Inhibition of LPS-Induced Nitric Oxide Production in RAW 264.7 Cell Lines, DPPH Radical Scavenging and Total Phenolic Content of Banana (*Musa sapientum*) Blossom Extracts

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Background: *Musa sapientum L. (Male bud, banana blossom) has been used in Thai health food and Thai traditional medicine preparation as part of postpartum breastfeeding care.*
Objective: *The extracts were tested for anti-inflammatory activity by inhibition of NO production by RAW 264.7 cell lines, anti-oxidant activity by DPPH radical scavenging assay and total phenolic content by Folin-Ciocalteu's method.*
Material and Method: *Both dry and fresh banana blossom were extracted by decoction, maceration with 50%, 95% EtOH and residue from maceration were extracted by decoction. The fresh young male bud was also soaked in water and dried by freeze drying. The extracts were tested for anti-inflammatory, anti-oxidant activity and the total phenolic content determined.*
Results: *The results of anti-inflammatory assay by inhibitory activity against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cell lines showed that dried male flower by decoction exhibited the most potent effect with* IC_{50} *value 5.9791±0.1908 µg/ml. The DPPH radical scavenging assay showed that fresh male flower by decoction had the most potent anti-oxidant activity with* EC_{50} *value 5.775±0.118 µg/ml. The total phenolic content of fresh male flower by decoction had the highest total phenolic content with value 269.313±0.719 mg GAE/g.*
Conclusion: *Therefore, this result is the basis for using decoction method for preparing food from dried male flower for postpartum care.*

Keywords: *Musa sapientum, Banana blossom, Anti-inflammatory, Anti-oxidant, Total phenolic content*

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Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are significant factors relating to several pathological processes including aging, atherosclerosis and inflammatory diseases[1]. Imbalance between the generation of ROS, free radicals and anti-oxidants abilities to scavenge them or to repair cell damage reflecting body to a condition call oxidative stress. Nitric oxide has several biological roles including modulation of vascular tone, memory formation and inflammation[2,3].

Blossom of *Musa sapientum* L. called in Thai "Huaplee" has long been used in Thai functional food and Thai traditional medicine. Banana blossom are showed maceration of dried flower by 50%, 95% ethanol and fresh flower by 50% ethanol gave good results for anti-oxidant activity by 2, 2-diphenyl-2-picrylhydrazy (DPPH) radical scavenging assay, total phenolic content by Folin-Ciocalteu's method and anti-inflammatory assay by determination of inhibitory activity against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cell lines[2,3]. *Musa x paradisiaca* (*M. x paradisiaca*) of dried flower aqueous extract has been produced more milk for treated rats[4]. *Musa sapientum* L. have been showed pulp, peel, bract, flower to study of anti-oxidant and anti-

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inflammatory on decoction, maceration by 50%, 95% ethanol and water[5,6]. The present study showed *Musa sapientum* L. (banana blossom) were tested bract, flower and young male bud on dried and fresh of anti-oxidant and anti-inflammatory. Banana blossom were extracted by decoction, maceration of 50%, 95% ethanol, and residue from maceration was re-extracted by decoction. The fresh young male bud was blend in water only.

The results from this research will support Thai functional food industry and Thai traditional medicine and will also improve postpartum breastfeeding care.

Material and Method

Plant of material

Plant materials were collected from Pathumthani province in Thailand in October, 2013. The blossom was divided into three parts: bract, male flower and young male bud. Bract is a dark violet and red color part outside banana blossom. Male flower are anthers and ovary inside the bract, and the young male bud is inside the dark violet and red color of banana blossom.

Collection and preparation of plant materials

Banana blossom of *Musa sapientum* L. (Male bud, banana flower) were divided to three parts, male flower, bract and young male bud. Each part of banana blossom was further separated for preparing prior to extraction as fresh type and dried type. The dried type obtained from treating of fresh type in hot air oven at 50°C and the fresh was used as fresh sample. Both dried and fresh type of male flower and bract were then extracted by decoction, maceration with 50% ethanol and 95% ethanol, and residue from maceration give the extracted of re-extraction. The fresh young male bud was blend in water only. The extracts were further evaporate under reduced pressure. Ethanolic extracts were evaporate using rotary evaporator and aqueous extracts were dried using freeze dryer.

