The Thai Journal of Pharmaceutical Sciences

Volume 36
Issue 0 *supplement issue*

Article 13

1-1-2012

BIOLOGICAL ACTIVITIES OF A PATENTED MIX EXTRACT FROM FRUITS OF PHYLLANTHUS EMBLICA AND ZANTHOXYLUMLIMONELLA

Buppachart Potduang

Bundit Fungsin

Isara Keeta

Natthachest Ketmanee

Anintitha Intharungsri

See next page for additional authors

BIOLOGICAL ACTIVITIES OF A PATENTED MIX EXTRACT FROM FRUITS OF PHYLLANTHUS EMBLICA AND ZANTHOXYLUMLIMONELLA

Authors

Buppachart Potduang, Bundit Fungsin, Isara Keeta, Natthachest Ketmanee, Anintitha Intharungsri, Sitthiphong Soradech, Cholticha Niwaspragrit, and Sayan Tanpanich

BP-3

BIOLOGICAL ACTIVITIES OF A PATENTED MIX EXTRACT FROM FRUITS OF *PHYLLANTHUS EMBLICA* AND *ZANTHOXYLUM LIMONELLA*

Buppachart Potduang[1*], Bundit Fungsin[2], Isara Keeta[1], Natthachest Ketmanee[1], Anintitha Intharungsri[1], Sitthiphong Soradech[1], Cholticha Niwaspragrit[3] and Sayan Tanpanich[3]

[1]*Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Technopolis, Klong 5, Klong Luang, Pathumthani, 12120, Thailand*
[2]*Biosciences Department, Thailand Institute of Scientific and Technological Research (TISTR), Technopolis, Klong 5, Klong Luang, Pathumthani, 12120, Thailand*
[3]*Agricultural Technology Department, Thailand Institute of Scientific and Technological Research (TISTR), Technopolis, Klong 5, Klong Luang, Pathumthani, 12120, Thailand*

KEYWORDS: *Phyllanthus emblica*, *Zanthoxylum limonella*, Biological activity, Mix extract

INTRODUCTION

Many edible fruits distributed in the tropical forests of Thailand are of medicinal important. Research on cosmeceutical products comprising of these fruits extract will strengthen the Thai herbal market and reduce possible damages from the ASEAN Free Trade Agreement.

This research aimed to select the appropriate mix extract from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* to be used as anti-microbial active extract of cosmeceutical products for dry and itchy skin. This report revealed the anti-microbial, anti-oxidant and anti-tyrosinase effects of the mix extract.

Phyllanthus emblica L. (EUPHORBIACEAE) is locally called Ma-khampom in Thailand. Its edible fruits are used as anti-oxidant, anti-inflammatory and anti-microbial agent in herbal medicines. It is used to treat diarrhea, jaundice, skin disorders, inflammations and premature graying. The fruit is rich in vitamin C, tannins and flavonoids. Its chemical constituents include vitamin C, gibberellins, lupeol, kaempferol, quercetin, emblicain A and B, punigluconin, pedunculaginn, phyllanthin, zeatin, amlaic acid, corilagin, ellagic acid, putranjivain A, digallic acid, phyllemblic acid, emblicol and galactaric acid[2)]. It is reported that its isolated phenolic compounds exhibit anti-inflammatory activity[1, 2, 3, 4, 5)]. We have found that the ethanol fruit extract of *P. emblica* was anti-microbial (MIC 10-20 mg/ml), anti-oxidant (EC_{50} 0.0187 μg/ml) and anti-tyrosinase (IC_{50} 898.67 μg/ml) effective. But the extract was not economically enough to be used as an anti-microbial active. So we have further investigated to find appropriate fruit extract to be added to enhance the anti-microbial potency of the active extract, and we have found that the ethanol extract of fruits of *Z. limonella* was suitable.

