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Natural Compounds Isolated from African Mistletoes (Loranthaceae) Exert Anti-Inflammatory and Acetylcholinesterase Inhibitory Potentials: *In Vitro* and *In Silico* Studies

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Abstract: Despite the medicinal uses of *Phragmanthera capitata*, *Globimetula dinklagei* and *Tapinanthus bangwensis* against memory loss, convulsions and pain, their efficacy against brain-dysfunction diseases and inflammation models has not yet been studied. Therefore, this study aims to investigate the anti-inflammatory and acetylcholinesterase (AChE) inhibitory potentials of their crude extracts and isolated natural compounds by combining *in vitro* and *in silico* experiments. Crude extracts and isolated compounds were tested *in vitro* for their AChE inhibitory activity by using Ellman's method. Additionally, their anti-inflammatory activity was determined by evaluating the nitric oxide (NO) production inhibitory activity in lipopolysaccharide-stimulated RAW 264.7 macrophage cells and the 15-lipoxygenase (15-LOX) inhibitory activity by using the ferrous oxidation xylenol orange assay. Furthermore, the *in silico* efficacy of natural compounds was investigated against ten putative target enzymes relevant in Alzheimer's disease (AD) pathogenesis and inflammation. It was found that the crude extracts had weak to moderate inhibitory potential against AChE, with the extract of *T. bangwensis* being the most active (50% inhibitory concentration (IC_{50}) = 48.97 µg/mL). Six natural compounds, namely, 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**2**), apigenin- 8-C-β-D-glucopyranoside (**3**), globimetulin B (**5**), globimetulin C (**6**), bangwaursene B (**8**) and 3β-acetoxy-11,12-epoxytaraxerol (**9**), were identified for the first time as having anti-AChE potential, among which (**9**) had the highest efficacy with an IC_{50} of 13.89 µM. Among the anti-AChE compounds, (**5**) was also efficient against NO production and 15-LOX, and the data are in agreement with the docking score. In summary, compounds (**5**) and (**9**) are the most prominent lead compounds that should be further tested experimentally against molecular targets of AD and inflammation.

Keywords: acetylcholinesterase; nitric oxide; 15-lipoxygenase; globimetulin B; molecular docking; African mistletoes

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1. Introduction

Plants and natural products are indeed rich sources of biologically active compounds that can be applicable in the discovery and development of new medicines against various diseases. African mistletoes (Loranthaceae) are hemi-parasitic plants that grow on many tree crops including citrus species and a variety of other plants with economic and medicinal values [1]. These plants depend on their host mainly for water and mineral nutrients [2]. Additionally, the host constituents may be exchanged with the mistletoes and vice versa, therefore enhancing their medicinal properties. In fact, according to traditional

medicine practices, mistletoes growing on guava, kola nuts and citrus are mostly used for the treatment of diseases such as cancer, hypertension, nervousness and insomnia, while those growing on cocoa are specifically efficient against hyperglycaemia [3]. African mistletoes are known as "all-purpose plants" because they were beneficial in folklore medicine as a remedy for many health problems [4]. An investigation carried out to interview 150 traditional healers on the medicinal uses of hemi-parasitic plants growing in Cameroon led to the identification of *Phragmanthera capitata* and *Globimetula dinklagei,* which are recommended for the treatment of 22 different diseases, including convulsions, nerves attacks, diabetes, epilepsy, back pains, kidney pains, chronic muscular pains, respiratory dysfunctions, headache, rheumatism, dizziness, uterine haemorrhage, hypertension, menopause, heart palpitations, general purifications, irregular menstruations and nose bleeding [5]. On the other hand, *Tapinanthus bangwensis* is used by most communities in Nigeria for the treatment of epilepsy, asthma, diabetes, blood pressure, acquired immunodeficiency syndrome and breast and ovary cancers [6]. To date, various extracts, fractions and natural compounds from *T. bangwensis*, *G. dinklagei* or *P. capitata* have been evaluated for their antibacterial [6,7], antioxidant [8–10], antipyretic and analgesic [11], hepatoprotective and hepatocurative [3,12], steroidogenetic and spermatogenetic [13], anti-hyperglycaemic and anti-hyperproteinaemic [14], anti-hepatitis C virus [15], antidiabetic [16] and anticancer [17] activities. Among their possible medicinal uses explored so far, African mistletoes have been used in the management of memory loss, a major cognitive hallmark of Alzheimer's disease (AD) [18]. However, their biological effect against AD and inflammation models has not yet been studied. For this reason, these plants may represent potential sources for the discovery of new acetylcholinesterase inhibitors (AChEIs) and anti-inflammatory compounds.

Approximately 40 million people, mostly older than 65 years, suffer from AD or related dementia [19]. The exact mechanism of AD is not completely elucidated, and full therapy has not yet been found. AD is known as a disease associated with the progressive degeneration of memory and cognitive functions due to a deficit of cholinergic function in the brain [20]. The loss of cholinergic function in the central nervous system has been proposed within the "cholinergic hypothesis" to contribute significantly to the cognitive decline observed in AD [21]. In fact, AChE and butyrylcholinesterase (BuChE) are two enzymes that play a crucial role in AD therapy by increasing the levels of brain acetylcholine (ACh) and therefore enhancing the deficient brain cholinergic neurotransmission [22]. AChE is the main enzyme that catalyses the breakdown of the ACh into acetate and choline [23,24]. Therefore, the inhibition of AChE will result in the elevation of the ACh level within the brain and thus can serve as a strategy for the treatment of AD and other related diseases such as Parkinson's disease. Moreover, it is also known that inflammation can significantly contribute to the progression of AD, accelerating the appearance of the disease. The nitric oxide (NO) radical is recognized to play a central role in inflammatory and immune reactions [25,26]. The elevation of NO within the central nervous system has been associated with the pathogenesis of AD [27]. In addition, the role of a specific brain enzyme called 15-lipoxygenase (15-LOX) has been recognized in the pathogenesis of AD [28], and its level is up-regulated in patients with AD or mild cognitive impairments [29]. In fact, 15-LOX is widely expressed in the central nervous system where it modulates amyloid beta (Aβ) and amyloid precursor protein (APP) processing [30]. Studies on patients undergoing long-term treatment of nonsteroidal anti-inflammatory drugs (NSAIDs) showed that these patients had a lower prevalence of AD [31]. It is then evident that a drug with both AChE inhibitory and anti-inflammatory effects will be beneficial in the treatment of AD. Different drugs including donepezil, galantamine and rivastigmine are AChEIs that have obtained regulatory approval in most countries [32,33]. Galantamine and rivastigmine are plant-derived alkaloids, together with physostigmine (eserine), which was the first AChEI investigated for the treatment of AD [33]. However, current approved drugs used against AD are effective only in the treatment of symptoms; these drugs are unable to cure or prevent AD [34]. Therefore, medicinal plants are now gaining more interest as alternative

sources for the discovery of drug candidates against AD, and scientific studies have proven the beneficial use of medicinal plants and their isolated phytochemicals for the treatment of AD [35]. The present study goes toward this perspective by searching new lead compounds from medicinal plants used against AD.

