Original Article

TJPS
The Thai Journal of Pharmaceutical Sciences
39 (3), July-September 2015: 94-101

In vitro biological activities of black pepper essential oil and its major components relevant to the prevention of Alzheimer's disease

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Abstract

Black pepper oil and its major components were investigated for the biological activities related to the prevention of AD. The inhibition of acetylcholinesterase (AChE) and β-amyloid aggregation, anti-inflammatory and antioxidant activities were examined. GC-MS analysis of black pepper oil identified 33 constituents with δ-3-carene, limonene, (-)-β-pinene, α-pinene and caryophyllene as the main components. The AChE inhibitory activity by microplate assay revealed that black pepper oil exhibited potent AChE inhibition with an IC_{50} value of 5.97 μg/ml. Among the compounds tested, δ-3-carene exhibited AChE inhibitory activity with an IC_{50} value of 20.50 μg/ml. β-Amyloid aggregation inhibitory activity was determined by a thioflavin T assay. Black pepper oil exhibited a weak inhibitory activity with 33.17 ± 6.67 % inhibition at 100 μg/ml, while limonene possessed stronger inhibitory activity with an IC_{50} value of 3.77 μg/ml. Anti-inflammatory activity was determined by a COX-2 activity assay in THP-1 cells. The oil and caryophyllene showed strong COX-2 inhibition. The weak antioxidant activity of black pepper oil and its major components was observed in TLC/DPPH, microplate assays and ESR analysis. These findings suggest that black pepper oil may be beneficial in lowering the risk of Alzheimer's disease via AChE inhibition and anti-inflammatory activity via COX-2 inhibition.

Key Words: Acetylcholinesterase, β-Amyloid aggregation, Anti-inflammatory activity, Antioxidant activity, *Piper nigrum* L.

Introduction

Alzheimer's disease (AD) is the leading neurodegenerative disease characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions. The exact cause of AD is still uncertain. Current therapeutic strategies for treating AD are based on the pathogenesis found in AD patients. Significant changes, that are observed in the brains of AD patients, include a decrease in hippocampal and cortical levels of acetylcholine (ACh), which plays a major role in learning and memory functions, and the appearance of neuritic plaques, which are composed of β-amyloid

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Received: 8 May 2015
Revised: 29 June 2015
Accepted: 29 June 2015

Academic Editor: Pithi Chanvorachote

proteins $A\beta_{1-40}$ and $A\beta_{1-42}$, and tau-based neurofibrillary tangles [1-3]. Oxidative stress and inflammation are also reported to be involved in the pathogenesis of AD [4, 5]. Thus, the inhibition of acetylcholinesterase (AChE), the enzyme involved in the metabolic hydrolysis of ACh at cholinergic synapses in the central and peripheral nervous system, β-amyloid aggregation, oxidative stress and inflammation are needed to decrease the progression of AD. Currently, many groups of compounds are under research and development processes for the treatment of AD. These include: (i) AChE inhibitors, (ii) anti-inflammatory agents, (iii) antioxidants, (iv) β-secretase and γ-secretase inhibitors, (v) γ-secretase modulators, and (vi) β-amyloid aggregation inhibitors [6]. In this study, AChE and β-amyloid aggregation inhibitory, anti-inflammatory and DPPH radical scavenging assays were selected to determine the potential of black pepper oil as an AD preventing agent.

Recently, food plants have gained increasing attention in researches related to AD. Blueberry, strawberry, pomegranate, papaya, apple, green tea, walnut, saffron, cinnamon, garlic and ginger are among edible plants exhibiting neuroprotective effects against AD [7]. Various essential oils from spices have shown potent AChE inhibition, such as the essential oils of *Salvia* species [8], turmeric, galanga and fingerroot [9]. Moreover, the constituents of essential oils have been reported to possess many biological activities related to AD [10-16]. Their lipophilicity and small molecular size which facilitate the passage through the blood-brain barrier is responsible for their activities [17]. Our ongoing program has found that black pepper oil exhibits a strong AChE inhibition by microplate assay. This finding and the recent trend of consuming functional foods to decrease the risk of degenerative diseases led us to perform extensive studies on other biological activities related to the prevention of AD. To verify the potential of black pepper oil in the prevention of AD, the inhibition of β-amyloid aggregation, the anti-inflammatory and the antioxidant activities of black pepper oil, as well as its major compounds were investigated.

