Antibacterial, Antimalarial and Cytotoxic Activities Screening of Methanolic Extract from the Seeds of Litchi (*Litchi chinensis* L.)

การตรวจกรองฤทธิ์ต้านเชื้อจุลชีพ ฤทธิ์ต้านเชื้อมาลาเรีย และความเป็นพิษต่อเซลล์ของสารสกัดจากเมล็ดลิ้นจี่ด้วยเมทานอล

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Abstract

The methanolic extract from seeds of Litchi *(Litchi chinensis* L.*)* was tested for antimicrobial activity with five strains of pathogenic bacteria including *Staphylococcus aureus, Bacillus cereus, Psuedomonas aeruginosa, Enterococcus fecalis* and *Escherichia coli.* The antimicrobial activities of extract were screened by agar diffusion before conducted with broth macrodilution methods for determining minimal inhibitory concentration (MIC). Quantitative assessment of antimalarial activities of the extract was determined by microculture radioisotope techniques. *Plasmodium falciparum* (K1, multi-drug resistant strain) cultured *in vitro* used as malarial parasite for this test. The methanolic extract was screened for cytotoxicity with Vero cells (African green monkey kidney cell line) by Sulforhodamine B (SRB) colorimetric assay.

The results show that the methanolic extract of Litchi seeds (10 mg/ml) inhibited growth of *S. aureus, B. cereus, P. aeruginosa* and *E. faecalis* (inhibition zone = 10, 10, 12 and 11 mm, respectively). MIC values of Litchi seed extract were 3.47, 6.93, 3.47 mg/ml for *S. aureus, B. cereus* and *E. faecalis* (*P. aeruginosa* was not inhibited growth by tested with broth macrodilution methods). The extract of Litchi seeds possessed preferable high antimalarial activities with IC_{50} = 2.70 ± 0.23 μg/ml. There was no cytotoxic effect to Vero cells for this extracts.

The antimicrobial and antimalarial activities may be due to the types and content of phenolic compounds present in the extract. Biological models to confirm such activities and the characterization of unknown compounds may be selected for further study.

Keywords: Antibacterial, Antimalarial, Cytotoxicity, Litchi seed, *Litchi chinensis*

บทคัดย่อ

สารสกัดด้วยเมทานอลจากเมล็ดลิ้นจี่ (*Litchi chinensis* L.) นำมาทดสอบฤทธิ์ต้านเชื้อจุลชีพกับเชื้อแบคทีเรียก่อโรค 5 ชนิด คือ *Staphylococcus aureus, Bacillus cereus, Psuedomonas aeruginosa, Enterococcus faecalis, Escherichia coli* โดยตรวจกรองด้วยวิธี Agar diffusion จากนั้นทดสอบด้วยวิธี Broth macrodilution methods เพื่อหาค่าความเข้มข้นของสารน้อยที่สุด ที่สามารถยับยั้งการเจริญของเชื้อแบคทีเรียและยีสต์ได้

สารสกัดได้นำมาทดสอบฤทธิ์ต้านเชื้อมาลาเรียโดยวิธี Microculture radioisotope techniques ซึ่งทดสอบกับเชื้อ *Plasmodium falciparum* (K1, multi-drug resistant strain) ที่เลี้ยงในหลอดทดลอง และทดสอบความเป็นพิษต่อเซลล์โดยวิธี Sulforhodamine B (SRB) colorimetric assay โดยทดสอบความเป็นพิษต่อเซลล์โดยใช้ Vero cells (African green monkey kidney cell line)

ผลการทดลองพบว่าสารสกัดด้วยเมทานอลจากเมล็ดลิ้นจี่ (10 mg/ml) สามารถยับยั้งการเจริญของ *S. aureus, B. cereus, P. aeruginosa* and *E. faecalis* (inhibition zone = 10, 10, 12 and 11 mm, ตามลำดับ) ค่า MIC ของสารสกัดด้วยเมทานอลจากเมล็ดลิ้นจี่เท่ากับ 3.47, 6.93, 3.47 mg/ml สำหรับ *S. aureus, B. cereus* และ *E. faecalis* ตามลำดับ (แต่ *P. aeruginosa* ไม่ถูกยับยั้งการเจริญจากการทดสอบโดยวิธี Broth macrodilution methods) สารสกัดด้วยเมทานอลจากเมล็ดลิ้นจี่มีประสิทธิภาพสูงในการต้านเชื้อมาลาเรียโดยให้ค่า IC50 = 2.70 ± 0.23 μg/ml โดยที่สารสกัดไม่มีความเป็นพิษต่อเซลล์เมื่อทดสอบกับ Vero cells

ผลในการต้านเชื้อจุลชีพ และฤทธิ์ต้านเชื้อมาลาเรียสารสกัดด้วยเมทานอลจากเมล็ดลิ้นจี่ อาจเนื่องมาจากชนิดและปริมาณของ Phenolic compounds ที่เป็นองค์ประกอบอยู่ ดังนั้น การใช้การทดสอบฤทธิ์ดังกล่าวด้วยวิธีอื่นเพื่อยืนยัน และการจำแนกสารที่ยังไม่ทราบชนิดเป็นสิ่งที่ควรจะศึกษาต่อไป

คำสำคัญ: ฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านเชื้อมาลาเรีย ความเป็นพิษต่อเซลล์ เมล็ดลิ้นจี่

Introduction

Human malaria is one of the most important health problems in tropical and subtropical regions. The widespread resistance of *Plasmodium falciparum* against classical antimalarial drugs through the tropics (1) has led to a search of new drugs with new modes of action. The search for new remedies includes investigation of from plant species used will be trend to interesting.

