SHORT REPORT

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In Vitro and *In Silico* Evaluation of Compounds from *Washingtonia filifera* as Acetylcholinesterase Inhibitors

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Abstract: Nine known compounds were isolated from the fruitless bunches of *Washingtonia filifera*, an underutilized agricultural waste, using NMR and mass spectrometry. The identified compounds included β-sitosteryl oleate (**1**), oleic acid (**2**), β-sitosterol (**3**), *threo*-2,3-*bis*-(4-hydroxy-3-methoxyphenyl)-3-methoxypropanol (**4**), syringaresinol (**5**), diosmetin (**6**), tricin (**7**), daucosterol (**8**), and luteolin-7-*O*-β-D-glucoside (cynaroside) (**9**). *In vitro* acetylcholinesterase (AChE) inhibition assays revealed that compound **5** exhibited the strongest activity (IC_{50} = 29.75 µM), followed by compounds **4** and **6**. Docking studies indicated significant interactions of the active compounds with key AChE residues, particularly Trp86 and Tyr341. ADME predictions further supported the drug-likeness of compounds **4** and **5**. These results highlight the significance of *W. filifera* agricultural waste as a source of bioactive compounds, particularly with neuroprotective effects.

Keywords: *Washingtonia filifera*; california fan plam; fruitless bunch; acetylcholinesterase; phenolics; lignan.

1. Plant Source

Limited studies have been conducted on the agricultural waste of *W. filifera*, particularly the plant grown in Egypt. This study investigated the phytochemical composition of fruitless bunches of *Washingtonia filifera* (Linden ex André) H. Wendl., an underexplored agricultural waste, particularly in Egypt. Samples were collected from the garden of the Faculty of Pharmacy, Mansoura University,

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on November 24, 2022, and identified by Assoc. Prof. Dr. Mahmoud Makram Qassem. A voucher specimen (WF-1-2020) is deposited in the Pharmacognosy Department, Faculty of Pharmacy, Mansoura University.

2. Previous Studies

The aerial parts of *W. filifera* showed the presence of flavonols, C-glycosyl flavones, and flavonoid sulfates. While flavonoid sulfates are rare in plants, they are present in several palm species, particularly *Washingtonia*. The plant contains chalconoids (chalcone-like compounds) in its leaf basal tissues, which exhibit antilarval activity, antioxidative, antimalarial, and anticancer activities [1]. Previous reports showed that the alcoholic seed extract of *W. filifera* exhibited strong antioxidant and enzyme inhibitory properties towards various enzymes involved in aging-related disorders, such as butyrylcholinesterase, xanthine oxidase, elastase, collagenase, and tyrosinase [2,3]. However, there is no previous research on the AChEI activity of the other parts of *W. filifera* or for the pure isolated compounds.