Materials and Methods

Plant material: Essential oil of black pepper fruit (*Piper nigrum* L.) was obtained from Thai-China Flavours and Fragrances Industry Co., Ltd. (Bangkok, Thailand).

Enzymes and chemicals: 6-Hydroxy-2,5,7,8-tetra methylchroman-2-carboxylic acid (Trolox), δ-3-carene, *trans*-caryophyllene, curcumin, 2,2-diphenyl-1-picryl hydrazyl (DPPH), limonene, lipopolysaccharide (LPS; from *E. coli* strain O55:B5), β-myrcene, phorbol 12-myristate-13-acetate, α-pinene, (-)-β-pinene and *R*-(-)-α-phellandrene were purchased from Sigma-Aldrich. Roswell Park Memorial Institute (RPMI) 1640 medium (with L-glutamine without $NaHCO_3$) was obtained from GIBCO BRL. Fetal bovine serum (FBS) and penicillin-streptomycin were obtained from JRS-JR Scientific. The COX activity assay kit was purchased from Cayman Chemical Company. Analytical grade methanol, ethanol, DMSO and Tween-20 were purchased from Merck. GC grade hexane was obtained from Fischer Scientific and analytical grade ethyl acetate and toluene were purchased from JT Baker.

Acetylthiocholine iodide (ATCI), lyophilized powder of AChE (a purified enzyme from eel [*Electrophorus electricus*] type VI-s, 425.94 units/mg, 687 U/mg protein), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), galanthamine and bovine serum albumin (BSA), were obtained from Sigma-Aldrich. Fifty mM Tris-HCl (pH 8.0) was used as a buffer throughout the experiment. The lyophilized enzyme was prepared in the buffer to obtain 100 U/mL. It was then further diluted with the buffer containing 0.1% BSA to yield 0.22 U/ml for the microplate assay, or diluted with the buffer to 3 U/mL for the TLC assay.

β-$Amyloid_{1-42}$ and thioflavin T (ThT) were purchased from American Peptide Company (Sunnyvale, CA, USA) and Sigma-Aldrich, respectively. A stock ThT solution (1 mg/mL; 3.14 mM) was prepared in deionized, distilled water. Forty microliters of ThT solution was added to 50 mM Tris buffer, pH 7.4 to yield a final 25 mL of 5.0 μM ThT solution. β-Amyloid stock solution was prepared by dissolving β-amyloid in 10 mM NaOH (0.5 mg/mL) and stored at -70°C until used. β-Amyloid stock solution (216.4 μl) was added to the Tris-HCl buffer (738.6 μl) to yield a final concentration of 25 μM β-amyloid solution.

Cell culture: THP-1, a promonocytic cell line, was purchased from the American Type Culture Collection (ATCC No. TIB-202). Cells were maintained in a RPMI-1640 medium, which was supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10 % heat inactivated FBS and incubated at a temperature of 37°C in a humidified atmosphere of 5 % CO_2.

Determination of chemical compositions of essential oil: Black pepper oil was analyzed by GC-MS (Shimadzu QP2010) using a DB-5ms bonded phase fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific). Helium was used as carrier gas at a flow rate of 0.68 mL/min. The injection port temperature was set at 250°C. The oven temperature was increased from 40°C (5 min hold) to 220°C in three different steps as follows: step 1 from 40°C to 80°C at a rate of 3.0°C/min, step 2 from 80°C to 135°C at a rate of 1.5°C/min, and step 3 from 135°C to 220°C at a rate of 10.0°C/min. Finally, it was held isothermally at 220°C for 5 min. The following significant quadrupole MS operating parameters were used: an interface temperature of 250°C and electron impact ionization was set at 70 eV, with a scan mass range of 40-400 m/z at a sampling rate of 1.0 scan/s. Compounds were identified by computer search using the Wiley and NIST digital libraries of mass spectral data and by comparison of their retention times and authentic mass spectra with reference standards.

