Comparison of Antimicrobial, Antioxidant Activities and Total Phenolic Content of *Antidesma Thwaitesianum* Fruit Extracts by Different Methods

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Background: *Antidesma thwaitesianum Mull.Arg. is a tropical fruit in Northeast Thailand and its fruits are used for soft drink and healthy food. The biological activities of extracts from Antidesma thwaitesianum using different extraction methods have not been reported.*

Objective: *Extracts from Antidesma thwaitesianum using different extraction methods were tested for antibacterial and antioxidant activities as well as were determined for total phenolic content. Ten extracts were tested for antimicrobial activity by disc diffusion, MIC, and MBC methods. DPPH assay was used to test antioxidant activity and Folin-Ciocalteu's reagent was used to determine total phenolic content.*

Results: *The extract obtained by decocting residue after maceration process of dried marc (MRW) exhibited stronger antioxidative power (EC_{50} = 11.73 μg/ml) than BHT (EC_{50} = 13.36 μg/ml). This antioxidant activity was related to total phenolic content of 85.77 ± 0.34 mg GAE/g. This extract also exhibited antimicrobial activity against Staphylococcus aureus (inhibition zone = 8 mm, MIC = 2.5 mg/ml). Moreover, the extract obtained by macerating dried marc (MME) exhibited antimicrobial activity against Bacillus subtilis with the same MIC value of 10 mg/ml.*

Conclusion: *All of the extracts of Antidesma thwaitesianum had less potential for antimicrobial activity than Gentamicin and Amphotericin B. On the other hand, the water extract especially obtained by decocting residue after maceration of dry marc had good antioxidant power and the highest total phenolic content. Thus, such water extract should be recommended for a good source of natural antioxidants for commercial uses.*

Keywords: *Antidesma thwaitesianum Mull.Arg., Antimicrobial, Antioxidant, DPPH, Total phenolic contents*

J Med Assoc Thai 2012; 95 (Suppl. 1): S147-S153
Full text. e-Journal: *http://jmat.mat.or.th*

Antidesma thwaitesianum Mull.Arg. (Mamao luang in Thai) is a tropical fruit distributed in Northeast Thailand and is classified in the family Euphorbiaceae[1]. It is a shrub of 60-120 cm in height with leaves 12-15 cm long and 3.7-5 mm in diameter. The dark green, leathery, evergreen, alternate leaves sprout a single leaf that curls into the shape of a spade with parallel or oval-shaped sides. Leaf edges and base are sharp. Flowers are tiny, yellowish-white in May-July. Its small and round fruits are clustered like a grape, which mature in August-October[2]. These fruits are used for soft drink and healthy food because they contain slightly higher amounts of some nutrients, such as catechin, epicatechin, rutin, quercetin, procyanidin B1, procyanidin B2, gallic acid, and ferrulic acid[3]. These phenolic compounds are well-known for health benefits. They also serve as plant defense mechanisms to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damages[4]. The extract from the wood of *Antidesma thwaitesianum* induced apoptosis of breast cancer cell lines (MDA-MB-435) by 44 ± 14%. Moreover, it induced estrogen receptor-negative MDA-MB 435 cells xenografted in athymic nude mice[5]. However, there are no reports which studied the relationship between the different extraction methods and biological activities of its fruits such as antimicrobial and antioxidant activities. Thus, the aim of the present study was to investigate these activities of the extracts from *Antidesma thwaitesianum* fruits obtained by different methods. Also, the total phenolic

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content of the extracts was determined.

Material and Method

Chemicals and reagents

Antioxidant assay and total phenolic content

All the chemicals and reagents were of analytical grade. Folin-Ciocalteu's phenol reagent (Fluka, Germany), Gallic acid (Sigma, USA), 2,2-Diphenyl-1-picrylhydrazyl (Fluka, Germany), Butylated hydroxytoluene (BHT) (Fluka, Germany), Sodium carbonate (Merck, Germany), Resazurin (Sigma, USA).