The combination of *in silico* with *in vitro* methods is essential in drug discovery, as this multidisciplinary approach represents the best way to generate accurate results. Despite the fact that *in silico* screening and molecular docking will facilitate the prediction of potential targets and make lead compound discovery faster and less expensive, the confirmation of predicted biological activity is still required [36]. Several reports have effectively used these methods simultaneously or in parallel to predict the molecular targets of natural compounds that correlate well with experimental data [37–39]. Therefore, owing to the medicinal uses of African mistletoes against memory loss, the present study aimed to investigate the AChE and anti-inflammatory inhibitory activities of their crude extracts and isolated compounds. Additionally, a molecular docking study was carried out with the isolated natural compounds against ten putative molecular targets that are involved in AD pathogenesis and inflammation.

2. Materials and Methods

2.1. Chemicals

Bovine serum albumin (BSA), sodium chloride (NaCl), magnesium chloride ($MgCl_2 \cdot 6H_2O$), acetylthiocholine iodide (ATCI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 15-lipoxygenase from *Glycine max*, acetylcholinesterase (AChE) from *Electrophorus electricus* (type VI-S lyophilized powder), ferrous (II) sulfate ($FeSO_4 \cdot 7H_2O$), xylenol orange, sulphuric acid, sodium nitrite ($NaNO_2$), lipopolysaccharide (LPS) and eserine were purchased from Sigma (Berlin, Germany). Tris(hydroxymethyl) aminomethane was purchased from Sigma (Switzerland). Quercetin and Griess reagent were purchased from Sigma–Aldrich (St. Louis, MO, USA) while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Separations (South Africa). Foetal bovine serum (FBS) was obtained from Highveld Biological Products (Modderfontein, South Africa). Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), penicillin/streptomycin/fungizone (PSF) and trypsin-EDTA (0.25%) were purchased from Whitehead Scientific (South Africa). Linoleic acid was purchased from Merck (Berlin, Germany).

2.2. Plant Material

The entire plant of *G. dinklagei* on *Manihot esculenta* (Euphorbiaceae) was harvested in October 2012 in Bandjoun (Cameroon). The leaves of *P. capitata* and the seeds of *T. bangwensis* were collected on *Pachyra insignis* (Bombacaceae) and *Cassia occidentalis* (Caesalpiniaceae), respectively, in April 2013 on the campus of the University of Yaounde I (Yaounde, Cameroon). Voucher specimens were prepared and identified by Mr. Jiofack, plant taxonomist, after comparison with the specimens already available in the library of the Herbarium. Our plant materials were then registered and deposited at the Cameroon National Herbarium under the numbers N°9960/SFR/CAM (*G. dinklagei*), N°24673/SFR/CAM (*P. capitata*) and N°38664/ HNC (*T. bangwensis*).

2.3. Extraction, Isolation Procedure and Characterization of Compounds

The leaves of *Phragmanthera capitata* were air-dried, and after grinding, the powder obtained (2.3 kg) was macerated with methanol (MeOH), followed by filtration with Whatman N°1 and evaporation of the solvent in vacuo to yield 210 g of a greenish extract (MLPC). Ten grams of this extract was kept for biological assays while the remaining 200 g was dissolved in ethyl acetate (EtOAc), and after filtration and evaporation, 23 g of ethyl acetate extract was obtained. This extract was then subjected to silica gel open column chromatography eluted with a gradient of *n*-Hexane–EtOAc. Two hundred fractions of 150 mL each were collected and grouped into 10 fractions (F_1–F_{10}) based on their thin-layer chromatography (TLC) profiles after evaporation. Fraction F_3 (0.6 g) was submitted to

column chromatography (CC) eluted with *n*-Hexane/EtOAc (80:20, *v*/*v*) to afford 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene (120 mg) (**1**) [40]; fraction F_6 (0.8 g) was submitted to column chromatography eluted with 100% EtOAc to afford 3-*O*-β-D-glucopyranosyl-28-hydroxy-α-amyrin (18 mg) (**2**) [41]. Fraction F_{10} (2.5 g) was further purified by column chromatography and eluted with 100% EtOAc to yield apigenin- 8-C-β-D-glucopyranoside (35 mg) (**3**) [42]. The structure of these isolated compounds was determined using spectroscopic techniques, that is, mass spectrometry (MS) and one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR) and by comparison with data from the literature.

Two kilograms of ground entire plants of *Globimetula dinklagei* and crushed powder from seeds of *Tapinanthus bangwensis* were separately extracted with a mixture of methylene chloride and methanol (CH_2Cl_2: MeOH, 1:1, *v*/*v*) at room temperature for 48 h to yield 120 g of a greenish oily extract (MEGD) and 170 g of an oily crude extract (MSTB), respectively. The three lupan-type triterpernoid derivatives [globimetulin A (**4**), B (**5**) and C (**6**)] were isolated from the entire plant extract of *G. dinklagei* [43] while the compounds bangwaoleanene A (**7**), bangwaursene B (**8**) [44] and 3β-acetoxy-11,12-epoxytaraxerol (**9**) [45] were isolated from the seed extract of *T. bangwensis.* The isolation procedure and structure elucidation of these compounds were previously described [17]. The chemical structures of all compounds are presented in Figure 1.