AChE inhibitory assay: In the TLC assay, black pepper oil and its major components were dissolved in methanol to the concentrations of 50 and 20 mg/mL, respectively. The suitable amount of each sample was

applied to two silica gel G60 F_{254} aluminium plates, and then developed with toluene-ethyl acetate (93:7). After the plates were dried, the first plate was viewed under ultraviolet light at the wavelengths of 254 nm and 366 nm and documented by photography. Then the plate was sprayed with anisaldehyde-sulfuric acid reagent and heated for 5 min on the TLC plate heater (CAMAG TLC Plate Heater III) at 110°C until optimal colorization was observed. The activity of the separated components was detected by spraying another plate with the substrate, dye and enzyme based on Ellman's protocol [18]. The plate was sprayed with ATCI reagent (1mM ATCI in buffer) followed by DTNB reagent (1mM DTNB in buffer) until the silica was saturated with the reagent. It was allowed to dry for 5 min and then sprayed with 3 U/mL of enzyme solution. A white spot, which indicates AChE inhibitory activity, appeared on the yellow background after 5 min. The results were recorded within 15 min.

The microplate assay was performed by the modified Ellman's coloric method using 96-well plates [19, 20]. Briefly, 125 µl of 3 mM DTNB in buffer containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$, 25 µl of 15 mM ATCI in deionized water, 50 µl of buffer containing 0.1 % BSA and 25 µl of sample (in buffer containing 50 % methanol) were added to the wells followed by 25 µl of 0.22 U/mL AChE. Methanol which has no effect on the assay was used as the solvent. The absorbance was measured at 405 nm before adding the enzyme and was measured again every 45 s for 5 cycles, after adding the enzyme, by a microplate reader (M200, Tecan, Switzerland). The assay was done in triplicate. Galanthamine was used as a positive control. The AChE inhibitory activity was calculated from the differences between the absorbance values of the control and the sample, and expressed as percentage inhibition. The percentage of enzyme inhibition was calculated as

$$\% \text{ AChE inhibition} = \{[(A-B) - (C-D)]/(A-B)\} \times 100$$

where A is the absorbance of control (reagent + methanol + enzyme), B is the absorbance of blank of control (reagent + methanol), C is the absorbance of sample (reagent + sample + enzyme), and D is the absorbance of blank of sample (reagent + sample + methanol). The concentration that inhibited 50 % of AChE activity (IC_{50}) was obtained from a graph that was plotted between the percentage of AChE inhibition and the concentration of the sample.

Thioflavin T assay: β-Amyloid aggregation inhibitory activity was assayed by the thioflavin T method [21, 22]. DMSO which has no effect on the assay was used as the solvent. Nine microliters of β-amyloid solution (25 µM) and 1 µl of DMSO were added to the wells. The solution was thoroughly mixed and incubated at 37°C in the dark for 48 h ($F_{A\beta}$). The reaction mixtures for the positive control and the samples were prepared as follows:

F_S (sample) = 1 µl of positive control, sample + 9 µl of Aβ solution

F_{Sb} (sample blank) = 1 µl of positive control, sample + 9 µl of Tris buffer

F_{Rb} (reagent blank) = 1 µl of DMSO + 9 µl of Tris buffer

After mixing, the reaction mixtures were incubated at 37°C in the dark for 48 h. Then 200 µl of 5 mM ThT solution was added into each well. Fluorescence absorption was measured at the excitation wavelength of 446 nm and emission wavelength of 500 nm by a microplate spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, USA). The assay was done in triplicate. Curcumin was used as a positive control. The percentage inhibition of β-amyloid aggregation was calculated as

$$\% \text{ inhibition} = \{1- [(F_S-F_{Sb})/(F_{A\beta}-F_{Rb})]\} \times 100$$

where F_S is the absorbance of sample, F_{Sb} is the absorbance of sample blank, $F_{A\beta}$ is the absorbance of free aggregation control, and F_{Rb} is the absorbance of reagent blank.