Antimicrobial assay

Microbial species (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 250309, and *Salmonella typhi* were kindly provided by Khun Sopa Kummee, Prince of Songkla University, Mueller-Hinton agar (Oxoid, England), Mueller-Hinton broth (Himedia, India), and DifcoTM plate count agar (BD, USA).

Plant materials and extraction

Antidesma thwaitesianum Fruits were collected from Sakonnakhon province, Thailand in September 2009.

Method of preparation

Fresh fruits were stored at -20°C and dried fruits were dried at 50°C. Fresh fruits were squeezed and the fruit juice was dried by lyophilization.

Water extraction

The fresh fruits were squeezed to yield juice (FSW) and dried marc. Fresh fruits, dried fruits, and dried marc were boiled in distilled water. The duration of decoction was 15 min. The extracts were filtered through a Whatman No. 1 filter paper and were dried by lyophilization to obtain FDW, DDW and MDW fractions, respectively.

Ethanolic extraction

Fresh fruits, dried fruits, and dried marc were macerated with 95% ethanol for 3 days. The extracts were then filtered through a Whatman No. 1 filter paper and were concentrated by evaporator to obtain the ethanolic extracts, *e.g.*, FME, DME, and MME fractions, respectively. Residue from maceration of fresh fruits, dried fruits, and dried marc were further boiled in water, and then the filtrate was dried by lyophilization to obtain FRW, DRW, and MRW fractions, respectively.

Determination of total phenolic content

Total phenolic contents in the extracts were determined by the modified Folin-Ciocalteu method[6] using a microplate reader[7]. An aliquot of the extracts (20 µl) was mixed with 100 µl of Folin-Ciocalteu's reagent and 80 µl of sodium carbonate in 96-well microplate. The plate was mixed well and allowed to stand for 30 min to develope colour. Absorbance values of sample were measured at 765 nm. Total phenolic content was expressed as mg gallic acid equivalent (GAE)/g, obtained from a calibration curve of gallic acid standard solutions (ranging from 5 to 100 µg/ml) shown in Fig. 1. All measurements were performed in triplicate.

Antioxidant assay

DPPH radical scavenging assay[8]

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Yamasaki et al 1994. Sample for testing was dissolved in absolute ethanol or distilled water in various concentrations. 100 µl of extracts were transferred into a 96-well microplate. Then 100 µl of 6×10^{-5} M DPPH (in absolute ethanol) were added into each well. A portion of sample was mixed with an equal volume of 6×10^{-5} M DPPH. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 520 nm. BHT was used as a positive control. The concentration of antioxidant needed to decrease the initial DPPH concentration (EC_{50}) by 50% is a parameter widely used to measure the antioxidant activity[9]. The scavenging activity was calculated as percentage inhibition in the formulae below:

$$\text{Inhibition \%} = [(A_{Control} - A_{Sample})/A_{Control}] \times 100$$

Effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) was obtained by linear regression analysis of the dose-response curve of % inhibition versus concentration, and EC_{50} is calculated using prism program. All determinations were carried out in triplicate.

Antimicrobial assay

Antibacterial and antifungal activities were screened by the disc diffusion assay. The MIC and the MBC were determined using the micro-dilution technique.

Antimicrobial assay (disc diffusion method)[10]

