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Chemical compositions and antibacterial activity of essential oil from dill fruits (*Anethum graveolens* L.) cultivated in Thailand

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Abstract

Dill fruit (*Anethum graveolens* L.) is herbal medicine that was used in Thai traditional medicine. The objective of this study was to investigate the chemical compositions and antibacterial activity of essential oil of dill fruit that was cultivated in Thailand (Udon Thani province). Essential oils of dill fruits were obtained by hydrodistillation and steam distillation. The yield of hydro distilled essential oil was higher than those of steam distilled essential oil. The main constituents of dill oils examined by GC-MS were dillapiole (19.98-48.9%), D-carvone (18.05-28.02%) and D-limonene (26.96-44.61%). Minor components, β-pinene (0-0.79%), β-myrcene (0.16-0.21%), decane (0.44-0.49%), 1,5,8-p-menthatriene (0.19-0.27%), undecane (0.34-0.38%), naphthalene (1.63-2.11%), *cis*-dihydrocarvone (0.38-0.95%), *trans*-dihydrocarvone (1.49-1.57%) and myristicin (0.67-1.41%) were presented. The essential oil from steam distillation contained higher content of D-limonene and D-carvone than oil from hydrodistillation. The results of this study indicated that steam distillation method is suitable for isolating essential oil of dill fruits due to high amount of active compounds, D-limonene and D-carvone and low amount of dillapiole which its toxicity to insects has been reported. The antibacterial activity against eleven microorganisms of dill oils and their major compounds were evaluated by micro-broth dilution assay. Dill oils from two kinds of extraction methods exhibited antibacterial activity against five bacteria (*S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. typhimurium* ATCC 14028, *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 8739) with average MIC values of 10 mg/ml while their major constituents, D-limonene and D-carvone demonstrated strong to moderate activity against tested microorganisms.

Keyword: *Anethum graveolens* L., GC-MS, D-limonene, D-carvone, Dillapiole, Antibacterial

1. INTRODUCTION

Anethum graveolens L. is commonly known as dill and its Thai vernacular name is Phak chi lao or Thian ta takkataen. It is a biennial or annual aromatic herb belonging to Apiaceae (Umbelliferae) family, grows up to 30-120 centimeters height. Compound leaf with divide margin show thread-like shape. The stems are hollow. Inflorescences are arranged in umbels. Flowers have pale yellow color. Fruit is a schizocarp, comprises a pair of carpel that split apart as two mericarps at mature stage.

This herb is native to Southern Europe, Western Asia, Southern Russia and Mediterranean region[1, 2] but it is now widely cultivated in all different areas of the world. Dill cultivated in Japan, India and Egypt is called East Indian dill or Sowa *Anethum sowa Roxb. ex Fleming*). In Thailand, dill is cultivated crop in the Northeastern region for using aerial part (dill weed) as a favorite seasoning agent in food.

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The dried dill fruits are well known for their medicinal properties as carminative, tonic, appetite stimulant and lactation[3]. Ayurvedic treatment in India, dill water was used to relieve the flatulence of infants, colic pain, vomiting and hiccup. Indian people have been known to chew dill fruit after meal for clears bad breath (halitosis). Dill fruit in Thailand is used for substitution of caraway fruit (*Carum carvi* L.) due to its similar taste and smell. It has been commonly used as groups together with other fruits in Thai traditional medicine for certain purposes called Phikatthianthang ha, chet and kao. Normally, the three groups are commonly used as carminative, tonic and appetite stimulant. Moreover, dill fruit is often used as a basic ingredient of three Yahom recipes and seven traditional herbal formulas in the National Essential Herbal Drug List of Thailand and more than 100 formulas of Thai traditional medicine (Tamra Kanpaetthaidoem)[4].

Essential oil of dill fruits has a grass like smell and a pale yellow color. The yield of fruit oil varied from 1.2 to 7.7%[5]. Their main components were D-carvone (35-60%), D-limonene and α-phellandrene[5]. Other compounds such as dihydrocarvone, eugenol, β-phellandrene, α-pinene, anethole, dill apiole, myristicin, carveol, β-caryophyllene, and others were also found[6]. The quality of dill fruit is determined regarding their main components in the essential oil which are D-carvone and D-limonene[7-10]. British Pharmacopoeia 2014 determines D-carvone content of dill oil is ranges from 43 to 63% (using titration method)[11]. Factors affecting the main composition of the essential oil reported to be the geographic origin[12-20], extraction methods[18], harvest stage[18], cultivars[19] and storage period[20]. Variation in quality of herbal medicines will effect on therapeutic effect or safety for consumers. So, quality control of herbal crude drugs and their constituents is of great importance. According to cultivated varieties, variations of main components including D-carvone (1.68-75.92%) and D-limonene (14.69-47.8%)[12-20] have been found.

