Original Article

ฤทธิ์ต้านออกซิเดชั่นและต้านการกลายพันธุ์ของสมุนไพร 3 ชนิดในวงศ์ Annonaceae ในเขตอนุรักษ์พันธุกรรมพืชพื้นที่โคกภูตากา อำเภอภูเวียง จังหวัดขอนแก่น

Antioxidative Activity and Antimutagenicity of Three Plants in Annonaceae in Plant Genetics Conservation at Khok Phutaka, Amphur Phuwiang, Khon Kaen

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Abstract

This study aimed to investigate the antioxidative activity and antimutagenicity of six extracts of three plants in Annonaceae, collected from Khok Phutaka area, Amphur Phuwiang, Khon Kaen. The results showed the strong antioxidative activities of the extracts from stem-root of *Ellipeiopsis cherrevensis* and leaf of *Polyalthia evecta* with EC_{50} at 14.3 and 14.7 µg/ml, respectively. The other extracts with relative order of activity were leaf of *E. cherrevensis* > leaf of *P. debilis* > stem-root of *P. evecta* > stem-root of *P. debilis* (EC_{50} = 17.9, 22.8, 46.3 and 177.9 µg/ml, respectively). The plant total phenolic contents are well correlated with their antioxidative activities. For pre-incubation bacterial mutation test, the extracts from leaf and stem-root of *E. cherrevensis* showed mutagenic effect on *Salmonella typhimurium* TA98 and TA100 in the absence of S-9 mix. Whereas in the presence of S-9 mix, the mutagenicity was found in the extracts from leaf of *E. cherrevensis*, leaf and stem-root of *P. debilis* and leaf of *P. evecta*. The antimutagenicity was detected in both TA98 and TA100 in all extracts in the presence of S-9 mix. In contrast, in the absence of S-9 mix only the extracts from stem-root and leaf of *P. debilis*; and leaf of *P. evecta* showed the antimutagenicity to TA98. Therefore, these three plants are interesting to be further studied on their anticancer activity.

Keywords: Annonaceae, Antioxidation, Antimutagenicity

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บทคัดย่อ

งานวิจัยนี้มีจุดมุ่งหมายเพื่อศึกษาฤทธิ์ต้านออกซิเดชั่นและต้านการกลายพันธุ์ของสารสกัด 6 ชนิดจากพืช 3 ชนิด ในวงศ์ Annonaceae ที่เก็บจากพื้นที่โคกภูตากา อำเภอภูเวียง จังหวัดขอนแก่น พบว่าสารสกัดจากส่วน ลำต้น-รากของนมแมวป่า และใบของนมน้อย มีฤทธิ์ต้านออกซิเดชั่นสูงสุดโดยให้ค่า EC_{50} เท่ากับ 14.3 และ 14.7 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารสกัดที่มีฤทธิ์รองลงมา คือ สารสกัดจาก ใบของนมแมวป่า > ใบของกล้วยเต่า > ลำต้น-รากของนมน้อย > ลำต้น-รากของกล้วยเต่า ซึ่งมีค่า EC_{50} เท่ากับ 17.9, 22.8, 46.3, และ 177.6 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ฤทธิ์ต้านออกซิเดชั่นของสารสกัดเหล่านี้มีความสัมพันธ์กับปริมาณสารฟีโนลิค การทดสอบฤทธิ์ก่อกลายพันธุ์ในแบคทีเรีย *Salmonella typhimurium* ชนิด TA98 และ TA100 พบว่าสารสกัดที่มีฤทธิ์ก่อกลายพันธุ์ในภาวะไม่มี S-9 mix คือ ใบและลำต้น-รากของนมแมวป่า สำหรับภาวะที่มี S-9 mix สารสกัดที่มีฤทธิ์ก่อกลายพันธุ์ คือ สารสกัดจากใบของนมแมวป่า, ใบและลำต้น-รากของกล้วยเต่า และใบของนมน้อย ส่วนการทดสอบฤทธิ์ต้านการกลายพันธุ์ พบว่า สารสกัดส่วนใหญ่มีฤทธิ์ในภาวะที่มี S-9 mix ทั้งต่อ TA98 และ TA100 แต่ภาวะที่ไม่มี S-9 mix เฉพาะสารสกัดจาก ลำต้น-ราก, ใบของกล้วยเต่า และใบของนมน้อย จึงมีฤทธิ์ต้านการกลายพันธุ์ต่อ TA98 ดังนั้น พืชทั้ง 3 ชนิดนี้ จึงเป็นที่น่าสนใจที่จะศึกษาฤทธิ์ต้านมะเร็งต่อไป