Agar disc diffusion method was employed for

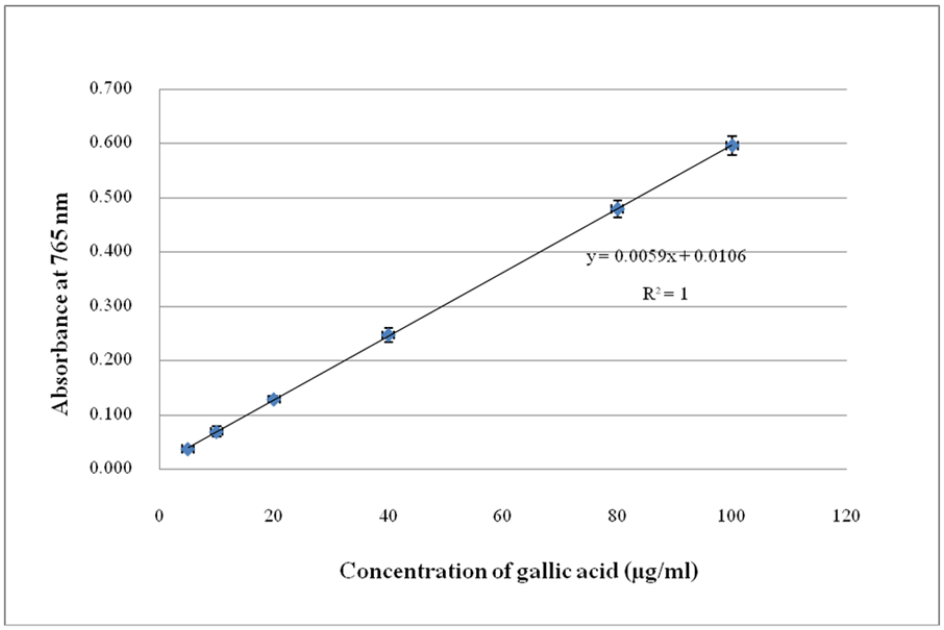

Fig. 1 Linear correlation between a series of gallic acid concentrations (µg/ml) as a standard and absorbance at 765 nm (n = 3)

the determination of antimicrobial activities of the extract, as described by Lorian 1996. Filter paper discs (6 mm in diameter) were impregnated with 10 µl of the extract (conc. 500 µg/ml). Air-dried discs were placed on inoculated Mueller-Hinton agar (MHA) surface (for bacteria) and Sabouraud Dextrose agar (SDA) surface (for yeast). Positive controls in the present study were Gentamicin and Amphotericin B (conc. 1 mg/ml). These plates were incubated at 37°C for 24 h for bacteria and at 30°C for 48 h for yeast. The zone of inhibition was calculated by measuring the diameter of the inhibition zone. Three different fixed directions were taken in triplicate and the average value was calculated.

Determination of minimum inhibitory and minimum bactericidal concentrations [11]

The minimal inhibitory concentration (MIC) values were determined by microdilution assay. This experiment was described by the method of Sarker et al 2007. The cultures were prepared from 18-24 h cultures of *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and from 36-48 h cultures of *Candida albicans* and *Cryptococcus neoformans* in broth cultures. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. This technique utilized the microdilution method in a 96-well microplate. Briefly, 50 µl of extract was diluted in two fold dilution in the well. The culture was diluted with Mueller-Hinton broth (MHB) medium to 0.5 McFarland standard using densitometer, and 50 µl was added into each well and mixed well. After 16-18 h at 37°C, 10 µl of resazurin solution were added in each well and incubated further for 2 h. MIC was defined as the lowest concentration of the extract that prevents a colour change in the 96-well microplate. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of the compound to kill the microorganisms. The MBC was determined by subculturing the test dilution (used in MIC) onto fresh solid medium and was incubated further for 24 h.

Statistical analysis

All experiments were carried out in triplicate. Statistical analysis was performed using Prism Software.

Results and discussion

The percent yield of the plant extracts in Table 1 showed that the decoction method gave the highest yield.

The total phenolic content of each extract was quantified using the Folin-Ciocalteu reagent. All the extracts showed significant amounts of phenolic compounds (22-85 mg GAE/g) (Table 1). The results demonstrated that the MRW and MDW contained the highest phenolic contents of 85.77 $\pm$ 0.34 mg GAE/g

Table 1. Percent yield, antioxidant activity, and total phenolic content of *Antidesma thwaitesianum* fruits (n = 3)

