Bioactivities of Ethanolic Extracts of Three Parts (Wood, Nutmeg and Mace) from *Myristica fragrans* Houtt.

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Background: *Myristica fragrans Houtt. is one of the spices that has long been used for treatment of various disorders. M. fragrans is known as Chan-thet and its three parts i.e. wood, seed (nutmeg) and aril (mace), are ingredients in various Thai traditional remedies such as anti-pyretic, anti-allergy, anti-inflammatory remedies.*
Objective: *To investigate the biological activities of ethanolic extracts obtained from wood, nutmeg and mace of M. fragrans according to the uses in Thai traditional medicine follow as: anti-inflammatory, anti-allergic and antioxidant activities.*
Material and Method: *Three parts of M. fragrans (wood, nutmeg and mace) were macerated with 95% ethanol. The extracts were examined for anti-inflammatory activity by determination of inhibitory effect on LPS induced nitric oxide production release in RAW 264.7 cell lines, anti-oxidant activity by inhibitory effect on PMA-induce superoxide radical in DMSO-differentiated from HL-60 cells, and anti-allergic activity by determining inhibitory activity of β-hexosaminidase release on RBL-2H3 cells.*
Results: *The ethanolic extract of wood presented potent anti-inflammatory activity more than nutmeg and mace (IC_{50} values = 40.26±0.58, 65.42±4.95 and 75.40±4.14 μg/ml, respectively). Nutmeg and mace showed high anti-oxidant activity while wood showed moderate activity (IC_{50} values = 21.164±1.03, 28.897±0.39 and 71.830±1.33 μg/ml, respectively). The extracts obtained from the three parts (wood, nutmeg and mace) showed strong anti-allergic activity (IC_{50} values = 13.29±0.28, 20.90±1.03 and 12.95±0.89 μg/ml respectively).*
Conclusion: *The extracts obtained from wood of M. fragrans showed high anti-inflammatory and anti-allergic activities but moderate anti-oxidant. The extracts of nutmeg and mace presented high anti-oxidant and anti-allergic activities but less anti-inflammatory activity. Therefore, extract of wood should be selected for treatment of diseases that related with inflammation while the extracts of nutmeg and/or mace should be used as an antioxidant. Finally, extracts of all 3 parts of M. fragrans could be used for allergy-related diseases because all parts showed potent activity in anti-allergy, anti-inflammatory and antioxidant roles. However, the further study should be performed in animal models for investigation of each activity of active compounds following bioassay guided isolation.*

Keywords: *Myristica fragrans, Houtt., β-hexosaminidase, Nitric oxide, Superoxide radical*

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Myristica fragrans Houtt. (Myristicaceae) has long been used as an herbal spice to treat various disorders[1]. In general traditional medicine, it is mostly used two parts i.e. seed and aril[2]. The seed (nutmeg) is widely used as carminative, astringent, hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac[3], anti-flatulence, anti-nausea, and anti-dyspepsia agents[4]. Mace (aril) is widely used as a flavoring agent, a hair dye, and a folk medicine. It also possesses antipapillomagenic, anticarcinogenic[5], anti-bacterial[6] and anti-inflammatory activities[7].

In Thai traditional medicine *M. fragrans* is known as Chan-thet. Three parts of this plant i.e. wood, seed (nutmeg) and aril (mace) are used as tonic, cardio-tonic and carminatives[8]. In addition, its three parts are also ingredients of various remedies such as for anti-pyretic, anti-allergy, anti-inflammatory, cardio-

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tonic, and other remedies. However, the most studies are found in nutmeg and mace, and only few studies found for its wood. Thus, this study was carried out to investigate the biological activities related to their uses in Thai traditional medicine as the followings: anti-inflammatory, anti-allergic and antioxidant activities of its extracts obtained from wood, nutmeg and mace. These three activities are important due to the fact that the common causes of illness are free radicals, inflammation and allergens.

The comparative biological studies of three parts of *M. fragrans* were also undertaken. The results are expected to support the use in Thai traditional medicine and to assist in selection of *M. fragrans* part which is the most appropriate to various diseases.