DPPH radical scavenging assay

Preparation of tested solution

DPPH solution was prepared at concentration of $6x10^{-5}$M, the aqueous extracts were deionized water and the ethanolic extracts were dissolved in absolute ethanol to produce stock sample solution at concentration of 1 mg/mL. The stock solution were then diluted with absoluted ethanol to produce working sample solution at concentrations of 1, 10, 50 and 100 µg/ml. Butylated hydroxytoluene (BHT) was used as positive control[3,6].

DPPH radical scavenging test

The anti-oxidant activity of plant extracts were evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The samples of crude extracts were prepared by dissolving in absolute ethanol to 1, 10, 50 and 100 µg/ml final concentrations for testing. 100 µl sample solutions were added to 96-well micro plates. Then, 100 µl of DPPH solution was added into each well. They were incubated for 30 minutes in the dark at room temperature. The absorbance was measured at 520 nm by spectrophotometer. The positive standard was tested in the same manner by BHT. All the results were reported as mean $\pm$ SEM of three replicates[3,6].

Total phenolic content assay

Total Phenolic Content was determined using Folin-Ciocalteu's method. 20 µl prepared extract solution was added to 96-well micro plates, and 100 µl Folin-Ciocalteu's reagent was added and mixed. After standing 5 min, 80 µl of sodium carbonate solution (7.5% w/v) was added. The samples were mixed and incubated at room temperature for 30 minutes. Then, the absorbance was measured at 765 nm by spectrophotometer. Standard solutions using gallic acid (12.5, 25, 50, 100, 200 and 400 µg/ml) were prepared and a calibration curve was generated. Results of samples were expressed on a weight basis as mg gallic acid equivalents/gram of sample (mg GAE/g)[6].

NO inhibitory effect test

Cell lines

RAW 264.7 murine macrophage leukemia cells line were culture in RPMI 1640 medium (Gibco, USA) and supplemented with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained in 5% CO_2 atmosphere with 95% humidity at 37°C and the cell culture medium was changed twice per week[7,8].

Preparation of sample solution

Aqueous extracts were dissolved in sterile distilled water and filtered by 0.22 µ to produce 10 mg/ml of concentration. Ethanolic extracts were dissolved in a quantity of sterile dimethylsulfoxide (DMSO) at 50 mg/ml of concentration. All samples were diluted in medium to produce working concentration of 1, 10, 30, 50 and 100 µg/ml[9,10] for calculation of percent inhibition

and 50 percent inhibitory concentation (IC_{50})[7,8].

Procedures

Cultured RAW 264.7 murine macrophage leukemia cells line were seeded for $1x10^5$ cells/well in 96 well plate, then accorded growth profiles in the optimal plating densities 75 cm^3 of flask in this assay of cells grown as a monolayer. They were washed with 3 ml phosphate-buffered saline (PBS) free of magnesium and calcium two times. The PBS was decanted and cells were detached by 3 ml of 0.25% trypsin-EDTA to produce a single cell suspension. Next, medium was added to the 3 ml in flask to stop trypsin-EDTA working. Then, the cells were centrifuged at 1,500 rpm for 5 min. The supernatants were removed, 10 ml of fresh medium were added and mixed with cells to make a single cell suspension. The viable cells counted in haemocytometer by Trypan blue stain 0.4% exclusion. Cultured RAW 264.7 cells were seeded in 96-well plate for $1x10^5$ cells/well and incubated at 37°C, 5% CO_2 atmosphere with 95% humidity for 24 hours. After that, the culture medium was replaced with fresh medium containing 10 ng/ml of LPS (Lipopolysaccharide) and incubated at 37°C in 5% CO_2 atmosphere with 95% humidity for 24 hours. Finally, NO production were determined using Griess reagent for measuring nitrite accumulation in the culture supernatant in which removing 100 µl from the well and 100 µl of Griess reagent were added. The 96 well plate was then incubated. The quantitaive amount of reaction were measured by spectrophotometer at wavelength of 570 nm. Finally, the % inhibition and IC_{50} were calculated by Prism program[6,7].

Cytotoxicity was also determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric method. After 24 hours incubation of cell lines with test sample, 10 µl of MTT solution was added into each well and incubated in 5% CO_2 atmosphere at 37°C with 95% humidity for 2 hours and then medium in each well was removed. Isopropranol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. Formazan formation was measured using spectrophotometer at wavelength of 570 nm. Prednisolone was used as positive control. Percent inhibition and IC_{50} values were calculated using Prism program[6,7].

Statistical analysis

All data were statistically analyzed as mean of three replications ± standard error of mean using SPSS software.

