Immunopharmacological Activity of Polysaccharide from the Pericarb of Mangosteen Garcinia : Phagocytic Intracellular Killing Activities

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Abstract

Polysaccharides from the pericarbs of mangosteen, *Garcinia mangostana* Linn., was obtained by treating the dried ground pericarbs with hot water followed by ethanol precipitation (M fraction). The extract was fractionated by anion exchange chromatography on a DEAE-cellulose column as MDE1-5 fractions. The fractions of MDE3 and MDE4 composed of mainly D-galacturonic acid and a small amount of neutral sugar (L-arabinose as the major one and L-rhamnose and D-galactose as the minor ones) were studied for immunopharmacological activities by phagocytic test to intracellular bacteria (*Salmonella enteritidis*) and nitroblue tetrazolium (NBT) and superoxide generation tests. The results showed that the number of *S. enteritidis* in cultured monocyte with extract of pericarb of mangosteen (MDE3) was killed. Activating score (mean ± SD) of NBT test of 100 polymorphonuclear phagocytic cells were 145±78, 338±58, 222±73, 209±77, 211±63, 372±19, 369±20, 355±34 in normal saline control, phorbol myristate acetate (PMA), MDE3, MDE4, indomethacin (I), PMA + MDE3, PMA + MDE4 and PMA + I, respectively. Superoxide generation test was also done by color reduction of cytochrome c. Both MDE3 and MDE4 stimulate superoxide production. The number of *S. enteritidis* in cultured monocyte with extract of pericarb of mangosteen was killed. This paper suggests that polysaccharides in the extract can stimulate phagocytic cells and kill intracellular bacteria (*S. enteritidis*).

Garcinia mangostana Linn. (Family *Guttiferae*) is known as mangosteen in Thailand. The fruit hull (pericarb) of the plant is effective for treatment of diarrhea, dysentery, skin disease and wound healing in Thai folklore. Antimicrobial activities of chemical constituents from *G. mangostana* Linn. were reported with the *in vitro* activities against *Staphylococcus aureus*, both pencillin-sensitive and penicillin-resistant strains, *Trichophyton mentagrophytes* and *Microsporum gypseum*[1].

Polysaccharides are the major constituents in the plant cell walls. The major xanthone of mangostin is presented in the pericarb of mangosteen. Mangostin which was extracted with methanol

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exhibited biological activities including anti-inflammatory function[2]. In the present study, we aimed to study immunopharmacological activities of polysaccharide from mangosteen's pericarb, which was primarily extracted with hot-water and DEAE-cellulose fractionation, on intracellular bacterial (*Salmonella enteritidis*) killing activity of monocyte. To elucidate the success of phagocytosis and killing activity the phagocytic polymorphonuclear cells were used for oxidative burst activity test by nitroblue tetrazolium (NBT) and superoxide generation tests.

MATERIAL AND METHOD

Materials : Ripe fruit (mangosteen) was collected in Thailand. The reddish-black pericarbs were finely ground after being sun-dried.

Preparation of extracts : The powder (100 g) of mangosteen's pericarb was suspended in 1.5 l distilled water and boiled for 1 hour. The polysaccharide in hot water extract was precipitated with distilled ethyl alcohol then redissolved in a small amount of water and was kept after lyophilization as M fraction.

Fractionation of hot water extract : The M fraction (1 g) was fractionated on a DEAE-cellulose (Whatman DE-23, Cl^- form) column (2.6 x 45 cm) and eluted in stepwise gradient fashion with distilled water and 0.05 to 2.0 M NaCl at a flow rate of 3 ml per minute. Five fractions designated MDE1-5 were obtained.

Analysis of constituent sugar : Neutral sugars in each fraction were determined by the phenol-sulphuric acid method using D-glucose as standard[3]. Each of MDE was fractionated by thin layer chromatography after hydrolysis with 90 per cent formic acid. The hydrolysates were reduced with $NaBH_4$ and then chromatographed on Dowex 1x8 column (1.5 x 10 cm, acetate form) using Hitachi G-3000 gas chromatograph with flame ion detector.

