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Research Article

Apigenin and Kaempferol from *Ficus benjamina* Leaves as Potential Inhibitors of Enzymes Relevant to Alzheimer's Disease Pathology: An *In Silico* and *In Vitro* Study

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ABSTRACT

Alzheimer's disease is a progressive neurodegenerative disease. In this report, we identified and evaluated polyphenol constituents from the leaves of *Ficus benjamina*, targeting enzymes relevant to the pathology of Alzheimer's disease. Polyphenols were extracted from the leaves of *F. benjamina*, subjected to LC-MS and GC-MS analyses. The constituents were subjected to *In Silico* analyses, targeting acetylcholinesterase (AChE), butyrylcholinesterase (BUChE), and beta-secretase 1 (BACE1). *In vitro* antioxidant (ferrous ion-chelating, copper reducing antioxidant, ferric reducing antioxidant power (FRAP), and total antioxidant capacity), and enzyme inhibitory (AChE, BUChE, and BACE1) assays, were performed using standard procedures. *In silico* analysis revealed that Apigenin and Kaempferol showed strong functional interactions with AChE, BUChE, and BACE1. Apigenin and Kaempferol showed potent antioxidant capacity with IC_{50} values of 76.82 ± 0.95 µg/ml and 38.85 ± 0.68 µg/ml (Metal chelating assay); 63.77 ± 2.19 and 81.53 ± 2.54 mg AAE/g (FRAP assay); 166.90 ± 5.02 and 216.28 ± 8.20 mg AAE/g (Total antioxidant capacity); and 64.96 ± 0.80 and 86.09 ± 1.27 mg TE/g (CUPRAC assay) respectively. They also significantly inhibited AChE, BUChE, and BACE1 with IC_{50} values of 42.67 ± 0.76 µg/ml and 46.76 ± 0.61 µM (Apigenin) respectively. The evaluation of Apigenin and Kaempferol from *F. benjamina* confirmed their potential usefulness in Alzheimer's conditions, and this could be due to their inhibitory action against the functional activities of AChE, BUChE, and BACE1.

Keywords: *Ficus benjamina; Polyphenols; Alzheimer's Disease; Apigenin; Kaempferol.*

INTRODUCTION

Alzheimer's disease (AD) is a progressive multifaceted neurological abnormality that results in cognitive decline, loss of memory, unusual behaviour, and personality changes (Najeeb *et al.*, 2015). Globally, it is the major cause of dementia, especially in late adult life; accounting for about 60-80% of cases (Thies & Bleiler, 2013). World Alzheimer Report 2012 revealed that in 2019 about 55 million people were known to be afflicted with AD worldwide, a situation that may reach 139 million by 2050 (Gauthier *et al.*, 2022), necessitating the need for a continuous search for a more effective and affordable pharmacotherapy. It is concerning that globally, over 75% of people with dementia remain undiagnosed (Gauthier *et al.*, 2022). Though, the available evidence showed a higher incidence of AD in Latin America and Asia, as compared to sub-Saharan Africa and India (Hebert *et al.*, 2013), with a steady increase in the ageing population, the global

incidence of AD and associated dementia are expected to increase. Such increases may not be unconnected with environmental factors, including dietary habits and lifestyle modification, and social-cultural, as well as cardiovascular and genetic factors (Tripathi *et al.*, 2012).

Despite the availability of drugs for the management of AD, none can stop or at least slow down associated neurodegeneration. Oxidative stress or death is a critical factor in the development of neurodegenerative diseases such as AD (Padurariu *et al.*, 2013; Zhao & Zhao, 2013). The imbalance of antioxidants/oxidants in favour of oxidants, which usually occurs due to increased free radicals or a decrease in antioxidant defense, is mainly initiated and enhanced by oxidative stress (Huang *et al.*, 2016). Oxidative stress is the connecting bridge for all other hypotheses (tau protein, neuroinflammation, amyloid cascade, and metal ions) that seek to explain the pathogenic mechanisms of AD (Bai *et al.*, 2022). Therefore, to reduce inflammation, and mop up the reactive species, antioxidants are widely preferred. Available reports suggest that antioxidants may play critical roles in AD (Albarracin *et al.*, 2012; Vauzour, 2012; Bakir *et al.*, 2020). Interestingly, natural polyphenols are known for their potent antioxidant properties, and they can be found in plants, plant parts, or products, such as vegetables, fruits, essential oils, tea, and red wine. Flavonoids are the largest family of polyphenols known for their multiple biological activities, namely; antibacterial, antioxidant, antiviral, hepatoprotective, anti-inflammatory, anticancer, and neuroprotective activities (Kumar & Pandey, 2013; Jucá *et al.*, 2020; Dias *et al.*, 2021). Their neuroprotective activity may be linked to their antioxidant effect and this may explain their ability to significantly attenuate cognitive impairments and beta-amyloid burden (Wang *et al.*, 2017; Ayaz *et al.*, 2019). Polyphenols are also known to be associated with increased or improved brain function and longevity (Vauzour, 2012; Silva dos Santos *et al.*, 2021). For instance, administration of epigallocatechin gallate (EGCG) to mice inhibited the onset of neurological symptoms, leading to prolongation of life span (Xu *et al.*, 2006; Scholey *et al.*, 2012; Youn *et al.*, 2022). Also, EGCG enhanced movement abilities and increased life span in a transgenic Drosophila melanogaster model of Parkinson's Disease (PD) (Wagner *et al.*, 2015). Therefore, the use of natural polyphenols may constitute an essential strategy in our bid to enhance pharmacotherapeutic intervention in AD.