คำสำคัญ: วงศ์อะโนนาซี ฤทธิ์ต้านออกซิเดชั่น ฤทธิ์ต้านการกลายพันธุ์

Introduction

Thailand is a country with wide biodiversity of natural resources. Khok Phutaka area, resided in Amphur Phuwiang, Khon Kaen Province with total area of approximately 700 Rai, has varieties of plants in several families, including Annonaceae. From our survey, three plants in Annonaceae were collected, *i.e*, *Ellipeiopsis cherrevensis* (Pierre ex Finet & Gapnep) R.E. Fr (Nom-Maew-Pa), *Polyalthia debilis* (Pierre) Finet & Gepnep (Kloy-Tau), *P. evecta* (Pierre) Finet and Gepnep (Nom-Noi). These plants were traditionally used for blood and kidney tonic, abdominal pain and galactogogue, respectively (Kanokmedhakul et al., 2006; 2003; 1998.) Several chemical constituents such as polyoxygenated cyclohexenes zeylenol, ferrudiol, ellipiopsis A, B and C were reported in *E. cherrevensis* extract (Kijjoa et al., 2002) and evectic acid and furans were found in *P. evecta* (Kanokmedhakul et al., 1998; 2006). Consumption of plant-derived phenolic has been shown to be associated with reduced risk of degenerative diseases according to reactive oxygen species (ROS) such as aging, cancer, coronary heart disease and Alzheimer's disease (Kushi et al., 1999). In this study, we present the antioxidative activity, total phenolic and flavonoid contents of the crude alcohol extracts of these plants. Moreover, mutagenicity and anti-mutagenicity were studied by pre-incubation bacterial assay.

Materials and Methods

1. Plant extraction

Leaves and stem-root of three plants in Annonaceae (*Ellipeiopsis cherrevensis*, *Polyalthia debilis* and *P. evecta*) were collected from Khok Phutaka area, Amphur Phuwiang, Khon Kaen Province in November, 2002. The voucher specimens were kept at the Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University. After drying, the plants were pulverized then macerated in 50 % ethanol for seven days and filtration. The filtrate was centrifuged at 500 g for 5

min. The supernatant was collected and concentrated at 40 °C using rotary evaporator. Finally the residue was freeze dried and kept in an amber, colored air-tight container at 4 °C until further used.

2. Chemical and test bacteria

Reference mutagens, 2-aminoanthracene (2-AA) and 2-aminofluorene (AF2) were gifted from Professor T. Matsushima (Japan Bioassay Lab, Japan). Nicotinamide adenine dinucleotide reduced form (NADH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glucose-6-phosphate (G-6-P), tannic acid, α-tocopherol, D-biotin are products from Sigma Chemical Co. (USA). 2,2´-diphenyl-picrylhydrazine (DPPH), quercetin dihydrate, L-histidine are products of Fluka (USA). All other chemicals and reagents used were of analytical grade. Bacto agar is from Himedia (India) and nutrient broth is from Unipath (England). *Salmonella typhimurium* TA98 and TA100 were provided by Professor Usanee Vinitketkumnuen (Chiangmai University, Thailand).