3. Present Study

Extraction and Isolation: The shade-dried powdered fruitless bunches of *W. filifera* (5 Kg) were extracted with 70% methanol (MeOH) at room temperature (25°C) (3 × 12 L) and evaporated *in vacuo* to yield 500 g of total fruitless bunches extract (TFBE). This residue was dissolved in the minimum amount of MeOH, diluted with 700 mL of distilled water, and then fractionated using solvents of increasing polarity to provide a petroleum ether fraction (PEF, 90 g), CH_2Cl_2 fraction (MCF, 31 g), EtOAc fraction (EAF, 24 g), and *n*-butanol fraction (BUF, 22 g). About 50 grams of PEF fraction were purified on a silica gel CC packed in petroleum ether, eluted with petroleum ether/ EtOAc (gradient), to afford two major groups. Group I (F88-96), eluted with petroleum ether/ EtOAc (98.5:1.5, *v*/*v*), was further purified over a silica gel CC by gradient elution with petroleum ether/ EtOAc to afford two subgroups. Subgroup I-a (F101-103), eluted with petroleum ether/ EtOAc (99.5:0.5, *v*/*v*), was further purified on a silica gel CC, previously packed in petroleum ether, using isocratic elution with petroleum ether/ CH_2Cl_2 (70:30, *v*/*v*) to afford compound **1** (40 mg) in fractions (F4-10). Subgroup I-b (F297-324), eluted with petroleum ether/ EtOAc (98:2, *v*/*v*), was concentrated to yield an oily residue (Compound **2**, 1.4 g). Group II (F173-209) eluted with petroleum ether/ EtOAc (90:10, *v*/*v*) were concentrated to yield colorless needles (Compound **3**, 213 mg). The MCF fraction (31 g) was further purified using the VLC technique using different solvents (Petroleum ether, CH_2Cl_2, and MeOH). Fraction 7 (13 g), eluted with CH_2Cl_2/ MeOH (10:1, *v*/*v*), was further purified by a silica gel CC previously packed in CH_2Cl_2. Gradient elution with CH_2Cl_2/ MeOH afforded three groups (Groups I-III). Group I (F145-168), eluted with CH_2Cl_2/ MeOH (99.5:0.5, *v*/*v*), was further purified by a Sephadex LH-20 CC previously packed in CH_2Cl_2/ MeOH (50:50, *v*/*v*) using isocratic elution to afford two subgroups. Subgroup I-a (F22-24), eluted with CH_2Cl_2-MeOH (50:50, *v*/*v*) on Sephadex LH-20, was further purified over a Sephadex LH-20 CC previously packed in MeOH/ distilled H_2O (50:50, *v*/*v*) using isocratic elution to afford two subgroups (I-a1 and I-a2). Subgroup I-a1 (F23-26) was purified over a Sephadex LH-20 CC using MeOH (100 %) to afford a brown solid residue (Compound **4**, 8 mg). Subgroup I-a2 (F29-37) was purified over a silica gel CC using gradient elution with petroleum ether/EtOAc. Subfractions 106-129, eluted with petroleum ether/ EtOAc (40:60, *v*/*v*) yielded a brown solid residue (Compound **5**, 11 mg). Subgroup I-b (F32-39), eluted with CH_2Cl_2/ MeOH (50:50, *v*/*v*) on Sephadex LH-20, yielded a pale-yellow powder (Compound **6**, 7 mg). Group II (F169-181), eluted with CH_2Cl_2/ MeOH (99:1, *v*/*v*) yielded a yellow powder (Compound **7**, 30 mg). Group III (F252-277), eluted with CH_2Cl_2/ MeOH (96:4, *v*/*v*) yielded a white powder (Compound **8**; 134 mg). The BUF fraction (22 g) was purified on a silica gel CC eluted with CH_2Cl_2/ MeOH (gradient). Fractions (F38-44) eluted with CH_2Cl_2-MeOH (80:20, *v*/*v*) were pooled and repeatedly purified over Sephadex LH-20 CC using MeOH/ distilled H_2O (50:50, *v*/*v*) to afford a yellow powder (Compound **9**; 10 mg).

In vitro AChE Inhibitory Assay: The AChE inhibition of was assessed using a modified Ellman's method [4]. Briefly, in each well of a 96-well plate, 25 µL of acetylthiocholine iodide (15 mM), 125 µL of DTNB (3 mM in buffer B), 50 µL of buffer A, and 25 µL of the test sample (prepared in DMSO, 25%) were combined. Absorbance was recorded at 405 nm using a BioTek® microplate reader (Winooski, VT, USA) at 16-second intervals for a total of ten readings. Subsequently, 25 µL of AChE (0.25 U/mL in buffer A) was introduced, and absorbance measurements were repeated ten times at the same intervals. A 25% DMSO solution served as the negative control.

Docking Study: The protein structure used in this study was the crystal structure (Resolution: 2.35 Å) of recombinant human acetylcholinesterase complexed with donepezil, obtained from the Protein Data Bank (PDB ID: 4EY7) [5]. Docking studies were carried out using MOE software (version 2015.10) from Chemical Computing Group Inc. according to the previously published procedure [6].

*Prediction of ADME and Drug-Likeness Properties of Compounds **1-9**:* The physicochemical characteristics, pharmacokinetics, drug-likeness, and toxicity of the studied compounds (**1**-**9**) were analyzed using the SwissADME tool, provided by the Swiss Institute of Bioinformatics (http://www.sib.swiss) [7].