Material and Method

Chemicals and reagents

MEM medium, RPMI1640 medium, fetal bovine serum (FBS) were purchased from Gibco (NY, USA), Phosphate buffered saline (PBS) from Amresco (Ohio, USA), Sodium bicarbonate from BDH (Poole, England), Penicilin-Streptomycin (P/S), trypsin-EDTA; lipopolysaccharide from *E. coli* (LPS), 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2 H-tetrazolium bromide (MTT), N-(1-naphthyl) ethylenediaminedihydro chloride, phosphoric acid solution, sulfanilamide, anti-dinitrophenylated bovine albumin (DNP-BSA), anti-BNP IgE (Monoclonal Anti-DNP) and 4-Nitrophenyl N-acetyl-β-D-glucosaminnide (PNAG) from Sigma-Aldrich Inc. (MO, USA). Hydrochloric acid fuming 37% bought from Merck (Darmstadt, Germany), Dimethylsulfoxide (DMSO), Isopropanol from RCI Labscan Limited (Bangkok, Thailand), Hank's balanced salt solution (HBSS), Phorbol 12-myristate 13-acetate (PMA) and Nitrotetrazolium blue chloride (NBT) from Sigma (Germany).

Cell culture

Anti-inflammatory activity

Murine macrophage leukemia cell lines (RAW 264.7) [American type culture collection (ATCC) VA, USA)] were cultured in RPMI1640 and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and maintained at 37°C in 5% CO_2.

Anti-allergic activity

Rat basophilic leukemia cell lines (RBL-2H3) were purchased from the American type culture collection (ATCC) (CRL-2256, VA, USA). The cells were cultured in minimum essential medium eagle (MEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and maintained at 37°C in 5% CO_2.

Anti-oxidant activity

Human promyelocytic leukemia cell lines (HL-60) were obtained from Asst. Prof. Pintusorn Hansakul, Faculty of Medicine, Thammasat University, Thailand. The cells were cultured in RPMI1640 medium supplements with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml of streptomycin. The cells were maintained at 37°C in an incubator with 5% CO_2 and 95% humidity. To induce myeloid differentiation, HL-60 cells were cultivated for 7 days in RPMI1640 containing 1.3% DMSO.

Plant materials and extraction

Three parts of *M. fragrans* were collected from Chumphon. They were cleaned and dried at 45°C in an oven for 24 hours. Dried plant materials were evaluated for quality standard following Thai Herbal Pharmacopoeia protocols. They were ground to powder. Each crude powder was macerated with 95% ethanol for 3 days, 3 times and dried using an evaporator. The percentages of extraction yield were calculated.

Preparation of sample solution

Each sample was dissolved in sterile dimethyl sulfoxide (DMSO). Then it was diluted to various concentrations i.e. 1, 10, 50 and 100 µg/ml.

In vitro, anti-inflammatory activity by inhibitory effects on the release of nitric oxide (NO) from RAW 264.7 cell lines[9]

RAW 264.7 cell lines were cultured in 96-well plate with 100 µl completed RPMI ($1x10^5$ cells/well) and incubated at 37°C at 5% CO_2 atmosphere with 95% humidity for 24 hours. At the end of the incubation time, the medium was replaced with fresh medium that containing of 10 ng/ml LPS with samples at various concentrations and then incubated for 24 hours. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The absorbance was measured at 570 nm using a spectrophotometer.

Cytotoxicity was determined using MTT colorimetric method. Briefly, after 24 hours incubation with test sample, MTT solution (10 µl, 5 mg/ml in PBS) was added to the wells, incubated for 2 hours then the

medium removed. Isopropranol containing 0.04 M HCl then was added to dissolve the formazan production in the cells and the optical density of formazan solution measured with a microplate reader at 570 nm. The inhibition (%) was calculated using the following equation:

Inhibition (%) = $[(OD_{control} - OD_{sample})/OD_{control}]$ x 100

$OD_{control}$ = mean of control media (+LPS) - mean of control media (-LPS)

OD_{sample} = mean of sample (+LPS) - mean of sample (-LPS)

In vitro, anti-oxidant activity by inhibitory effect on PMA-induce superoxide radical in DMSO-differentiated from HL-60 cells (NBT reduction assay)[10]