Several plants have shown therapeutic potential for neurodegenerative diseases, among which are the Ficus species (Sharifi-Rad *et al.*, 2020; Andrade *et al.*, 2023; Sasidharan *et al.*, 2023; Tyler & Tyler, 2023). *Ficus benjamina* is a flowering tree plant, known to be native to Asia and Australia, and commonly referred to as weeping fig. Several biological and pharmaceutics activities have been attributed to different parts of *F. benjamina*. For instance, the extracts of leaves, stem and root of *F. benjamina* showed antioxidant, antimicrobial, and substantial hemolytic potentials (Imran *et al.*, 2014). Also, the aqueous extract of the figs of *F. benjamina* showed anticonvulsant activity (Singh *et al.*, 2023), while the aqueous extract of the leaves served as a good source of reducing and stabilizing agents, ensuring the faster synthesis of nanoparticles (Puente *et al.*, 2019). Other biological activities attributed to various parts of *F. benjamina* include hepatoprotective (Kanaujia, 2011), antimicrobial (Mehtab, 2009; Imran *et al.*, 2014; Truchan *et al.*, 2015), antioxidant (Abdel-Hameed, 2009; Abhishek et al., 2013; Saptarini & Herawati, 2015), antiviral (Yarmolinsky, 2012), antimycobacterial (Cruz, 2012), antinociceptive (Parveen, 2009) and antidiarrhoeal (Oladiji *et al.*, 2012). However, toxicity studies on the ethanol leave extract of F. benjamina showed significant alterations in ALT and AST following 60 days of repeated dosing (Hasti *et al.*, 2014), indicating the need for caution when using the extracts. Meanwhile, the most common biological activities relevant to neurodegenerative disorders that are linked to Ficus species and their phytoconstituents are antioxidant and anti-cholinesterase (Salehi *et al.*, 2021; Sieniawska *et al.*, 2022; Pahari *et al.*, 2022; Rani *et al.*, 2024). Among the identified phytoconstituents from several Ficus species are polyphenolic and triterpenoid compounds (Parveen, 2009; Imran *et al.*, 2014; Walia *et al.*, 2022; Kiralan *et al.*, 2023). *Ficus benjamina* Linn. (Moraceae) is known for its abundant polyphenolic compounds (Hassan *et al.*, 2003; Abhishek *et al.*, 2013; Ojha, 2013; Imran *et al.*, 2014), including syringic, p-coumaric, chlorogenic, and ferulic acids (Imran *et al.*, 2014). Interestingly, the inhibitory activity of isoflavonoids from F. benjamina on BACE1 has been documented (Dai *et al.*, 2012), paving the way for further studies on the potential biological effects of polyphenolic constituents of *F. benjamina*. Therefore, we identified and performed an *in silico* analysis of polyphenol constituents from the leaves of *F. benjamina*, targeting acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and beta-secretase 1 (BACE1) enzymes that are relevant to the pathology of AD. The selected polyphenols from the *in silico* analysis were further evaluated for their antioxidant, and enzyme inhibitory activities.

MATERIALS AND METHODS

Collection and authentication of the leaves of *Ficus benjamina*

The leaves of *F. benjamina* were obtained from Obafemi Awolowo University (OAU) campus in Ile-Ife, Nigeria, and were authenticated at the Department of Botany Herbarium, OAU, Ile-Ife, with herbarium voucher number IFE-17688. The plant name was also checked against http://www.theplantlist.org,andwww.worldfloraonline.org, which are extensive sources for the confirmation of medicinal plants.

Preparation of *ficus benjamina* polyphenolic-rich fractions

The polyphenols were extracted following earlier reported method (Karunrat & Izabela, 2012) with little modifications. The process involved three successive extractions, a concentration of combined extracts, and at least two rounds of purifications. The first two extractions from the finely grounded dried leaves of *F. benjamina* (250 g) were done in 2 L of acidified methanol (80 % methanol, 19 % H_2O, and 1 % acetic acid) at 4°C for 4 hrs. The third extraction was carried out overnight (16 h). The combined supernatant was evaporated under reduced pressure at 40°C using a rotary evaporator. The concentrated alcoholic extract was reconstituted in acidified water (99 % H_2O, 1 % acetic acid, v/v) and applied to an Amberlite XAD-16 resin column for purification. The polyphenols were eluted with 80 % ethanol (80 % ethanol, 19.9 % H_2O, 0.1 % trichloroacetic acid), and evaporated under reduced pressure at 40°C using a rotary evaporator. The final fine lyophilized powder (polyphenolic-rich fraction) was obtained by reconstitution of purified fraction in purified water, followed by freeze-drying under vacuum. The yield of the extraction was expressed as the fraction of the original powdered leaves of *F. benjamina*.

Liquid chromatography and mass spectrometry (LC-MS) analysis

A solution of a 10 mg sample of the polyphenolic-rich fraction was made in 1 mL of 80% methanol, 19.9% water, and 0.1% trichloroacetic acid. The sample was vortexed mix for 30 min, sonicated for 20 minutes, and spun down for 10 min at 10,000 rpm to obtain a clear supernatant for subsequent analysis. LC-MS analysis was carried out on an Agilent Technologies 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, USA) coupled to a 6540 UHD Q-TOF System. An Agilent Eclipse plus C18 analytical column (100 mm x 2.1 mm i.d., 1.8 µm), set at 40°C was used. The binary solvent system is composed of 0.1% formic acid in HPLC-grade water and 0.1% formic acid in acetonitrile, with a flow rate of 0.5 mL/min. The gradient increases from the starting point of 15 % to 25 % over 20 min, followed by an increase to 97 % over the next 50 min. The MS full scan mode with a range of m/z 100 to 1700, at a rate of 1.5 scans per second, was used for mass peak detection. The data was analyzed using Agilent Mass Hunter and Mass Profiler software. The identified compounds were checked against the Metlin metabolite database.

Gas chromatography and mass spectrometry (GC-MS) analysis

Purified polyphenolic-rich fraction (10 mg) was treated with 50 µL each of BSTFA (*N,O*-Bis(trimethylsilyl)trifluoroacetamide) and pyridine solutions for 1 hour at 60°C, followed by GC-MS analysis of the derivatized samples. The GC consists of a helium gas at a flow rate of 1 mL/min, and 1 µL injection, within 80° – 340°C at a rate of 15°C/min. The GC-MS consists of an Agilent Technologies Model 7890 gas chromatograph equipped with an Agilent DB-1ms Inert (15 m, 0.25 mm, 0.25 µm) column, and coupled to Model 5975C Mass Spectrometer, operating under standard EI ionization conditions. Automated Mass Spectral Deconvolution and Identification System (AMDIS) software was used for the deconvolution and integration of resulting spectra. The mass spectra and retention indices were checked against NIST17 and Wiley11 Mass Spectral Libraries for the identification of analyte peaks.

In silico studies

Preparation of ligand

The 2D structures of the polyphenolic compounds identified by GC-MS and LC-MS from *F. benjamina* were prepared using ChemDraw Ultra 12.0. The Chem3D Pro 12.0 was used for conversion from 2D to 3D conformation, and thereafter energy was minimized. The ligands (in pdb format) were prepared for docking using the VEGA ZZ platform (Pedretti, 2002) as earlier described (Daniyan and Ojo, 2019).

Preparation of target proteins

The selected target proteins (namely, beta-secretase 1 enzyme {BACE1, PDB code: 5DQC} (Ghosh *et al.,* 2016), acetylcholinesterase enzyme {AChE, PDB code: 6O4W} (Gerlits *et al.,* 2019) and butyrylcholinesterase {BUChE, PDB code: 3O9M} (Asojo *et al.,* 2011)), were obtained from RCSB PDB (Rose *et al.,* 2011). Selected target proteins were checked for correctness using SwissPdb viewer (Guex & Peitsch, 1997) and prepared by removal of co-crystalized hydrogens, normalization of coordinates, addition of new hydrogens, removal of incorrectly added hydrogens, and minimization using VEGA ZZ platform (Pedretti *et al.,* 2002), as previously described (Daniyan & Ojo, 2019).