In addition, medicinal plants essential oils have been used as alternative nature anti-microbial agent in food preservation because their antimicrobial activities have been reported previously[12, 14, 16, 18, 20, 26]. Thus, its activity depends on the bioactive components in essential oil. Some reports indicated that D-limonene and D-carvone generates antimicrobial activity against some microorganisms such as: *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Escherichia coli, Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Aspergillus niger* and *Candida albicans*[16, 27-30].

In Thailand, the aerial part of dill (dill weed) is normally used as a favorite seasoning agent in food, but dill fruit is not used. No quality information was provided for the dill fruit used in Thai traditional medicine industries which are mostly imported from China or India. So, the aim of this study was to investigate the chemical compositions and antibacterial activity against food-borne pathogens and especially oral pathogens which has never been studied before of essential oils from dill or *A. graveolens* L. fruits that cultivated in Thailand.

2. MATERIALS AND METHODS

2.1. Raw material

The dried mature fruits of cultivating dill (*A. graveolens* L.) in Udon Thani province were collected. The sample was checked and approved by Prof. Dr.Wongsatit Chuakul, Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. The contaminants such as insects, stones and branches were then removed from the samples before drying in the hot air oven at 50°C, 24h. Samples were kept in clean containers and stored in a refrigerator (4°C) until further use.

2.2. Isolation of essential oils

hydrodistillation method 50 g of dill fruits were grinded, and put into a 500-ml round bottom flask of Clevenger apparatus. Five hundred ml of water was added and the temperature was set at 100-110°C. Steam distillation with modified Clevenger apparatus was also used for isolation of volatile oil by 50 g of dill fruits were grinded and put into an upper glass chamber which exposed to steam after heating the water inside the lower round bottom flask. Essential oil from both extraction methods were distilled, until exhausted (5 h). The upper layer was separated and dried over anhydrous sodium sulphate (Na_2SO_4). The essential oil was kept in a light protected, well-closed container and stored in a refrigerator (4°C). Yield based on dried weight were calculated (triplicate experiments).

2.3. Gas chromatographic-mass spectral (GC-MS) analysis

The GC-MS analyses was performed on GC-2010 (Shimadzu Ltd., Kyoto, JAPAN) with AOC 20i auto injector; split injector was used with a 10-µl syringe. The sample solution at 0.125µl/ml was injected under column oven temperature program which was as follow. The initial oven temperature was kept constant at 60°C for 1 min, then heated to 100°C at 5°C/min, to 120°C at 2°C/min, to 180°C at 6°C/min. A split ratio was set at 50:1; helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. For the chromatographic separation a fuse-silica capillary column 30 m × 0.25 mm i.d. × 0.25 µm (film thickness), using DB-5MS column (J&W Scientific, USA) was employed. The compound was detected by a series QP 2010 Plus quadrupole mass spectrometer scanning in full-scan and SIM (selected ion monitoring) mode. The electron energy was 70 eV. The temperature of transfer line was 220°C; ion source temperature 200 °C and MS quad 250°C. The mass spectrometer was operated in electron-impact (EI) mode with 1.2 kV detection volts; the scan-range was 40 – 400 atomic mass units (amu); 0.50 s interval and 1000 amus the scan speed. The components of essential oils were identified by comparison of their mass spectra with those in the Wiley 275 and NIST (National Institute for standard and Technologies) 98 spectral library. Analytical data from GC/MS QP 2010 Plus were collected and processed using GC/MS solution software Version 2.21 running on Microsoft® Window XP. This condition of GC-MS has been validated. The qualitative analysis was based on the percentage of area under the curve of each peak of the sample. In the quantitative analysis, D-limonene and D-carvone were used as the standards for calculating the concentration and n-tetradecane was used as internal standard. Sample was dissolved with methanol.

2.4. Bacterial Cultures

Essential oils of dill fruit and their main components, D-limonene and D-carvone were tested on ten reference strains representing for foodborne pathogenic bacteria: *Staphylococcus aureus* (ATCC 6538), *S. aureus* (ATCC 25923), *S. aureus* (ATCC 29213), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300), *Bacillus subtilis* (ATCC 6633), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) and *P. aeruginosa* (ATCC 27853). One reference strain representing for oral pathogenic bacteria: *Streptococcus sorbrinus* (ATCC 33478) was also used. The microorganisms were grown and maintained on Tryptic Soy Agar (TSA). The inoculated plates were incubated at 37°C for 24 h. The isolated colony was inoculated to Mueller Hinton broth (MHB) and incubated overnight at 37°C. After incubation, the microorganism cultures were used for the assay.

