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Enzyme inhibitors from the aril of *Myristica fragrans*

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ABSTRACT

The fruit aril of *Myristica fragrans*, commonly known as "mace", is a popular spice in worldwide. Chromatographic separation of the combined ethyl acetate (EtOAc) and the methanol (MeOH) extracts of the aril of *Myristica fragrans* furnished malabaricone C (**1**), 3-(3-methyl-5-pentyl-2-furanyl)-2(*E*)-propenoic acid (**2**), licarin A (**3**), maceneolignan B (**4**) and elemicin (**5**). Compounds **1**–**5** were screened for acetylcholinesterase (AChE), and α-glucosidase enzyme inhibitory activities and DPPH radical scavenging activity. Compound **1** showed the highest AChE inhibitory activity (IC_{50} 2.06 ± 0.04 μg/mL) and antioxidant activity (IC_{50} 6.56 ± 0.02 μg/mL) while compound **2** displayed the most potent α-glucosidase inhibitory activity (IC_{50} 50.91 ± 0.01 μg/mL). This is the first report of α-glucosidase inhibitory activity of **2**. Results indicate that the aril of *M. fragrans* showed good anticholinesterase and α-glucosidase inhibitory activities and antioxidant effect *in-vitro* that have a potential to be used as the treatment of Alzheimer's disease.

1. Introduction

Herbal products are becoming popular as cognitive enhancers hence studies on plant based products on neurological disorders are of interest (Masondo et al., 2019). Neurological disorders have drawn a worldwide concern due to its increased manifestation especially in the aging population. Age-related neurological disorders such as Parkinson's and Alzheimer's diseases (AD) have common pathological features including oxidative stress, neuronal inflammation, neuronal loss and abnormal protein aggregation in the central nervous system. AD is a common cause of dementia that ultimately leads to death (Francis et al., 1999). Currently, 47 million people worldwide are estimated to be affected by dementia out of which 60% are from low and middle income family (WHO, 2017, 2018). There is an intriguing connection between type II diabetes and dementia (Sridhar et al., 2015) There are epidemiological, cognitive and neuropathological evidence of the link between type II diabetes and neurodegenerative diseases (WHO, 1999). Brain insulin resistance, insulin dysregulation, amyloidogenesis, neuroinflammation, oxidative stress, and mitochondrial dysfunction are some of the underlying mechanisms. As a result, neurodegeneration progresses, causing impaired cognitive function especially in elderly population (Lee et al., 2018). Inhibition of acetylcholinesterase enzyme (AChE) is one of the key approaches in AD drug discovery. Currently AChE inhibitors donepezil, tacrine and metrifonate are used for symptomatic treatment of AD causing temporary relief of the symptoms that last for 12–24 months. Due to the lack of effective treatment strategy, scientists have paid attention for many years to search for AChE inhibitors from natural sources with high potency and less side effects (Masondo et al., 2019).

Nutraceuticals derived from spices such as *Curcuma longa* L., *Piper nigrum* L., *Syzygium aromaticum* L. Merr., *Zingiber officinale* Roscoe, *Allium sativum* L., *Coriandrum sativum* L., and *Cinnamomum zeylanicum* Blume, are used to treat neurodegenerative diseases (Kannappan et al., 2011). There is an increase in demand for herbal drugs including AChE inhibitors. Natural products based AChE inhibitors approved by FDA include rivastigmine and galantamine. Huperzine, curcumin and resveratrol are in phase III clinical trials (Kumar et al., 2017).

In a continuation of our search for bioactive compounds from Sri Lankan grown spices, the present investigation was carried out on the aril ("mace") of *Myristica fragrans* of the family Myristicaceae. *M. fragrans* is a popular spice used worldwide. Here we report the isolation and identification of α-glucosidase and AChE and inhibitors from the aril of *M. fragrans*.