Drug-likeness and ADMET prediction

The prediction of the biological activities of the ligands was done using an online web server, Molinspiration (https://molinspiration.com). Further prediction of drug-likeness by "Lipinski's rule of five" (Lipinski, 2004; Lipinski et al., 2001), and ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties were conducted using the pkCSM online prediction server (https://biosig.lab.uq.edu.au/pkcsm/) (Pires et al., 2015). Briefly, a local database (sdf format) of the identified polyphenolic compounds was prepared and converted to SMILE representations using ADSV (Dassault Systèmes BIOVIA, 2015). The text file containing the SMILE representations was uploaded to the pkCSM server for auto-generation of drug-likeness and ADMET properties.

The results (downloaded in excel format) were analyzed and ligands that passed Lipinski's rule of five (molecular mass < 500 Daltons, Log *P* < 5, hydrogen bond donors < 5, hydrogen bond acceptors < 10, and polar surface area between 40 and 130), and have favourable ADMET properties were selected for further molecular docking study.

Molecular docking

Docking procedures were first validated in a preliminary assessment using the co-crystalized ligands as earlier reported (Daniyan and Ojo, 2019). Briefly, the re-docked co-crystalized ligand-protein complex was generated for each scored pose by Autodock vina, and subsequently superimposed on / structurally aligned with the original downloaded structure using ADSV (Dassault Systèmes BIOVIA, 2015) and/or YASARA View (Vriend, 2014) as earlier reported (Daniyan & Ojo, 2019). The best-aligned re-docked complex, as judged by RMSD of aligned structures, was selected. Also, the selection was further checked by rescoring the poses generated by Autodock vina using PLANTS (Korb et al., 2006), XScore version 1.2 (Obiol-Pardo & Rubio-Martinez, 2007), and NNScore versions 2.0 python script (Durrant & McCammon, 2010), followed by consensus analysis as earlier reported (Daniyan & Ojo, 2019). Thereafter, the selected polyphenols (as per Lipinski's rule of five and ADMET), prepared as earlier described (Daniyan & Ojo, 2019), and the corresponding co-crystallized ligand of each downloaded structures, which serve as controls, were docked into the active site of BACE1, AChE and BUChE, using AutoDock vina (Trott & Olson, 2010) interface in VEGA ZZ platform (Pedretti et al., 2002), with binding modes and exhaustiveness set at 9 and 8 respectively as earlier described (Daniyan & Ojo, 2019). Docking results were analyzed for the selection of the most promising ligands for further molecular dynamic (MD) simulation.

Molecular dynamic simulation

Selected polyphenolic compounds (from the analysis of druglikeness, ADMET, and molecular docking) in complex with target proteins (BACE1, AChE, and BUChE) were checked for interaction stability with molecular dynamic (MD) simulations using GROMACS package (Abraham et al., 2015; Berendsen et al., 1995). The downloaded structures with their co-crystallized ligands, served as control. Each protein – ligand complex was prepared for MD simulation using pyGROMODS (available from https://github.com/Dankem/pyGROMODS;https://doi.org/10.5281/zenodo.7912747; and https://doi.org/10.5281/zenodo.8087090) (Daniyan, 2023). In this study, complex preparation by pyGROMODS involves ligand parameterization with GAFF (General Amber Force Field), complex reconstruction and solvation (cubic box, tip3p water, dimension 0.15), and system neutralization with counter ions (Na+/CL-) as earlier described (Daniyan & Ojo, 2019), as well as generation of needed MD simulation input files (structures and topology files) (Daniyan, 2023). The MD simulations process using GROMACS was conducted by using the MDS interface of pyGROMODS, which allowed the upload of generated MD input files and MDS parameter files. Briefly, the system was energy minimized (20,000 steps of steepest descent / conjugate gradient), and thermally equilibrated (0 to 300 K at 10 K/psec) in a 5 ns each of NVT {Canonical ensemble, in which number of particle/substance (N), volume (V), and temperature (T) are constant} and NPT {Isothermal – isobaric ensemble, in which number of particle/substance (N), pressure (P), and temperature (T) are constant} simulations. The resultant structures were then equilibrated in 15 ns MD simulation steps, followed by 100 ns production MD simulation. Basic MD simulation trajectory analysis, including root mean square deviation (RMSD), energy, radius of gyration (RG), and root mean square fluctuation (RMSF), were performed on the last 30 ns of MD simulation using inbuilt analysis codes in GROMACS package and VEGA ZZ platform (Abraham et al., 2015; Berendsen *et al.*, 1995; Pedretti *et al.*, 2002). Also, ligand interaction profiles were done using LigPlot (Laskowski & Swindells, 2011).

In vitro studies

The assays presented below were done in triplicates using at least three differently prepared samples.

Ferrous *Ion-chelating* assay

The procedure described by Singh and Rajini (2004) (Singh & Rajini, 2004) was used for the ferrous ion-chelating assay. The percentage inhibition of complex formation (ferrozine–Fe^{+2}) was calculated as:

$$\text{Chelating Effect (\%)} = [((A\_control - A\_sample)) / A\_control] \times 100$$

where A_ control and A_ sample represent the absorbance of the control and samples respectively.

Copper reducing antioxidant capacity (CUPRAC) assay

The procedure for the determination of the ability of the test compounds to reduce cupric ions (Cu^{2+}) was as reported by Apak *et al.* (2006) (Apak *et al.,* 2006) and presented by Gulcin (2012) (Gulcin, 2012). The final absorbance was read at 450 nm, and the reducing capability of the test compounds was expressed as Trolox equivalent (TEAC).

Determination of ferric reducing antioxidant power (FRAP)

The FRAP assay measures the reducing capability of antioxidants in a redox-linked colorimetric method using a spectrophotometer (Benzie & Strain, 1996). The procedure for FRAP assay was as presented by Iris and Strains (1996)

(Iris and Strains, 1996). The reducing power, which is a measure of the concentration of compounds with ferric reducing ability that is comparable or equivalent to that of the standard, was expressed as equivalent concentration (EC).

Determination of total antioxidant capacity

The procedure used to determine total antioxidant capacity at acidic pH was as described by Prieto *et al.,* (1999) (Prieto *et al.,* 1999). The total antioxidant capacities of the test compounds were expressed as an ascorbic acid equivalent.

Anti-cholinesterase assay

The inhibitory activities of the identified polyphenols (Apigenin and Kaempferol) against selected cholinesterase (AChE and BUChE) were determined using the procedure presented by Ellman *et al.* (1961) as described by Jung *et al.* (2010) (Ellman *et al.,* 1961; Jung *et al.,* 2010).