Zanthoxylum limonella Alston (RUTACEAE) is called "Ma-khwaen" in Thailand. The plant is a deciduous tree, up to 18 m tall. It was found distributed in subtropical and tropical regions through China, India, Indonesia, Malaysia and Northern Thailand. The bark of young trees is green with spines, becoming grey with woody prickles of 2-3 cm on older trees. The edible fruit is non-toxic and has been traditionally used as food flavor in the northern part of Thailand. The extraction of roots, stem-barks, stems and fruits are widely used for antibacterial, anti-inflammatory and anesthetic properties. The fruit consists of various substances e.g. alkaloids, amides, coumarins, sterols and phenylpropanoid-ligans. The essential oil from *Z. limonella* fruits exhibits the anti-oxidative potential. The oil contains Sabinene which is a potent bactericidal against the multi-drug resistant bacteria[6, 7, 8)]. We have reported that the ethanol extract from fruits of *Z. limonella* has total flavonoids of 3.61 mg rutin/g extract. The extract is effective as anti-microbial (MIC 2.5-10 mg/ml) and anti-oxidant (EC_{50} 5.94 μg/ml) [9)]. We have also found that the extract was a potent anti-tyrosinase (IC_{50} 0.33 μg/ml).

The crude ethanol extracts from fruits of *Z. limonella* and *P. emblica* were mixed in a suitable ratio to give the best anti-microbial effective mix extract. The anti-inflammatory efficacy of the mix extract was separately reported. This report reveals the biological efficacy of the patented mix extract on anti-microbial, anti-oxidant and anti-tyrosinase. The mix extract is further used to develop various cosmeceutical products for dry and itchy skin benefit from the extract's antimicrobial and anti-inflammatory efficacy. The antioxidant and anti-tyrosinase activities of the mix extract also benefit the products. The antioxidant agents help to provide protection against free radicals induced by UV. The anti-tyrosinase agents inhibit melanin biosynthesis, resulting in brightening of the skin [10, 11)].

MATERIALS AND METHODS

Plant material: The dry fruit powder of *P. emblica* and *Z. limonella* were provided by the Agricultural Technology Department, Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the crude ethanol extract: *P. emblica* extract was prepared by macerated the fruit powder 500 g with ethanol-water for 4 nights, filtered through Whatman paper No.41 and rinse with the

same solvent. The solvent was removed under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision) at 45 °C. *Z. limonella* was extracted with another appropriate proportion of ethanol-water for 3 nights, filtered through small amount of TISTR-S (Trade secrets), rinsed and evaporated under reduced pressure. The crude ethanol extracts were kept at 5-10 °C for further investigations.

Anti-microbial assay: The mix extracts of various ratios were dissolved in 40% ethanol to be subjected to paper disk agar diffusion assay against *Propionibacterium acnes*, *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes* and *Candida albicans*. Stock of microorganism was prepared by cultivation on nutrient agar. When microorganism reveals good, it was separated to sterile water and adjusted to the concentration of 0.5 McFarland. Twenty milliliters of nutrient agar was pipette into a petri dish and allowed to set. The diluted microorganism was then added and distributed evenly over the agar surface. The microbial agar plate was let dry in aseptic conditions, and then placed the filter paper disks on the agar surface. Added 20 µl of sample solution onto the disk, then closed the lids and incubated the culture plate at 37°C for 18-24 h. Measured the diameter in cm of the clear zone of inhibition [12, 13]. Ketoconarzole 3 mg/ml was used as an external standard. The most effective mix extract against tested microbial was selected for further investigations and product development.

Antioxidant assay: The selected mix extract was dissolved in absolute ethanol to be subjected to the antioxidant assay using DPPH radical scavenging micro-plate method as described by Potduang *et al.*[14]. Equal volumes of absolute ethanol solutions of the extract and 0.06 mM DPPH (2, 2-diphenyl-1-picrylhydrazyl (Sigma) were mixed for 30 min in a micro-well plate, Absorbance was measured at 517nm with a micro-plate reader (TECAN, Sunrise remote). All samples were run in triplicate. BHA was used as reference standard. The % scavenging activity of test samples was determined as follows:

$$\% \text{ Scavenging} = 100 \times [C-(A-B)] / C$$

Where A, B and C represent the absorbance of DPPH in the reaction mixture, blank, and control respectively. Plot the %scavenging *vs.* log concentration. Calculated the EC_{50} (conc. of 50% scavenging) using the resulted linear equation.

