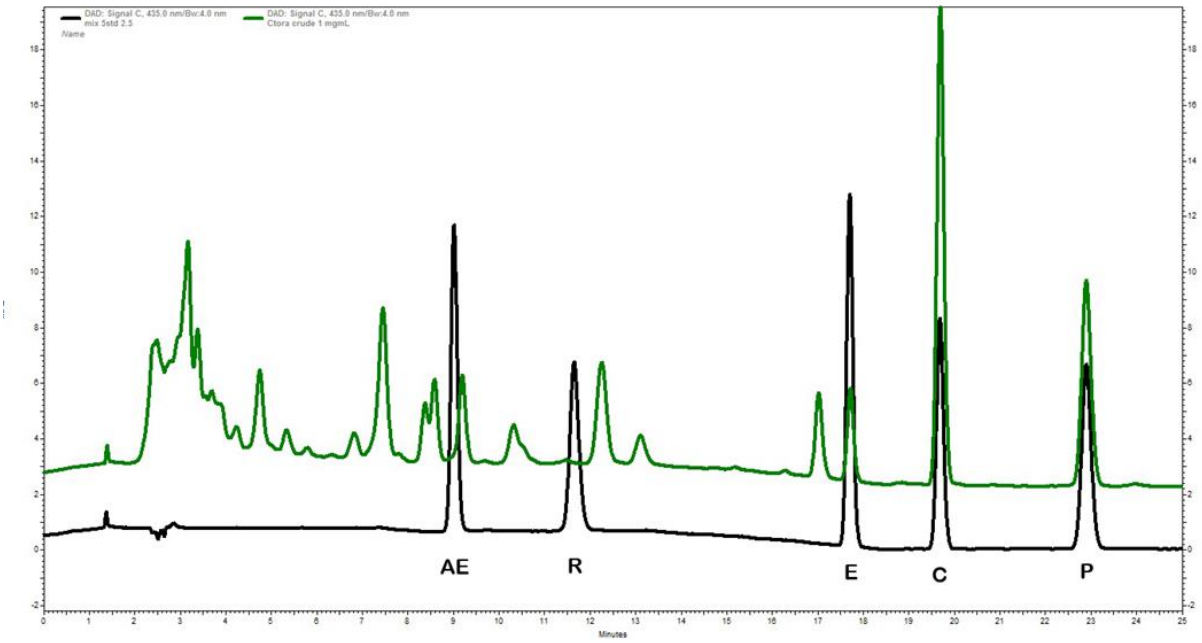


Figure 2 Overlay HPLC chromatogram of mix 5 anthraquinone standards and crude *C. tora* seed extract