Beta-secretase 1 **enzyme (BACE1) inhibitory assay**

The procedure for the determination of *in vitro* BACE1 enzyme inhibition capacity of the test compounds was as presented by Francesca *et al* (2007) (Francesca *et al.,* 2007). The percent inhibition of enzyme activity by a range of concentrations of selected polyphenolic compounds was determined as earlier reported (Jung *et al.,* 2010, 2016).

Data presentation and analysis

Quantitative data, such as free energy of binding, and in vitro antioxidant and enzyme inhibition assays results, are presented as Mean ± SD (standard deviation) and analyzed using either Student's T-test for pairwise comparison and/or one-way analysis of variance (ANOVA) followed by Dunnet post hoc test for pairwise comparison, as implemented in GraphPad Prism version 8/9 (GraphPad, La Jolla, CA) with significant level set to $p < 0.05$.

RESULTS AND DISCUSSION

Phenolic composition of *ficus benjamina* leaves

The yield of polyphenol extract obtained from the leaves of *F. benjamina* was 3.57 ± 0.86% based on the dry weight of the leaves, indicating a moderate content of polyphenolic compounds. A total of 24 phenolic and related compounds were detected in the leaves of *F. benjamina* using LC-MS analytical methods and literature comparison (Table 1). The polyphenols include Apigenin-7-xylosyl-(1-6)-glucoside, blumenol-C-o-[apiosyl-(1-6)-glucoside], salicylaldehyde, kaempferol-3-neohesperidoside, 2(1H)-Quinolinone and 7-hydroxy-4-methylphthalide-o-[arabinosyl-(1-6)-glucoside] (Table 1). On the other hand, a total of 21 phenolic and related compounds were detected using the GC-MS analytical method and literature comparison. The major constituents detected were quininic acid, 4-hydroxybenzoic acid, benzeneacetic acid, 4-hydroxybenzeneacetic acid, aucubin, and 4-[oxy]-benzaldehyde (Table 1). The other components were present at less than 0.1%. The representative chromatograms for LC-MS and GC-MS are presented in Figure 1. Overall, these analyses showed that the leave extract of *F. benjamina* contained a complex mixture, consisting mainly of phenolic compound which constitutes over 50%, and while other components were present at less than 1.0 %, it should be noted that a significant portion of total composition could not be identified.

In silico **predictions**

Drug-likeness and ADMET

The results of pkCSM (Pires et al., 2015), which predicted drug-likeness of selected F. benjamina-derived compounds, were analyzed based on "Lipinski's rule of five" (Lipinski, 2004; Lipinski *et al*., 2001), in the light of proposed amendment of polar surface areas of not greater than 140 A2, and partition coefficient of $-1 < \log P < 6$ (Egan et al., 2000; Ghose et al., 1999; Veber et al., 2002). Ten compounds, including salicylaldehyde, 2(1H)-Quinolinone, 3'-Methoxyfukiic acid, Salicylic acid, (10R,11R)-Pterosin L, 2-Hydroxy-5-methylquinone, bisbynin, deidaclin and the aglycone part of Apigenin-7-xylosyl-(1-6)-glucoside and Kaempferol-3-neohesperidoside i.e Apigenin and Kaempferol respectively, fulfilled drug-relevant properties (Table S1). Therefore, these compounds were further subjected to an ADMET study to determine if the compound(s) is/are suitable for in vitro studies.

In the ADMET study (Table 2), the absorption capacity of the test compounds was assessed using Caco-2 and skin permeability index, as well as intestinal absorption (human). A compound with an apparent permeability coefficient (Papp) > 8 x 10^6 cm/s or log Papp values > 0.90 cm/s, is regarded as having a high Caco-2 permeability (Pires *et al.,* 2015). The results showed that the log Papp of the polyphenols ranges from -0.109 to 1.73 cm/s, and there are 5 compounds, namely, salicylaldehyde, 2(1H)-quinolinone, salicylic acid, 2-hydroxy-5-methylquinone, and Apigenin, that have log Papp > 0.9 cm/s, suggesting high Caco-2 permeability, similar to E20 (donepezil) and BEZ co-crystalized controls. The remaining 5 compounds, namely, 3'-methoxyfukiic, (10R,11R)-pterosin L, kaempferol, bisbynin, and deidaclin, have log Papp < 0.9 cm/s, and thus are predicted to have low Caco-2 permeability, similar to 5E7 co-crystalized control. Moreover, though nearly all the compounds possess moderate ability to cross the blood-brain barrier (BBB) and CNS permeability, salicyaldehyde, 2(1H)-quinolinone, salicylic acid, and 2-Hydroxy-5-methylquinone, are better able to cross the blood-brain barrier, similar to BEZ. Interestingly, Apigenin and Kaempferol also showed similar absorption, CNS permeability, metabolism excretion, and toxicity profiles with BEZ.

Table 1. The Polyphenol Constituents of *Ficus benjamina* Leave Using LC-MS and GC-MS

S/N	LC-MS		GC-MS	
	Phenolic and related compounds	%	Phenolic and related compounds	%
1	Apigenin-7-xylosyl-(1-6)-glucoside (isomer 1)	9.47	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.001
2	Kaempferol-3-neohesperidoside	1.69	Quininic acid	44.272
3	Apigenin-7-xylosyl-(1-6)-glucoside (isomer 2)	0.96	Protocatechoic acid	0.072
4	Datiscin (isomer 1)	0.82	4-Hydroxybenzoic acid	1.453
5	Datiscin (isomer 2)	0.73	Benzoic acid, 3-methoxy-4-[oxy]-	0.061
6	6-C-Glucopyranosylkaempferol	0.44	3-Hydroxybenzoic acid	0.089
7	Blumenol-C-o-[apiosyl-(1-6)-glucoside]	5.89	4-Hydroxybenzeneacetic acid	0.439
8	Salicylaldehyde	2.04	Hydroquinone	0.077
9	2(1H)-Quinolinone	1.54	Tyrosol	0.049
10	7-Hydroxy-4-methylphthalide O-[arabinosyl-(1-6)-glucoside]	1.34	3-(2-oxanyloxy) benzoic acid methyl ester	0.003
11	3'-Methoxyfukiic acid	1.06	3H-Cyclopenta - [1,3] - cyclopropa-[1,2]-benzen-3-one,1,2,3a,3b,6,7 hexahydro-3a,6,6-trimethyl-	0.013
12	Benzyl O-[arabinofuranosyl-(1-6)-glucoside]	0.71	4,4,5,8-Tetramethylchroman-2-ol	0.027
13	Salicylic acid	0.66	4-[oxy]-benzaldehyde	0.134
14	(10R,11R)-Pterosin L	0.63	4-Hydroxybutanoic acid	0.042
15	Amlodipine Dimethyl Ester	0.52	4-Vinylphenol	0.031
16	2-Hydroxy-5-methylquinone (isomer 1)	0.46	Arbutin	0.035
17	Phenylethyl primeveroside	0.43	Aucubin	0.297
18	6-O-p-Coumaroyl-D-glucose (isomer 1)	0.42	Benzaldehyde, 3-hydroxy-	0.038
19	Bismahanine	0.41	Benzaldehyde, 4-hydroxy-	0.041
20	6-O-p-Coumaroyl-D-glucose (isomer 2)	0.39	Benzeneacetic acid	1.001
21	2-Hydroxy-5-methylquinone (isomer 2)	0.37	Blumenol A	0.027
22	Bisbynin	3.33		
23	Deidaclin	3.44		
24	5-hydroxysebacate	3.12		