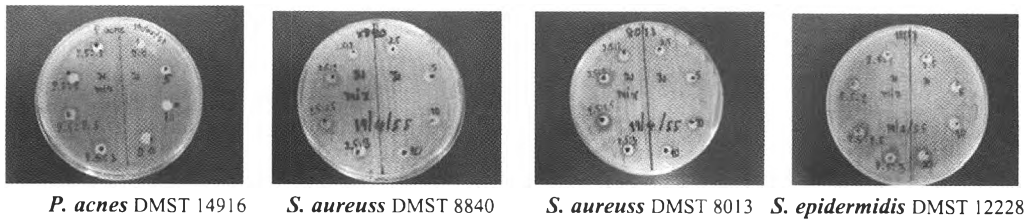
Tyrosinase inhibition assay: The selected mix extract was dissolved in 20% ethanol to be subjected to the tyrosinase inhibition assay using the dopa-chrome micro-plate method modified from Iida *et al.*[14] as described by Potduang *et al.*[15]. The 20% ethanol solution of the mix extract was pipette 50 µl to mix with 50 µl of 314.8U/ml mushroom tyrosinase enzyme (Fluka) solution in buffer and 100 µl of 0.02 M sodium phosphate buffer (pH 6.8). The mixture was pre-incubated at room temperature for 10 min before adding 50 µl of the substrate (0.34 mM L-Dopa, Sigma) solution in buffer , mixed well and measured the absorbance at 492 nm by a micro-plate reader (TECAN, Sunrise remote). Incubated the reaction mixture for 2 min in the micro-plate reader, and then re-measured the absorbance. All samples were run in triplicate. Kojic acid, a well-known tyrosinase inhibitor, was used as reference standard. Calculated the % tyrosinase inhibition using the differences of absorbance as follows:

$$\%\text{Tyrosinase Inhibition} = [(A-B)-(C-D)] \times 100/ (A-B)$$

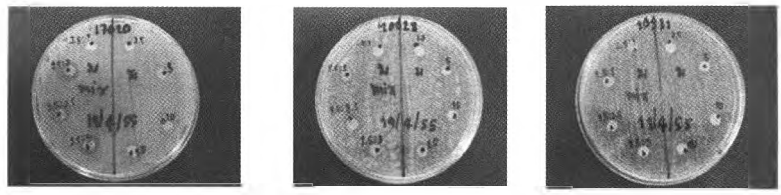
A, B, C and D are the differences of absorbance at 492 nm. Where A = control (L-Dopa mixed with enzyme in buffer); B = blank (L-Dopa in buffer); C = reaction mixture; and D = blank of C (L-Dopa mixed with test sample in buffer). Plot the %tyrosinase inhibition *vs.* log concentration. The resulted linear equation was used to calculate the IC_{50} (conc. of 50% inhibition).

RESULTS AND DISCUSSION

Anti-microbial activity: Figure 1 and Table 1 shows clear zones of *in vitro* microbial inhibition of Z. *limonella* extract (1, 2, 2.5 and 3 mg/ml) adding to 2.5 mg/ml of *P. emblica* extract. The mix extract was active against *P.* acne, *S. aureus, S. epidermidis* and *S. pyogenes*, but not active against *C. albicans*. The result exhibited that 2 mg/ml of Z. *limonella* extract adding to 2.5 mg/ml of *P. emblica* extract was the most anti-microbial effective.