HL-60 cell lines ($1x10^6$) cells were incubated with various dilutions of the extract which was mixed in 500 µl of HBSS at 37°C in an incubator with 5% CO_2 and 95% humidity for 15 minutes. Next, they were incubated with 500 ng/ml PMA and 1.25 mg/ml NBT solution for another 1 hour. At the end of the incubation time, 2 ml of 1 N HCl was added. After vortex and centrifugation at 4,000 rpm for 10 min, supernatant was removed and mixed in 300 ml DMSO. Then, 100 µl of sample solution was added into 96 well-plates. The plates were measured at 572 nm using a microplate reader and compared to the control (without the extract). The inhibition was calculated by the following equation:

NBT reduction (%) = $[(OD_{control} - OD_{sample})/OD_{control}]$ x 100

$OD_{control}$ was mean of control (not added sample), OD_{sample} was mean of test sample (added sample)

In vitro, inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cell lines[11]

RBL-2H3 cells were dispensed in 24-well plates at a concentration of $2x10^5$ cells/well and allowed to adhere for 1 hour at 37°C in 5% CO_2. The cells were sensitized with anti-dinitrophenylimmunoglobulin E (anti-DNP IgE) (0.45 g/ml), then incubated overnight. The cells were washed twice with 500 µl of Siraganian buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Piperazine-N,N-bis (2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA) and 40 mM NaOH, pH 7.2). After that, 20 µl of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 µl of antigen (DNP-BSA, final concentration is 10 g/ml) to stimulate the cells to degranulate. The supernatant was transferred into a 96-well plate and incubated with 50 µl of substrate PNAG in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 hour. The reaction was stopped by adding 200 µl of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was added to Siraganian buffer (final DMSO concentration was 0.1%). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation:

Inhibition (%) = 1 - (T - B - N) x 100/(C - N)

Control (C) was DNP-BSA (+) and test sample (-); Test (T) was DNP-BSA (+) and test sample (+); Blank (B) was DNP-BSA (–) and test sample (+); normal (N) was DNP-BSA (–), test sample (–).

Statistical analysis

The values were expressed as mean ± SEM of three determinations. The EC_{50} and IC_{50} values were calculated using the Prism program. The statistical significance was calculated using unpaired t-test.

Results

The percentage yield of three parts showed that the mace had the highest extraction yield but the wood had least yield, its yield values were shown in Fig. 1.

The anti-inflammatory activity of the extracts obtained from three parts of *M. fragrans* was lower than that of the reference standard drug, prednisolone (*p*-value <0.05). However, the wood extract showed stronger anti-inflammatory activity than that of the extracts obtained from nutmeg and mace, respectively.

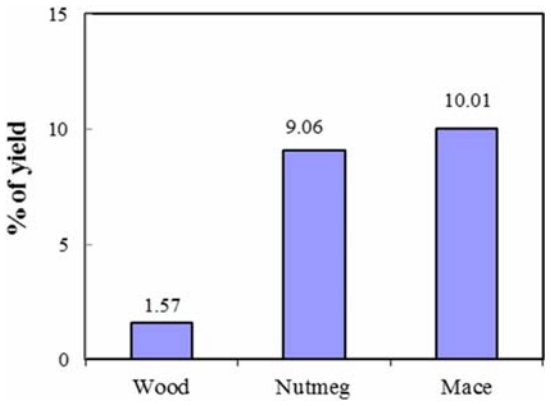

Fig. 1 Percentage yields of ethanolic extracts from three parts of *M. fragrans.*

The wood extract could well inhibit nitric oxide while the extracts obtained from nutmeg and mace had moderate nitric oxide inhibition activity. The cytotoxicity showed less than 30% of RAW 264.7 cells were dead after incubation with the extracts. This indicate that the extract were not toxic to the cells. The results of anti-inflammatory activity by inhibitory effects on the release of nitric oxide (NO) from RAW 264.7 cell lines are shown in Table 1.

The anti-oxidant activity of the extracts obtain from three parts was lower activity than reference standard agent, propyl gallate (*p*-value <0.05). However, nutmeg and mace showed high anti-oxidant activity by PMA-induced superoxide radical in DMSO-differentiated from HL-60 cells (NBT reduction assay) while the wood extract had moderate activity. The results of anti-oxidant activity are shown in Table 2.