2. Materials and methods

2.1. General

All chemicals and solvents were of analytical grade and purchased from Sigma-Aldrich. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-AL300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer

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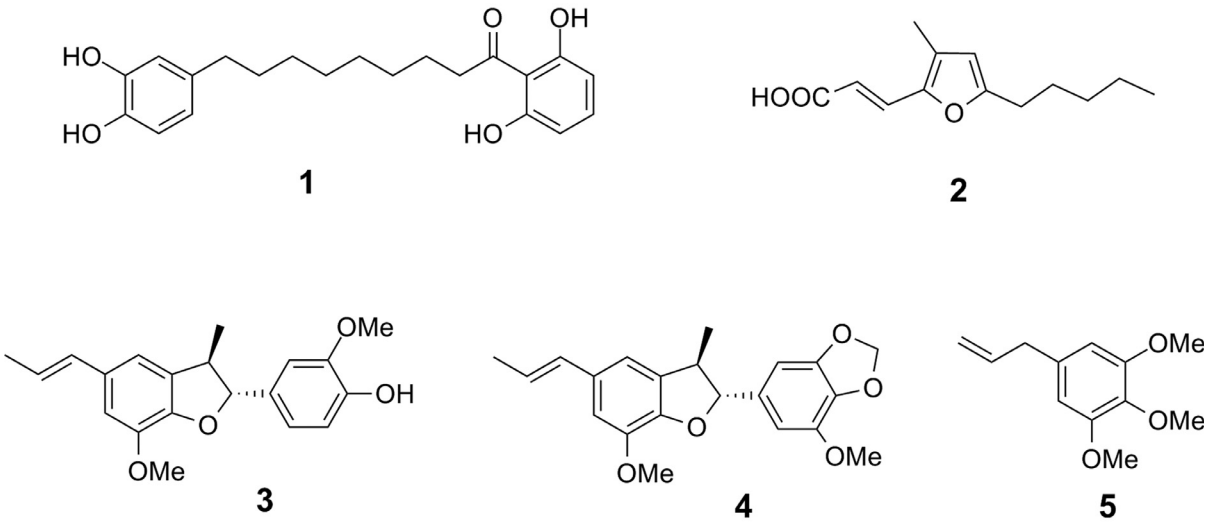

Fig. 1. Structures of compounds **1**–**5**.

in $CDCl_3$ or CD_3OD solution with Me_4Si as the internal standard. The mass spectra were obtained with Thermo Scientific LCQ Fleet MS Instrument. UV absorbance of the samples was taken using an Elisa micro plate reader (Synergy HTX multi-mode reader, BioTek instruments USA).

2.2. Plant material, extraction & isolation compounds

Arils of *M. fragrans* were purchased from a local market in Kandy, Sri Lanka. A voucher specimen (SS/2016/MF) is deposited at the Department of Pharmacy, Faculty of Allied Health Sciences, University of Peradeniya, Sri Lanka. The air dried aril (100 g) was sequentially extracted with *n*-hexane, dichloromethane (DCM), EtOAc and MeOH using ultrasonicator at room temperature. Solvents were evaporated using rotary evaporator to give *n*-hexane (18.20 g), DCM (8.60 g), EtOAc (5.61 g) and MeOH (3.37 g) extracts. All extracts were subjected to anticholinesterase assay (Ellman et al., 1961). Based on the similarity of TLC, both MeOH and EtOAc extracts were combined and chromatographed over silica gel (*n*-hexane-EtOAc-MeOH) to yield 6 fractions F_1-F_6 which screened for AChE activity. Chromatographic separation of these column fractions with silica gel column chromatography, Sephadex LH-20 and PTLC furnished five compounds **1** (200 mg), **2** (40 mg), **3** (240 mg), **4** (80 mg) and **5** (80 mg).

Malabaricone C (**1**): ^{1}H NMR (300 MHz, CD_3OD) δ: 7.18 (1H, t, J = 8.1 Hz, H-19), 6.66 (1H, d, J = 8.1 Hz, H-14), 6.60 (1H, d, J = 2.0 Hz, H-11), 6.47 (1H, dd, J = 8.1, 2.0 Hz, H-15), 6.34 (2H, d, J = 8.1 Hz, H-18, H-20), 3.11 (2H, t, J = 6.9 Hz, H_2-2), 2.44 (2H, t, J = 6.9 Hz, H-9), 1.67 (2H, quin, J = 6.9 Hz, H_2-3), 1.53 (2H, quin, J = 6.9 Hz, H_2-8), 1.41–1.25 (8H, m, H_2-4, H_2-5, H_2-6, H_2-7); ^{13}C NMR (75 MHz, CD_3OD) δ: 209.7 (C-1), 163.3 (C-17, C-21), 145.9 (C-12), 143.9 (C-13), 136.8 (C-19), 135.8 (C-10), 120.6 (C-15), 116.5 (C-11),116.2 (C-14), 108.3 (C-16, C-18, C-20), 45.6 (C-2), 36.2 (C-9), 32.8 (C-8), 30.5 (C-5, C-6), 30.4 (C-4), 30.2 (C-7), 25.7 (C-3). ESI-MS (negative mode) m/z: 357 $[M-H]^-$.