LC-MS and GC-MS means Liquid Chromatography and Gas Chromatography Mass Spectrometry respectively

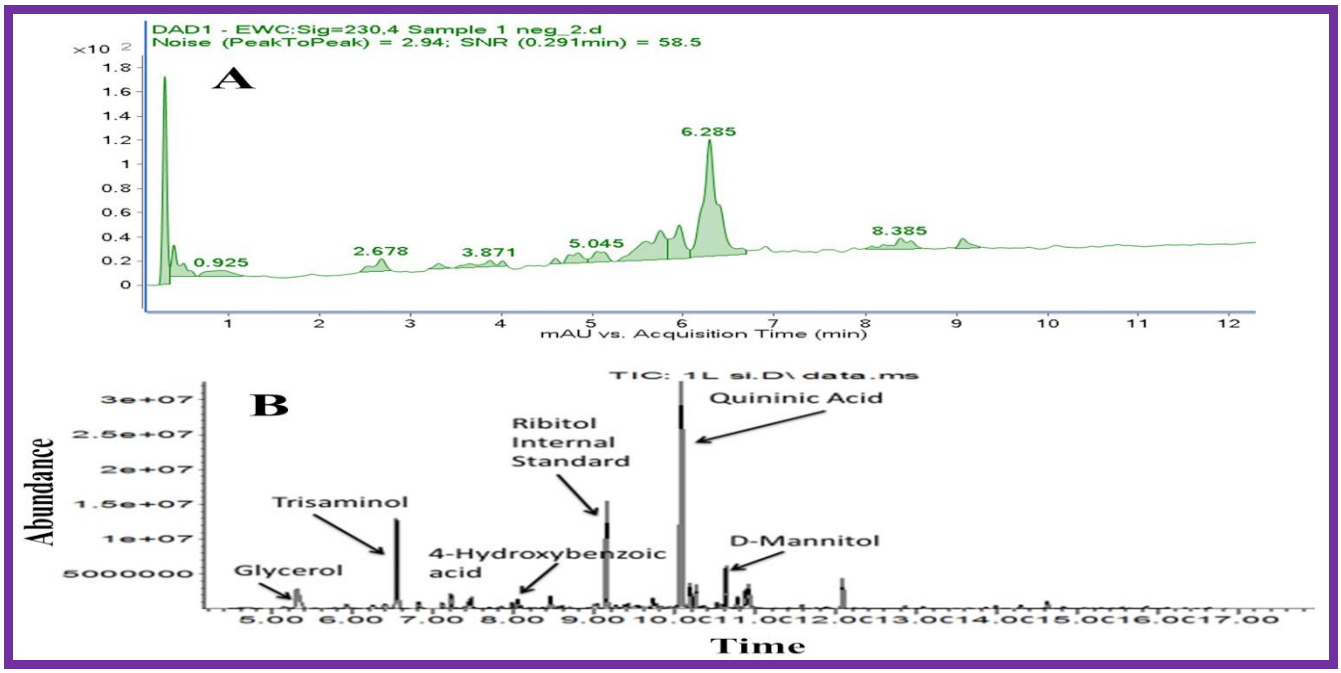

Figure 1. The representative (A) LC-MS and (B) GC-MS chromatograms of *Ficus benjamina* leaves

Table 2. Table of ADMET Prediction of Compounds that Passed Lipinski's Rules of Five

S/NO.	Compounds	Absorption		Distribution / Permeability				Metabolism (Inhibition)		Excretion (Total Clearance)	Toxicity		
		Caco-2	Intestinal	Skin	VDss	BBB	CNS	CYP 2D6	CYP 3A4		Ames	LD_{50} (mg/kg)	Hepatic
1	Salicylaldehyde	1.730	87.225	-2.539	-0.069	-0.217	-2.002	No	No	0.147	No	1.941	No
2	2(1H)-Quinolinone	1.617	95.245	-2.539	0.001	-0.187	-2.098	No	No	0.319	No	2.181	No
3	3'-Methoxyfukiic acid	-0.677	0.000	-2.735	-0.694	-1.234	-3.931	No	No	0.481	No	2.284	No
4	Salicylic acid	1.151	83.887	-2.723	-1.570	-0.334	-3.210	No	No	0.607	No	2.282	No
5	(10R,11R)-Pterosin L	0.780	79.098	-2.792	0.265	-0.502	-2.712	No	No	0.193	No	1.754	No
6	2-Hydroxy-5-methylquinone	1.174	91.527	-3.903	-0.081	-0.276	-2.930	No	No	0.526	Yes	1.837	No
7	Apigenin	1.007	93.250	-2.735	0.822	-0.734	-2.061	No	No	0.566	No	2.45	No
8	Kaempferol	0.032	74.290	-2.735	1.274	-0.939	-2.228	No	No	0.477	No	2.449	No
9	Bisbynin	0.227	52.064	-3.425	0.044	-0.441	-3.058	No	No	1.247	Yes	2.944	No
10	Deidaclin	-0.109	45.894	-3.246	-0.104	-0.865	-3.657	No	No	1.46	No	1.865	No
11	5E7 *	0.358	51.668	-2.735	0.377	-1.159	-3.601	No	Yes	0.720	No	2.568	Yes
12	E20 (Donepezil) *	1.273	93.707	-2.585	1.266	0.157	-1.464	Yes	Yes	0.987	No	2.753	Yes
13	BEZ *	1.707	100.000	-2.728	-1.640	-0.220	-2.002	No	No	0.707	No	2.170	No

BBB and CNS mean Blood-brain barrier and Central nervous system respectively * Control Co-crystalized ligands