3. Determinations of total phenolic and flavonoid contents

The total phenolic content (TPC) was determined by Folin-Ciocalteu method (Makkar et al., 1993). The extract was dissolved in 50% ethanol (5 mg/mL concentration), the solution was sonicated for 30 min and filtered. Various concentrations of the extract solution (0.5 mL) were separately mixed with 0.5 ml of Folin-Ciocalteu reagent and 0.25 mL of 20% sodium carbonate. After standing at room temperature (RT) for 40 min, the optical density of the blue-colored samples was measured at 725 nm. The TPC was expressed as mg tannic acid equivalent (TAE) /g dried extract.

Flavonoid contents were measured using colorimetric method (Woisky and Salatino, 1998). The plant extract was dissolved in 99% ethanol at 1 mg/mL concentration. Then 0.5 ml of the extract solution was mixed with 0.5 ml of 2% aluminium chloride solution. After standing at RT for 1 h, the absorbance at 420 nm was determined. The flavonoid contents were expressed as mg quercetin equivalent (QE)/g dried extract.

4. Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity was used for the determination of the plant antioxidative activity (Aruoma et al., 1997). The extract was dissolved in methanol to make various sample concentrations (5-200 µg/mL). Each 2,800 µl of the extract solution was mixed with 200 µL of 1mM DPPH dissolved in methanol. The mixture was vigorously shaken and kept at RT for 15 min. The absorbance (A) was measured at 515 nm. The absorbance of the control was obtained by replacing the sample with ethanol. Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) were used as standard references. The scavenging activity (SA) was calculated using the formula, SA (%) = 100 [A control-A sample] / A control. Then the data were expressed as 50% effective concentration (EC_{50}) of SA or inhibition of oxidation in term of µg/mL.

5. S-9 mixture preparation

Chemical can cause mutagenicity either direct or indirect activation by microsomal enzymes. In general, the rat liver S-9 fraction was used for the metabolic activation. The liver S-9 fraction was prepared from Sprague-Dawley rats as described by Matsushima et al (1976). The S-9 mixture (S-9 mix) contained 10% S-9, 4 mM NADPH, 4 mM NADH, 5 mM G-6-P, 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer, pH 7.4.

6. Determinations of mutagenic and anti-mutagenic activities

For mutagenesis assay, the modified pre-incubation bacterial mutation assay (Ames, 1972; Araki et al., 1984; Sripanidkulchai et al., 2002) was carried out in both the presence and absence of S-9 mixture in order to detect indirect and direct mutagenicity of the plant extracts. The two standard test strain, *Salmonella typhimurium* TA98 and TA100, were used. The extract was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mg/mL. The mixture of plant extract (0.01-0.1 ml) with 0.5 ml of S-9 mix or 0.1 M phosphate buffer (pH 7.4) and 0.1 mL of the test strain of bacteria was incubated at 30°C for 30 min. Then this mixture was rapidly mixed with 2 ml of molten top agar containing 0.1 µmol of histidine and biotin, and poured rapidly into a minimal glucose agar plate and incubated at 37°C for 48 hr. The background or negative control was included in each experiment by using DMSO alone instead of the plant extract. All tests were duplicated. The revertant colonies were counted, and the toxic effects were determined by viewing the background lawn under a stereo microscope. The results with revertant colonies at 2x background and dose-response manner of both TA98 and TA100 were reported to be positive mutagenicity.

For antimutagenicity test, the plant extract was pre-incubated as describe above, but with the addition of the positive mutagens, either 2-AA in the presence or AF2 in the absence of S-9 mix. 0.5 µg 2-AA were used for both TA98 and TA100, AF2 was used at concentration of 0.1 and 0.01 µg for TA98 and TA100, respectively. The data were expressed as 50% inhibitory concentration (IC_{50}) obtained from the plotting of % inhibition [100 (a—b)/a, where a= number of revertant colonies of mutagen, b= number of revertant colonies of mutagen plus the plant extract] and the extract concentration.