Intracellular bacterial killing activity test[4] : Mononuclear cells were separated from 20 ml heparinized blood by Ficoll-Hypaque technique and were resuspended in Hank's balance solution with Ca^{+2} and 10 per cent autologous plasma. Cell suspension (10^5 cells/ml) 0.5 ml was pipetted into a culture plate (24 well microtiter) with a glass cover in each well. The mononuclear cells were incubated at 37°C in 5 per cent CO_2 for 2 hours. The monocytes were adhered to the glass covers, then the non-adherent cells were removed.

The microorganisms, *S. enteritidis*, in trypticase soy broth (0.5 ml of McFarland No. 0.5 equivalent cell suspension) was inoculated into each well and the mixture was incubated for 2 hours. After phagocytosis, the supernatant was removed. Cultured media with Pen G - streptomycin; chloramphenicol and Pen G - streptomycin; Pen G - streptomycin and MDE3 were added into separate well. Pen G - streptomycin was used as extracellular killing control and chloramphenicol as intracellular control. After incubation for 0, 4, 8, 12, 16, 20 and 24 hours, the glass cover was removed and stained with Wright's Giemsa stain. Bacteria in monocytes were examined and counted from 100 monocytes under microscope. After incubation the phagocytic monocyte cells were lysed with 0.05 per cent Triton X-100 and then were cultured for colony count determination by pour plate technique.

NBT test[5] : 0.1 ml of EDTA blood was mixed with 0.1 ml of NBT (0.2% in NSS) and then added with 20 µl of stimulating agent (phorbol myristate acetate (PMA), MDE3, MDE4, indomethacin, PMA + MDE3, PMA + MDE4, PMA + indomethacin) using NSS as control. The mixture was incubated at 37°C for 60 minutes, then the mixture was smeared on a slide and stained with Wright's Giemsa stain.

Criteria of NBT score :

0 = no color of NBT in cytoplasm
1 = small blue-black dot $\geq$ 1 in a half of cytoplasm
2 = medium blue-black dot in more than half of cytoplasm
3 = large blue-black dot in cytoplasm
4 = very large blue-black dot in cell

Superoxide generation test[6,7] : Superoxide was measured as the reduction of ferricytochrome c. Superoxide production was investigated in the presence of activators : PMA, MDE3, MDE4, indomethacin, PMA + MDE3, PMA + MDE4 and PMA + indomethacin. To assess the specificity of ferricytochrome c reduction, superoxide dismutase was added to selected ferricytochrome c-containing tubes with or without polymorphonuclear cell suspension before activators were added. Superoxide production was calculated from the reduced ferricytochrome c formed using the extinction coefficient $\varepsilon_{550} = 2.1 \times 10^{-3}\ M^{-1}cm^{-1}$

RESULTS

Hot water extract (M) from the pericarbs of mangosteen consisted of several acidic polysaccharide components which can be easily demonstrated by electrophoresis on cellulose acetate in pyridine-acetic acid medium and visualized with toluidine blue stain. When M was fractionated on DE-23 column, it gave 5 fractions, MDE1-5. The respective yield was 1.7, 3.0, 28.7, 18.0 and 7.4 per cent. Sugar content from thin layer chromatography and gas liquid chromatography techniques revealed that D-galacturonic acid was detected in all fractions except for MDE1 which consisted mostly as L-arabinose with a small amount of L-rhamnose and D-galactose. The major sugar in MDE2 was L-arabinose and the minor ones were D-glucose and D-galactose. In MDE3, 4 and 5 L-arabinose was also plenty while L-rhamnose and D-galactose were also found. D-xylose and D-glucose were very low in these three fractions.

The number of intracellular bacteria (*S. enteritidis*) was more than 15 organisms per cell from 100 infected monocyte cells in media without chloramphenicol added after 24 hour-incubation and was 3.1 and 3.2 in media while chloramphenicol or MDE3 was added, respectively (see Table 1). MDE3 had less killing activities than chloramphenicol in the same concentration (8 μg/well) as shown in Table 2. The killing property of MDE3 may be either antimicrobial activity or production of reactive oxygen intermediate (superoxide, hydroxyl radicals, hydrogenperoxide) by NADPH oxidase catalysis or both.

Table 1. An average number of intracellular bacteria (*S. enteridiidis*) per cell from 100 infected monocyte cells under microscope examination.