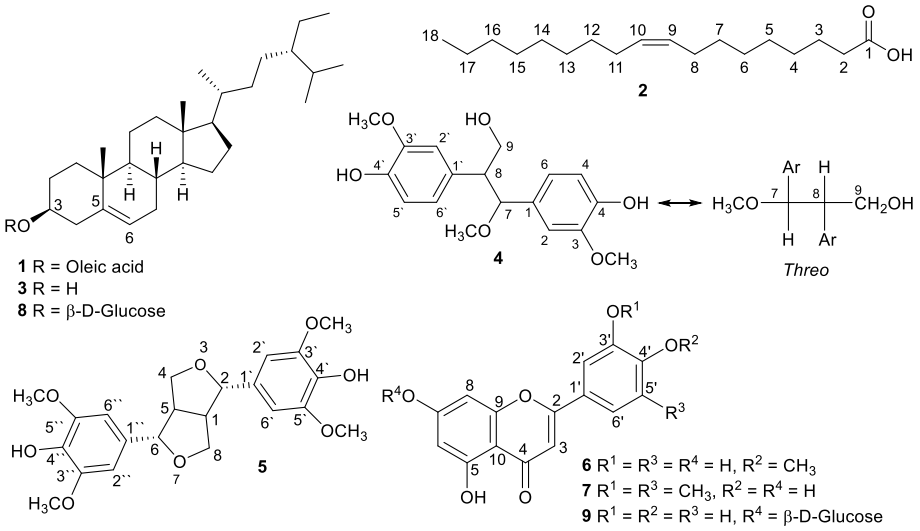

Figure 1. Structures of the isolated compounds (**1**-**9**) from *W. filifera* fruitless bunches

Compound **1** (Figure 1) was identified as β-sitosteryl oleate based on ^{1}H-NMR, APT, HMBC, and mass spectral data (Table S1; Figures S2-S4), consistent with literature [8]. Compound **2** was confirmed as oleic acid via ^{1}H-NMR, APT, and mass spectral data (Table S2; Figures S5-S7) [9], a compound previously identified in the seed oil of *W. filifera* [10,11] and the fruit oil of *W. robusta* [12]. Compound **3** was verified as β-sitosterol using IR spectrum (Figure S8) and co-chromatography with an authentic standard [12]. Compound **4** was identified as 9-hydroxy-7-methoxy-8-[4'-hydroxy-3'-methoxyphenyl]-4-hydroxy-3-methoxyphenylpropane, also named 2,3-*bis*-(4-hydroxy-3-methoxyphenyl)-3-methoxypropanol based on ^{1}H-NMR, APT, HSQC, HMBC, and ESI-MS (Table S3; Figures S9-S13). This compound has been reported from various plant sources, including *Aralia bipinnata* [13], *Irvingia malayana* [14], and *Solanum melongena* [15]. The stereochemistry of compound **4** was determined to be the *threo* isomer based on a comparison of its ^{13}C chemical shifts (δ_C) with those reported by Yang et al. [15] for both *erythro* and *threo* configurations. Specifically, the chemical shift of carbon C-7 in compound **4** was observed at 87.42 ppm, closely corresponding to that of the *threo* isomer (87.4 ppm) and differing from the *erythro* isomer (δ_C 84.9 ppm). Compound **5** was identified as syringaresinol, supported by its ^{1}H-NMR, APT, HSQC, and ESI-MS spectral data (Table S4; Figures S14-S17) [16]. Compound **6** was elucidated as diosmetin (5,7,3'-trihydroxy-4'-

methoxyflavone) based on comprehensive spectral analysis, including ^{1}H-NMR (Table S5; Figures S18, S19), APT spectrum (Table S5; Figure S20), HSQC, and HMBC spectra (Figures S21-S23) [17]. Compound **7** was identified as tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone), as evidenced by its ^{1}H-NMR (Table S6; Figure S24), APT (Table S6; Figure S25), HSQC (Figure S26) and HMBC (Figure S27) spectra [18]. This compound has previously been reported in *W. filifera* leaves [19]. Compound **8** was characterized as β-sitosterol-3-*O*-β-D-glucoside based on its IR spectral data (Figure S28) and confirmed by co-chromatography with an authentic standard [20]. Finally, compound **9** was identified as luteolin 7-*O*-β-D-glucoside through analysis of its ^{1}H-NMR, APT, HSQC, and HMBC spectral data (Table S7; Figures S29-S32) [21]. These findings significantly contribute to the chemotaxonomic understanding of the genus *Washingtonia* and the family Arecaceae by documenting several compounds for the first time. β-Sitosteryl oleate (**1**), luteolin-7-*O*-β-D-glucoside (**9**), syringaresinol (**5**), diosmetin (**6**), and daucosterol (**8**) are reported here for the first time in the *Washingtonia* genus. Notably, compound **4** [*threo*-2,3-*bis*-(4-hydroxy-3-methoxyphenyl)-3-methoxypropanol] is reported here for the first time from the Arecaceae family.