Parts used	Methods	Extract	Code	% Yield	Percent inhibition at various concentration (µg/ml)				Antioxidant activity* EC_{50} (µg/ml)	Total phenolic content* (mg GAE/g)
					1	10	50	100		
Fresh Fruits	Squeeze	Water	FSW	8.39%	1.53 ± 1.73	6.79 ± 0.40	23.58 ± 4.25	56.99 ± 1.40	91.11 ± 0.39	22.67 ± 0.06
	Maceration	EtOH	FME	7.39%	1.89 ± 1.15	5.31 ± 1.84	22.80 ± 1.93	36.31 ± 1.64	>100	35.77 ± 0.56
	Decoction of Residue after Maceration	Water	FRW	1.94%	14.86 ± 0.46	41.35 ± 0.88	70.36 ± 7.70	74.02 ± 5.92	13.91 ± 0.50	48.88 ± 0.10
	Decoction	Water	FDW	15.48%	9.23 ± 8.18	30.37 ± 3.33	57.39 ± 5.58	70.40 ± 6.71	24.53 ± 0.26	30.18 ± 1.00
Dried Fruits	Maceration	EtOH	DME	18.23%	2.05 ± 0.91	12.97 ± 1.84	43.90 ± 0.29	69.72 ± 2.02	61.24 ± 1.04	25.77 ± 0.80
	Decoction of Residue after Maceration	Water	DRW	30.78%	10.56 ± 3.78	22.01 ± 3.05	65.40 ± 2.49	86.51 ± 3.83	33.60 ± 0.21	32.38 ± 0.23
	Decoction	Water	DDW	44.33%	0.56 ± 0.44	19.40 ± 0.35	74.91 ± 1.61	92.45 ± 1.61	27.33 ± 0.49	40.91 ± 0.52
Dried marc	Maceration	EtOH	MME	12.74%	7.06 ± 2.90	24.55 ± 1.99	64.88 ± 3.19	77.35 ± 6.89	28.96 ± 1.11	33.63 ± 0.35
	Decoction of Residue after Maceration	Water	MRW	14.26%	0.02 ± 1.84	43.40 ± 1.72	82.66 ± 4.02	91.52 ± 2.16	11.73 ± 0.52	85.77 ± 0.34
	Decoction	Water	MDW	25.32%	0.46 ± 7.52	28.32 ± 3.14	73.99 ± 6.07	86.63 ± 2.88	19.49 ± 0.24	59.50 ± 0.32
BHT	-	-	-		10.14 ± 2.00	40.83 ± 0.87	83.10 ± 6.53	86.64 ± 4.07	13.36 ± 0.18	-

* Means of three measurements ± SEM (n = 3); BHT was used as a positive control for antioxidant activity

and 59.50 ± 0.32 mg GAE/g, respectively. In contrast, FSW contained the lowest phenolic content of 22.67 ± 0.06 mg GAE/g. Therefore, high phenolic contents were found in water extracts of dried marc. In addition, these high total phenolic contents in the extracts corresponded to strong antioxidant activity. As shown in Table 1, the MRW exhibited stronger antioxidant activity with an EC_{50} of 11.73 ± 0.52 µg/ml than BHT with an EC_{50} of 13.36 ± 0.18 µg/ml. This result was related to high total phenolic content. Thus, the *Antidesma thwaitesianum* juice which has been promoted as the health products, surprisingly showed less antioxidant activity and total phenolic content than marc.

Based on these results, marc is consequently more beneficial for health than fruits and juice and should be kept for preparing health products. Decoction method should be recommended for preparing marc with potent antioxidant activity because it extracted anthocyanin glycoside, a chemical group with strong antioxidant properties[12-14].

From disc diffusion method used to screen antimicrobial activity of all the extracts, MRW and MDW inhibited growth of *S. aureus* better than all of the other extracts in the range of 7-8 mm (Table 2). Only MME exhibited antimicrobial activity against *B. subsitis* with an inhibition zone of 6.88 mm, but all other extracts showed no inhibitory activity against *E. coli, S. typhi, C. albicans,* and *C. neoformans.* Moreover, the minimal inhibitory concentrations (MIC) of the active extracts were in the range of 2.5-10 mg/ml (Table 2). The MRW showed the highest inhibitory activity against *S. aureus* with the same lowest MIC and MBC value of 2.5 mg/ml. This supports previous reports that high phenolic contents were associated with growth inhibition against both gram-positive and gram-negative bacteria[15].