IC_{50} values were determined by testing in 5-6 concentrations at a range of 0.01 - 100 mM for the major components and positive control, and at concentrations between 0.001 - 10 mg/mL for black pepper oil. The IC_{50} value was calculated from a graph that was plotted between the percentage inhibition of β-amyloid aggregation and the log concentration of the sample, using GraphPad Prism 4.0 software.

COX-2 activity assay: COX-2 inhibitory activity was quantified using a COX activity assay kit and performed according to the manufacturer's instructions [23]. Briefly, THP-1 cells were treated with 40 µg/mL PMA for 48 h to differentiate monocytes into macrophages. After washing the cells twice with PBS, it was cultured in FBS-free RPMI-1640. Black pepper oil, its major compounds and celecoxib, the positive control, were added to each well. DMSO at the final concentration of 0.1 % which showed no effect on the assay was used as the solvent. One hour later, the inflammation was induced by adding 1 µg/ml LPS and the reaction mixture was incubated for 24 h. Then, the cells were scraped and homogenized in Tris-HCl, pH 7.8, containing 1 mM EDTA. After centrifugation, the supernatant obtained was used as the source for COX enzyme. Then, 60 µM SC-560 [5-(4-methoxyphenyl)-3-trifluoromethylpyrazole, a highly selective COX-1 inhibitor, was added to eliminate all COX-1 activity. The reaction was initiated with the addition of arachidonic acid for 5 min at 25°C. COX-2 inhibitory activity was evaluated according to the manufacturer's instructions.

DPPH scavenging activity: In the TLC/DPPH assay, black pepper oil and its major constituents were subjected to TLC analysis using the same conditions mentioned in the AChE inhibitory assay section. The first plate was sprayed with anisaldehyde-sulfuric acid reagent and heated for 5 min on the TLC plate heater at 110°C until optimal colorization was observed. Another plate was sprayed with a 0.2 % DPPH reagent in methanol and was

left for 8 h in the dark at room temperature. The plate was observed under white light. Active radical scavengers appeared as yellow color zones against a purple background [24].

The microplate assay was performed using a microplate reader (Tecan M200, Switzerland) as follows [24]. Methanol which has no effect on the assay was used as the solvent. Reaction mixtures containing test samples (dissolved in methanol) at various concentrations (at least five) and 20 µl of 1 mM DPPH methanolic solutions in 96-well microtiter plates were incubated at room temperature for 10 min, and absorbances were measured at 517 nm (A_{sample}). The degree of discoloration indicates the scavenging activity of the compound tested. A blank experiment was also carried out using the same procedure without the test material and the absorbance was recorded as A_{blank}. The assay was done in triplicate and Trolox was used as the positive control. The free radical scavenging activity of each solution was then calculated as $100 \times ((A_{blank} - A_{sample})/A_{blank})$.

The DPPH scavenging assay using the ESR trapping technique was carried out according to the method of Nanjo et al. [25] with a slight modification. Methanol which has no effect on the assay was used as the solvent. The test samples were dissolved in methanol to the concentrations of 4 mg/ml for black pepper oil and 10 mM for its major components. A reaction mixture containing 100 µl of 0.25 mM DPPH in methanol, 50 µl of methanol and 50 µl of the test sample was transferred to a capillary tube, placed into an ESR tube and fitted into the cavity of the X-band ESR spectrometer (E 500, Bruker Corporation, USA). After it had been incubated at an ambient temperature for 60 s, the ESR spectrum was recorded. The ESR measurement conditions were described herein: central field 3505 ± 50 G, modulation frequency 100 kHz, modulation amplitude 4.0 G, microwave power 49.88 mW, receiver gain 6.32×10^2 dB, conversion time 40.96 ms and time constant 1.28 ms. Trolox, the standard antioxidant, was used as a positive control. The assay was performed in triplicate and the free radical scavenging activity was calculated as percentage inhibition.