2.5. Bacterial activity assay

Antibacterial activity of dill oils was evaluated using micro-broth dilution assay[31] to determine the minimal inhibitory concentration (MIC): Stock solution of each sample was prepared by dissolve in 100% DMSO to give a concentration of 250 mg/ml. Various concentrations of sample solutions were prepared with two-fold serial dilutions using Mueller Hinton Broth (MHB) containing 0.5% Tween 20 and final concentration at DMSO in the assay was less than 8%, to give concentrations ranging from 20 mg/ml to 0.625 mg/ml.

Suspensions of each test microorganism with turbidity equivalent to 0.5 MacFarland standard was prepared by cultured and diluted as mentioned above to yield 10^8 CFU/ml. And then each test microorganism was diluted again using MHB to give concentration of 10^6 CFU/ml before tests. The final volume of each dilution in a 96-well microtiter plate was one hundred microliter was prepared by mixing between fifty microliter of tested bacterial and fifty microliter of samples, which was incubated at 37°C for 24 h.

The inhibition of bacterial growth or the minimal inhibitory concentration (MIC) was observed by choosing clear well in a 96-well microtiter plate. The minimal bactericidal concentration (MBC) was also determined after MIC test by all concentrations of substance that inhibited growth of bacteria were performed by transferring and streak on agar plate (MHA) and then incubated at 37°C for 24 h. The lowest concentration of each substance that destroy particular microorganisms test was recorded. The negative growth controls was inoculated growth medium in MHB and MHB + 0.5% tween 20 without samples. Ciprofloxacin was used for positive growth control which prepared by serial two-fold diluted to give final concentration from 100 µg/ml to 0.78 µg/ml.

2.6. Statistical Analysis

Analysis of variation was performed by one-way ANOVA with a 95% confidence interval (Significantly different at $p < 0.05$).

3. RESULTS AND DISCUSSION

The oil yields of dill fruit that cultivated in Thailand isolated by two extraction methods is summarized in **Table 1**. The essential oils have a pale yellow color. The hydrodistillation produced the higher oil yields when comparing to steam distillation method. These results are consistent with report of Sefidkon *et al*[21] about the effect of distillation method on oil yield. It may be explained that hydrodistillation method was carried out by immersing samples in boiling water which making its exposed to higher temperature than steaming, consequently breaking down the vittae structures containing volatile oil and let the volatile oil come out easier. The yield of essential oil from dill fruit cultivated in Thailand was range 1.05 to 2.01%. However, these results were lower than previous reports of Yili *et al*[13] and Vokk *et al*[17].

Table 1. Yields of essential oils from dill fruits (*Anethum graveolens* L.) that cultivated in Thailand (Udon Thani province) distilled by two methods (n=3)

Method	Yield of essential oil* (% w/w)
Hydrodistillation	2.01±0.25**
Steam distillation	1.05±0.07**

*Express as mean±SD (n=3)
**Significantly different at $p < 0.05$ in one-way ANOVA

D-limonene and D-carvone concentrations of dill fruits oils from cultivated in Thailand distilled by two difference methods is summarized in **Table 2**. Essential oil isolated by steam distillation contained higher content of D-limonene and D-carvone than oil isolated by hydrodistillation. D-limonene concentrations of dill oils obtained by hydrodistillation and steam distillation were 26.44 and 44.14 w/v, respectively. Whereas, D-carvone contents in dill oils using hydrodistillation and steam distillation were 17.1 and 27.14 w/v, respectively. D-carvone contents in our samples considering to be lower than the standard requirement provided by British pharmacopoeia 2014[11] which are 43-63% w/w.

The chemical compositions of essential oils of dill fruits cultivated in Thailand (Udon Thani province) distilled by two methods is summarized in **Table 3**. Twelve constituents of dill oils by both processes were identified by GC-MS. The oils showed similar main constituents including dillapiole (19.98-48.9%), D-carvone (18.05-28.02%) and D-limonene (26.96-44.61%). Other components such as β-pinene (0.79%), *β*-myrcene (0.16-0.21%), decane (0.44-0.49%), 1,5,8-p-menthatriene (0.19-0.27%), undecane (0.34-0.38%), naphthalene (1.63-2.11%), *cis*-dihydrocarvone (0.38-0.95%), *trans*-dihydrocarvone (1.49-1.57%) and myristicin (0.67-1.41%) were presented.