Conclusion

In conclusion, the results revealed that the water extracts of dried marc possessed high biological activity. These water extracts contained high phenolic compounds which corresponded to their antioxidant and antimicrobial activities. On the other hand, extracts of fresh fruits obtained by maceration method displayed the least potential for antioxidant and antimicrobial activities. These results support previous findings that the water extract of residue of dried marc (MRW) has the greatest antioxidant and antimicrobial activities as well as the highest phenolic content, thereby being good for health. This extract should thus be recommended as a good source of natural antioxidants

Table 2. Antimicrobial activity against six microorganisms of *Antidesma thwaitesianum* fruit extracts shown by inhibition zone, MIC (mg/ml), and MBC (mg/ml) (n = 3)

Parts of Used	Methods	Extract	Code	Inhibition zone (mm) (Conc. 5 mg/disc), MIC (mg/ml), MBC (mg/ml)					
				S. aureus	*B. subtitis*	*E. coli*	*S. typhi*	*C. albicans*	*C. neoformans*
Fresh Fruits	Squeeze	Water	FSW	0	0	0	0	0	0
	Maceration	EtOH	FME	0	0	0	0	0	0
	Decoction of Residue after Maceration	Water	FRW	7 ± 0,5,5	0	0	0	0	0
	Decoction	Water	FDW	7 ± 0,5,5	0	0	0	0	0
Dried Fruits	Maceration	EtOH	DME	7.33 ± 0.33, > 20, > 20	0	0	0	0	0
	Decoction of Residue after Maceration	Water	DRW	0	0	0	0	0	0
	Decoction	Water	DDW	7 ± 0,10,10	0	0		0	0
Dried marc	Maceration	EtOH	MME	7 ± 0,10,20	6.83 ± 0.17, 10, 10	0	0	0	0
	Decoction of Residue after Maceration	Water	MRW	8 ± 0, 2.5, 2.5	0	0	0	0	0
	Decoction	Water	MDW	8 ± 0,5,10	0	0	0	0	0
Gentamicin (10 µg/disc)		-	-	22 ± 0,1.25, 1.25	26 ± 0,0.156, 0.156	20 ± 0	20 ± 0	NT	NT
Amphotericin B (10 µg/disc)		-	-	NT	NT	NT	NT	16 ± 0	15.17 ± 0.28

Water and ethanolic extracts were used at 5 mg/disc; 0 represents no inhibition zone; NT, Not tested; The diameter of the filter disc (6 mm) is included

for commercial uses.

Acknowledgements

The authors are grateful to the National Research Council of Thailand (NRCT), the National Research University Project of Thailand Office of Higher Education Commission and faculty of Medicine, Thammasat University, for financial support. We also thank for Thailand Institute of Drug Research and Development (TIDRD) for providing equipments on biological assay. The help and support of Professor Dr. Somboon Kietinun and Associate Professor Sukanya Jesadanont are highly appreciated.

Potential conflicts of interest

None.

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ฤทธิ์ต้านเชื้อจุลชีพ ฤทธิ์ต้านอนุมูลอิสระ และปริมาณสารกลุ่มฟีนอลิก จากสารสกัดจากผลมะเม่าด้วยวิธีการสกัดต่าง ๆ

ภาณัฐ เดชะยนต์, พินทุสร หาญสกุล, อรุณพร อิฐรัตน์

ภูมิหลัง: *มะเม่า (Antidesma thwaitesianum) อยู่ในวงศ์ Euphorbiaceae ปัจจุบันได้นำผลมะเม่าสุกมาแปรรูปเป็นผลิตภัณฑ์ชนิดต่างๆ เช่น น้ำมะเม่า ไวน์ และ แยม นอกจากนี้ผลมะเม่ามีสารประกอบของสาร กลุ่มฟีนอลิกสูง ซึ่งเป็นที่ทราบกันอย่างแพร่หลายว่าสารกลุ่มนี้มีฤทธิ์ในการต้านอนุมูลอิสระ ชะลอการเสื่อมของเซลล์ร่างกาย ฤทธิ์ต้านแบคทีเรีย ต้านการอักเสบ และป้องกันโรคมะเร็ง ดังนั้นควรศึกษาการสกัดผลมะเม่าด้วยวิธีการต่าง ๆ และวิเคราะห์ปริมาณสารกลุ่มฟีนอลิก และศึกษาฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ต้านเชื้อจุลชีพ*