Results

The percentage of yield are shown in Table 1. The extract obtained from extra fresh male flower by decoction and residue dried male flower by 50% ethanol with 3.91% and 38.20%.

The results of antioxidant activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay are shown in Table 1. Fresh male flower by decoction exhibited the most potent antioxidant activity with EC_{50} value 5.775±0.118 µg/ml, followed by fresh young male bud by decoction and dried male flower by decoction with EC_{50} values 6.28±0.088 and 10.601±0.979 µg/ml respectively. The fresh male flower by decoction showed the best value for antioxidant activity. BHT is 14.12±0.81 µg/ml.

The total phenolic contents are shown in Table1. Fresh male flower by decoction exhibited the most potent total phenolic content with values 269.313±0.719 mgGAE/g., followed by fresh young male bud by decoction and dried male flower by decoction with values 201.160±1.359 and 146.847±0.667 mgGAE/g, respectively. The fresh male flower by decoction showed the best value for total phenolic content.

The NO inhibition are shown in Table1. The results indicate that dried male flower by decoction exhibited the most potent NO inhibition with IC_{50} value 5.979±0.191 µg/ml, followed by dried young male bud by decoction and residue dried male flower by 50% ethanol with IC_{50} values 20.9133±0.2345 and 31.1656±1.3674 µg/ml, respectively. Dried male flower by decoction showed inhibitory effect on nitric oxide (NO) production lower than positive control, Prednisolon (IC_{50} = 0.164±0.011 µg/ml).

Discussion

The percentage yields of plant extracts, anti-oxidant activity by DPPH radical scavenging assay, Total phenolic content and anti-inflammatory activity are shown in Table 1. The highest percentage of yields of extract obtained from extra fresh male flower by decoction with 3.91%. The highest radical scavenging assay by fresh male flower decoction with EC_{50} value 5.775±0.118 µg/ml, followed by fresh young male bud decoction and dried male flower decoction with EC_{50} values 6.28±0.088 and 10.601±0.979 µg/ml, respectively. Total phenolic content by Folin-Ciocalteu's are shown fresh male flower decoction exhibited the highest total phenolic content with value 269.313±0.719 mgGAE/g., followed by fresh young male bud decoction and dried male flower decoction with values 201.160±1.359 and 146.847±0.667 mgGAE/g, respectively. Gallic acid

Table 1. Table showed the %yield, Total phenolic content, antioxidant activities and anti-inflammatory activities of *Musa sapientum* Extracts

Part for used	Part used in extraction	% yield		Total Phenolic content (mg GAE/g)		Anti-oxidant activities (EC_{50} µg/ml)		NO-inhibitory activities (IC_{50} µg/ml)	
		Fresh	Dried	Fresh	Dried	Fresh	Dried	Fresh	Dried
Male flower	Decoction	3.91	23.30	269.31±0.72	146.85±0.66	5.78±0.12	10.60±0.98	>100	5.98±0.19
	50%EtOH	1.88	23.28	8.54±0.57	11.67±0.37	>100	>100	>100	>100
	Residue 50% EtOH	0.48	38.20	15.15±0.19	91.13±1.45	>100	18.04±0.14	>100	31.17±1.37
	95% EtOH	3.51	8.97	55.33±0.76	3.60±1.16	33.76±0.50	>100	>100	>100
	Residue 95% EtOH	0.82	23.43	13.60±0.26	123.74±0.76	>100	12.85±0.36	>100	43.46±0.72
Bract	Decoction	2.35	34.83	42.96±0.74	25.50±0.42	41.35±0.93	23.32±0.58	>100	>100
	50% EtOH	1.50	23.71	3.27±0.92	2.73±1.28	>100	>100	>100	>100
	Residue 50% EtOH	0.27	6.22	8.90±0.34	23.69±0.56	>100	56.30±0.36	>100	>100
	95% EtOH	1.55	12.13	9.67±0.63	23.33±1.20	>100	>100	>100	>100
	Residue 95% EtOH	0.69	16.78	45.96±0.96	46.13±0.65	>100	44.85±0.18	>100	>100
Young male bud	Decoction	3.02	18.07	201.16±1.36	36.25±0.56	6.28±0.09	59.16±0.04	>100	20.91±0.23
	50%EtOH	0.75	16.09	2.31±1.35	27.90±1.40	>100	>100	>100	>100
	Residue 50% EtOH	0.34	12.85	13.27±0.36	51.27±0.43	>100	54.55±1.18	>100	>100
	95%EtOH	1.62	4.92	44.75±0.60	58.79±0.75	36.89±1.37	39.70±1.26	>100	>100
	Residue 95% EtOH	0.80	25.97	18.96±0.77	75.58±0.67	26.76±1.25	>100	>100	>100
	soaked in water	1.35	-	19.72±0.78	-	>100	-	>100	-