3-(3-Methyl-5-pentyl-2-furanyl)–2(*E*)-propenoic acid (**2**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.51 (1H, d, J = 15.6 Hz, H-2), 6.14 (1H, d, J = 15.6 Hz, H-3), 5.97 (1H, s, H-4′), 2.60 (2H, t, J = 7.5 Hz, H_2-1′′), 2.12 (3H, s, CH_3-3′), 1.65 (2H, quin, J = 7.5 Hz, H-2′′), 1.40–1.27 (4H, m, H_2-3′′, H_2-4′′), 0.91 (3H, t, J = 6.9 Hz, H_3-5′′); ^{13}C NMR (75 MHz, CD_3OD) δ: 172.5 (C-1), 159.9 (C-5′), 145.6 (C-2′), 131.0 (C-2), 128.6 (C-3′), 110.8 (C-3), 110.7 (C-4′), 31.3 (C-3′′), 28.2 (C-1′′), 27.4 (C-2′′), 22.3 (C-4′′), 13.9 (C-5′′), 10.4 (C-3′-CH_3). ESI-MS (negative mode) m/z: 221 $[M-H]^-$.

Licarin A (**3**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.97 (1H, brs, H-2), 6.89 (2H, brs, H-5, H-6), 6.78 (1H, brs, H-2′), 6.76 (1H, brs, H-6′), 6.36 (1H, brd, J = 15.6 Hz, H-7′), 6.10 (1H, dd, J = 15.6, 6.6 Hz, H-8′), 5.62 (HO-4′), 5.10 (1H, d, J = 9.3 Hz, H-7), 3.90 (3H, s, CH_3O-3′), 3.87 (3H, s, CH_3O-3), 3.44 (1H, m, H-8), 1.87 (3H, d, J = 6.6 Hz, H_3-9′), 1.38 (3H, d, J = 6.9 Hz, H_3-9); ^{13}C NMR (75 MHz, CD_3OD) δ: 146.7 (C-4′), 145.8 (C-3), 144.2 (C-3′), 140.9(C-4), 133.3 (C-5′), 132.2 (C-1), 132.2 (C-1′), 131.0 (C-7′), 123.4 (C-8′), 119.9 (C-6), 114.1 (C-5), 113.4 (C-6′), 109.4 (C-2′), 109.0 (C-2), 93.8 (C-7), 56.0 (OCH_3-3), 56.0 (OCH_3-3′), 45.6 (C-8), 18.3 (C-9′), 17.6 (C-9). ESI-MS (negative mode) m/z: 325 $[M-H]^-$.

Maceneolignan B (**4**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.78 (1H, brs, H-2′), 6.75 (1H, brs, H-6′), 6.61 (2H, brs, H-2, H-6), 6.36 (1H, brd, J = 15.9 Hz, H-7′), 6.09 (1H, dq, J = 15.9, 6.6 Hz, H-8′), 5.96 (2H, s, OCH_2O), 5.07 (1H, d, J = 9.0 Hz, H-7), 3.89 (3H, s, CH_3O-3′), 3.89 (3H, s, CH_3O-5), 3.41 (1H, m, H-8), 1.86 (3H, d, J = 6.6 Hz, H_3-9′), 1.39 (3H, d, J = 6.6 Hz, H_3-9); ^{13}C NMR (75 MHz, CD_3OD) δ: 149.0 (C-3), 146.5 (C-4′), 144.2 (C-3′), 143.6 (C-5), 135.2 (C-4), 135.1 (C-1), 133.0 (C-5′), 132.4 (C-1′), 130.9 (C-7′), 123.5 (C-8′), 113.4 (C-6′), 109.5 (C-2′), 106.3 (C-6), 101.5 (OCH_2O), 100.7 (C-2), 93.5 (C-7), 56.7 (OCH_3-5), 56.0 (OCH_3-3′) 45.9 (C-8), 18.3 (C-9′), 17.9 (C-9). ESI-MS (negative mode) m/z: 353 $[M-H]^-$.