Molecular docking and selection of compounds for *In vitro* study

A preliminary assessment of the validity and reproducibility of the docking procedure revealed that the best-docked complex was generated using the best-scored pose from Autodock Vina. Also, the interaction analysis of this re-docked complex with LigPlot (Laskowski & Swindells, 2011) showed that the downloaded and docked structures interacted with similar residues (Figures S1, S2, and S3). Therefore, Autodock vina was adopted for the molecular docking of the selected compounds based on drug-likeness and ADMET analysis against the target proteins. The results of molecular docking using Autodock vina are presented in Table 3. Docking analysis revealed that a total of 5 and 8 compounds showed greater than or equal binding energy with AChE and BUChE respectively when compared with their respective co-crystalized ligands (Table 3). While the binding energy of all the compounds with BACE1 was less than that of its co-crystalized ligand, it was found that Apigenin and Kaempferol showed significantly higher binding energy with all three proteins when compared with other selected compounds (Table 3). It should also be noted that though the insilico analysis revealed that salicyaldehyde, 2(1H)-quinolinone, salicylic acid, (10R,11R)-Pterosin L and 2-Hydroxy-5-methylquinone, are better able to cross the blood-brain barrier, they have significantly lower binding energy with AchE, BUChE, and BACE1 when compared with Apigenin and Kaempferol (Table 3). Therefore, Apigenin and Kaempferol were selected for further in silico and in vitro investigations.

Analysis of protein-ligand interaction of docked complexes

The protein–ligand interaction analysis for the interaction of Apigenin, Kaempferol, and the control co-crystalized ligands with BACE1, AChE, and BUChE as plotted using LigPlot (Laskowski & Swindells, 2011), revealed varying degrees of interactions. In Figure 2, BACE1-ligand complexes showed a higher number of amino acids interacting with the co-crystalized ligand (5E7) and forming hydrogen bonds (Gly34, Gln73, Asp228, Gly230, Thr232, Asn233), consistent with reported data (Ghosh *et al.,* 2016), than Apigenin (Ile126 and Thr231) and Kaempferol (Ile126, Arg128 and Thr231), suggesting weaker interaction compared to the control. Also, while Apigenin formed a hydrogen bond with only Tyr341, both Kaempferol and E20 formed a hydrogen bond with Phe295, when interacting with AChE (Figure 3), suggesting similar functional effects. However, a higher number of hydrogen bonds forming residues were observed with Apigenin (Ser198, Leu286 and His438) and Kaempferol (Thr120, Tyr128, Ala328 and His438) in BUChE when compare with the co-crystalized ligand (BEZ: Ser198 and His438), suggesting a much better interaction with BUChE (Figure 4). Also, though both Apigenin and Kaempferol showed a higher number of residues with hydrophobic interactions, similar to controls, the relatively higher number of hydrogen bond-forming

interacting residues in all the Kaempferol – protein complexes (Figures 2 – 4), and their similarities to those formed by co-crystalized ligands, may be an indication that Kaempferol is a better-interacting partner than Apigenin.

Table 3. Binding Energies (Kcal/Mol) of the Selected Compounds

S/N	Compounds	Binding Energy (Kcal/mol)		
		BACE1	AChE	BUChE
1	Apigenin	-9.1	-10.4	-11.2
2	Kaempferol	-9.7	-9.2	-10.2
3	Bisbynin	-7.2	-8.3	-7.8
4	(10R,11R)-Pterosin L	-6.9	-7.7	-7.9
5	Deidaclin	-6	-7.4	-7.4
6	2(1H)-Quinolinone	-6.1	-7.3	-7.1
7	2-Hydroxy-5-methylquinone	-5	-6.7	-6.2
8	3'-Methoxyfukiic acid	-5.9	-6.7	-7
9	Salicylic acid	-5.3	-6.6	-6
10	Salicylaldehyde	-5.2	-5.9	-5.5
13	5E7 *		-7.4	
11	E20 (Donepezil) *			-6.1
12	BEZ *	-12		

* Co-crystalized ligands as controls. Compounds were those selected based on drug-Likeness and ADMET.

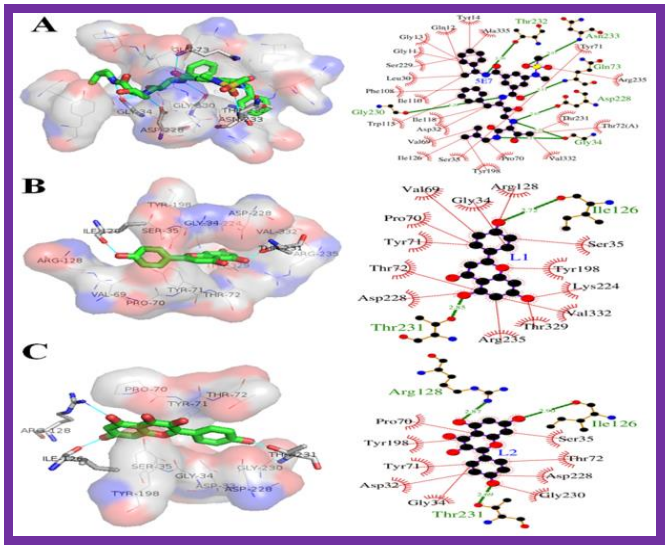

Figure 2. BACE1 – ligand Interaction analysis. (A) Interaction scheme of the BACE1 with its co-crystalized ligand (PDB: 5DQC); (B) Interaction scheme of the docked BACE1-Apigenin Complex; (C) Interaction scheme of the docked BACE1-Kaempferol Complex. L1 and L2 are Apigenin and Kaempferol respectively. Protein residues are shown as surface, hydrophobic interacting residues as lines, hydrogen bond forming residues as stick, and ligands as either stick or ball. Hydrogen bonds and their residues are shown in green, hydrophobic interactions in pink, and ligand oxygen atoms in red. Analysis was performed using LigPlot (Laskowski & Swindells, 2011) and ADSV (Dassault Systèmes BIOVIA, 2015). Images were setup using Microsoft PowerPoint and prepared using GIMP version 2.10.14 (The GIMP Development Team, 2019)