Results and Discussion

Gas chromatography-mass spectrometry (GC-MS) analysis of black pepper essential oil identified thirty three constituents. δ-3-Carene was the major component (30.75 %) followed by limonene (25.41 %), (-)-β-pinene (12.18 %), α-pinene (6.32%) and caryophyllene (2.66 %) (**Table 1** and **Figure 1**). The results from previous reports indicated that there are three types of black pepper oil: sabinene/caryophyllene, caryophyllene and δ-3-carene/ caryophyllene/limonene types [26]. Our study suggests that Thai black pepper oil is the δ-3-carene/ caryophyllene/limonene type. The five major components obtained from GC-MS analysis were subjected to biological assays.

Table 1 Chemical constituents of black pepper essential oil by GC-MS analysis

Peak No.	Components	Retention time (min)	Area percentage[a]
1	α-Thujene	13.133	0.12 ± 0.00
2	α-Pinene	13.478	6.32 ± 0.06
3	Camphene	14.320	0.11 ± 0.01
4	β-Phellandrene	15.619	0.56 ± 0.02
5	(-)-β-Pinene	15.836	12.18 ± 0.09
6	β-Myrcene	16.619	2.64 ± 0.03
7	δ-3-Carene	17.561	30.75 ± 0.19
8	Cymene	18.424	4.81 ± 0.02
9	Limonene	18.697	25.41 ± 0.14
10	Dehydro-*p*-cymene	21.675	0.61 ± 0.02
11	Linalool	22.944	0.17 ± 0.00
12	2-Pinen-4-ol	23.655	0.11 ± 0.01
13	Limonene oxide	24.951	0.26 ± 0.01
14	*trans*-Pinocarveol	25.432	0.18 ± 0.02
15	Pinocarvone	26.917	0.17 ± 0.01
16	*p*-Cymen-8-ol	28.341	0.23 ± 0.01
17	Myrtenol	29.364	0.21 ± 0.01
18	Estragole	29.582	0.11 ± 0.01
19	(-)-*cis*-Sabinol	29.906	0.67 ± 0.04
20	Eucarvone	30.380	0.32 ± 0.01
21	Carvone	32.921	0.21 ± 0.02
22	Carvacrol	37.304	0.13 ± 0.01
23	2-Pinen-4-one	37.717	1.78 ± 0.04
24	*exo*-2-Hydroxycineole	38.838	0.60 ± 0.02
25	Limonene dioxide	39.674	0.73 ± 0.02
26	δ-Elemene	40.042	2.31 ± 0.02
27	α-Copaene	43.095	0.13 ± 0.01
28	β-elemene	44.278	0.23 ± 0.02
29	Caryophyllene	46.422	2.66 ± 0.02
30	α-Humulene	49.210	0.18 ± 0.07
31	(-)-Caryophyllene oxide	57.605	4.22 ± 0.07
32	Spathulenol	59.159	0.23 ± 0.03
33	Tetracyclo[6.3.2.0(2,5). 0(1,8)] tridecan-9-ol, 4,4-dimethyl	59.366	0.90 ± 0.08

[a]Each value is expressed as a mean ± SD of three injections.

In this study, both TLC and microplate assays were performed to determine the AChE inhibitory activity of black pepper oil and its major components. The TLC