Special attention is focused on extraction and biological tests of inexpensive residues from agricultural and food industries. This study speculates on increasing the values of the waste by-products of fruit industries. Because of Litchi fruit is one of the popular fruits, which is manufactured to canning fruits. Thus, Litchi seeds meet our criteria.

Litchi (*Litchi chinensis* Sonn.) originated from South China and is extensively grown in subtropical South East Asia. The fruits are borne in clusters and are round to ovoid, up to 4 cm in diameter. The color of the peel ranges from yellow to red, and is thin and, leathery and needs to be removed before eating. The pulp is called aril which is translucent, juicy and sweet with acid taste. The single seed is shiny, dark brown in the middle of the fruit and occupies up to half of the fruits weight approximately 50 g/fruit.

The methanolic extract of Litchi seeds was evaluated for antibacterial and antimalarial activities.

The study was conducted using cytotoxicity tests with Vero cells (African green monkey kidney cell line).

Materials and methods

Fruits

Fresh Litchi fruits were purchased from a local market in Bangkok, Thailand.

Extraction protocols

The seeds were carefully separated from the fruits. Air-dried samples were homogenized by blending to a fine homogeneous powder prior to extraction.

Air-dried material (5 g) was extracted with hexane in a Soxhlet apparatus (3 h) to remove lipid. The material was dried under a stream of nitrogen and extracted further with methanol (3 h) as modified from Sudjaroen *et al.*, 2005 and Owen *et al.*, 2000 (2, 3). Organic solvent was removed by rotary evaporation at 35- 40˚C in vacuo.

Biological assays

Antibacterial tests (4)

Inoculum development

The inoculum was prepared in Trypticase Soy Broth (TSB) and the number of cfu/ml in the inoculum was determined and standardized. For this, McFarland standards provided laboratory guidance for the number of bacteria for susceptibility testing. A 0.5 McFarland standard is comparable to a bacterial suspension of 10^8cfu/ml.

Agar diffusion method

The suspension was streaked entirely on Mueller-Hinton Agar (MHA) surface. Holes were made on MHA by sterile Pastuer's pipette. 25 µl of each extract (2.5, 5.0, 7.5 and 10 mg/ml were diluted by 0.025% Dimethyl sulfoxide (DMSO) in sterile water) was added into the holes of MHA. After incubation for 18-24 hours at 35˚C, zones of inhibition were measured in millimeter (mm) and calculated in triplicate from independent experiments.

Appropriate antimicrobial drugs were used as positive control and 0.025% DMSO as negative control.

Broth macrodilution method

Extracts that showed inhibition zone against pathogens in agar diffusion method were subjected to assay with macrodilution method for MIC determination. The extract (10 mg/ml) was diluted in two-fold serial dilution by TSB containing 1% 2,3,5-Triphenyl tetrazolium chloride (TTC) as growth indicator. A 0.5 McFarland standard is comparable to a bacterial suspension of 10^8 cfu/ml for all tests and negative control. The extract control was prepared similarly, without pathogen suspension. The TSB and suspension were used as positive (no pathogen growth) and negative control (pathogen growth), respectively. After incubation period, the positive control had yellow color (no bacterial growth) and negative control had red color (bacteria still growth). Comparing with controls, titer of test samples, which can inhibit pathogen, also had yellow color.

Antimalarial activity test

Plasmodium falciparum (K1, multidrug resistant strain), were maintained in continuous cul-

ture in human erythrocytes incubated at 37 °C in RPMI 1640 medium with human serum under an atmosphere with 7% CO_2 and low oxygen (1 or 5%). The parasites continued to reproduce in their normal asexual cycle of approximately 48 hours (5).

Antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), using the microculture radioisotope technique based on the method described by Desjardins *et al.* (6). The extract concentration at 1 and 10 µg/ml were tested in triplicate. The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. The Dihydroartemisinin (DHA) was standard compound, and 0.1% DMSO was used as negative control.

***In vitro* cytotoxicity assay**

African green monkey kidney fibroblast (Vero) cells were performed employing the calorimetric method as described by Skehan and co-workers. The sulforhodamine B (SRB) assay was performed to assess growth inhibition using a colorimetric assay, which estimates cell number indirectly by staining total cellular protein with the dye SRB (7). Briefly, 100 µl/each well of cell suspensions (0.5-2.0 x 10^5 cells/ml) were seeded in 96-well microtiter plates and incubated at 37 °C to allow for cell attachment. After 24 h, the cells were treated with the extract by adding 100 µl/well of each concentration in triplicate to obtain a final concentration of 50.0, 25.0, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/well for the extracts (two-fold serial dilution). The Ellipticine 0.603 µg/ml and 0.5% DMSO were used as positive and negative control, respectively.