Table 2. D-limonene and D-carvone concentrations of dill fruits (*Anethum graveolens* L.) oils cultivated in Thailand (Udon Thani province) obtained by two extraction methods

Methods	Concentration * (w/v)	
	D-limonene	D-carvone
Hydrodistillation	26.44±0.23**	17.1±0.05**
Steam distillation	44.14±0.17**	27.14±0.09**

*Express as mean±SD (n=3)
**Significantly different at $p < 0.05$ in one-way ANOVA

Table 3. Chemical compositions of dill oils (*Anethum graveolens* L.) that cultivated in Thailand (Udon Thani province) by using hydrodistillation and steam distillation methods

Peak no	Compounda	Formular	Relative content % (Rt (min))	
			Hydrodistillation	Steam distillation
1	*β*-pinene	$C_{10}H_{16}$	no	0.79 (6.368)
2	*β*-myrcene	$C_{10}H_{16}$	0.21 (6.607)	0.16 (6.619)
3	Decane	$C_{10}H_{22}$	0.44 (6.870)	0.49 (6.876)
4	1,5,8-p-menthatriene	$C_{10}H_{14}$	0.19 (7.042)	0.27 (7.028)
5	D-limonene	$C_{10}H_{16}$	**26.96 (7.656)**	**44.61 (7.661)**
6	Undecane	$C_{11}H_{24}$	0.34 (9.524)	0.38 (9.512)
7	Naphthalene	$C_{10}H_8$	1.63 (12.301)	2.11 (12.307)
8	*Cis*-dihydrocarvone	$C_{10}H_{16}O$	0.38 (12.785)	0.95 (12.794)
9	*Trans*-dihydrocarvone	$C_{10}H_{16}O$	1.49 (13.034)	1.57 (13.042)
10	D-carvone	$C_{10}H_{14}O$	**18.05 (14.560)**	**28.02 (14.568)**
11	Myristicin	$C_{11}H_{12}O_3$	1.41 (25.125)	0.67 (25.132)
12	Dillapiole	$C_{12}H_{14}O_4$	**48.9 (27.580)**	**19.98 (27.585)**

[a]Peak identifications are base on MS comparisons with file spectra and retention time (Rt)
no : could not detected

In this study, dillapiole was the highest content of essential oil as well as in the studies of Babri *et al*[22] and Ashraf *et al*[23]. It has been reported that dillapiole could be used as insecticide synergist[24] which our dill oil might be used as alternative for new source of dillapiole. D-carvone and D-limonene are the major constituents of dill fruits oil. In this case, D-carvone contents of oil (18.08-27.57%) were higher than previous reports[13] but less than report of Jianu *et al* (D-carvone 34.17%)[18], Mahmoodi *et al* (D-carvone 36.09%). Another one compound found in high content in the oil was D-limonene (23.57-44.5%) which was more than other reports[13-17].

Our result indicated that dill fruits oil cultivated in Thailand is qualitatively agreeable with previous reported by *Kelly et al.* (D-carvone 17.9–64.0% and D-limonene 28-47.8%)[19]. But it is not desirable when compare with main constituent of caraway fruit (*Carum carvi* L.) oil from report of Laribi *et al* (D-carvone 61.58–77.35% and D-limonene 16.15–29.11%)[25]. The result of this study indicated that steam distillation method is suitable for isolating essential oil of dill fruits (*Anethum graveolens* L.) because the content of dillapiole, compound with insecticidal effects was low while the contents of active compounds, D-limonene and D-carvone were high when comparing with hydrodistillation method. This may be due to the temperature of steam distillation was lower than hydrodistillation method and plant materials were not immersed in water. Some components with high boiling points like dillapiole (around 294°C) cannot vaporize from plant materials when distilled with steam.