Elemicin (**5**): ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.41 (2H, s, H-4, H-6), 5.96 (1H, ddt, J = 16.9, 10.3, 6.6 Hz, H-2′), 5.12 (1H, brd, J = 16.9 Hz, Ha-3′), 5.08 (1H, m, Hb-3′), 3.85 (6H, s, OCH_3-1, OCH_3-3), 3.83 (3H, s, OCH_3-2), 3.34 (2H, brd, J = 6.6 Hz, H_2-1′); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 153.1 (C-1, C-3), 137.2 (C-2′), 136.3 (C-2), 135.7 (C-5), 115.9 (C-3′), 105.5 (C-4, C-6), 60.8 (OCH_3-2), 56.0 (OCH_3-1, OCH_3-3), 40.5 (C-1′). ESI-MS (negative mode) m/z: 207 $[M-H]^-$.

2.3. Bioassays

2.3.1. Anticholinesterase activity

Modified Ellman's method was used for quantitative analysis of AChE inhibitory activity, the alternative substrate acetylthiocholine iodide is cleaved by AChE enzyme producing thiocholine and acetic acid (Ellman et al., 1961). Thiocholine produced by this reaction then reacts with DTNB to produce mixed disulphide and 5-thio-2-nitrobenzoic acid which is yellow in colour. In this assay, samples were dissolved in 50% ethanol in buffer. First phosphate buffer pH 8.0 (0.1 M, 100 μL) was added to all the wells. Then AChE enzyme (0.2 U/mL, 50 μL) dissolved in phosphate buffer pH 8.0 was added to the wells except the blank where buffer was added instead of enzyme. Subsequently sample (25 μL) was added and incubated at room

temperature (RT) for 10 min; meantime the solvent that was used to dissolve the sample was used in control instead of sample. Finally acetylthiocholine iodide (15 mM, 25 μL), dissolved in deionized water was added followed by DTNB (3 mM, 50 μL), in phosphate buffer pH 7.0 (0.1 M) and sodium bicarbonate (0.2 mg/mL), was added to all the wells including control and blank. Absorbance was measured at the end point at 412 nm after incubation in room temperature for 10 min. Donepezil hydrochloride was used as the positive control. The experiment was done in triplicate for each concentration. The percentage of anticholinesterase activity (AA) was calculated as follows. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the enzyme, was calculated using log dose inhibitory activity curve.

$$AA\% = \frac{\delta Acontrol - \delta Asample}{\delta Acontrol} \times 100$$

Where

$$\delta A_{control} = A_{control} - A_{control\text{–}blank}$$
$$\delta A_{sample} = A_{sample} - A_{sample\text{–}blank}$$

2.3.2. DPPH radical scavenging activity

Free radical scavenging activity of pure compounds were tested qualitatively and quantitatively using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Tepe et al., 2007). Test samples were prepared in MeOH to obtain concentration ranging from 6.25 to 100 μg/mL. DPPH in MeOH (0.3 mM, 60 μL) was added to sample (150 μL). After 30 min incubation at RT in the dark, absorbance (A) was measured against the control at 517 nm by using micro-plate reader. DPPH (0.3 mM, 60 μL), methanol (150 μL), without the test compound was used as the control whereas methanol (210 μL) was used as the control-blank. Test sample (150 μL) with methanol (60 μL) was used as the sample-blank. The experiment was done in triplicate. Ascorbic acid was used as the positive control. Radical scavenging activity (RSA) was calculated using the following equation. The IC_{50} value of the sample, which is the concentration of sample required to scavenge 50% of the DPPH free radicals, was calculated using Log dose scavenging activity curve.

$$RSA\% = \frac{\delta Acontrol - \delta Asample}{\delta Acontrol} \times 100$$

Where

$$\delta A_{control} = A_{control} - A_{control\text{–}blank}$$
$$\delta A_{sample} = A_{sample} - A_{sample\text{–}blank}$$

