ฤทธิ์ต้านอะเซทิลโคลีนเอสเทอเรสของน้ำมะตูม
Acetylcholinesterase Inhibitor Activities of *Aegle marmelos* Fruit Beverage

นิพนธ์ต้นฉบับ

Original Article

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ต้านอะเซทิลโคลีนเอสเทอเรสของน้ำมะตูม *Aegle marmelos* (Family Rutaceae) ที่เตรียมจากมะตูมนิ่ม มะตูมไข่ และมะตูมบ้าน และวิเคราะห์ปริมาณสารมาร์มีโลซินซึ่งเป็นฟูราโนคูมารินที่เป็นสารสำคัญในผลมะตูม **วิธีการศึกษา:** เตรียมน้ำมะตูมโดยต้มผลแห้งของมะตูมนิ่ม มะตูมไข่ และมะตูมบ้านในน้ำ แล้วทำให้เป็นผงแห้งโดยใช้เครื่องทำแห้งแบบพ่นฝอย ทดสอบฤทธิ์ต้านอะเซทิลโคลีนเอสเทอเรสตามวิธีของเอลแมน โดยใช้กาลันตามีนเป็นกลุ่มควบคุมเชิงบวก และวิเคราะห์ปริมาณสารมาร์มีโลซินโดยเปรียบเทียบปริมาณจากกราฟสารมาร์มีโลซินมาตรฐาน **ผลการศึกษา:** ปริมาณสารมาร์มีโลซินในน้ำมะตูมที่ทำเป็นผงแห้ง 100 มก. ที่เตรียมได้จากมะตูมนิ่ม มะตูมไข่ และมะตูมบ้านอยู่ในช่วง 600-1000 มคก. และมีฤทธิ์โดยคำนวณเป็นค่าความเข้มข้นของสารที่สามารถยับยั้งการทำงานของเอนไซม์ได้ครึ่งหนึ่ง (IC_{50}) อยู่ในช่วง 125-136 มคก./มล. สารมาร์มีโลซินและสารกาลันตามีน แสดงค่า IC_{50} 942.81 มคก./มล. และ 114.64 มคก./มล. ตามลำดับ และไม่พบความสัมพันธ์ของปริมาณสารมาร์มีโลซินและฤทธิ์ยับยั้งการทำงานของเอนไซม์ **สรุป:** น้ำมะตูมที่เตรียมจากมะตูมนิ่ม มะตูมไข่ และมะตูมบ้านมีฤทธิ์ยับยั้งการทำงานของเอนไซม์อะเซทิลโคลีนเอสเทอเรส

คำสำคัญ: อะเซทิลโคลีนเอสเทอเรส, มะตูมนิ่ม, มะตูมไข่, มะตูมบ้าน

Abstract

Objective: To investigate acetylcholinesterase inhibitor activity of *Aegle marmelos* (commonly known as matoom, Family Rutaceae) fruit beverage derived from matoomnim, matoomkhai and matoomban, as well as to determine the content of marmelosin, a furanocoumarin found in the fruit. **Methods:** The beverage was separately prepared by boiling the dried fruits of matoomnim, matoomkhai and matoomban in water. Their powdered form was prepared by a spray drying process. Acetylcholinesterase inhibitor activity was determined based on Ellman's method and galantamine was tested as a positive control. The content of marmelosin was determined based on marmelosin standard curve. **Results:** Marmelosin content in 100 mg beverage powder obtained from matoomnim, matoomkhai and matoomban ranged from 600 to 1000 µg, while the activity reported as half-maximal inhibitory concentration (IC_{50}) was 125 - 136 µg/ml. Marmelosin and galantamine exhibited acetylcholine esterase activity with IC_{50} values of 942.81 and 114.64 µg/ml, respectivelt. There was no apparent relationship between marmelosin content and the activity. **Conclusion:** *A. marmelos* fruit beverage of matoomnim, matoomkhai and matoomban exhibited acetylcholinesterase inhibitor activity.