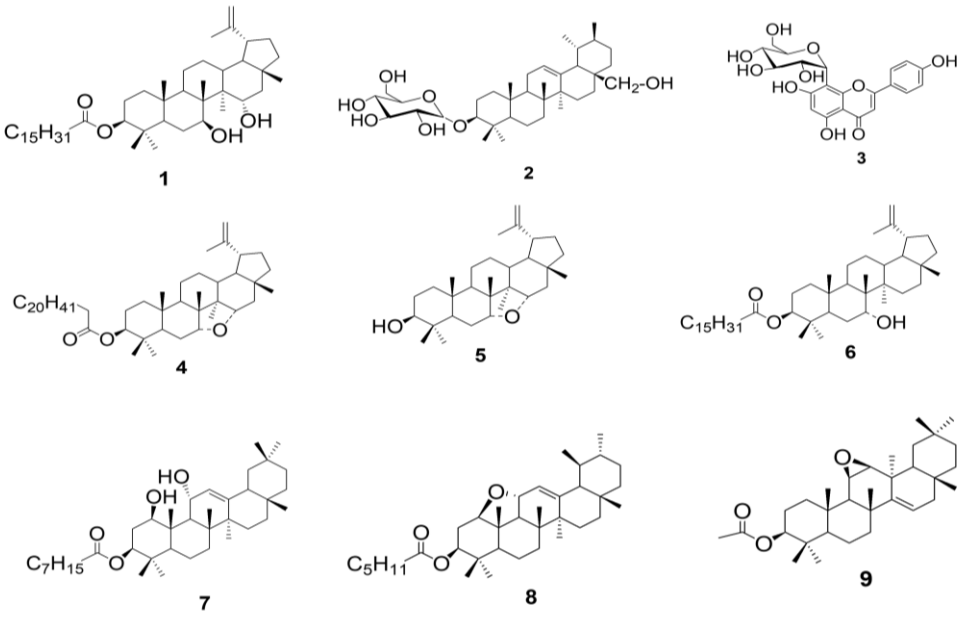

Figure 1. Chemical structures of isolated compounds: 7β,15α-dihydroxy- 3β-palmitatelup-20(29)-ene (**1**), 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**2**) and apigenin- 8-C-β-D-glucopyranoside (**3**) were isolated from the leaf extract of *P. capitata*. Globimetulins A (**4**), B (**5**) and C (**6**) were isolated from the entire plant extract of *G. dinklagei*. Bangwaoleanene A (**7**), bangwaursene B (**8**) and 3β-acetoxy-11,12-epoxytaraxerol (**9**) were isolated from the seed extract of *T. bangwensis*.

2.4. Acetylcholinesterase Inhibitory Assay

The acetylcholinesterase inhibitory activity was determined by using Ellman's spectrophotometric method [46] with slight modifications adapted to a 96-well plate. On this plate, 25 µL of tested samples and a positive control (eserine), all prepared at 1 mg/mL, were serially diluted (1:1) with dimethylsulfoxide (DMSO) at 10% and used as a negative control. Thereafter, 125 µL of 3 mM DTNB in Buffer A (50 mM Tris–HCl, pH 8.0, containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$), 50 µL of Buffer B (50 mM, pH 8.0, containing 0.1 % bovine serum albumin) and 25 µL of AChE (0.2 U/mL) prepared in Buffer A were added successively to all wells. After 5 min of incubation at room temperature, the absorbance was measured at 405 nm on a microplate reader (Epoch, BioTek, Winooski, VT, USA), and the readings were used as blanks for the test samples and negative control. Then, the enzymatic

reaction was initiated by adding to all wells 25 µL of 15 mM of acetylthiocholine iodide dissolved in water. The rate of acetylthiocholine hydrolysis was measured by reading the absorbance on a microplate reader (Epoch, BioTek, Winooski, VT, USA) at 405 nm after 5 min of incubation at room temperature. After the readings were subtracted from the blank at each concentration used, the percentage of inhibition was calculated using the following formula:

$$AChE\ inhibitory\ activity\ (\%) = 100 - [\frac{\text{Abs (sample)} - \text{Abs (sample blank)}}{\text{Abs (negative control)} - \text{Abs (negative control blank)}} \times 100] \quad (1)$$

Abs: absorbance.

2.5. Anti-Inflammatory Assays

2.5.1. Nitric Oxide Inhibitory Assay

RAW 264.7 macrophage cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), were grown at 37 °C in a 5% CO_2 humidified environment using DMEM with high glucose (4.5 g/L) containing L-glutamine (4 mM) and supplemented with 10% FBS and 1% PSF solution. The cells at their exponential phase were seeded at a density of 20,000 cells per well in 96 well-microtitre plates and they were incubated overnight at 37 °C with 5% CO_2 to allow their attachment. The cells were pre-treated with different concentrations (100, 50, 25, 10 and 5 µg/mL) of tested samples and incubated for 1 h at 37 °C in a 5% CO_2 humidified environment. Thereafter, culture medium containing 2 µg/mL of LPS was added to each well followed by further incubation for 24 h at 37 °C in a 5% CO_2 humidified environment. The negative control was the RAW 264.7 cells treated with LPS and DMSO at a concentration of 0.5% (*v*/*v*), while quercetin (100, 50, 25, 10 and 5 µg/mL) was used as a positive control. Then, 100 µL of cell supernatant from each well of the 96 well-microtitre plate was transferred into another 96-well microtitre plate, and an equal volume of Griess reagent was added according to the protocol described by the manufacturer (Sigma Aldrich, St. Louis, MO, USA). The absorbance of the mixture was measured at 550 nm on a microplate reader (Synergy Multi-Mode Reader, BioTek, Winooski, VT, USA) after 10 min of incubation at room temperature in the dark. The quantity of nitrite was determined from a sodium nitrite standard curve, and the percentage of NO inhibition was calculated based on the ability of each sample to inhibit nitric oxide production by RAW 264.7 macrophage cells compared with the negative control after subtracting the blank made of 100 µL of culture medium with an equal volume of Griess reagent.

$$NO\ production\ inhibition\ (\%) = 100 - [\frac{\text{Abs (sample)} - \text{Abs (blank)}}{\text{Abs (negative control)} - \text{Abs (blank)}} \times 100] \quad (2)$$

Abs: absorbance.

2.5.2. Determination of Cell Viability of Treated RAW 264.7 Macrophage Cells

The cell viability of treated RAW 264.7 macrophages was determined to know whether the reduction in NO production by the tested samples was related to their cytotoxicity. Therefore, the viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay as described by Mosmann (1983) [47]. After the cell supernatant from the 96-well microtitre plates mentioned above was discarded, 200 µL of fresh medium with 30 µL of MTT (5 mg/mL) dissolved in phosphate-buffered saline was added to each well. These plates were further incubated for 4 h, and the culture medium was gently aspirated. Then, the formazan crystals were dissolved in 50 µL of DMSO and kept in the dark for 15 min at room temperature. The absorbance was measured at a wavelength of 570 nm on a microplate reader (Synergy Multi-Mode Reader, BioTek, Winooski, VT, USA). The percentage of cell viability was calculated with the control (LPS-treated cells without tested samples) taken as 100% viability.