Culture condition	No. of intracellular organisms at indicated time (hours)						
	0	4	8	12	16	20	24
Media (without drug)	3.5	3.3	5.1	9.6	> 10	> 15	> 15
Media with chloramphenicol	4.1	4.0	3.5	3.6	3.4	3.3	3.1
Media with MDE3	4.2	3.6	3.9	3.8	3.5	3.6	3.2

Table 2. Baterial number (CFU/plate) of pour plate culture after phagocytic killing on intracellular *S. enteritidis* by MDE3.

Substance added on adherent cell on slip after 2, 24 hours incubation	Expt. No.	Intracellular *S. enteritidis* (CFU/plate) at indicated time	
		2 hours	24 hours
1. Media with Pen G and streptomycin	1	TNTC*	TNTC
	2	TNTC	TNTC
	3	TNTC	TNTC
	4	TNTC	TNTC
2. Media with Pen G, streptomycin and chloramphenicol	1	2	0
	2	9	5
	3	0	4
	4	4	2
3. Media with Pen G, streptomycin and MDE3	1	5	5
	2	56	26
	3	17	10
	4	30	22

* TNTC = Too numerous to count

(By 2 hours incubation of intracellular *S. enteritidis* on adherent cells in media with Pen G/strep.; Pen G, strep/chloram.; or Pen G, strep./MDE3 and then pour plate technique was performed, the colony count was determined after 24 hours incubation)

Table 3. Average NBT score (mean ± SD) from 19 healthy individuals after stimulation with various activators in 100 phagocytic cells

Tube No.	Activators	NBT score
1	Normal saline solution	145 ± 78
2	Phorbol 12-myristate 13-acetate (PMA)	338 ± 58
3	MDE3	222 ± 73
4	MDE4	209 ± 77
5	Indomethacin	211 ± 63
6	PMA + MDE3	372 ± 19
7	PMA + MDE4	369 ± 20
8	PMA + Indomethacin	355 ± 34

Table 4. Superoxide generation (nmol/10^6 PMN) under various stimulants.

No.	1	2	3	4	5	6	Mean	SD
PMA	28.5	13.6	13.6	16.7	27.9	28.7	21.5	6.9
MDE3	18.5	8.6	18.6	2.8	8.5	5.1	10.35	6.1
MDE4	0	3.3	0.4	16.6	5.9	14.1	6.7	6.5
Indomethacin	9.9	20	11.8	6.9	18.6	-1.7	10.9	7.3
PMA + MDE3	26.1	23.4	38.6	2.7	22.1	18.1	21.8	7.3
PMA + MDE4	24.8	6.1	29.8	4.6	13.8	37.9	19.8	12.3
PMA + Indo.	14.6	27.8	12.7	23.1	21.6	6.90	17.8	7.04

MDE3, the major component of hot water extract, can stimulate white blood cells to produce oxidative bursts as demonstrated by NBT test. The results showed that activating score (mean±SD) of NBT score of 100 polymorphonuclear phagocytic cells were 145±78, 338±58, 222±73, 209±77, 211±63, 372±19, 369±20, 355±34 when incubated with NSS, phorbol myristate acetate (PMA), MDE3, MDE4, indomethacin (I), PMA + MDE3, PMA + MDE4, and PMA + I, respectively (Table 3).

Superoxide generation was indirectly measured by color reduction of cytochrome c. It was revealed that both MDE3 and MDE4 can stimulate superoxide production in polymorphonuclear cells. MDE3 produced slightly more superoxide than MDE4. However, the stimulation effect of both MDE3 (0.066 µg/tube) and MDE4 (0.066 µg/tube) were still less than PMA in the same amount (0.066 µg/tube) as shown in Table 4.

DISCUSSION

Pathogenic microorganisms such as *Mycobacterium tuberculosis, M. leprae, S. typhi, S. enteritidis* have been found to remain viable intracellularly. In this study MDE3 (polysaccharide mainly D-galacturonic acid) can penetrate into cell and kill intracellular *S. enteritidis*. However, its efficiency is less than that of chloramphenicol while Pen G and streptomycin have no intracellular killing activity. Killing activity may be either direct effect of antimicrobial activities or respiratory burst activities, or both.