2.3.3. α-Glucosidase inhibitory activity

The α-glucosidase inhibition was determined by quantifying the *p*-nitrophenol liberated under the assay conditions (Sharp et al., 2007). α-Glucosidase from *Saccharomyces cerevisiae* (Sigma-Aldrich, G0660) (1.2 U/mL) was dissolved in phosphate buffer pH 6.5 (30 mM) and 4-nitrophenyl α-D-glucopyranoside (*p*NPG) were used as the enzyme and the substrate. First phosphate buffer pH 6.5 (30 mM, 100 μL) was added to each well. Then enzyme (10 μL) dissolved in phosphate buffer pH 6.5 was added to wells except the blank where buffer was added instead of enzyme. Subsequently sample (40 μL) was added and incubated at 37 °C for 5 min. Meantime the solvent 4% acetone in water that was used to dissolve the sample was used in control. Finally, *p*-NPG (2.65 mM, 10 μL), dissolved in phosphate buffer pH 6.5 was added to all the wells including control and blank. The mixture was incubated at 37 °C for 30 min and then the absorbance was measured at 410 nm. Acarbose was used as the positive control while the experiment was done in triplicate. The α-glucosidase inhibitory activity was calculated as follows. The IC_{50} value of the sample was calculated using log dose inhibitory activity curve. Test sample was prepared by dissolving the sample in 4% acetone in water concentrations ranging from 6.25 to 100 μg/mL.

$$\%\ \alpha\text{–}Glucosidase\ inhibition\ activity = \frac{\delta Acontrol - \delta Asample}{\delta Acontrol} \times 100$$

Where,

$$\delta A_{control} = A_{control} - A_{control\text{–}blank}$$
$$\delta A_{sample} = A_{sample} - A_{sample\text{–}blank}$$

3. Results and discussion

The aril of *M. fragrans* seeds was sequentially extracted with *n*-hexane, DCM, EtOAc and MeOH, and the extracts were subjected to anticholinesterase assay with a slight modification of Ellman's method (Ellman et al., 1961). The inhibition of AChE leads to the accumulation of acetylcholine in the synapse resulting increased neurotransmission. Therefore inhibition of AChE remains a promising treatment strategy for AD (Zengin et al., 2019) Among the four extracts MeOH extract exhibited the highest activity (96.75%) at the concentration of 100 μg/mL and EtOAc extract (87.51%) was followed. Combined EtOAc and MeOH extracts were chromatographed over silica gel eluting with *n*-hexane-EtOAc-MeOH to give six fractions F_1 - F_6. AChE inhibitory activity of the extracts and fractions is given in Table 1. The fraction F_3, which showed the highest activity, was further separated over silica gel to afford compounds **1**–**3**. Similarly, the combined fractions F_1 and F_2 yielded compounds **4** and **5**. Compounds **1**–**5** were identified as malabaricone C (**1**) (Maia et al., 2008), 3-(3-methyl-5-pentyl-2-furanyl)–2(*E*)-propenoic acid (**2**) (Kimura et al., 2010), licarin A (**3**) (Leon-Diaz et a al., 2010), maceneolignan B (**4**) (Morikawa et al., 2016) and elemicin (**5**) (Jayasinghe et al., 2003) by comparing their spectroscopic data with literature values. Compounds **1**–**5** were tested for their AChE inhibitory activity (Ellman et al., 1961), α-glucosidase inhibitory activity (Sharp et al., 2007) and DPPH radical scavenging activity (Tepe et al., 2007). Malabaricone C (**1**) showed the highest AChE inhibitory activity (IC_{50} 2.06 ± 0.04 μg/mL) while it was inactive in α-glucosidase inhibition assay. The highest α-glucosidase inhibitory activity was observed for the furan acid (**2**) (IC_{50} 50.91 ± 0.01 μg/mL). Malabaricone C also exhibited potent antioxidant property (IC_{50} 6.56 ± 0.02 μg/mL). Acetylcholinesterase and α-glucosidase inhibitory activities and DPPH radical scavenging activity of compounds **1**–**5** are given in the Table 2.