2.5.3. Soybean 15-Lipoxygenase Inhibitory Assay

This assay was performed according to the previously described procedure of Pinto et al. (2007) [48] with slight modifications in 96-well microtitre plates [49]. The assay is based on the formation of the Fe^{3+}/xylenol orange (FOX) complex with maximal absorption at 560 nm. The 15-LOX (final concentration: 200 UI/mL) from *Glycine max* (Sigma, Berlin, Germany) was incubated with tested samples or quercetin (100, 50, 25, 10, 5 and 1 µg/mL) used as a positive control at room temperature (25 °C) for 5 min, while DMSO at 10% (*v*/*v*) was used as a negative control. The substrate, linoleic acid (final concentration, 140 µM) prepared in Tris-HCl buffer (50 mM, pH 7.4), was added, and the plates were incubated at 25 °C for 20 min in the dark. The assay was terminated by adding 100 µL of FOX reagent [sulfuric acid (30 mM), xylenol orange (100 µM), ferrous (II) sulfate (100 µM), dissolved in methanol/water (9:1)]. The plates were further incubated at 25 °C for 30 min in the dark, and the absorbance was measured at 560 nm on a microplate reader (Epoch, BioTek, Winooski, USA). The blanks were made in the same way as samples except that the substrate was added after the FOX reagent. The 15-LOX inhibitory activity was determined by calculating the percentage of the inhibition using the following formula:

$$15LOX\ inhibitory\ activity\ (\%) = 100 - [\frac{\text{Abs (sample)} - \text{Abs (blank)}}{\text{Abs (negative control)} - \text{Abs (blank)}} \times 100] \quad (3)$$

Abs: absorbance.

2.6. Statistical Analysis

The 50% inhibitory concentration (IC_{50}) values of each tested sample were determined by plotting a non-linear regression curve of bioactivity against the logarithm (log10) of the sample concentrations by using the software GraphPad Prism 6.0 (GraphPad software Inc., San Diego, CA, USA). All experiments were performed in triplicate, and the results are presented as mean ± standard deviation (SD) values. The comparison of data among tested samples and/or controls was done using one-way analysis of variance (ANOVA) and Student–Newman–Keuls or Dunnett's tests in GraphPad Prism 6.0 software. Results were considered significantly different when the p value was greater than 0.05.

2.7. Molecular Docking Study

The potential therapeutic efficacy of the natural compounds used as ligands, namely, 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene (Ligand_**1**), 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (Ligand_**2**), apigenin- 8-C-β-D-glucopyranoside (Ligand_**3**), globimetulin A (Ligand_**4**), globimetulin B (Ligand_**5**), globimetulin C (Ligand_**6**), bangwaoleanene A (Ligand_**7**), bangwaursene B (Ligand_**8**) and 3β-acetoxy-11,12-epoxytaraxerol (Ligand_**9**), against ten putative target enzymes, namely, acetylcholinesterase (PDB: 4ARB), β-secretase (PDB ID:4L7J), Tau-protein kinase-I (PDB ID: 1J1C), cyclin-dependent kinase (PDB ID: 7VDQ), glycogen synthase kinase-3beta (PDB ID:1H8F), human 15-lipoxygenase-2 (PDB ID: 4NRE), murine inducible nitric oxide synthase (PDB ID: 1QW5), human inducible nitric oxide synthase (PDB ID: 4NOS), cyclooxygenase-1 (PDB ID: 3N86) and cyclooxygenase-2 (PDB ID: 4PH9), was investigated. Briefly, the ligand 3D-chemical structures were retrieved from the PubChem database, and some of the ligand structures were designed via ChemDraw 21.0 software due to their unavailability in PubChem. As per the requirement and to obtain reliable docking results, all ligands in 3D were optimized using Avogadro software and saved in a dot.pdb (.pdb) file. Similarly, all target enzyme 3D protein structures were retrieved from the protein data bank with individual PDB IDs, and mostly the fragmented protein structures were rebuilt through the SWISSMODEL tool before molecular docking [50]. In addition, all attached heteroatoms from each retrieved protein structure were removed and saved in .pdb format for docking studies. Following our previous report, we carried out the molecular docking study using AutoDock 4.2 [51,52]. Based on the minimum docking score (kcal/mol), we selected the best docking pose of the proposed

ligands. Then, BIOVIA-Discovery Studio Visualizer (BIOVIA-DSV) software was employed for protein–ligand molecular interaction visualisation of the docking complexes [53].

3. Results and Discussion

3.1. Acetylcholinesterase Inhibitory Activity

Acetylcholinesterase (AChE) is the enzyme that serves to break down acetylcholine (ACh) into acetate and choline, and acetylcholine deficiency is correlated with the severity of AD, the most common type of dementia [54]. The inhibition of AChE by natural compounds will induce a high level of ACh in the cholinergic synapses, which enhances its bioavailability and thus prolongs the duration of the neurotransmitter action within the brain [23,55]. In this study, nine compounds, i.e., eight terpenoids (**1**), (**2**), (**4**), (**5**), (**6**), (**7**), (**8**) and (**9**) and one flavonoid (**3**), were isolated from three African mistletoes (Loranthaceae), and the AChE inhibitory activity of the crude extracts and the isolated compounds was investigated. We found that all crude extracts and six isolated compounds (**2**, **3**, **5**, **6**, **8** and **9**) had a concentration-dependent inhibitory effect on AChE (Figure 2). After the IC_{50} values of active samples were determined by non-regression linear using the software GraphPad Prism 6.0 (see Table 1), it was observed that if compared to eserine (IC_{50} =1.23 µg/mL or 4.47 µM) used as the positive control in this study, the crude extracts had weak to moderate inhibitory potential against AChE, with the seed extract of *T. bangwensis* (MSTB) having the highest activity (IC_{50} of 48.97 µg/mL). The compound 3β-acetoxy-11,12-epoxytaraxerol (**9**) isolated from this active extract (MSTB) was the most potent, with an IC_{50} value of 6.92 µg/mL (13.89 µM). Previous studies have reported the AChE inhibitory activity of taraxerol isolated from *Clitoria ternatea* with an IC_{50} value of 69 µM [56] or from the twigs of *Vaccinium oldhami* with an IC_{50} value of 33.6 µg/mL (79 µM) [57]. Therefore, the current study identified a new taraxerol derivative, namely, 3β-acetoxy-11,12-epoxytaraxerol (**9**), as being more potent than taraxerol against AChE, which warrants further investigations on structure–activity relationships with taraxerol derivatives. Structural optimization is always more ideal for drug chemistry concepts that explore potency based on their composition and later inspire the development of a number of lead candidates [53]. Three other compounds, 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (**2**), apigenin- 8-C-β-D-glucopyranoside (**3**) and globimetulin C (**6**), also had good AChE inhibitory effect, with IC_{50} values of 18.63 µg/mL (30.84 µM), 25.06 µg/mL (57.81 µM) and 24.64 µg/mL (36.18 µM), respectively. It is therefore clear that the active compounds (**2**), (**3**), (**5**), (**6**), (**8**) and (**9**) were responsible for the efficacy of their respective plant extracts against AChE, which further confirmed the medicinal usage of *P. capitata*, *T. bangwensis* and *G. dinklagei* against brain dysfunction related diseases as previously reported in an ethnopharmacology survey [5]. Additionally, this study also revealed an increase in the AChE inhibitory activity after the purification of more active compounds from bioactive extracts although we did not use a bioassay-guided method for their isolation. Despite the fact that most of the potent AChEIs from plants are alkaloids, several non-alkaloidal compounds including terpenoids, flavonoids or phenolic compounds have been identified as efficient against AChE [58]. In this study, we report one flavonoid (**3**) and five terpenoids, (**2**), (**5**), (**6**), (**8**) and (**9**), which are identified for the first time as having promising AChE inhibitory potential.