Results

1. Antioxidative activity, total phenolic and flavonoid contents

The investigation of 6 extracts from 3 plants in Annonaceae family revealed the strong antioxidative activities in correlation with their total phenolic contents (TPC). The extracts from stem-root of *E. cherrevensis* and leaf of *P. evecta* showed highest antioxidative activities with EC_{50} of 14.3 and 14.7 µg/ml, respectively. Whereas the EC_{50} of vitamin C and vitamin E were 3.61 and 6.12 µg/ml, respectively. The other extracts with relative order of activity were leaf of *E. cherrevensis* > leaf of *P. debilis* > stem-root of *P. evecta* > stem-root of *P. debilis* with EC_{50} at 17.9, 22.8, 46.3 and 177.9 µg/ml, respectively. In general, their TPC were associated with the antioxidative activities. High TPC was found in extracts from leaf of *P. evecta*; leaf of *E. cherrevensis* and *P. debilis*; and stem-root of *E. cherrevensis* (198.8±10.4; 134.9±4.2; 134.9±1.5; 129.3±5.5 TAE mg/g, respectively). In contrast, the plant flavonoid contents were not related to their antioxidative activities. (Table 1)

Table 1 Antioxidative activity, total phenolic, and flavonoid contents of plant extracts

Name[1]	plant used[2]	% yield	total phenolic[3] content (TAE, mg/g)	Flavonoid[4] content (QE, mg/g)	EC_{50} (r^2)[5] (µg/mL)
1. *Ellipeiopsis cherrevensis*	L	10.2	134.9±4.2	30.9±3.9	17.9 (0.991)
(Pierre ex Finet & Gagnep.) R.E.Fr.	S-R	9.3	129.3±5.5	6.9±1.5	14.3 (0.991)
(นมแมวป่า)					
2. *Polyalthia debilis* (Pierre)	L	8.4	134.9±1.5	27.8±4.0	22.8 (0.940)
Finet and Gapnep	S-R	5.0	35.7±1.5	5.1±2.0	177.9 (0.975)
(กล้วยเต่า)					
3. *Polyalthia vecta* (Pierre)	L	10.4	198.8±10.4	38.7±5.3	14.7 (0.995)
Finet and Gapnep	S-R	5.6	88.6±8.9	1.4±0.5	46.3 (0.990)
(นมน้อย)					

[1] names as described by Samitinanta (2001).

[2] L = Leaf, S-R = stem and root.

[3] expressed as mean ± SD of duplicate experiments (each test came from seven different concentrations of the plant extract).

[4] expressed as mean ± SD of duplicate experiments (each test came from 3 different concentrations of the plant extract).

[5] expressed as 50% effective dose of inhibition (EC_{50}) and correlation (r^2) of DPPH scavenging activity.

2. Mutagenicity test

With the criteria that the extract possesses positive mutagenicity must show number of revertant colonies 2 times of the background and dose-dependent manner in both TA98 and TA100, the results demonstrated that the extracts from leaf and stem-root of *E. cherrevensis,* leaf of *P. evecta* had mutagenicity in the absence of S-9 mix. In the presence of S-9 mix, the extract from leaf of *E. cherrevensis*, leaf and stem-root of *P. debilis* and leaf of *P. evecta* had mutagenicity. However, extract from stem-root of *P. debilis* showed toxicity to the test bacterial. The extracts that possess weak mutagenicity were the extracts from the leaf and stem-root of *P. debilis*. (Table 2)

Table 2 Number of revertant colonies in the mutagenicity test of plant extracts from Annanaceae.