The antibacterial activity of essential oils from dill fruits (*Anethum graveolens* L.) cultivated in Thailand distilled by two methods and their major constituents against tested microorganisms on minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) is summarized in **Table 4.** Our results indicated that their major compounds as D-limonene and D-carvone exhibited antibacterial activity against some Gram positive and some Gram negative bacteria. *S. aureus* (MRSA) ATCC 43300 was the most sensitive strain to both D-limonene and D-carvone with MIC values of 0.3125 and 1.25 mg/ml, respectively and MBC values of 1.25 and 2.5 mg/ml, respectively. Strains of *S. aureus* ATCC 25923*, S. sorbrinus* ATCC 33478, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739 and *S. typhimurium* ATCC 14028 were more sensitive to D-limonene than D-carvone and dill oils with the MIC values of 1.25-5 mg/ml and MBC values of 1.25-10 mg/ml. Whereas, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213 and *K. pneumoniae* ATCC 700603 had quite the same MIC and MBC values with rang 5-10 mg/ml in both D-limonene and D-carvone. *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 9027 were not killed by all tested concentrations of samples.

The EOUH showed antibacterial activity against *E. coli* ATCC 8739, *K. pneumoniae* ATCC 700603 and *S. typhimurium* ATCC 14028 with MIC values of 10 mg/ml (equivalent to D-limonene content of 2.64 mg/ml and D-carvone content of 1.71 mg/ml by calculation) whereas the EOUS showed antibacterial activity against *S. aureus* ATCC 25923, *S. aureus* ATCC 29213 and *S. typhimurium* ATCC 14028 with MIC values of 10 mg/ml (equivalent to D-limonene content of 3.82 mg/ml and D-carvone content of 2.4 mg/ml by calculation). It has been reported by Jianu *et al.*[18] that dill oil (D-carvone 34.17% and D-limonene 40.19%) had an inhibitory effect against Gram positive bacteria (*S. aureus*, *Streptococcus pyogenes* and *Clostridium perfringens*) and Gram negative bacteria (*Shigella flexneri*, *K. pneumoniae*, *S typhimurium* and *E. coli*). However, the dill oils showed no activity against some strains even though the concentrations of D-limonene and D-carvone in the oils were higher than those of standard compound. Regarding to the MIC values of EOUH and EOUS in comparison with equivalent content of D-limonene and D-carvone, anti-bacterial activity of dill oils against tested bacteria might not only depend on major components, but also depend on the other composition existing in oils. The various compounds existing in dill oils may play roles as synergist or antagonist for antibacterial activity. Moreover, the characteristic of each bacterial strain is different and effects on antibacterial susceptibility. According to many factors, the antibacterial activity of dill oils cannot be directly compared with pure compound.

4. CONCLUSION

The dill fruit (*Anethum graveolens* L.) oil contents and compositions were varied depending on the extraction method. The anti-bacterial activity of dill oils was also reported. In the future, dill cultivated in Thailand may be used instead of imported samples along with the improvement of varieties and the cultivation method based on the active compound content which should be used as the quality control of dill fruit products.

Table 4. Antibacterial activities of dill oils (*Anethum graveolens* L.) obtained from two extraction methods as well as some of their major compounds representing as on MICs and the MBCs values

Strains		MIC[a] and MBC[b] value (mg/ml)								Ciprofloxacin (µg/ml)
		D-limonene		D-carvone		EOUHc		EOUSd		
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
Gram positive	*Staphylococcus aureus* ATCC 6538	5	10	5	5	>10	>10	>10	>10	<0.78
	S. aureus ATCC 25923	2.5	5	2.5	2.5	>10	>10	10	>10	25
	S. aureus ATCC 29213	5	10	10	10	>10	>10	10	>10	< 0.78
	Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300	0.3125	1.25	1.25	2.5	>10	>10	>10	>10	50
	Bacillus subtilis ATCC 6633	1.25	1.25	10	10	>10	>10	>10	>10	< 0.78
	Streptococcus sorbrinus ATCC 33478	1.25	5	10	10	>10	>10	>10	>10	12.5
Gram negative	*Salmonella typhimurium* ATCC 14028	2.5	5	5	5	10	>10	10	>10	< 0.78
	Pseudomonas aeruginosa ATCC 27853	>10	>10	>10	>10	>10	>10	>10	>10	< 0.78
	P. aeruginosa ATCC 9027	>10	>10	>10	>10	>10	>10	>10	>10	< 0.78
	Klebsiella pneumoniae ATCC 700603	10	10	10	10	10	>10	>10	>10	< 0.78
	Escherichia coli ATCC 8739	2.5	5	5	5	10	>10	>10	>10	< 0.78

[a]The minimal inhibitory concentration (MIC)
[b]The minimal bactericidal concentration (MBC)
[c]EOUH = Essential oil of dill fruit from Udon Thani province distilled by hydrodistillation
[d]EOUS = Essential oil of dill fruit from Udon Thani province distilled by steam distillation

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