The results of AChE inhibition assay of the isolated compounds (Table 1) revealed that compound **5 (**syringaresinol) showed the strongest inhibitory activity against AChE among the isolated compounds with an IC_{50} of 29.75 µM, followed by compound **4** (IC_{50} of 72.49 µM) and **6** (IC_{50} of 76.90 µM). Lignans were previously reported to exhibit protective effects against neurodegenerative diseases, such as AD, Parkinson's, stroke, and other conditions linked to neuronal cell damage [22,23]. Acetylcholinesterase inhibition was previously reported as one of the mechanisms through which lignans exert neuroprotective effects against Alzheimer's disease (AD). On the other hand, compounds **7** (tricin) and **9 (**cynaroside) showed modest activity. However, compounds **8** (β-sitosterol-3-*O*-β-D-glucoside)**, 2** (oleic acid), and **3** (β-sitosterol) showed weak activity. Whereas compound **1** (β-sitosteryl oleate) exhibited the lowest inhibitory activity.

Table 1. AChE inhibitory activity and docking results of compounds **1-9** against AchE protein

Compound	IC_{50} (µM)	Score (kcal/mol)	RMSD[b]	Interactions[c]
1	› 100[a]	-10.9898	1.4618	
2	› 100[a]	-7.6073	1.4174	Trp86, Arg296
3	› 100[a]	-7.6745	1.5253	Trp86, Glu202, Tyr133
4	72.49±1.24	-7.6684	0.8996	Trp86, Tyr341
5	29.75±0.69	-8.0811	1.7034	Trp86, Tyr341
6	76.90±4.57	-6.5626	1.0250	Phe295, Tyr341
7	› 100 (166.21±8.21)	-6.9222	1.0912	Ser203, Trp286, Tyr341
8	› 100[a]	-8.1962	1.3087	Trp86
9	› 100 (360.01±11.02)	-8.3806	1.4742	Ser203, Ser293, Tyr341
Donepezil[d]		-8.3431	1.2587	Trp86, Trp286, Tyr337
Donepezil[e]				Trp86, Trp286, Phe295
TFBE	89.60±3.15			
Galnthamine	1.44 ± 0.25			

[a] Inactive up to concentration of 500 µM; [b] RMSD: Root mean square deviation. A value < 2 Å suggests acceptable; docking result; [c] Acetylcholinesterase (AchE) protein, PDB code: 4EY7 [5]; [d] Donepezil (docked ligand); [e] Donepezil (co-crystallized ligand). TFBE: Total fruitless bunches extract.

To explain the results of the *in vitro* AChE assay, a docking study of isolated compounds (**1-9**) with human AChE at its active site (PDB code 4EY7) was conducted (Table 1). Cheung et al. (2012) described the structural details of the human AChE binding site, emphasizing the unique conformations of its active site residues, particularly Trp86, Tyr337, Tyr341, and Phe338, which play a critical role in ligand binding [5]. Donepezil, a standard AChE inhibitor, showed strong interactions involving π-π stacking with Trp86 and Trp286, similar to the observations in the study of Cheung et al. [5] validating our docking results (Figure S42). Compounds such as β-sitosteryl oleate (**1**) demonstrated the strongest binding score (-10.9898 kcal/mol), though no specific interactions were

listed. This can be potentially explained by the bulkiness or hydrophobicity of this compound, which occupied the hydrophobic region of the active gorge effectively. Compounds **4** and **5** showed significant arene-H and π-π stacking interactions with Trp86, respectively (Figures S36, S37). They also showed significant interaction with Tyr341 residue (π-π stacking). These results align with the reported data of these residues as key contributors to ligand binding and stabilization [5]. Interestingly, syringaresinol (**5**) shared donepezil its molecular sizes and several structural features, which can be noticed from their superimposed structures compared to **4** (Figure S43). This result could explain the remarkable higher AChE inhibitory activity of **5** compared to the other tested compounds. Dosmetin (**7**) showed strong interactions with the key amino acid residues of Phe295 (H-bond) and Tyr341 (π-π stacking), which were previously identified as critical residues at the active site of AChE, anchoring diosmetin at the entrance of the active site gorge [5]. This can clarify why diosmetin showed *in vitro* AChE inhibitory activity.

The early evaluation of ADME properties of a potential drug is crucial to ensure its bioavailability and reflects its medicinal chemistry compatibility. The results of analysis of ADME and drug-likeness properties of compounds **1-9** using the SwissADME web tool [7] (Table S8; Figures S44, S45) indicated that compounds **4** and **5** exhibited a balanced profile of solubility, bioavailability, and GI absorption, making them promising candidates for further exploration.

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Supporting Information

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