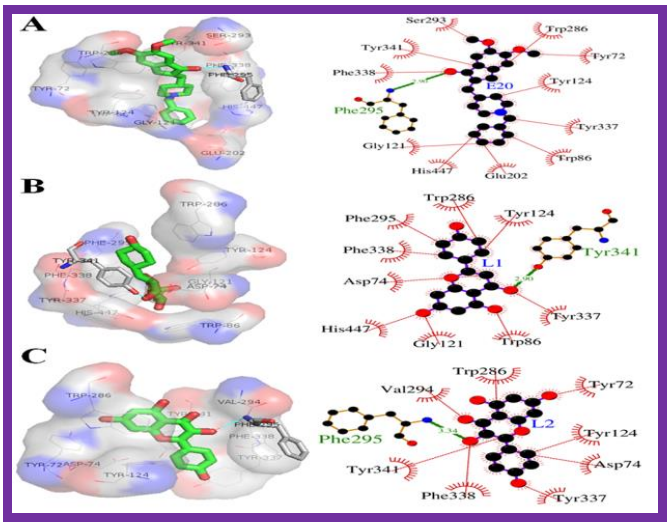

Figure 3. AChE – ligand Interaction analysis. (A) Interaction scheme of the AChE with its co-crystalized ligand (PDB: 6O4W); (B) Interaction scheme of the docked AChE-Apigenin complex; (C) Interaction scheme of the docked AChE-Kaempferol complex. L1 and L2 are Apigenin and Kaempferol respectively. Protein residues are shown as surface, hydrophobic interacting residues as lines, hydrogen bond forming residues as stick, and ligands as either stick or ball. Hydrogen bonds and their residues are shown in green, hydrophobic interactions in pink, and ligand oxygen atoms in red. Analysis was performed using LigPlot (Laskowski & Swindells, 2011) and ADSV (Dassault Systèmes BIOVIA, 2015). Microsoft PowerPoint was used to setup images and the final figure was prepared using GIMP version 2.10.14 (The GIMP Development Team, 2019)

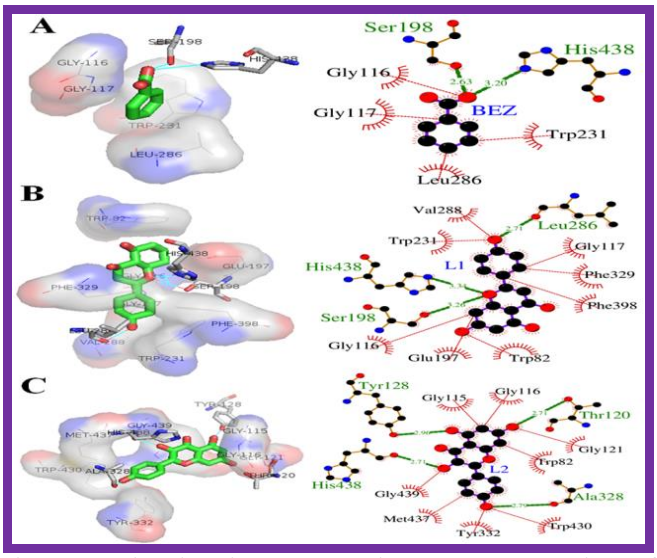

Figure 4. BUChE – ligand Interaction analysis. (A) Interaction scheme of the BUChE with its co-crystalized ligand (PDB: 3O9M); (B) Interaction scheme of the docked BUChE-Apigenin complex; (C) Interaction scheme of the docked BUChE-Kaempferol complex. L1 and L2 are Apigenin and Kaempferol respectively. Protein residues are shown as surface, hydrophobic interacting residues as lines, hydrogen bond forming residues as stick, and ligands as either stick or ball. Hydrogen bonds and their residues are shown in green, hydrophobic interactions in pink, and ligand oxygen atoms in red. Analysis was performed using LigPlot (Laskowski & Swindells, 2011) and ADSV (Dassault Systèmes BIOVIA, 2015). Images were arranged in Microsoft PowerPoint and prepared using GIMP version 2.10.14 (The GIMP Development Team, 2019).

Molecular dynamic simulation of docked complexes

The functional interaction of apigenin and kaempferol with selected protein targets was stable

The stability of functional interactions of Apigenin and Kaempferol with target proteins, following molecular dynamic simulation, was evaluated from the trajectories derived from the last 30 ns of simulation through the analysis of root mean square deviation (RMSD), root mean square fluctuations (RMSF), radius of gyration (RG) and hydrogen bonds using the in-built packages in GROMACS and VEGA ZZ (Abraham et al., 2015; Maiorov & Crippen, 1995; Pedretti et al., 2002). In general, the analysis of RMSD, RG, and hydrogen bonds (Figure 5) revealed that Apigenin and Kaempferol showed a significantly higher degree of stability, especially in their interaction with BACE1 and AChE, when compared with their respective co-crystalized ligands (5E7 and E20). It is interesting to note that in their interaction with BUChE, RMSD analysis revealed that while Apigenin was relatively stable throughout the timeframe, it took Kaempferol a significantly longer time to attain the same level of stability. Nevertheless, they are still in a better stable interaction when compared with the co-crystalized control (BEZ) (Figure 5). Meanwhile, of all three co-crystalized ligands, only BEZ showed a significantly lower RG when compared to Apigenin and Kaempferol. These inferences are supported by generally higher hydrogen bonds (hb/ps), smaller deviation in the mean values of RMSD and RG, with a close range of values for both Apigenin and Kaempferol, when compared with controls (5E7, E20, and BEZ) (Table S2). Therefore, taken together, both Apigenin and Kaempferol showed a comparable degree of interaction with BACE1 and AChE, but Apigenin had a better interaction with BUChE.

Free energy of binding of apigenin and kaempferol with selected protein targets

The free energy of binding was estimated from the ensembles of trajectories from the molecular dynamic simulation using gRINN (Residue Interaction energies and Networks), a tool for the calculation of energy of interaction between a given residue and the protein (Serçinoğlu & Ozbek, 2018), and MMPBSA.py python script (Miller *et al.,* 2012), using both the Generalized Born (GP) (Lee *et al.,* 2005; Nguyen *et al.,* 2013) and the Poisson Boltzmann (PB) (Baker, 2004; Fogolari *et al.,* 2002) methods, as implemented in AmberTools22 (Case *et al.,* 2022). Apart from providing insights into the molecular and conformational states of drug targets, as well as the correlation between protein structure and functions, estimation of free energy of binding can also assist in the identification of novel molecular scaffolds, and optimization of lead compounds in rational drug design (Dolenc *et al.,* 2011; Reddy *et al.,* 2013).

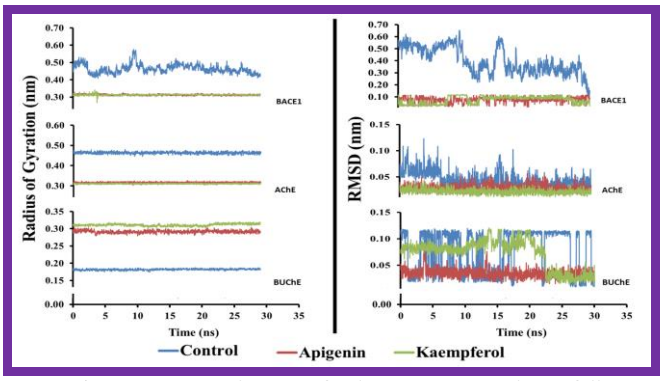

Figure 5: Apigenin and Kaempferol trajectory analysis following Molecular Dynamic simulation of their docked complex with BACE1, AChE, and BUChE. Analysis was performed using the GROMACS package and VEGA ZZ (Abraham et al., 2015; Pedretti et al., 2002). Microsoft PowerPoint was used to arrange the images for final preparation using GIMP version 2.10.14 (The GIMP Development Team, 2019).