Since bacterial killing is associated with ability to develop all of the reactions associated with the respiratory burst, the oxygen-dependent mechanism of white blood cells. MDE3/MDE4 may activate the hexose monophosphate shunt and produce $NADP^+$ and superoxide (O_2^-) by using NADPH. This activity can be demonstrated by NBT dye reduction test (Table 3). The reaction may be as follows :

$$2O_2 + NADPH \text{ ------ MDE3/oxidase } \text{----->} 2O_2^- + NADP^+ + H^+$$

$$2O_2^- + 2H^+ \text{ ----------> } O_2 + H_2O_2$$

$$\text{NBT (clear yellow) ---- } O_2^-, H_2O_2 \text{ -----> deep blue formazan}$$

Stimulation of PMN, monocytes by inflammatory compounds, (e.g. immune complexes, opsonized particles, indomethacin), microorganism, tumor promotors (e.g. PMA) results in activation of an oxidative burst. The catalytic formation of O_2^- and H_2O_2 is commenced, and then HOCl is formed. HOCl is a unique function of neutrophils for host defence against infection. Our finding elucidated that polysaccharide from mangosteen pericarb stimulated phagocytic cells to produce superoxide.

In the present study, indomethacin, nonsteroid anti-inflammatory drug (NSAID), can produce superoxide corresponding to a previous finding[8]. Indomethacin employed to treat chronic inflammatory conditions such as rheumatoid arthritis and gout had a strong inhibitor of myeloperoxidase-H_2O_2-Cl^- system, especially HOCl. Hutadilok[2] found that anti-inflammatory activity of mangostin isolated by benzene extraction was not able to prevent hyarulonic acid degradation. Further studies are needed for clarification of the anti-inflammatory and extracellular bacterial killing activities of polysaccharide fractions from hot water extract, both major (MDE3, MDE4) and the minor ones (MDE1, MDE2 and MDE5).

(Received for publication on June 24, 1997)

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ฤทธิ์ทางภูมิคุ้มกันเชิงเภสัชวิทยา ของสารสกัดโพลีแซคคาไรด์จากเปลือกมังคุด : การทำลายเชื้อที่อยู่ในเซลล์

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สารโพลีแซคคาไรด์ที่ได้จากการสกัดเปลือกมังคุด *Garcinia mangostana* Linn. ด้วยน้ำร้อนแล้วตกตะกอนด้วยเอธานอล นำมาแยกส่วนโดยใช้ DEAE-cellulose anion exchange column chromatography ได้เป็น 5 ส่วน โดยส่วนที่มีปริมาณมากที่สุดคือ MDE3 และ MDE4 ซึ่งมีส่วนประกอบส่วนใหญ่เป็น D-galacturonic acid และมี neutral sugar เป็น L-arabinose มากที่สุด ส่วน L-rhamnose กับ D-galactose มีเพียงเล็กน้อย เมื่อนำสารสกัดดังกล่าวมาศึกษาฤทธิ์ทางภูมิคุ้มกันเชิงเภสัชวิทยา โดยใช้วิธีการจับกินแบคทีเรีย (*Salmonella enteritidis*) เข้าในเซลล์โมโนซัยท์ พบว่า *S. enteritidis* ในเซลล์โมโนซัยท์ที่เลี้ยงไว้ถูกทำลายได้ เมื่อมี MDE3 อยู่ด้วย จะกระตุ้นให้เกิดซุปเปอร์ออกไซด์ใน polymorphonuclear cell และเมื่อศึกษาการตรวจโดย Nitroblue tetrazolium (NBT) test นับจำนวนคะแนนการเกิดผลบวก NBT ต่อ polymorphonuclear 100 เซลล์ พบว่า เมื่อใช้ normal saline, phorbol myristate acetate (PMA), MDE3, MDE4, indomethacin (I), PMA + MDE3, PMA + MDE4, และ PMA + I มีคะแนนเป็น 145±78, 338±58, 222±73, 209±77, 211±63, 372±19, 369±20, 355±34 ตามลำดับ ผลการเกิดซุปเปอร์ออกไซด์ โดยใช้ปฏิกิริยารีดิวส์สีของ cytochrome c พบว่าทั้ง MDE3 และ MDE4 สามารถกระตุ้นให้มีการสร้างซุปเปอร์ออกไซด์ได้เช่นเดียวกัน รายงานนี้เสนอแนะว่า โพลีแซคคาไรด์ในสารสกัดดังกล่าว สามารถกระตุ้นเซลล์ phagocyte ให้ทำลายเชื้อแบคทีเรีย (*S. enteritidis*) ได้

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