Keywords: Acetylcholinesterase, *Aegle marmelos, matoomnim*, matoomkhai, matoomban

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Introduction

The fruit of *Aegle marmelos* (Family Rutaceae), commonly known as bael or matoom, has been long and widely used in traditional Thai medicine. It is a component in herbal formulas such as "Tri-Kesorn-Mas" and "Look Plaag Mae." Marmelosin (Figure 1, European community (EC) number 207-581-1[1]) also known as imperatorin is one of the active substances found in the fruits and it has been reported to prevent memory impairment and oxidative stress in the brain induced by scopolamine in mice.[2] Marmelosin can also easily cross the blood brain barrier[3], therefore, it is possible to act in the brain and there have been many reports showing that *A. marmelos* fruit possesses acetylcholinesterase inhibitory activity.[4,5] Acetylcholinesterase has been the main target for the treatment of Alzheimer's disease (AD) since the discovery of cholinergic deficits in patients suffering from AD.[6] In this study, we aimed to examine anti-acetylcholinesterase activity of *A. marmelos* fruit beverage. The beverage in tea form is a widely popular drink among Thai people for refreshment. To make a *A. marmelos* beverage, unripe fruits are either cut into pieces, dried and boiled in water or pulverized and packed as tea bags.

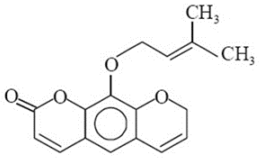

Figure 1 Chemical structure of marmelosin.

The fruits of *A. marmelos* can be different in shape and size. They are characterized by thin or hard woody shell with tiny aromatic glands with pleasant smell. The shell is green in colour when raw and becomes woody and hard and yellowish colour when ripe.[7] The varieties of *A. marmelos* are diverse and there is no standard name for each cultivar.[8] In Thailand, there are three local cultivars with dietary use including matoomnim, matoomkhai and matoomban[9] (Figure 2). The fruit of matoomnim is round in shape with thin and soft, woody shell, and it is known to be an ingredient in several recipes of traditional Thai medicine. The fruit of matoomkhai is oval or oblong in shape. It possess thin but hard, woody shell. The fruit of matoomban can be round or oval with thick, hard, woody shell.

Figure 2 Matoomnim (A), matoomkhai (B) and matoomban (C). The scale bar represents 1 cm.

The objectives of this study were to explore the acetylcholinesterase inhibition activity of *A. marmelos* fruit beverage of matoomnim, matoomkhai and matoomban, and to determine marmelosin content in those beverages.

Methods

Chemicals

Galantamine hydrobromide, bovine serum albumin lyophilized powder, acetylthiocholine iodide, AChE (type VI-S) from the electric eel (*Electrophorus electricus*), and 5,5´-dithiobis (2-nitrobenzoic acid) were obtained from Sigma-Aldrich. Marmelosin was obtained from Aktin Chemicals, Inc. All solvents were of analytical grade.

Preparation of powder from *A. marmelos* fruit beverage

The fruits of matoomnim and matoomban were obtained from agroforestry learning center in Sanam Chai Khet district, Chachoengsao province, and matoomkhai was obtained from cultivated site in Burirum province, Thailand. The specimens were deposited at Department of Pharmacognosy, Srinakharinwirot University, Ongkarak, Nakonnayok, Thailand. The collection numbers were WSAENIM01, WSAEKHAI01, WSAEBAN01 for matoomnim, matoomkhai and matoomban, respectively. The fruits were rinsed with clean water, finely chopped and dried at 50 °C. The dried specimens were finely powdered and boiled in water for 5 minutes, left until cooled at room temperature before filtering and drying by spray dryer (Buchi mini spray dryer B-290, Germany). The conditions for spray drying were as follows: inlet temperature 155 °C, outlet temperature 122 °C, feed rate of 5 ml/min, air flow rate of 30 m^3/h, and spray flow rate of 473 L/h. The spray dried powder was stored in desiccator until use.

Determination of marmelosin content by HPTLC[10]

A CAMAG HPTLC system (Switzerland) consisting CAMAG Linomat 5 applicator, CAMAG TLC Scanner 4 and CAMAG winCATS software was used for the study. Spray dried *A. marmelos* powder (100 mg) was dissolved in 1 ml methanol and sonicated for 15 minutes. The standard solution of marmelosin (1 mg/ml) was prepared by dissolving with methanol. The standard solution of marmelosin (2, 4, 8, 16, 32 µl) and the sample solutions (20 µl) were applied on a pre-coated HPTLC plate (E. Merck) with CAMAG Linomat 5 applicator. The band width was 7 mm. The plates were developed in a solvent system of toluene: ethyl acetate: glacial acetic acid (7: 3: 0.1 v/v/v) at 25 ± 20 °C temperature, up to 8.5 cm. After development, the plate was dried and scanned at 310 nm using absorbance reflectance mode by CAMAG Scanner 4. The peak area was recorded. Calibration curve was performed by plotting peak area versus marmelosin amount. Marmelosin amount in the sample was calculated using the respective calibration curve, y = 617.33*x + 7367.00, R^2 = 0.9993.