The results of anti-allergic activity test showed that the ethanolic extracts of three parts, exhibited significantly stronger anti-allergic activity than that of the reference standard, chlopheniramine (*p*-value <0.05). The extracts obtained from wood and mace exhibited higher inhibitory effect on beta-hexosaminidase releases than that of the extract of nutmeg. However, all three parts showed high anti-allergic activity. The results of anti-allergic activity are shown in Table 3.

The comparisons of all activities of three parts

Table 1. % inhibition and IC_{50} of anti-inflammatory activity by inhibitory effects on the release of nitric oxide (NO) from RAW 264.7 cell lines of three parts from *M. fragrans* and prednisolone (n = 3)

Part	% inhibition of concentration (µg/ml) ± SEM					IC_{50} (µg/ml) ± SEM
	0.1	1	10	50	100	
Wood	-	4.05±3.63	16.85±1.19	57.78±0.30	86.14±1.22	40.26±0.58
Nutmeg (seed)	-	-2.53±1.91	-0.68±1.20	41.89±2.12	74.05±3.69	65.42±4.95
Mace (aril)	-	-3.69±0.36	-0.71±0.22	33.57±2.24	65.11±2.19	75.40±4.14
Prednisolone	40.08±1.95	53.13±0.85	60.48±0.67	76.70±2.53	-	0.84±0.07

Table 2. % inhibition and EC_{50} of PMA-induced superoxide radical in DMSO-differentiated from HL-60 cells (NBT reduction assay) of three parts from *M. fragrans* and propyl gallate (n = 3)

Part	% inhibition of concentration (µg/ml) ± SEM				EC_{50} (µg/ml) ± SEM
	1	10	50	100	
Wood	2.90±2.69	3.65±0.42	37.52±2.06	54.59±2.50	71.83±1.33
Nutmeg (seed)	26.20±1.85	32.64±1.83	90.48±4.01	98.54±1.21	21.16±1.03
Mace (aril)	3.10±2.02	11.76±1.31	96.47±1.22	101.16±0.25	28.90±0.39
Propyl gallate	20.83±2.83	55.56±2.12	74.62±0.73	79.16±2.10	8.25±0.53

Table 3. % of inhibition and IC_{50} on the release of β-hexosaminidase from RBL-2H3 cell lines (anti-allergic activity) of three parts from *M. fragrans* and chlopheniramine (n = 3)

Part	% inhibition of concentration (µg/ml) ± SEM				IC_{50} (µg/ml) ± SEM
	1	10	50	100	
Wood	-10.78±3.39	34.74±1.00	59.26±1.35	104.54±4.09	13.29±0.28
Nutmeg (seed)	-4.42±3.98	26.97±1.39	74.68±3.14	90.81±1.75	20.90±1.03
Mace (aril)	14.32±0.35	41.71±1.06	61.56±2.82	81.18±0.21	12.95±0.89
Chlopheniramine	-5.83±2.06	16.71±0.76	66.36±1.47	77.88±2.99	28.10±1.08

from *M. fragrans* are presented in Fig. 2.

Discussion

The anti-inflammatory activity by inhibitory effects on nitric oxide (NO) release from RAW 264.7 cell lines and inhibitory effects on β-hexosaminidase release from RBL-2H3 cell lines of *M. fragrans* has been studied. Prasaprohyai, a remedy for colds, asthma and fever contains wood, nutmeg and mace[12]. They inhibited nitric oxide (IC_{50} values = 30.42, 47.23 and 78.38 μg/ml, respectively[13]. Previous results related similarly with this study that wood showed potent anti-inflammatory activity more than nutmeg and mace. Three parts in Prasaprohyai remedy from previous study inhibited β-hexosaminidase (IC_{50} values = 59.89, 13.89 and 11.65 μg/ml, respectively[14]; the results were related with this study, except wood. This study showed higher anti-allergic activity than previous study which may differ source and age of wood. Anti-oxidant activity of tree parts of *M. fragrans* on PMA-induce superoxide radical in DMSO-differentiated HL-60 cells is the first study. However, these parts were found and many reported anti-oxidant activities by another assay. The nutmeg and mace extracts showed moderate anti-oxidant activity by determining DPPH scavenging activity[15] while the extracts of both parts had high anti-oxidant activity in this study. However, some results might not be as same as the previous studies because the place and age of the herb collection were different.