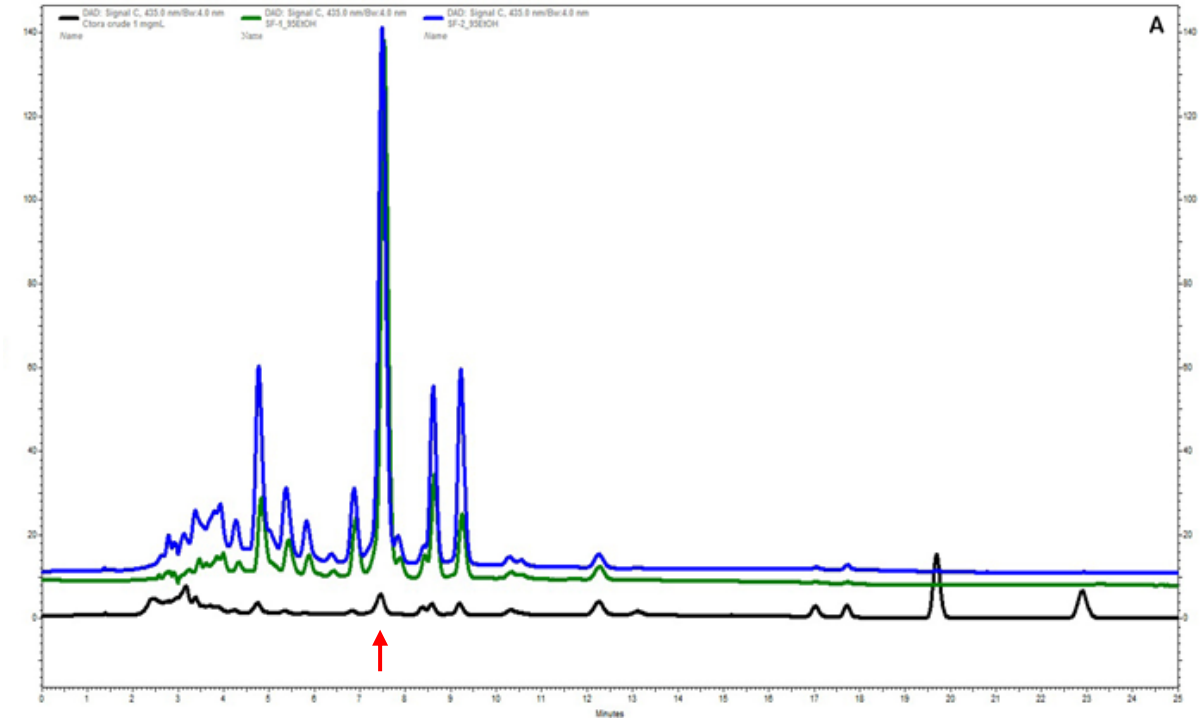


Figure 3. Overlay HPLC chromatogram of crude *C. tora* seed extract and ethanolic subfractions (SF1, SF2)