equivalents are used as the standard of analysis and UV absorbance at 765 nm (n = 3), $R^2 = 0.999$. The anti-inflammatory assay of determination of inhibitory activity against lipopolysaccharide (LPS) of induced nitric oxide (NO) production in RAW 264.7 cell lines are shown the dried male flower decoction with the strongest anti-inflammatory effect with IC_{50} value as 5.979±0.191 µg/ml, followed by dried young male bud decoction and dried male flower residue in 50% ethanol with IC_{50} values as 20.9133±0.2345 and 31.1656±1.3674 µg/ml, respectively. Therefore, this result shows the decoction method most suitable for preparing functional food from dried male flower for postpartum care.

Musa sapientum L. in Indian folk medicine has been used to treat diabetes mellitus for the unripe pulp has tannin[10], used to treat of the diarrhea[11], the leucocyanidins are the part of flavonoid group[9], used to treat the peptic ulcers[12,13], unripe pulp and unripe peel has been used to the wound healing[1,6]. Ripe pulp has been used for the laxative, root used to treat for the muscle sprains, burns and scald. Banana sap has been used for the haemostasis, to treat the thrush from moniliasis to treat insect of the stings and the female flowers have been used as the post partum care. Fruit peel has been used for an antibacterial agent and the astringent. Leaves have been used as protect from the allergies and to prevent of burns[14-17].

The fresh flower of decoction extract showed the EC_{50} as 5.16±0.34 µg/ml, followed by the fresh flower of water extract (24-hr soaking) showed the EC_{50} as 5.82±0.28 µg/ml[6]. The fresh bract of decoction extract showed EC_{50} as 18.92±1.51 µg/ml, followed by the fresh bract of water extract had EC_{50} of 33.06±3.18 µg/ml[5].

The fresh flower of water extract had total phenolic content of 218.75 mg GAE/g, followed by the fresh flower of decoction extract with 185.86 mg GAE/g[12]. The fresh bract of decoction extract had total phenolic content as 73.00±0.45 mg GAE/g,[5].

The dried flower of water extract as exhibited NO-inhibitory activity of IC_{50} as 9.75±0.93 µg/ml. The dried bract of the 95%EtOH extract had exhibited NO-inhibitory activity as IC_{50} as 93.93±3.88 µg/ml. The cytotoxic effects of extracts were determined have been used as using the MTT assay[3].

Musa x paradisiaca (*M. x paradisiaca*) has been tested of the galactagogue activity. Dried flower aqueous extract has been produced more milk for treated rats than control and ethanol groups. The aqueous extract has been increased of milk production by 25%, the petroleum ether extract by 18%. The aqueous, the petroleum ether, the ethanol and control extracts has been showed 4.62±2.45, 4.37±1.93, 3.65±1.89 and 3.69±1.79 g/pub/day, respectively[4].

The dried flowers of 95% ethanol extract of the 'Khao nuan' variety has been showed the highest *in vitro* anticancer effect against in lung cancer cells showed IC_{50} as 4.14 µg/ml. The fresh flower of decoction extract as 'Khao nuan' showed the highest inhibition on HIV-1integrase showed IC_{50} as 8.7 µg/ml. The peel from the end of flower stalk exhibited the highest inhibition on HIV-1 protease showed IC_{50} as 26.3 µg/ml. The fresh flower extract of 95% ethanol have been showed the highest anti-allergic activity by inhibitory effects on the release of β-hexosaminidase showed IC_{50} as 15.587 µg/ml. The fresh flower extract of 95% ethanol of the 'La-ong nam' variety have been showed the highest anti-acetylcholinesterase activity (% inhibition as 5.53±1.85 µg/ml). The fresh flower of decoction have been showed the highest MIC by Disc diffusion against *S. aureus* as 7.00±0.00 1,250 µg/ml[5].