The results presented different activities of the extracts of the three parts. They may depend on the active compound consisting of *M. fragrans* such as trimyristin, macelignan and myristicin[16], which are in differing proportions in each plant part, with results accordingly. For example, macelignan (isolate from mace) inhibited β-hexosaminidase release in RBL-2 H3 cells treated with A23187 (10 μM) in a dose-dependent manner (5 μM macelignan, 8% inhibition; 10 μM, 14% inhibition; and 20 μM, 32 % inhibition)[17]. Myristicin which was isolated from mace and nutmeg has anti-inflammatory property related with its inhibition of NO, cytokines, chemokines and growth factors in RNA-stimulated macrophages via the calcium pathway[18]. However, anti-allergy activity of *M. fragrans* wood extract should be further studied to isolate the active anti-allergic compounds. The combination of three parts should be extracted and all activity compared with single part extract because three parts of this plant are combined for use in Thai traditional medicine.

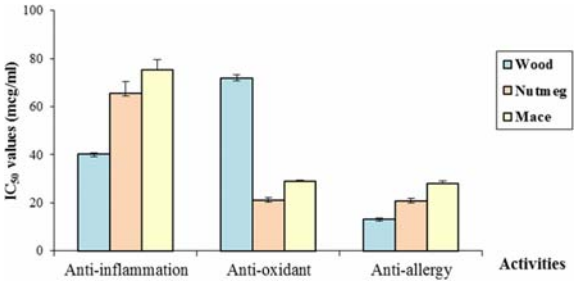

Fig. 2 Comparison IC_{50} values of all activities of ethanolic extract from three parts of *M. fragrans.*

Conclusion

The ethanolic extract of *M. fragrans* wood showed high anti-inflammatory and anti-allergic activities, but moderate anti-oxidant activity. The nutmeg and mace extracts possessed high anti-oxidant and anti-allergic activities with moderate anti-inflammatory activity. Therefore, remedies for inflammation-related diseases should contain the wood part of *M. fragrans* while anti-oxidant related diseases should contain nutmeg or/and mace. However, all parts of *M. fragrans* could be used for allergy-related diseases. Further work should be focused on the combination of the three parts of *M. fragrans* and test for activities of anti-inflammatory, anti-oxidant and anti-allergic properties including the isolation of active compounds possessing the anti-allergic activity from the wood. In conclusion, this knowledge supports using all parts of *M. fragrans* as ingredients in Thai traditional remedies to treat chronic diseases caused by inflammation, allergens and free radicals.

What is already known on this topic?

Myristica fragrans Houtt. (Myristicaceae) as an herbal spice and has long been used to treat various disorders. Ingeneral traditional medicine mostly used two parts of *M. fragrans*. The seed (nutmeg) is widely used as carminative, astringent, hypolipidaemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, treating flatulence, nausea, and dyspepsia. Mace is widely used as a flavoring agent, a hair dye, and a folk medicine. It also possesses antipapillomagenic, anticarcinogenic, antibacterial and anti-inflammatory activities. In Thai traditional medicine *M. fragrans* is known as Chan-thet. Three parts of this plant are used as drugs including wood, seed (nutmeg) and aril (mace). Its three parts are also ingredients of various remedies such as for anti-pyretic, anti-allergy, anti-inflammatory and other remedies.

What this study adds?

This study was carried out to investigate the biological activities related to its use in Thai traditional medicine including anti-inflammatory, anti-allergic and antioxidant activities of its extracts obtained from wood, nutmeg and mace. These three activities are important because the causes of illness are free radicals, inflammation and allergens. The comparative biological studies of three parts of *M. fragrans* were also undertaken. The results are expected to support the use in Thai traditional medicine and to assist in selection of part of *M. fragrans* most appropriate to various diseases.

Acknowledgements

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Potential conflicts of interest

None.