Table 1. Inhibitory concentrations (IC_{50} in µg/mL and µM) of crude extracts of *Phragmanthera capitata*, *Globimetula dinklagei* and *Tapinanthus bangwensis* and their isolated compounds with reference controls (eserine) or (quercetin).

Extracts and Compounds	IC_{50} Values					
	AChE		NO		15-LOX	
	µg/mL	µM	µg/mL	µM	µg/mL	µM
MLPC	58.73 ± 5.63 [a]	N/A	67.02 ± 2.27 [a]	N/A	57.30 ± 9.38 [a]	N/A
1	>100	>143.67	>100	>143.67	>100	>143.67
2	18.63 ± 5.28 [b]	30.84 ± 8.74 [a]	>100	>165.56	>100	>165.56

Table 1. *Cont.*

Extracts and Compounds	IC_{50} Values					
	AChE		NO		15-LOX	
	µg/mL	µM	µg/mL	µM	µg/mL	µM
3	25.06 ± 1.56 [b]	57.81 ± 3.59 [b]	>100	>230.69	>100	>230.69
MEGD	142.61 ± 13.45 [c]	N/A	27.85 ± 0.20 [b]	N/A	36.96 ± 8.71 [b]	N/A
4	>100	>130.94	>100	130.94	>100	>130.94
5	33.59 ± 2.80 [d]	76.34 ± 6.36 [c]	67.64 ± 4.25 [a]	153.72 ± 9.65 [a]	7.14 ± 0.53 [c]	16.22 ± 1.20 [a]
6	24.64 ± 4.06 [b]	36.18 ± 5.96 [a]	>100	>146.84	>100	>146.84
MSTB	48.97 ± 3.53 [a]	N/A	24.02 ± 2.27 [b]	N/A	56.93 ± 4.78 [a]	N/A
7	>100	>171.52	>100	>171.52	>100	>171.52
8	87.47 ± 11.54 [e]	161.98 ± 21.37 [d]	>100	>185.18	23.31 ± 3.77 [c]	43.16 ± 6.98 [b]
9	6.92 ± 1.75 [f]	13.89 ± 3.51 [e]	>100	>200.80	>100	>200.80
Eserine	1.23 ± 0.09 [g]	4.47 ± 0.32 [f]	N/A	N/A	N/A	N/A
Quercetin	N/A	N/A	12.51 ± 0.51 [c]	41.12 ± 1.69 [b]	23.97 ± 2.78 [c]	79.37 ± 9.20 [c]

Data are presented as the means of triplicate measurements ± standard deviation; superscript letters [a–g] represent statistical difference between data obtained, and for each column of the above table, data with same letters are statistically not different while data with different letters are significantly different at $p < 0.05$. IC_{50}: concentration required to inhibit the biological effect by 50% compared to untreated controls. MLPC: methanol leaf extract of *P. capitata*, MEGD: methylene chloride/methanol (1:1) entire plant extract of *G. dinklagei*, MSTB: methylene chloride/methanol (1:1) seed extract of *T. bangwensis*. Compounds (**1**–**9**) were isolated from the above-mentioned plant extracts. IC_{50}: 50% inhibitory concentration. AChE: acetylcholinesterase, NO: nitric oxide, 15-LOX: 15-lipoxygenase. N/A: Not Applicable.

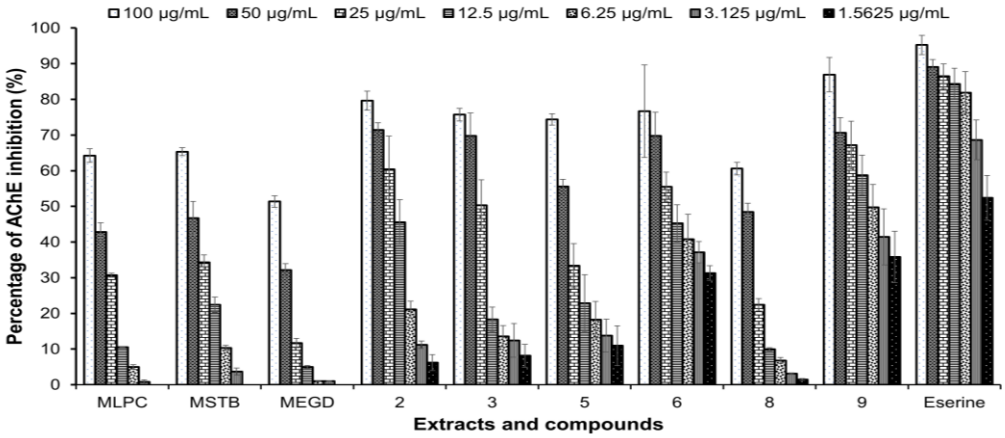

Figure 2. Acetylcholinesterase inhibitory activity of active extracts and compounds at different concentrations. Eserine was used as a positive control. Data are presented as the mean ± standard deviation of three independent experiments. MLPC: methanol leaf extract of *P. capitata*, MEGD: methylene chloride/methanol (1:1) entire plant extract of *G. dinklagei*, MSTB: methylene chloride/methanol (1:1) seed extract of *T. bangwensis*. Compounds (**2**), (**3**), (**5**), (**6**), (**8**) and (**9**) were isolated from the above-mentioned plant extracts.