Determination of acetylcholinesterase inhibitory activity[11]

The reaction was performed in 96 well plate by mixing either 20 µl of galantamine standard (1.95, 3.90, 7.81, 15.63, 31.25, 62.5, 125 and 250 µg/ml), marmelosin (39.06, 78.13, 156.50, 312.50, 625.00 and 1250.00 µg/ml), or 20 µL of *A. marmelos* powdered sample in methanol (9.765, 19.53, 39.06, 78.13 and 156.25 µg/ml) in Tris buffer pH 7.8 with 20 µl of acetylcholine esterase (0.27 U/ml) solution in Tris buffer pH 7.8. The plate was slowly shaken on shaker for 30 minutes, then Ellman's reaction mixture consisting of 50 µL of 5, 5'-dithio-bis (2-nitro benzoic acid) (10 mM) and 20 µl of

acetylthiocholine (75 mM), was added. The plate was shaken on shaker and allowed to stand at room temperature for 30 minutes in order to complete the reaction. Absorbance was measured at 412 nm by using microplate reader (BioTek PowerWave^{TM} XS, USA). The experiment was done in triplicate starting from mixing the Ellman's reaction mixture with the test sample. The percentage of acetylcholine esterase inhibition (percentage of inhibition) was calculated according to the following equation:

Percentage of inhibition = $(A_0 - A_1) / A_0 \times 100$,

where A_0 was the absorbance of control reaction (all solutions without tested compound) and A_1 was the absorbance of standard or tested compound. Half-maximal inhibitory concentration (IC_{50}) of galantamine was calculated from the graph of inhibition percentage versus log concentration. IC_{50} of tested compound was calculated from the graph of inhibition percentage versus concentration.

Results and Discussion

Aegle marmelos fruit beverage was prepared by boiling the dried fruits in water, and was made to powdered form by a spray dryer. HPTLC chromatogram revealed that matoomnim, matoomkhai and matoomban had similar chemical fingerprints with marmelosin at hR_f 5 (Figure 3). The chromatogram suggested that marmelosin was not a major constituent in *A. marmelos* fruit beverage. The content of marmelosin varied among fruit cultivars (Table 1).

The content of marmelosin in matoomnim was 1.6 times and the content in matoomban was 1.5 times higher than that in matoomkhai. The previous study revealed that marmelosin content obtained from methanol extraction of the fruit was 270 µg/100 mg dry fruit.[10] Another study estimated marmelosin content by extraction of the dry fruits using a Soxhlet apparatus and reported that marmelosin content was 500-650 µg/100 mg dry fruit.[12] It was apparent that marmelosin content in the fruits differed by fruit cultivars and extraction methods.

To explore acetylcholinesterase inhibitory activity, the beverage powdered was prepared in methanol. When the powdered was re-dissolved in water, it could not homogenously mix with the acetylcholinesterase assay system. Therefore, the result of the inhibitory activity could not be obtained. On the other hand, methanolic solution of the powdered could exhibit activity in a concentration-dependent manner and the IC_{50} was shown in Table 1.

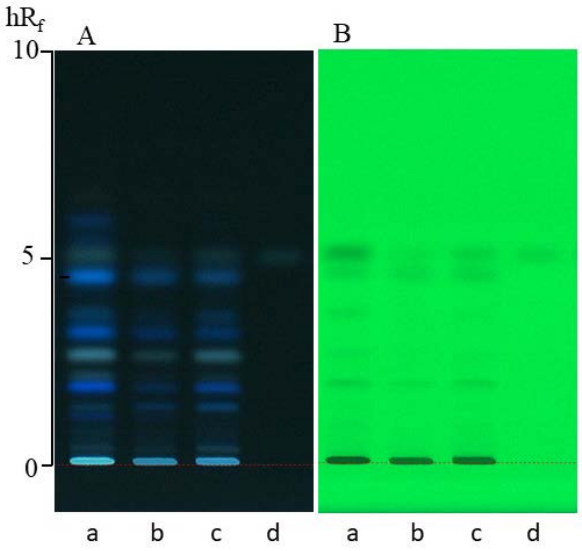

Figure 3 HPTLC chromatogram of *A. marmelos* fruit beverage derived from matoomnim (a), matoomkhai (b), matoomban (c) and marmelosin (d) visualized in UV 366 nm (A) and UV 254 nm (B).