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ฤทธิ์ทางชีวภาพของสารสกัดเอทานอลจากจันทน์เทศ (แก่นจันทน์ ลูกจันทน์ และดอกจันทน์)

แสงนภา จำปาสุริ, อรุณพร อิฐรัตน์

ภูมิหลัง: *จันทน์เทศ เป็นสมุนไพรที่มีรสร้อน ใช้ในการรักษาโรคต่างๆ มายาวนาน ในทางการแพทย์แผนไทยใช้จันทน์เทศ 3 ส่วน ได้แก่ ส่วนแก่นเม็ด และเยื่อหุ้มเม็ด (ดอกจันทน์) มาเป็นส่วนประกอบในตำรับยารักษาโรคต่างๆ เช่น ตำรับยาแก้ไข้ ตำรับยาต้านการแพ้ ตำรับยาต้านการอักเสบ*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ทางชีวภาพของจันทน์เทศ โดยศึกษาฤทธิ์ที่สอดคล้องกับการใช้ในทางการแพทย์แผนไทย ได้แก่ ฤทธิ์ต้านการอักเสบ ฤทธิ์ต้านการแพ้ และฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากแก่นจันทน์ ลูกจันทน์ และดอกจันทน์*

วัสดุและวิธีการ: *แก่นจันทน์ ลูกจันทน์ และดอกจันทน์ สกัดโดยการหมัก 95% เอทานอล จากนั้นจะนำสารสกัดที่ได้มาทดสอบฤทธิ์ต้านการอักเสบโดยศึกษาฤทธิ์ยับยั้งการหลั่งไนตริกซ์ออกไซด์จากเซลล์ RAW 264.7 ทดสอบฤทธิ์ต้านอนุมูลอิสระโดยศึกษาฤทธิ์ยับยั้งอนุมูลอิสระซุปเปอร์ออกไซด์จากเซลล์ HL-60 และทดสอบฤทธิ์ต้านการแพ้โดยศึกษาการยับยั้งเอนไซม์ β-hexosaminidase จากเซลล์ RBL-2H3*

ผลการศึกษา: *สารสกัดชั้นเอทานอลจากแก่นจันทน์มีฤทธิ์ต้านการอักเสบที่ดีกว่าลูกจันทน์และดอกจันทน์ค่า IC_{50} = 40.26±0.58, 65.42±4.95, 75.40±4.14 μg/ml ตามลำดับ ฤทธิ์ต้านอนุมูลอิสระพบว่าลูกจันทน์และดอกจันทน์มีฤทธิ์ต้านอนุมูลอิสระที่ดี ส่วนแก่นจันทน์ต้านอนุมูลอิสระได้ปานกลางค่า IC_{50} = 21.164±1.03, 28.897±0.39 และ 71.830±1.33 μg/ml ตามลำดับ ฤทธิ์ต้านการแพ้พบว่าทั้งแก่นจันทน์ ลูกจันทน์ และดอกจันทน์มีฤทธิ์ต้านการแพ้ที่ดีค่า IC_{50} = 13.29±0.28, 20.90±1.03 และ 12.95±0.89 μg/ml ตามลำดับ*

สรุป: *แก่นจันทน์เทศมีฤทธิ์ต้านการอักเสบและต้านการแพ้ที่ดี แต่ต้านอนุมูลอิสระปานกลาง ส่วนลูกจันทน์และดอกจันทน์มีฤทธิ์ต้านอนุมูลอิสระและต้านการแพ้ที่ดีแต่มีฤทธิ์ต้านการอักเสบปานกลาง ดังนั้นโรคที่เกี่ยวกับการอักเสบควรเลือกใช้ส่วนแก่นจันทน์ ส่วนการต้านอนุมูลอิสระควรเลือกใช้ลูกจันทน์และ/หรือดอกจันทน์ และโรคที่เกี่ยวกับการแพ้เลือกใช้ได้ทั้งสามส่วนเพราะมีฤทธิ์ต้านการแพ้ ต้านการอักเสบและต้านอนุมูลอิสระ อย่างไรก็ตามควรมีการศึกษาฤทธิ์ทางชีวภาพของจันทน์เทศในสัตว์ทดลองและควรศึกษาการแยกสารเพื่อหาสารออกฤทธิ์ตามหลัก bioassay guided ต่อไป*