3.2. Nitric Oxide Production Inhibitory Activity and Cytotoxic Effect on LPS-Stimulated RAW 264.7 Macrophages

Nitric oxide (NO) plays an important role in inflammation, and its overproduction within the central nervous system is associated with the pathogenesis of AD [27]. Therefore, any AChEI with NO production inhibitory effect will be beneficial to decrease inflammation observed during the pathogenesis of AD. Crude extracts and isolated compounds were tested at 100 µg/mL in LPS-stimulated RAW 264.7 macrophage cells, and all three crude extracts and globimetulin B (**5**) had a good NO production inhibitory effect (at least 50% inhibitory effect) (Figure 3A). The results of the IC_{50} values obtained from these active samples are presented in Table 1. The seed extract of *T. bangwensis* (MSTB) had the highest AChE inhibitory potential and also exhibited the highest NO production inhibitory potential

(IC_{50} of 24.02 µg/mL). The combination of these two biological activities within the same extract confirms the medicinal use of *T. bangwensis* for the management of brain dysfunction-related diseases and in our case, its efficacy as a potential treatment of AD.

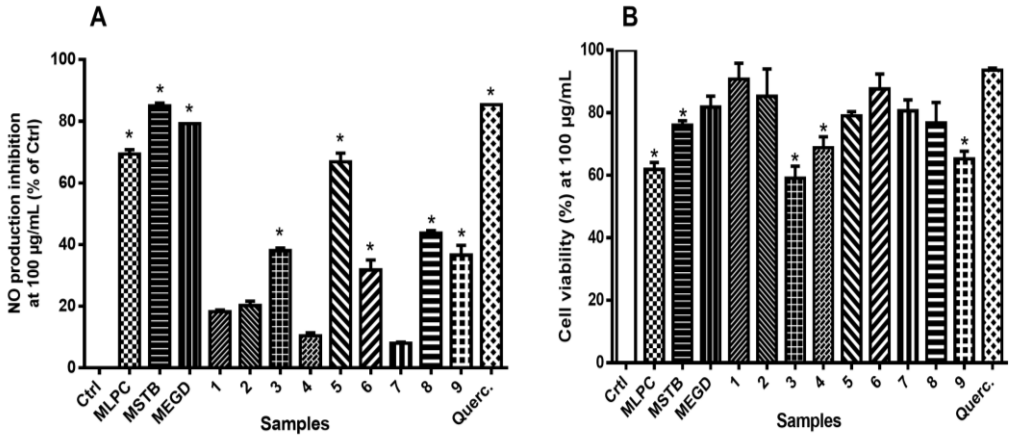

Figure 3. Nitric oxide inhibitory activity of crude extracts and isolated compounds in LPS-stimulated RAW 264.7 macrophage cells (**A**) and their respective percentage of cell viability (**B**). The percentage of NO inhibition was determined based on the efficacy of test samples to inhibit NO production by RAW 264.7 cells compared with the negative control (RAW 264.7 cells treated with LPS and DMSO at 0.5% without test samples), which was considered to be 0% inhibition. Quercetin was used as a positive control. Data represent the mean ± standard deviation of three independent experiments, MLPC: methanol leaf extract of *P. capitata*, MEGD: methylene chloride/methanol (1:1) entire plant extract of *G. dinklagei*, MSTB: methylene chloride/methanol (1:1) seed extract of *T. bangwensis*. Compounds (**1**–**9**) were isolated from the above-mentioned plant extracts. Querc. (Quercetin). Data are presented as means of triplicate measurements ± standard deviation; (*) means a statistical difference ($p < 0.05$) between the tested samples versus the negative control (Crtl).

The cytotoxicity of crude extracts at 100 µg/mL and isolated compounds was determined in LPS-stimulated RAW 264.7 macrophage cells by using the MTT assay. All crude extracts and compounds had a cytotoxic effect when compared to the LPS-stimulated RAW 264.7 cells treated with DMSO 0.5% used as a negative control (Figure 3B). The most important result was obtained with MSTB, MEGD, globimetulin B (**5**) and bangwaursene B (**8**), which had about 80% viable cells at 100 µg/mL, a concentration that showed a good NO production inhibitory effect. This means that the NO production inhibitory effect of these tested samples was not related to their cytotoxic effect.

3.3. Lipoxygenase Inhibitory Activity

Lipoxygenases catalyse the stereo selective addition of oxygen to polyunsaturated fatty acids such as linoleic and arachidonic acids, forming leukotrienes and lipoxins. The role of 15-LOX in the regulation of AD has been recognized [59]. In fact, the level of 15-LOX increases during the early phase of AD, thereby indicating its implication in the pathogenesis of AD [60]. It therefore makes sense that 15-LOX can be considered another target used to stop the progression of AD. This study identified three crude extracts (MLPC, MEGD and MSTB) and two compounds, (**5**) and (**8**), with inhibitory effect against 15-LOX. The results of the IC_{50} values determined with the software GraphPad Prism 6.0 are presented in Table 1. It was found that two active compounds, globimetulin B (**5**) and bangwaursene B (**8**), were highly potent against 15-LOX with IC_{50} values of 7.14 µg/mL (16.22 µM) and 23.31 µg/mL (43.16 µM), respectively. These compounds were even more effective than quercetin, which had an IC_{50} value of 23.97 µg/mL (79.37 µM). As 15-LOX has been recognized to be involved in the progression of various inflammation-related diseases such as psoriasis, atherosclerosis and rheumatoid arthritis [61,62] and the discovery of very potent human 15-LOX inhibitors is still underway [63], our study therefore identified

two terpenoids, globimetulin B (**5**) and bangwaursene B (**8**), which can be considered potential anti-15-LOX agents for the management of these diseases.