P. acnes DMST 14916 ***S. aureuss*** DMST 8840 ***S. aureuss*** DMST 8013 ***S. epidermidis*** DMST 12228



S. pyogenes DMST 17020 *C albicans* DMST 90028 *C albicans* DMST 10231

Figure 1 Microbial inhibition of mix extracts comprising of Z. *limonella* extract (1, 2, 2.5 and 3 mg/ml) and 2.5 mg/ml *P. emblica* extract

Table 1 Inhibition zones diameter (cm) of mix extracts comprising of various ratios of *Z. limonella* extract adding to 2.5 mg/ml *P. emblica* extract

Microorganisms	Inhibition zones (cm) of added *Z. limonella* extract			
	1 mg/ml	2 mg/ml	2.5 mg/ml	3 mg/ml
*P .acne*DMST 14916	-/+	-/+	-/+	-/+
S. aureus DMST 8013	-/+	+(1)	+(0.8)	+(0.8)
S. aureus DMST 8840	-/+	-/+	-/+	-/+
S. epidermidis DMST 12228	+(0.7)	+(1)	+(0.8)	+(1)
S. pyogenes DMST 17020	-	+(1)	+(0.8)	+(1)
C. albicans DMST 10231	-	-	-	-
C. albicans DMST 90028	-	-	-	-

*The concentration of *P. emblica* extract is 2.5 mg/ml and *Z. limonella* extract are 1, 2, 2.5 and 3 mg/ml.
- = can not to see clear zone
-/+ = clear zone is not clear (cm)
+ = clear zone is clear (cm)

Table 2 DPPH radical scavenging effect of the patented mix extract from fruits of *P. emblica* and *Z. limonella*

Substances	Concentration of sample (ppm)	Log concentration	%Scavenging	EC_{50} (µg/ml)
Mix extract	1.25	0.09691	15.68047	0.00790
P. emblica	12.5	1.09691	52.07101	
+ *Z. limonella*	37.5	1.57403	90.23669	
	62.5	1.79588	92.30769	
	125	2.09601	91.12426	
BHA	1.25	0.09691	8.28402	0.01127
	12.5	1.09691	38.75739	
	37.5	1.57403	85.20710	
	62.5	1.79588	91.42012	
	125	2.09691	91.71598	

Table 3 Anti-tyrosinase effect of the patented mix extract from fruits of *P. emblica* and *Z. limonella*

Substances	Concentration of sample (ppm)	Log concentration	%Tyrosinase inhibition	IC_{50} (µg/ml)
Mix extract	400	2.6021	25.56	5517.22
P. emblica	800	2.9031	43.33	
+ *Z. limonella*	1200	3.0792	50.00	
	1600	3.2041	38.89	
	2000	3.3010	35.56	
Kojic acid	7.57	0.8788	37.78	0.03
	15.13	1.1798	81.11	
	31.25	1.4949	96.67	
	62.50	1.7959	96.67	
	125.00	2.0969	96.67	

Anti-oxidation activity: Table 2 shows the scavenging effect of the patented mix extract on DPPH radicals. The plotted graph of %scavenging *vs.* log concentration gave the resulted linear equation: y = 42.102X + 12.199. Substituted y = 50 to obtain X = 0.898, which gave the antilog 0.898 = 0.00790. So the mix extract scavenged DPPH radicals at the EC_{50} 0.0079 µg/ml compared to std. BHA (EC_{50} 0.0113 mg/ml).

Anti-tyrosinase activity: Table 3 shows the anti-tyrosinase effect of the patented mix extract. The plotted graph of %Tyrosinase inhibition *vs.* Log concentration gave the resulted linear equation: y = 15.659X - 8.5916. Substituted y = 50 to obtain X = 3.7417, which gave the antilog 3.7417 = 5517.2215. So the mix extract inhibited enzyme tyrosinase at the IC_{50} 5517.22 µg/ml, compared to std. Kojic acid (IC_{50} 0.03 µg/ml).

CONCLUSION

The mix extract from fruits of *P. emblica* and *Z. limonella* comprising 2 mg/ml of Z. *limonella* extract and 2.5 mg/ml of *P. emblica* extract was the most anti-microbial effective. The mix extract had the MIC 4.5 mg/ml against *P. acnes*, *S. aureus*, *S. epidermidis* and *S. pyogenes*. It scavenged DPPH radicals at the EC_{50} 0.0079 µg/ml, and inhibited enzyme tyrosinase at the IC_{50} 5517.22 µg/ml. These anti-microbial, anti-oxidant and anti-tyrosinase efficacies of the mix extract could benefits various cosmeceutical products.