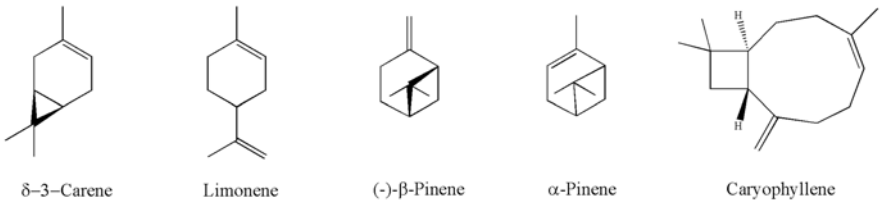

Figure 1 Structures of major compounds of black pepper oil

bioassay showed weak AChE inhibitory activity of black pepper oil and α-pinene (**Table 2**). AChE inhibitory activity of black pepper oil from this study corresponded with previous study [9]. However, black pepper oil exhibited the strong AChE inhibition using the microplate assay with an IC_{50} value of 5.97 μg/ml, which was 0.29-fold weaker than that of the positive control, galanthamine. The difference in the results obtained by two assays can be explained by a change in enzyme affinity for the compound when using the TLC method, which was caused by an interaction of the silica from the TLC plates with either AChE or the test samples [27]. Among the major compounds tested, δ-3-carene was the most potent component with an IC_{50} value of 20.50 μg/ml followed by α-pinene and caryophyllene. α-Pinene showed stronger inhibition than (-)-β-pinene. These results supported previous studies, which stated that limonene exhibited weak AChE inhibitory activity with 17.50 ± 1.14 % inhibition at 50 μg/ml [12] and was inactive at 1.36 mg/ml [11].

Miyazawa and Yamafuji [12] and Miyazawa et al. [10] explain the structure-activity relationship (SAR) of monoterpenoids such that 3.1.1 and 4.1.0 bicyclic hydrocarbons with an allylic methyl group exert strong AChE inhibition. Our results agree with this hypothesis; δ-3-carene, the 4.1.0 bicyclic monoterpenoid, was the strongest AChE inhibitor among the major compounds tested. The 3.1.1 bicyclic monoterpenoid, (-)-β-pinene, which has a terminal olefin, showed weaker AChE inhibition than α-pinene, which possesses an allylic methyl group. The presence of an isopropenyl group in limonene decreases the potency of it as an AChE inhibitor [10]. The AChEI activity of black pepper oil itself was more potent than each of its major constituents alone. Thus, it can be concluded that the AChEI activity of black pepper oil is not only as a result of the major components tested, but also other minor components. This study is the first to report on the strong AChEI activity of black pepper oil and its major active compound was identified as δ-3-carene.

A thioflavin T assay revealed the inhibition of β-amyloid aggregation of black pepper oil and its major compounds as shown in Table 2. Black pepper oil exhibited weak β-amyloid aggregation inhibitory activity. Limonene possessed strong inhibitory activity with an IC_{50} value of 3.77 μg/ml. Thus, limonene is the active compound that is thought to attribute to the activity of black pepper oil in this assay. Congo red, curcumin, indole derivatives, kaempferol, quercetin, resveratrol and bis-styrylbenzene derivatives (**Figure 2**) are compounds that have been isolated from plants that are reported to inhibit β-amyloid aggregation [3, 28]. Although their SARs are not clearly understood, the key features of some β-amyloid aggregation inhibitors have been reported. For example, the thiazolidine moiety is a key feature of thiazolidine derivatives [29]. However, the key features of curcumin include, an aromatic group at each end, a hydroxyl substitution on these aromatic rings, an optimum length (8-10 Å) and flexibility of the linker region [22]. The weak activity of other major components tested may be due to the structural differences between them and the aforementioned known inhibitors. The major compounds of black pepper oil lacked the key structural features, therefore they exhibited weak β-amyloid aggregation inhibitory activity. This is the first report on β-amyloid aggregation inhibitory activity of black pepper oil and its main components.