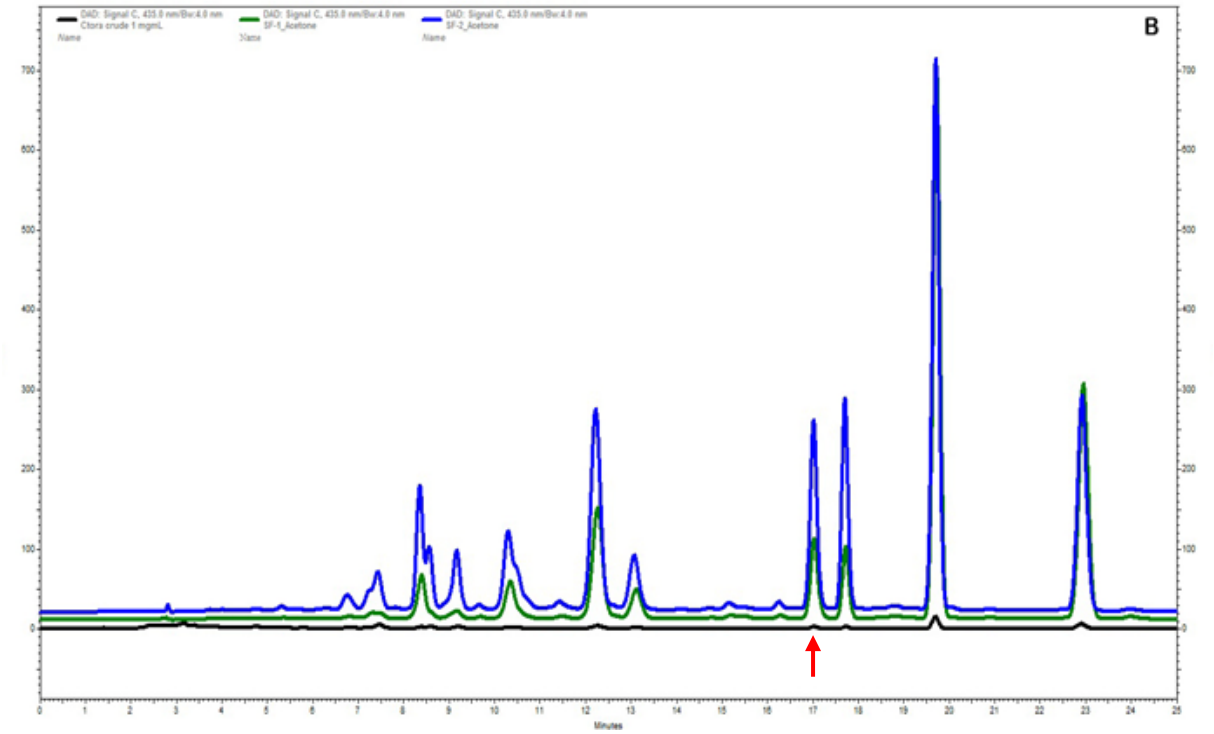


Figure 4. Overlay HPLC chromatogram of crude *C. tora* seed extract and acetone subfractions

Partial purified using nonpolar polymer based column (Diaion HP20^{TM}) increases some anthraquinone contents although total anthraquinones were not increased that much. Diaion HP20^{TM} was a synthetic adsorbents made from poly(styrene-divinylbenzene) that had relatively small pore volumes, small particle sizes and a large specific surface area (Diaion^{TM} Technical Manual). The compounds were separated by adsorption and desorption mechanism, molecular weight and increase hydrophobicity. It can apply to industry separation and food grade chemical was available.

The peaks were observed at RT 6.9 and 7.5 min (arrow) in 95% ethanolic subfractions and RT 17 min (arrow) in acetone subfractions could not be identified in liquid chromatography. However, LC-MS/MS can characterize the molecular ions were 315 and 330 which were corresponding to the molecular weight of 1-desmethylaurantio-obtusin and aurantio-obtusin, respectively. (MS spectra are showed in supplement document.)

In this study minimum inhibition concentration (MIC) of 95% ethanolic and acetone subfractions of crude *C. tora* seeds were 125-250 and 250-500 µg/mL against *T. mentagrophytes* and *M. gypseum*, respectively. In addition, dealcoholized of *C. tora* leaf extracts showed antifungal activity against *T. mentagrophytes* with inhibitory response in concentration dependent (100-300 µg/mL) by turbidity method (Mukherjee *et al*., 1996). In comparison, anthraquinone high-yielding extract of *Senna alata* leaves showed MIC of 62.5 µg/mL against *T. mentagrophytes* (Sakunpak *et al*., 2009). Another study reported that anthraquinone aglycone from ethanol extract of *Senna alata* leaves inhibited *T. mentagrophytes* with MIC of 0.88 mg/mL (880 µg/mL) (Wuthi-udomlert *et al*., 2010).

In *Senna alata* the main compounds that may responsible for antifungal activity were aloe-emodin and rhein (Sakunpak *et al*., 2009). The previous study of *Cassia tora* reported chrysophanic acid-9-anthrone as the major compound for antifungal activity (Acharya & Chatterjee, 1975). In this study, chrysophanol, physcion and emodin that were increased in acetone subfraction may be related to antifungal activity. In addition, 1-desmethylauranttio-obtusin and aurantio-obtusin were found in 95% ethanolic subfraction. These two compounds

may be involved in antifungal activity. The increasing in antifungal activity of subfractions may result from the combination of these anthraquinones.

CONCLUSION

This study has identified six anthraquinones in *C. tora* seed extracts using RP-HPLC with PDA detector. Aloe-emodin, emodin, chrysophanol and physcion were detected in crude ethanolic extracts and acetone subfractions. 1-Desmethylaurantio-obtusin and aurantio-obtusin were observed in 95%ethanolic and acetone subfractions, respectively. These anthraquinone contents increased significantly in eluted subfractions. These anthraquinoes may involve in inhibition of *T. mentagrophytes* and *M. gypseum*. The separation process can be adapted to increase yield and purity. High content of anthraquinones can be useful for medicinal research or as pesticide in organic farm.

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