Treatment	Concentration (mg/ plate)	TA98				TA100			
		–S-9 mix		+S-9 mix		–S-9 mix		+S-9 mix	
		L	S-R	L	S-R	L	S-R	L	S-R
1. *Ellipeiopsis cherrevensis*	0	0	0	0	0	0	0	0	0
	1	15	10	12	0	11	2	29	4
	2.5	19	18	34	0	69	76	51	16
	5	28	44	115	1	81	133	87	0
	10	73	49	191	2	149	197	139	0
2. *Polyalthia debilis*	0	0	0	0	0	0	0	0	0
	1	0	0	25	125	12	22	18	198
	2.5	0	0	42	193	60	34	23	188
	5	10	8	53	160	118	48	20	113
	10	41	33	70	49, t	180	106	25	13, t
3. *Polyalthia evecta*	0	0	0	0	0	0	0	0	0
	1	8	7	82	17	36	0	16	41
	2.5	25	7	126	0	38	13	61	24
	5	51	7	215	0	37	22	82	0
	10	62	0	185	0	83	22	143	24
Background (n=4)		24±7		30±4		153±38		150±30	
AF2 (n=5)		485±93		-		500±285		-	
2-AA (n=5)		-		313±50		-		241±105	

data expressed as an average of two separated experiments of each plant extract, for the background and the reference mutagens, n=4 and n=5, respectively.

t = toxicity was observed according to the clear background lawn under the microscopic observation.

3. Antimutagenicity test

As shown in Fig 1 and Table 3, in the condition without S-9 mix, the extracts of stem-root and leaf of *P. debilis* and leaf of *P. evecta* showed strong antimutagenicity in TA98 with IC_{50} at 1, 7.3 and 3.2 mg/plate, respectively. In contrasts, all extracts did not show antimutagenicity in TA100 without S-9 mix. In the presence of S-9 mix, all extracts demonstrated strong antimutagenicity to TA98 and TA100, with IC_{50} 1.8-8.4 and 1.6-2.4, respectively.