วัตถุประสงค์: *ศึกษาปริมาณสารในกลุ่มฟีนอลิก ฤทธิ์ต้านอนุมูลอิสระ (Antioxidant activity) และฤทธิ์ต้านเชื้อจุลชีพของสารสกัดผลมะเม่าที่ได้จากการสกัดด้วยวิธีต่างๆ*

วัสดุและวิธีการ: *สกัดสารจากผลมะเม่าด้วยวิธีการต่างๆ จากนั้นนำสารสกัดไปศึกษาหาปริมาณสารในกลุ่มฟีนอล (Total phenolic content) ด้วยวิธี Folin-Ciocalteu's reagent ฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH radical scavenging assay และหาค่า EC_{50} และหาฤทธิ์ต้านเชื้อจุลชีพ 6 สายพันธุ์ คือ Bacillus subtilis Staphylococcus aureus, Escherichia coli, Salmonella typhi, Candida albicans และ Cryptococcus neoformans ด้วยวิธี disc diffusion assay, minimal inhibitory concentration (MIC) และ The minimal bactericidal concentration (MBC)*

ผลการศึกษา: *จากการหาปริมาณสารในกลุ่มฟีนอลพบว่า เมื่อนำกากหลังจากขั้นน้ำจากผลสุกไปหมักเอทธานอล แล้วนำกากที่ได้หลังจากการหมักเอทธานอลไปต้มน้ำอีกครั้ง สารสกัดน้ำที่ได้จากการต้มครั้งหลังนี้ (MRW) มีปริมาณสารกลุ่มฟีนอลิกสูงสุด (GAE = 85.77 mg GAE/g) ซึ่งพบว่ามีความสัมพันธ์กับมีฤทธิ์ต้านอนุมูลอิสระซึ่งวิธีการสกัดนี้ได้สารสกัดสามารถรีดิวซ์สามารถทำให้สีของ DPPH จางลงได้ดีที่สุด ซึ่งให้ค่า EC_{50} = 11.73 μg/ml ซึ่งมีฤทธิ์ดีกว่าสารมาตรฐาน BHT ให้ค่า EC_{50} = 13.36 μg/ml เมื่อศึกษาฤทธิ์ต้านเชื้อจุลชีพพบว่า สารสกัดของผลมะเม่ามีฤทธิ์ยับยั้งเชื้อ gram-positive คือ S. aureus และ B. subtilis ได้โดยสารสกัด MRW มีฤทธิ์ในการยับยั้งเชื้อ S. aureus ได้ดีที่สุด (MIC 2.5 mg/ml) ซึ่งสัมพันธ์กับปริมาณสารกลุ่มฟีนอลิกที่พบปริมาณมากที่สุด แต่สารสกัดกากหลังจากการขั้นน้ำหมักด้วยเอทธานอล (MME) เท่านั้นที่สามารถยับยั้งเชื้อ B.subtilis ได้ (MIC 10 mg/ml) เมื่อทดสอบเชื้อ gram-negative (Escherichia coli, Salmonella typhi) และ เชื้อรา (Candida albicans, Cryptococcus neoformans) พบว่าสารสกัดของผลมะเม่าไม่สามารถยับยั้งเชื้อทั้ง 4 ชนิดได้*

สรุป: *สารสกัดผลมะเม่าโดยการต้มน้ำมีฤทธิ์ต้านอนุมูลอิสระที่สูง แต่มีฤทธิ์ในการยับยั้งเชื้อได้น้อย ซึ่งคุณสมบัติเหล่านี้สัมพันธ์กับปริมาณสารกลุ่มฟีนอลิก ซึ่งสารกลุ่มนี้มีประโยชน์ต่อสุขภาพ สารสกัดจากมะเม่าจึงเหมาะสมที่จะนำมาพัฒนาเป็นผลิตภัณฑ์เสริมอาหารจากผลิตภัณฑ์ธรรมชาติ เพื่อเป็นการเพิ่มคุณค่าของพืชสมุนไพรไทย*