3.4. Binding Efficacy of Natural Compounds against Selected Target Enzymes

Ten enzymes were selected for molecular docking based on their relevance as therapeutic targets in the management of AD and inflammation. Acetylcholinesterase (AChE), β-secretase 1 (BACE1), Tau-protein kinase-I (TPK-I), cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase-3β (GSK-3β) are most attractive targets for anti-Alzheimer drug development [64] whereas 15-Lipoxygenase (15-LOX), inducible nitric oxide synthase (iNOS) and cyclooxygenases 1 and 2 (COX1 and COX2) have been implicated in the pathogenesis of many inflammation-related diseases including AD [31]. As per our hypothesis, we calculated the docking score or binding efficacy (kcal/mol) of each ligand against the ten selected enzymes (Table 2). According to the recorded molecular docking score, all ligands exhibited activity within the range of −6 to −11 kcal/mol, where −10.8 kcal/mol was the most effective result for bangwaursene B against 15-LOX-2, and −6.4 kcal/mol was the lowest potential score for 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene against GSK-3β. Individually, 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene (−10.6 kcal/mol) exhibited the greatest potential against 15-LOX-2, 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin (−9.6 kcal/mol) against M_iNOS, apigenin- 8-C-β-D-glucopyranoside (−11.4 kcal/mol) against M_iNOS, globimetulin A (−9.8 kcal/mol) against 15-LOX-2, globimetulin B (−10.7 kcal/mol) against 15-LOX-2, globimetulin C (−10.4 kcal/mol) against 15-LOX-2, bangwaoleanene A (−10.4 kcal/mol) against 15-LOX-2, bangwaursene B (−10.8 kcal/mol) against 15-LOX-2 and 3β-acetoxy-11,12-epoxytaraxerol (–9.9 kcal/mol) against BACE1 (Table 2).

Table 2. Therapeutic efficacy of nine natural products against ten target enzymes based on protein–ligand binding or the molecular docking score (kcal/mol).

Ligands	AChE	BACE1	TPK-I	CDK5	GSK-3β	15-LOX-2	M_iNOS	H_iNOS	COX-1	COX-2
Ligand_**1**	−9.1	−7.8	−6.9	−8.2	−6.4	−10.6	−8.6	−8.0	−8.4	−8.8
Ligand_**2**	−8.8	−9.3	−7.7	−7.4	−8.3	−9.2	−9.6	−8.8	−8.7	−8.7
Ligand_**3**	−9.4	−10.1	−8.2	−10.4	−8.7	−9.6	−11.4	−10.9	−9.5	−8.5
Ligand_**4**	−9.0	−7.7	−7.5	−8.2	−7.9	−9.8	−8.4	−8.8	−7.4	−6.8
Ligand_**5**	−8.6	−10.0	−9.9	−8.0	−8.6	−10.7	−8.5	−8.6	−8.1	−8.3
Ligand_**6**	−9.3	−7.8	−6.9	−7.5	−7.0	−10.4	−9.2	−9.4	−8.4	−8.0
Ligand_**7**	−9.1	−8.4	−7.9	−8.1	−8.1	−10.4	−10.2	−9.4	−8.8	−8.6
Ligand_**8**	−9.1	−9.0	−9.3	−7.5	−8.5	−10.8	−10.6	−9.2	−9.1	−8.7
Ligand_**9**	−8.7	−9.9	−8.5	−8.7	−8.8	−9.2	−9.7	−9.3	−8.9	−8.1

Ligand_**1**: 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene; Ligand_**2**: 3-O-β-D-glucopyranosyl-28-hydroxy-α-amyrin; Ligand_**3**: apigenin- 8-C-β-D-glucopyranoside; Ligand_**4**: globimetulin A; Ligand_**5**: globimetulin B; Ligand_**6**: globimetulin C; Ligand_**7**: bangwaoleanene A; Ligand_**8**: bangwaursene B; and Ligand_**9**: 3β-acetoxy-11,12-epoxytaraxerol; AChE: acetylcholinesterase (PDB: 4ARB); β-secretase or BACE1 (PDB ID:4L7J); TPK-I: Tau-protein kinase-I (PDB ID: 1J1C); CDK5: cyclin-dependent kinase (PDB ID: 7VDQ); GSK-3β: glycogen synthase kinase-3beta (PDB ID:1H8F); *15*-LOX-2: human 15-lipoxygenase-2 (PDB ID: 4NRE); *M_iNOS*: murine inducible nitric oxide synthase (PDB ID: 1QW5); H_iNOS: human inducible nitric oxide synthase (PDB ID: 4NOS); COX-1: cyclooxygenase-1 (PDB ID: 3N86); COX-2: cyclo-oxygenase-2 (PDB ID: 4PH9).

Overall, most candidates showed effectiveness against the 15-LOX-2 and M_iNOS targets, where apigenin- 8-C-β-D-glucopyranoside was the most prominent candidate followed by bangwaursene B as per the docking score. In addition, the protein–ligand interaction analyses indicated that apigenin- 8-C-β-D-glucopyranoside formed a greater number of hydrogen bond interactions with the respective target enzymes (Figure 4). Based on a comparison of *in silico* with *in vitro* data, globimetulin B and bangwaursene B, which showed the highest binding scores against 15-LOX-2 (−10.7 and −10.8 kcal/mol, respectively), exhibited a marked inhibitory effect against 15-LOX (IC_{50} of 16.22 and 43.16 μM, respectively). Additionally, bangwaursene B, with a binding score of −10.6 kcal/mol against M-iNOS, also exhibited an anti-inflammatory effect by inhibiting NO production (Figure 3A). These results indicate the accordance between the molecular docking

study and the experimental data obtained with globimetulin B (**5**) and bangwaursene B (**8**). By contrast, 3β-acetoxy-11,12-epoxytaraxerol, which had the highest inhibitory effect against AChE (IC_{50} of 13.89 µM), showed a binding score of −8.7 kcal/mol compared to −9.1 kcal/mol for 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene, which was inactive up to 143.67 µM. This indicates the importance of combining an *in silico* study with *in vitro* experiments for an efficient selection of potential lead compounds against any disease. In the current drug discovery process, bioinformatics tools, mostly molecular docking simulation, are the most widely used platform for both academic and industrial researchers to select the best potential leads targeting any desired target enzymes associated with any disease development and proliferation [50,53]. However, proper hypotheses and computational knowledge are required to minimize the error and produce reliable outputs.