The plates were incubated for 1 h (d 0) and 72 h (d 3) at 37 °C. At the end of each exposure time, the medium was removed. The cells were fixed with 20% (w/v) trichloroacetic acid (TCA) at 4 °C for 1 h, stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid for 30 min, and washed four times with 1% acetic acid. The protein-bound dye was solubilized with 10 mM/l Tris base, pH 10. The absorbance (OD) of each well was read on an ELISA plate reader (Amersham, Buckinghamshire, UK) at 492 nm. Percentage of cell survival was calculated using the formula: % cell survival = [(OD_{d3} of test -OD_{d0})/ (OD_{d3} negative control - OD_{d0})] x 100.

Results and discussion

Litchi seed extract possessed antibacterial activities, which inhibited *S. aureus, B. cereus, P. aeruginosa* and *E. faecalis* growth (inhibition zone = 10, 10, 12 and 11 mm, respectively). MIC values of Litchi seed extract were 3.47, 6.93, 3.47 mg/ml for *S. aureus, B. cereus* and *E. faecalis* (*P. aeruginosa* was not inhibited growth by tested sample with broth macrodilution methods). The extract of Litchi seeds possessed preferable high antimalarial activities with IC_{50} = 2.70 ± 0.23 µg/ml (Table 1). Furthermore, no any cytotoxic effect of this extract to Vero cells is shown in Table 2 (final concentration = 50 µg/ml).

The antibacterial and antimalarial activities may be due to phenolic compounds, which are contained in the extracts. Litchi seeds contain a

significant amount of polyphenolic compounds. The phenolic compounds display multifunctional activities as effective antimicrobial or as antioxidant agents (8).

Table 1 The IC_{50} of the methanolic extract of Litchi seeds against *Plasmodium falciparum*, K1 Strain *(In vitro)* by microculture radioisotope technique [a, b]

Methanol extract	Final conc. (mg/mL)	Count data (Mean ± SD)	%Uptake (Mean ± SD)	IC_{50} (Mean ± SD)
Litchi seed	10	2174 ± 783.33	8.63 ± 3.48	2.70 ± 0.23
	1	20825.33 ± 2444.7	81.43 ± 6.03	(mg/mL)
0.1% DMSO	-	25524.00 ± 1414.30	100	-
DHA	10 nM	183.33 ± 67.28	0.7 ± 0.26	4.20 ± 0.26
	2 nM	23814.00 ± 3191.96	92.73 ± 7.48	(nM)

[a] Negative control = 0.1% DMSO

[b] IC_{50} of positive control: Dihydroartemisinine (DHA)

Table 2 The cytotoxic effect of Litchi extract to Vero cells in each concentration by Sulforhodamine B (SRB) assay *

Methanol extract	Final concentration (mg/mL)	Mean OD	SD	% Growth	Cytotoxicity activity	IC_{50} (ml/mL)
Litchi seed	50.00	0.920	0.019	90.13	Non-cytotoxic	-
	25.00	0.949	0.004	93.79	Non-cytotoxic	-
	12.50	0.951	0.050	94.04	Non-cytotoxic	-
	6.25	0.973	0.018	96.82	Non-cytotoxic	-
	3.13	1.004	0.018	100.70	Non-cytotoxic	-
	1.56	0.999	0.017	100.09	Non-cytotoxic	-
	0.78	0.942	0.019	92.82	Non-cytotoxic	-

*Negative control: 0.5% DMSO

IC_{50} of positive control: Ellipticine = 0.603 μg/ml

In case of antiplasmodial or antimalarial activity, the methanolic extact was high and similar to African medicinal plants: *Cassia occidentalis* leaves, *Euphorbia hirta* whole plant, *Garcinia kola* stem bark and *Phyllanthus niruri* whole plant, which had $IC_{50} < 3$ μg/ml (9).

The methanolic extract of Litchi seeds and such African medicinal plants had similar phenolic compounds, procyanidins or condensed tannins as major components. The degree of solubility of extracts affects their activities, procyanidin oligomers had dissolved in buffer or culturing media better than procyanidin polymers. However, the procyanidin polymers possess higher antimicrobial activities due to the number of hydroxyl groups.

In the present study, we concluded that extract of Litchi seed is the good source of chemopreventive agents for antibacterial and antiplasmodial activities and no evidence of cytotoxicity by *in vitro* cytotoxic screening assay with Vero cells.

This finding gives incentive to search for more sources of the "low cost" chemopreventive agents in developing countries especially tropical region, which has high range of biodiversity.

However, more biological models for confirm such activities and the characterization of unknown compounds will need to be conducted in further studies.

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