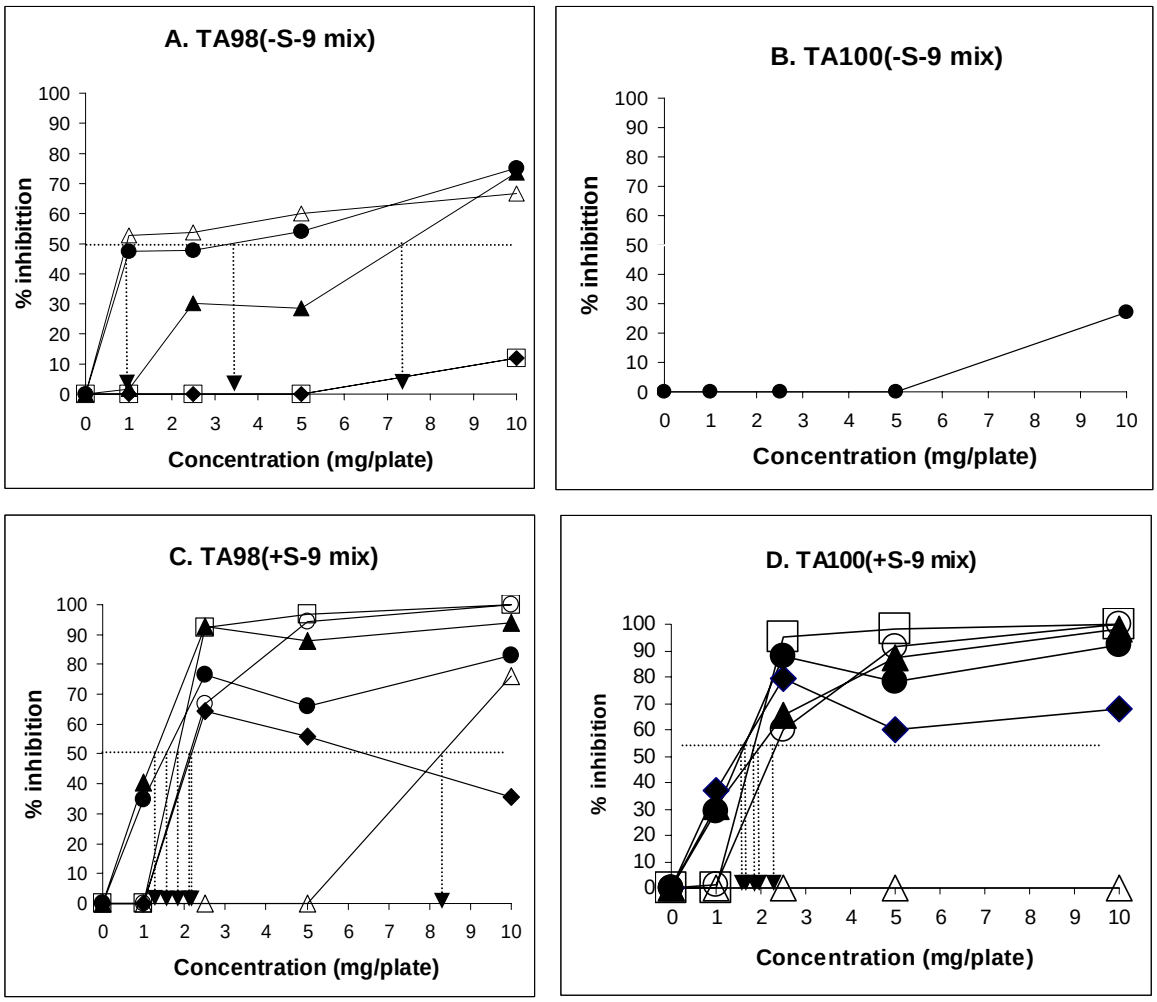

Figure 1 Antimutagenicity of plant extracts in the absence (A,B) and presence (C,D) of S-9 mix

leaf of *E. cherrevensis*
leaf of *P. debilis*
leaf of *P. evecta*
stem- root of *E. cherrevensis*
stem-root of *P. debilis*
stem-root of *P. evecta*)

Table 3 50% inhibition of mutagenesis (IC_{50}) of plant extract on *Salmonella typhimurium*.

Name	plant used[1]	IC_{50} (mg/plate)			
		-S-9 mix		+S-9 mix	
		TA 98	TA 100	TA 98	TA 100
1.*Ellipeiopsis cherrevensis*	L	>10	>10	2.2	1.6
(นมแมวป่า)	S-R	>10	>10	1.8	1.9
2.*Polyalthia debilis*	L	7.3	>10	1.2	2
(กล้วยเต่า)	S-R	1	>10	8.4, t	>10, t
3.*Polyalthia evecta*	L	3.2	>10	1.8	1.7
(นมน้อย)	S-R	>10	>10	2.1	2.4

[1]L = leaf, S-R = stem and root
t = toxicity

Discussion

The study demonstrated the strong antioxidative activity of three plants for Annonaceae family. Among 6 studied extracts, stem-root of *E. cherrevensis*, and leaf of *P. evecta*, had lowest EC_{50}, whereas stem-root of *P. debilis* had highest EC_{50}. Their total phenolic contents, but not flavonoid contents, were associated with their antioxidative activity, suggesting the important role of their phenolic constituents. Previous studies on *P. cerasoides* showed the significance ROS scavenging activity and contained high level of total phenolic contents (Ravikuman et al., 2008), indicating the rich sources of antioxidant of the plant in Annonaceae family. Our results on bacterial mutagenicity test suggest the possible action of the plant on DNA, which should be carefully considered for the safety use of these plants. However, these plant extracts also possess the antimutagenicity on *S. typhimurium* TA98 and TA100 in the condition of metabolic activities by S-9 mix of 2-AA mutagen. These data support the finding on moderate toxic effect of *P. cerasoides* (Ravikuman et al., 2008) and high toxicity of acetogenins of Annonaceae (Derbré et al., 2008). Therefore, the active constituents of plant in Annonaceae are needed to be further studied for future consideration as important leads for new anticancer drugs.

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