Table 1 The marmelosin content of A. marmelos powdered and their half-maximal inhibitory concentration (IC50) of acetylcholinesterase activity.

	Marmelosin content (µg) in 100 mg dried powder	IC_{50} of acetylcholinesterase activity (µg/ml) (%RSD)
matoomnim	1012.11	133.22 (2.93)
matoomkhai	651.00	136.27 (3.28)
matoomban	985.04	124.54 (3.93)
marmelosin	-	942.81 (4.46)
galantamine	-	114.64 (1.62)

The previous studies revealed contrast results of marmelosin activity determined based on Ellman's method. Kim et al reported that the IC_{50} was 63.7 µM (17.22 µg/ml)[13], whereas Sigurdsson and Gudbjarnason stated that inhibitory activity was above 274 µg/ml, i. e. , IC_{50} > 274 µg/ml. [14] Sigurdsson and Gudbjarnason described that their result differed from Kim et al since their study did not incubate marmelosin with the enzyme for 30 minutes before the reaction buffer was added. Both studies used mouse brain as the enzyme source and did not report catalytic activity of the enzyme. In our study, the activity of marmelosin was determined based on Ellman's method but using the

commercial enzyme from electric eel. The enzyme activity was 0.27 U/ml. Our result disagreed with those previous studies.

In this study, marmelosin content in 100 mg powder obtain from various types of the fruit ranged from 600 to 1000 µg, while the activity reported as IC_{50} was 125 to 136 µg/ml. The activity was close to that of galantamine, which is a potent acetylcholinesterase inhibitor,[15] and was higher than marmelosin alone. It was observed that there was no apparent relationship between marmelosin content and the activity (Figure 4). Marmelosin was a major compound in *Angelica* spp.[13,14] and has been reported to be one of the active principles that play a crucial role for the acetylcholinesterase inhibitory activity in these plants. According to HPTLC chromatogram (Figure 3), marmelosin was not a major constituent in *A. marmelos* fruit beverage.

The activity presented in the fruit beverage may be the synergistic effect of other active substances in the *A. marmelos* fruit beverage. In addition to marmelosin, psoralen and bergapten were also reported to be furocoumarin components in *A. marmelos* fruit.[12] Psoralen isolated from *Psoralea corylifolia* fruits[16] and bergapten obtained from *Heracleum crenatifolium* Boiss.[17] have been found to exhibit inhibitory activity of acetylcholinesterase enzyme. Thus, these compounds may be responsible for the activity in *A. marmelos* fruits. Several minor components such as flavonoids, phenolic compounds, and alkaloids were also found in *A. marmelos* fruits.[18-20] Thus, the composition of the active substances in the powdered fruit should be investigated and the inhibition of enzyme activity should be evaluated to establish the synergism of the active substances in the fruit powder.

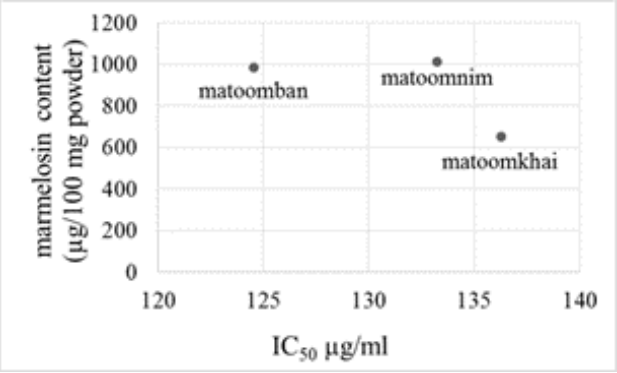

Figure 4 Scatter plot between marmelosin content and acetylcholinesterase inhibitory activity.

Conclusion

A common feature in the Alzheimer's disease (AD) brain is the presence of acetylcholinesterase which is commonly associated with oxidative stress caused by β-amyloid plaques.[6] The beverage obtained from *A. marmelos* dried fruit powder exhibited a prominent acetylcholinesterase inhibitory activity, thus it could be regarded as a potential food supplement to delay or prevent AD. In addition, since the activity obtained from each fruit cultivar was comparable, they could be alternative to each other. Further study should be performed to clarify the chemical constituents that exhibit inhibitory activity of acetylcholinesterase enzyme as well as to demonstrate the synergistic effect of marmelosin and other components.