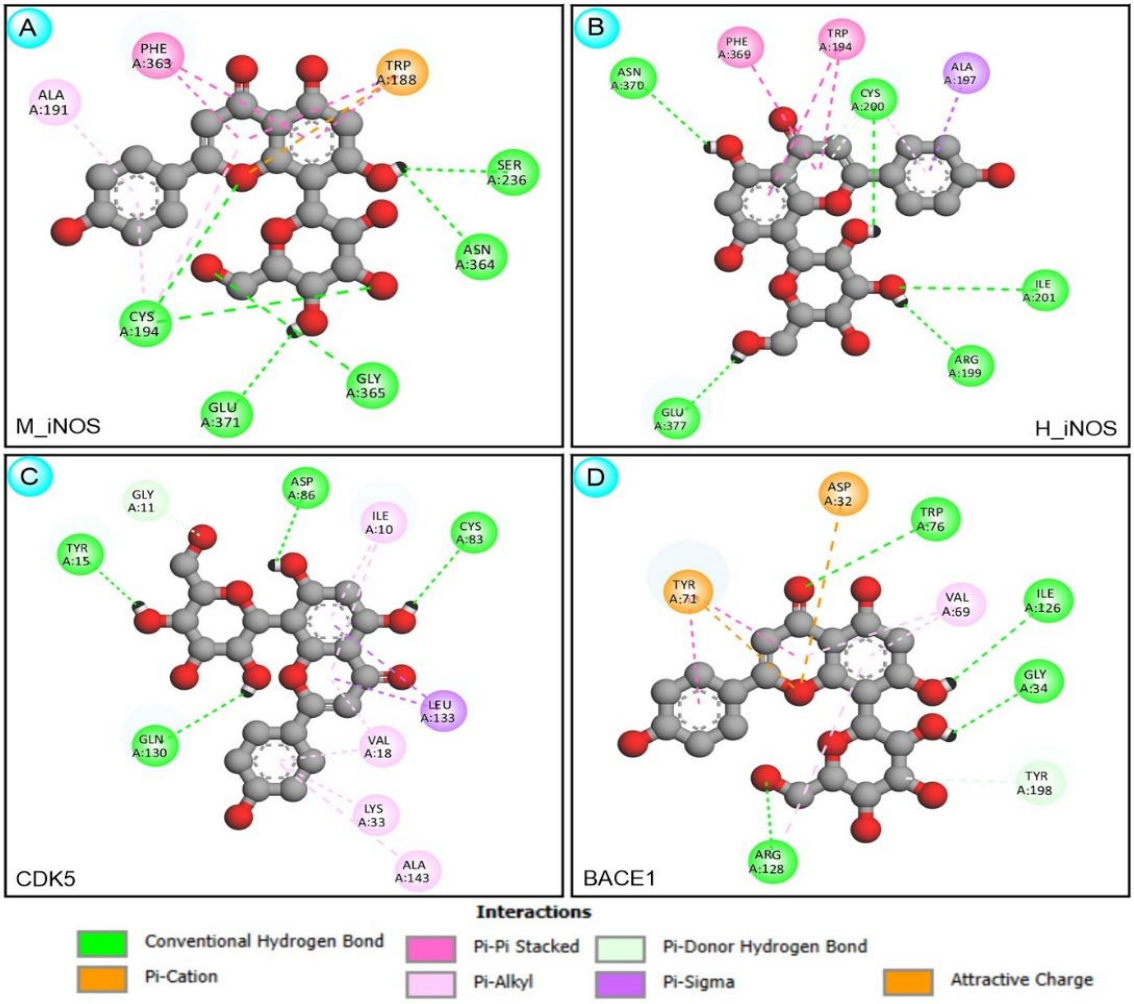

Figure 4. Visualization of protein–ligand interactions using BIOVIA-DSV software; (**A**): protein–ligand interactions between M_iNOS and ligand_**3** (−11.4 kcal/mol); (**B**): protein–ligand interactions between H_iNOS and ligand_**3** (−10.9 kcal/mol); (**C**): protein–ligand interactions between CDK5 and ligand_**3** (−10.4 kcal/mol) and (**D**): protein–ligand interactions between BACE1 and ligand_**3** (−10.1 kcal/mol). Ligand_**3**: apigenin-8-C-β-D-glucopyranoside.

4. Conclusions

Globimetulin B (**5**) isolated from the entire plant of *Globimetula dinklagei* was the only compound that simultaneously exhibited AChE, 15-LOX and NO production inhibitory activities, as well as high binding scores with BACE1, TPK-I and 15-LOX-2 targets. Globimetulin B can therefore represent a potential candidate for drug development against AD and other inflammation-related diseases. Additionally, other compounds such as 3-O-β-D-

glucopyranosyl-28-hydroxy-α-amyrin (**2**), apigenin- 8-C-β-D-glucopyranoside (**3**), globimetulin C (**6**) and 3β-acetoxy-11,12-epoxytaraxerol (**9**) that were active either against AChE and/or 15-LOX and which showed effectiveness against the AChE, 15-LOX-2 and M_iNOS targets can also be valorized with respect to their therapeutic value. The current study also opens a further research direction aiming to investigate the structure–activity relationship of taraxerol derivatives against AChE. Furthermore, the docking results were in agreement with in vitro experimental data for globimetulin B and bangwaursene B against 15-LOX-2 while a disagreement was observed for 7β,15α-dihydroxy-3β-palmitatelup-20(29)-ene and 3β-acetoxy-11,12-epoxytaraxerol against AChE. This finding confirmed the importance of combining *in silico* and *in vitro* methods to generate the most accurate and reliable results.

Author Contributions: Conceptualization, E.M.N. and H.L.D.M.; Methodology, E.M.N., S.S.S. and H.L.D.M.; software, E.M.N., S.S.S. and C.I.C.; validation, E.M.N., S.S.S., Y.M.N.M., P.M. and C.I.C.; formal analysis, E.M.N., S.S.S., H.L.D.M., P.M. and C.I.C.; investigation, E.M.N., H.L.D.M. and P.M.; resources, T.J.M., S.S.M. and L.J.M.; data curation, E.M.N., Y.M.N.M. and H.L.D.M.; writing—original draft preparation, E.M.N., S.S.S. and H.L.D.M.; writing—review and editing, E.M.N., S.S.S., H.L.D.M., P.M., C.I.C., T.J.M., S.S.M. and L.J.M.; visualization, E.M.N., H.L.D.M. and P.M.; supervision, E.M.N., P.M., T.J.M., S.S.M. and L.J.M.; project administration, E.M.N., H.L.D.M. and P.M.; funding acquisition, E.M.N., T.J.M., S.S.M. and L.J.M. All authors have read and agreed to the published version of the manuscript.

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