Conclusion

These results support using this Thai medicinal plant for preparing food for postpartum care especially breast feeding. Treatments requiring anti-oxidant and anti-inflammatory efficacy may also use this medicinal plant according to Thai traditional medical practice. The result shows the decoction method giving the most potent effect of anti-oxidant, Total phenolic content and anti-inflammatory assay of determination of inhibitory activities against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cell lines. As a prophylactic in common family cuisine, the male bud is convenient and appropriate.

What is already known on this topic?

Banana blossom is widely used in Thai traditional medicine for preparing food for postpartum care. Its blossom possess anti-inflammatory, anti-oxidant, anticancer, inhibition on HIV-1integrase, inhibition on HIV-1 protease, anti-allergic and antimicrobial especially strong anti-oxidant which relate to high content of total phenolic content. Its blossom have been reported on anti-inflammatory and anti-oxidant that showed 50%, 95% ethanol, decoction and water but its blossom have not been reported on for residue by 50%, 95% ethanol, soaked in water, three part of blossom: bract, flower and young male bud.

What this study adds?

The present study showed dried male flower

decoction of most commonly of result. The result showed the most potent effect of anti-oxidant and anti-inflammatory efficacy. In addition, dried young male bud decoction and dried male flower residue in 50% ethanol were also good the result of anti-inflammatory efficacy. The present study showed the decoction method that the most potent effect of anti-oxidant, Total phenolic content and anti-inflammatory. Hence, the present study support using this Thai medicinal plant for preparing food for postpartum care.

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Potential conflicts of interest

None.

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ฤทธิ์ยับยั้งการเหนี่ยวนำการสร้างไนตริกออกไซด์ด้วย LPS ในเซลล์เพาะเลี้ยง RAW 264.7 ฤทธิ์ต้านอนุมูลอิสระ DPPH และปริมาณสารฟีนอลิกรวมของสารสกัดหัวปลี

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ภูมิหลัง: *ในภูมิปัญญาทางแผนไทย หัวปลีถูกใช้ในการรักษาทางแพทย์แผนไทยและใช้ในการทำประกอบอาหาร เพื่อบำรุงน้ำนมให้แก่มารดาหลังคลอด*

วัตถุประสงค์: *เพื่อศึกษาการทดสอบสารสกัดสำหรับฤทธิ์ต้านการอักเสบโดยฤทธิ์ยับยั้งการเหนี่ยวนำการสร้างไนตริกออกไซด์ในเซลล์เพาะเลี้ยง RAW 264.7, ฤทธิ์ต้านอนุมูลอิสระ DPPH และปริมาณสารฟีนอลิกรวมโดยวิธี Folin-Ciocalteu's*

วัสดุและวิธีการ: *เตรียมหัวปลีทั้งแบบสดและแบบแห้ง จากนั้นนำมาสกัดด้วยวิธีการต้ม, สกัดด้วยวิธีหมัก 50% EtOH, นำกากของการสกัดด้วยวิธีหมัก 50% EtOH มาต้ม, สกัดด้วยวิธีหมัก 95% EtOH, นำกากของการสกัดด้วยวิธีหมัก 95% EtOH มาต้มและใช้หัวปลีอ่อนสดสกัดด้วยวิธีการแช่น้ำเพียงตัวเดียวเท่านั้น ส่วนของสารสกัดชั้นน้ำ นำมาทำให้แห้งด้วยวิธี freeze dry และส่วนของสารสกัดชั้น Ethanol นำมาระเหยแห้งด้วยเครื่อง Rotary evaporator แล้วนำมาทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH, หาค่า Total phenolic content และศึกษาฤทธิ์ต้านการอักเสบจากเซลล์ RAW 264.7*

ผลการศึกษา: *การศึกษาฤทธิ์ยับยั้ง การหลั่งไนตริกออกไซด์จากเซลล์ RAW 264.7 พบว่าสารสกัดดอกหัวปลีแห้งมีฤทธิ์ดีที่สุด คือ 5.9791±0.1908 μg/ml จากการทดลองในการศึกษาฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH และ Total phenolic content พบว่าสารสกัดดอกหัวปลีสดด้วยวิธีการต้มมีฤทธิ์ดีที่สุดคือ 5.775±0.118 mg/ml และ 269.313±0.719 mgGAE/g ตามลำดับ*

สรุป: *จากการศึกษาพบว่าสารสกัดดอกหัวปลีแห้งด้วยวิธีการต้มเหมาะแก่การเตรียมเป็นอาหารเพื่อบำรุงน้ำนมสำหรับมารดาหลังคลอด*