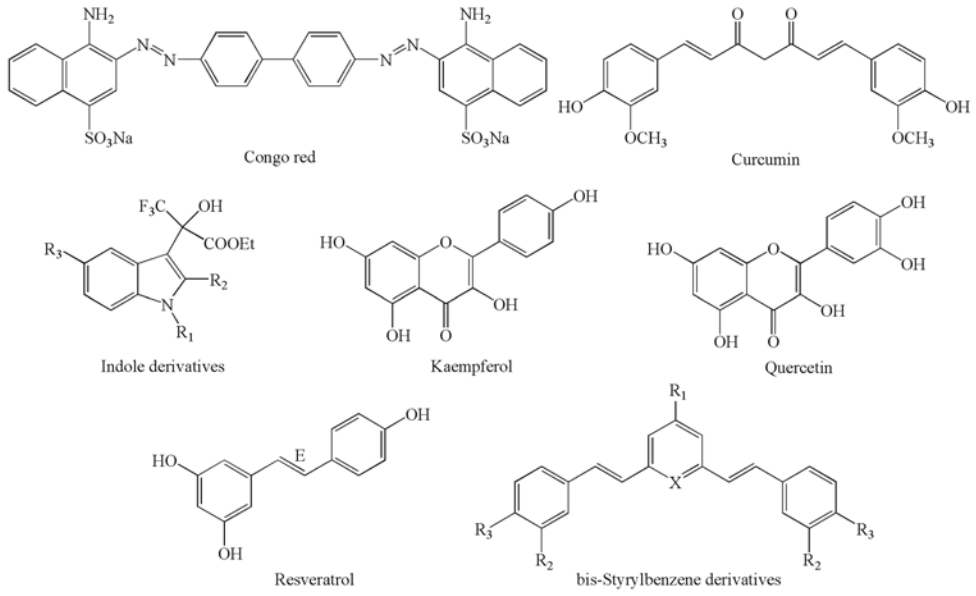

Figure 2 Structures of known β-amyloid aggregation inhibitors

Table 2 Biological activities related to AD prevention of black pepper oil and its major components.

	AChE inhibition			β-amyloid aggregation inhibition			Anti-inflammatory activity	Antioxidant activity				
		Microplate assay			IC_{50}		COX-2 inhibition	TLC/ DPPH assay	Microplate assay		ESR trapping technique	
Sample	TLC/AChE assay	% inhibition[a]	IC_{50} (μg/ml)	% inhibition[b]	μM	μg/ml	% inhibition[c]		% inhibition at 1 mg/ml	IC_{50} (μg/ml)	% inhibition[d]	IC_{50} (μM)
Black pepper oil	+	93.76 ± 2.12	5.97	33.17 ± 6.67	ND	ND	61.63 ± 19.73	++	inactive	ND	0.24 ± 0.42	ND
δ-3-Carene	–	62.75 ± 0.71	20.50	40.36 ± 0.87	ND	ND	inactive	–	7.76 ± 1.23	ND	2.60 ± 0.84	ND
Limonene	–	17.50 ± 1.14	ND	54.13 ± 1.76	27.66	3.77	31.40 ± 7.26	–	7.66 ± 0.17	ND	7.75 ± 0.46	ND
(-)-β-Pinene	–	13.54 ± 1.87	ND	40.12 ± 1.31	ND	ND	inactive	–	inactive	ND	1.07 ± 2.14	ND
α-Pinene	+	37.33 ± 0.24	ND	36.83 ± 0.94	ND	ND	inactive	+	inactive	ND	5.70 ± 0.44	ND
Caryophyllene	+	33.01 ± 2.92	ND	59.82 ± 1.26	228.00	46.59	67.90 ± 3.49	–	inactive	ND	inactive	ND
Galanthamine	+++	89.18 ± 2.84	1.73	NA	NA	NA	NA	NA	NA	NA	NA	NA
Curcumin	NA	NA	NA	93.22 ± 2.64	0.77	0.28	NA	NA	NA	NA	NA	NA
Celecoxib	NA	NA	NA	NA	NA	NA	73.84 ± 12.33	NA	NA	NA	NA	NA
Trolox	NA	NA	NA	NA	NA	NA	NA	+++	>100.0 ± 0.00	4.12	>100.0 ± 0.00	24.06

Values are presented as the mean ± SD (n = 3)
IC_{50} = the concentration of test sample that produces 50% inhibition.
NA = not assessed.
ND = not determined, due to the inhibition is below 50% at the concentration tested.
[a]Black pepper oil and its major components were tested at 50 μg/ml and the positive control, galanthamine, at 20 μg/ml.
[b]Black pepper oil was tested at 100 μg/ml, its major components and the positive control, curcumin, at 100 μM.
[c]Black pepper oil was tested at 100 μg/ml, its major components at 100 μM and the positive control, celecoxib, at 0.5 μM.
[d]Black pepper oil was tested at 1 mg/ml, its major components and the positive control, Trolox, at 2.5 mM.