ACKNOWLEDGMENTS

The authors express their gratitude to the Thailand Institute of Scientific and Technological Research (TISTR) for providing research funds and laboratory facilities. We would like to thank Mr. Apinan Srichuay[2], Biosciences Department laboratory of TISTR, for microbial culture and his kindly training for new researchers. We thank all TISTR colleagues who in one way or another help this project to succed.

REFERENCES

1. *Phyllanthus emblica* L. Available at http://www.globinmed.com/index.php?option=com_content&view=article&id=79255:phyllanthus-emblica-l&catid=718:p&Itemid=150, accessed 5 October 2012.
2. Emblica. Available at http://www.drugs.com/npp/emblica.html#ref19, accessed 10 October 2012.
3. Penning A. 2012. Beauty in layers: Multitasking ingredients. GCI Magazine, 5 pages. Available at http://www.gcimagazine.com/marketstrends/segments/antiaging/156350405.html?page=3, accessed 7 October 2012.
4. Liu X, Cui C, Zhao M, Wang J, Luo W, Yang B and Jiang Y. 2008. Identification of phenolics in the fruit of emblica (*Phyllanathus emblica* L.) and their antioxidant activities. Food Chem. 109: 909-915.
5. Muthuraman A, Sood S and Singla SK. 2011. The antiinflammatory potential of phenolic compounds From *Emblica officinalis* L. in rat. Inflammopharmacology 19(6): 327-34.
6. Mallikarjuna P, Uma Maheswara Rao V and Satyanarayana T. 1999. Antimicrobial activity of *Zanthoxylum limonella*. Indian Drugs 36 (7): 476-478.
7. Tangjitjaroenkun J, Supabphol R and Chavasiri W. 2012. Antioxidant effect of *Zanthoxylum limonella Alston*. J Medicinal Plants Res. 1407-1414.
8. Charoenying P, Laosinwattana C, Phuwiwat W, and Lomratsiri J. 2008. Biological activities of *Zanthoxylum limonella Alston* fruit extracts. KMITL Sci. J. 8(1): 12-15.
9. Ngamnon Y, Potduang B*, Fungsin B, Phasuk S, Takolpuckdee P, Rerk-am U, Kaewduang M and Tanpanich S. 2012. Antimicrobial, antioxidant activities and total flavonoids of fruit extracts from Ma–khwaen (*Zanthoxylum limonella*). Thai J Pharm Sci. 35 (Suppl.): 36-37.
10. Itchy skin cause. Available at http://www.buzzle.com/articles/itchy-skin-causes.html, accessed 10 October 2012.
11. Penning A. 2012. Beauty in layers: Multitasking ingredients. GCI Magazine, 5 pages. Available at http://www.gcimagazine.com/marketstrends/segments/antiaging/156350405.html?page=3, accessed 7 October 2012.
12. Raahave D. 1974. Paper disk agar-diffusion assay of penicillin in the presence of streptomycin. Antimicrobial Agents and Chemotherapy (Nov): 603-605.
13. Wilkins TD and Chalgren S. 1976. Medium for use in antibiotic susceptibility testing of anaerobic bacteria. Antimicrobial Agents and Chemotherapy 10(6): 9265-28.
14. Potduang B, Chongsiriroeg C, Benmart Y, Giwanon R, Supatanakul W, Tanpanich S. 2007. Biological Activities of *Schefflera leucantha*. Afr J Trad CAM. 4(2):157–164.
15. Iida K, Hase K, Shimomura K, Sudo S, Katota S, Namba T. 1995. Potent inhibitors of tyrosinase activity and melanin biosynthesis from *Rheum officinale*. Planta Med. 61:425–428.