Malabaricone C (**1**) belongs to a diarylnonanoid class of secondary metabolites, characteristically contained in Myristicaceae plants. AChE inhibitory activity of **1** (IC_{50} 44.0 μM) (Cuong et al., 2014) was previously reported, which is in agreement with our data. In addition, anti-fungal, antimicrobial (Dzoyem et al., 2017), anti-inflammatory and antioxidant activities (Kang et al., 2012) of malabaricone C have been reported. The furan acid **2** was previously isolated only from mace (Kimura et al., 2010), although its structure homologue mumiamicin (3-(3-methyl-5-heptyl-2-furanyl)–2(*E*)-propenoic acid) was

Table 1
Anticholinesterase activity of the extracts and fractions.

Crude extract/Fractions	Percentage inhibition at the concentration of 100 μg/mL
n-Hexane	62.99
DCM	85.09
EtOAc	87.51
MeOH	96.75
F_1	84.48
F_2	80.34
F_3	87.19
F_4	45.81
F_5	−1.18
F_6	−1.73

Table 2
Anticholinesterase, α-glucosidase inhibitory and DPPH radical scavenging effect of compounds **1**–**5**.

Compounds	Anticholinesterase activity		α-Glucosidase inhibitory activity		Antioxidant activity	
	Percentage inhibition[a)]	IC_{50} (μg/mL)	Percentage inhibition[a)]	IC_{50} (μg/mL)	Percentage inhibition[a)]	IC_{50} (μg/mL)
1	100.09	2.06 ± 0.04	NA	NA	96.33	6.56 ± 0.02
2	14.09	ND	90.63	50.91 ± 0.01	27.98	213 ± 0.08
3	43.66	111.3 ± 0.07	NA	NA	73.34	24.74 ± 0.09
4	NA	NA	30.60	ND	NA	NA
5	20.02	ND	NA	NA	NA	NA

ND - not determined; NA - not active.
IC_{50} values of positive controls, donepezil HCl, acarbose and ascorbic acid, for anticholinesterase, α-glucosidase inhibitory and antioxidant activities were 0.03±0.00, 265.3 ± 0.13 and 5.76±0.01 μg/mL, respectively.
[a)] assayed at 100 μg/mL.

reported from a marine actinomycete strain *Mumia* sp. Mumiamicin was shown to have antioxidant and antimicrobial activities (Kimura et al., 2018). A dihydrobenzofuran type neolignan, licarin A (**3**) was found in various plants. Somewhat more potent DPPH radical scavenging activity of **3** (IC_{50} 56.1 μg/mL) has been reported (Barros et al., 2009). Also, it is reported that compound **3** protects primary cultured neuronal cells against glutamate-induced oxidative stress via antioxidative activities (Choong et al., 2005). Maceneolignan B (**4**) is a rare dihydrobenzofuran type neolignan that has been isolated only from the aril and seeds of *M. fragrans* and reported to have an inhibition activity of nitric oxide production in lipopolysaccharide activated macrophage RAW264.7 (Morikawa et al., 2016; Cao et al., 2013). Elemicin (**5**) has been found in essential oil of various plants including *M. fragrans* (Simamora et al., 2018) and showed a potent antifungal and larvicidal activity (Marston et al., 1995).

4. Conclusion

In conclusion five secondary metabolites, malabaricone C (**1**), 3-(3-methyl-5-pentyl-2-furanyl)−2(*E*)-propenoic acid (**2**), licarin A (**3**), maceneolignan B (**4**) and elemicin (**5**) have been isolated from the aril of *M. fragrans*. Malabaricone C (**1**) was found to show potent AChE inhibitory activity with an IC_{50} value of 2.06 ± 0.04 μg/mL which further confirmed the results of previous report. DPPH radical scavenging activity of **1** (IC_{50} value of 6.56 ± 0.02 μg/mL) was confirmed. Furthermore, α-glucosidase inhibitory activity of 3-(3-methyl-5-pentyl-2-furanyl)−2(*E*)-propenoic acid (**2**) is reported here for the first time. These results proved the potential of the aril of *M. fragrans* as a natural source of anticholinesterase, α-glucosidase inhibitory and antioxidant active compounds that will benefit in the development of new therapeutic against AD and type II diabetic.

Declaration of Competing Interest

No potential conflict of interest was reported by the authors.

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