The anti-inflammatory activity of black pepper oil and its major components was determined by a COX-2 activity assay in human monocytic (THP-1) cells. The results showed that black pepper oil and caryophyllene exhibited strong anti-inflammatory activity through COX-2 inhibition (**Table 2**). The activity of the oil and caryophyllene was close to that of celecoxib, the positive control, at the concentration tested. Limonene weakly inhibited COX-2 activity, while other major compounds were inactive. Thus, caryophyllene is the active compound responsible for the anti-inflammatory activity of black pepper oil in this assay. The major compounds of black pepper oil have been reported to possess anti-inflammatory activity via different mechanisms. δ-3-Carene has the ability to reduce carrageenan-induced pedal edema in rats [30]. Limonene has been found to decrease the productions of NO, PGE_2, IL-1β, IL-6 and TNF-α in LPS-activated RAW 264.7 cells [13]. α-Pinene has shown anti-inflammatory activity in carrageenan-induced paw edema model [31] and was able to inhibit the nuclear translocation of NF-κB induced by LPS in THP-1 cells [14]. β-Pinene has been shown to be active in the inhibitions of carrageenan-induced edema [32] and NO production in LPS-induced RAW 264.7 macrophages [33]. Caryophyllene was able to decrease TNF-α release, PGE production, as well as iNOS and COX-2 expression induced by platelet activating factor or carrageenan in rats [15]. This study, however, is the first to report on the inhibitory activity of black pepper oil and its major compounds on COX-2 in THP-1 cells.

Previous studies have stated that DPPH radical scavenging activity showed positive correlations with antioxidant activity, reducing power and total phenolic contents [34]. Therefore a DPPH assay, which is a simple and rapid method [35], was used to evaluate antioxidant activity in this study. Black pepper oil was found to be active in the TLC/DPPH assay but it was inactive at 1 mg/ml when using a microplate assay. The positive control Trolox exhibited a 50 % inhibition at 4.12 ± 0.30 μg/ml (**Table 2**). The radical scavenging activity of Thai black pepper oil was weaker than that of black pepper oil from India [36]. The difference between their activities resulted from the variation in their chemical compositions. The oil from India contained β-caryophyllene (29.90 %) as the major component [36], while Thai black pepper oil is rich in δ-3-carene (30.75 %). The major compounds of black pepper oil, at the concentrations presented in the essential oils, were applied on a TLC plate and determined for scavenging activity. δ-3-Carene, limonene, (-)-β-pinene and caryophyllene were inactive in the TLC/DPPH assay. δ-3-Carene and limonene exhibited weak antioxidant activity in the microplate assay. Although α-pinene, one of the major compounds, demonstrated weak antioxidant activity in the TLC/DPPH assay, it was inactive in the microplate assay. The activity of the major components, therefore, correlates with a previous report [16]. Black pepper oil and its major components were also tested for the DPPH scavenging activity using an electron spin resonance (ESR) trapping technique. It was found that all test samples exerted weak activity at the maximum solubility in methanol. Based on the results it can be concluded that black pepper oil and its major constituents exhibit weak radical scavenging activity. This is due to the lack of an active hydroxyl group on the monoterpenes in the oil [37]. Since the radical scavenging activity of black pepper oil and its major components were weak, other antioxidant methods were not performed. It can be concluded from the results of this study that the weak antioxidant activity of black pepper oil will not be attributed to the prevention of AD.

Conclusion

Black pepper oil could prove to be a useful agent for the prevention of AD, due to AChE inhibition and anti-inflammatory activity via COX-2 inhibition.

Acknowledgement

The authors are grateful to Mullika Traidej Chomnawang for her suggestion on the anti-inflammatory assay and Gita Shah for the language editing of the manuscript. This work was supported by the Co-ordinating Center for Research and Development to Increase Value of the Plants Indigenous to Thailand, Mahidol University and the Thailand Research Fund.

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