Acknowledgements

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References

1. European Chemical Agency. (Accessed on Mar. 2, 2020, at https://echa.europa.eu/information-on-chemicals/ec-inventory)
2. Budzynska B, Boguszewska-Czubara A, Kruk-Slomka M, et al. Effects of imperatorin on scopolamine-induced cognitive impairment and oxidative stress in mice. *Psychopharmacology* 2015;232(5):931-942.
3. Tun T, Kang YS. Imperatorin is transported through blood-brain barrier by carrier-mediated transporters. *Biomol Ther* (Seoul) 2017;25(4):441-451.
4. Paulbabu K, Deepak Singh K, Prashanti P, Padmaja M. Neuroprotective potential and efficacy of neurodegenerative disorders of fruit extract of *Aegle marmelos*. *Int J Pharm Pharm Sci* 2015;7(1):155-159.
5. Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. *Phytomedicine* 2007;14(4):289-300.
6. Hodges JR. Alzheimer's centennial legacy: origins, landmarks and the current status of knowledge concerning cognitive aspects. *Brain* 2006;129(pt 11):2811-2822.
7. Baliga M, Bhat H, Joseph N, Fazal F. Phytochemistry and medicinal uses of the Bael fruit (*Aegle marmelos* Correa): A concise review. *Food Res Int* 2011;44(7):1768-1775.
8. Sharma N, Dubey W. History and taxonomy of *Aegle marmelos*: A review. *Int J Pure App Biosci* 2013;1(6):7-13.
9. Thai Herbal Pharmacopoeia. (Accessed on Mar. 2, 2020, at https://bdn.go.th/thp//ebook/qQqcZKtkpR9gC3q0GT5gMJq0qT5co3uw)
10. Shailajan S, Menon S, Hande H. Method validation of marmelosin from fruit pulp of *Aegle marmelos* (L.) Correa using HPTLC technique *J Pharm Res* 2011;4(5):1353-1355.
11. Asaduzzaman M, Uddin M, Kader M. et al. *In vitro* acetylcholinesterase inhibitory activity and the antioxidant properties of *Aegle marmelos* leaf

extract: implications for the treatment of Alzheimer's disease. *Psychogeriatrics* 2014;14(1):1-10.

12. Shinde PB, Laddha KS. Simultaneous quantification of furanocoumarins from Aegle marmelos fruit pulp extract. *J Chromatogr Sci* 2015; 53(4):576–579.
13. Kim DK, LimJP, Yang JH, Eom DO, Eun JS, Leem KH. Acetylcholinesterase inhibitors from the roots of *Angelica dahurica*. *Arch Pharm Res* 2002;25(6):856–859.
14. Sigurdsson S, Gudbjarnason S. Inhibition of acetylcholinesterase by extracts and constituents from *Angelica archangelica* and *Geranium sylvaticum. Z Naturforsch C J Biosci* 2007;62(9-10):689-693.
15. Hopkins TJ, Rupprecht LE, Hayes MR, Blendy JA, Schmidt HD. Galantamine, an acetylcholinesterase inhibitor and positive allosteric modulator of nicotinic acetylcholine receptors, attenuates nicotine taking and seeking in rats. *Neuropsychopharmacology* 2012;37(10):2310–2321.
16. Somani G, Kulkarni C, Shinde P, Shelke R, Laddha K, Sathaye S. In vitro acetylcholinesterase inhibition by psoralen using molecular docking and enzymatic studies. *J Pharm Bioallied Sci* 2015;7(1):32–36.
17. Orhan I, Tosun F, Sener B. Coumarin, anthroquinone and stilbene derivatives with anticholinesterase activity. *Z Naturforsch C J Biosci* 2008;63(5-6):366-370.
18. Tagad VB, Sahoo AK, Annapure US. Phytochemical study and GC-MS analysis of bael (*Aegle marmelos*) fruit pulp. *Res J Life Sci Bioinform Pharm Chem Sci* 2018;4(5):779-791.
19. Nirupama G, Padmasri G, Ramesh R, Vasanthi M. Comparative analysis of phytochemical constituents present in various parts of *Aegle marmelos*. *Asian Pac J Trop Dis* 2012;2(suppl 2):S774-S777.
20. Jannat K, Jahan R, Rahmatullah M. *Aegle Marmelos* (L.) Correa, A potential source for the treatment of diabetes associated alzheimer's disease: A review. *Pharm Res* 2019;3(4):000186. (doi: 10.23880/oajpr-16000186)