The results of the free energy of binding are presented in Table 4. The gRINN results showed that Apigenin and Kaempferol have significantly higher energy of binding with AChE and BUChE when compared with their respective co-crystalized ligands (E20 and BEZ), and with their interactions with BACE1. On the other hand, generalized born (GB) and Poisson Boltzmann (PB) analysis revealed stronger biding of Apigenin and Kaempferol with BACE1 and BUChE, when compared with respective co-crystalized ligands (5E7 and BEZ), and with their interaction with AChE. Therefore, while the degree of interaction differs, and may have been influenced by other factors, including protein targets, both gRINN and MMPBSA revealed the ability of Apigenin and Kaempferol to establish functional interactions with AChE, BACE1, and BUChE.

Table 4. Free Energy of Binding for the Interaction of Apigenin and Kaempferol with target Proteins

		BACE1	AChE	BUChE
gRINN (kJ/mol)	5E7	-20.725 ± 1.112*		
	E20		-36.053 ± 0.193*	
	BEZ			-17.016 ± 0.188*
	Apigenin	-8.000 ± 0.004	-53.749 ± 0.295	-40.659 ± 0.367
	Kaempferol	-7.020 ± 0.010	-47.526 ± 0.262	-42.497 ± 0.659
GB (Kcal/mol)	5E7	-8.356 ± 1.065*		
	E20		-46.698 ± 0.471*	
	BEZ			-16.579 ± 0.419*

Table 4. Continued

	Apigenin	-13.501 ± 0.726	-42.79 ± 0.536	-31.787 ± 0.480
	Kaempferol	-11.639 ± 0.473	-34.181 ± 0.441	-22.953 ± 0.610
PB (Kcal/mol)	5E7	-2.618 ± 0.343*		
	E20		-16.181 ± 0.331*	
	BEZ			-7.190 ± 0.154*
	Apigenin	-4.141 ± 0.308	-5.758 ± 0.254	-9.653 ± 0.284
	Kaempferol	-3.599 ± 0.324	-12.98 ± 0.283	-8.910 ± 0.390

Data were expressed as Mean ± SD. The gRINN is 'get Residue Interaction energies and Networks'. GB is Generalized Born, and PB is Poisson Boltzmann. The * represents a significant difference when compared with corresponding Apigenin and Kaempferol at $p < 0.05$.

In Vitro assay

In Vitro antioxidant assays

The results of the antioxidant assays carried out on the polyphenols (Apigenin and Kaempferol) are shown in Figure 6. In the metal chelating assay, the percentage chelating activity increased with elevation in concentration, suggesting concentration-dependent activities (Figure 6A). At 100 µg/ml, Kaempferol showed significantly higher percentage chelating activity (72.67 ± 0.14%, $p < 0.05$) and significantly lower IC50 values (38.85 ± 0.68 µg/ml, $p < 0.05$) when compared with apigenin which showed percentage chelating activity of 57.35 ± 0.60 %, with the IC50 values of 76.82 ± 0.95 µg/ml. However, both were significantly lower than the standard, EDTA ($p < 0.05$), which has the percentage chelating activity of 94.82 ± 1.86 % and IC50 value of 15.72 ± 0.80 µg/ml (Figure 6A).

In addition, the ability of the polyphenols to reduce Fe3+ to Fe2+ (FRAP assay) and their total antioxidant capacity, were measured in terms of ascorbic acid equivalent concentration. The results showed 81.53 ± 2.54 (Kaempferol) and 63.77 ± 2.19 (Apigenin) mg AAE/g for FRAP assay, and 216.28 ± 8.20 (Kaempferol) and 166.90 ± 5.02 (Apigenin) mg AAE/g for total antioxidant capacity (Figure 5B). Furthermore, the ability of the polyphenols to reduce Cu2+ to Cu+ (CUPRAC assay), measured in terms of Trolox equivalent per gram, were 86.09 ± 1.27 and 64.96 ± 0.80 mg TE/g for Kaempferol and Apigenin respectively (Figure 6B). These results show that the antioxidant capacities of Kaempferol are significantly higher ($p < 0.05$) than those of Apigenin.

In Vitro enzymes inhibitory assays

The AChE and BUChE inhibitory assays revealed a concentration-dependent increase in inhibition by both Kaempferol and Apigenin. While Kaempferol showed an IC50 of 42.67 ± 0.76 and 15.74 ± 0.04 µg/ml, Apigenin gave an IC50 of 50.03 ± 1.58 and 20.10 ± 0.08 µg/ml for AChE and BUChE respectively (Table 5). However, Kaempferol showed significantly ($p < 0.05$) higher inhibitory activities compared to Apigenin in both AChE and BUChE assays. Also, both Apigenin and Kaempferol showed more inhibitory activities towards BUChE than AChE. This may be a result of the wider active site gorge of BUChE which makes the compounds have easier access to the catalytic centre of BUChE resulting in a more efficient inhibition of BUChE than of AChE.

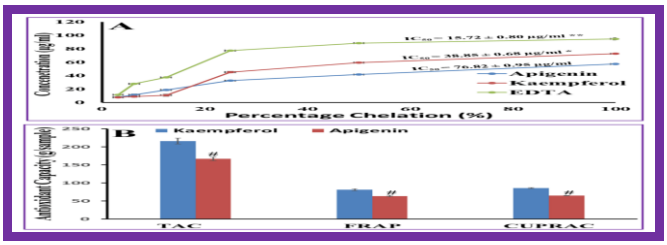

Figure 6: Antioxidant capacities of Apigenin and Kaempferol. TAC is Total Antioxidant Capacity; FRAP is Ferric Reducing Antioxidant Power; CUPRAC, Copper Reducing Antioxidant Capacity. The **, *, and # represent significant differences at $p < 0.05$ when compared with Apigenin and Kaempferol, Apigenin alone, and Kaempferol alone respectively. Microsoft PowerPoint was used to arrange the images for final preparation using GIMP version 2.10.14 (The GIMP Development Team, 2019).

Table 5. *In Vitro* Enzymes' Inhibitory Activities at Different Concentrations of Apigenin and Kaempferol

Anti-cholinesterase Activity					BACE1 Inhibitory Assay				
Conc. (µg/ml)	AChE % Inhibition		BUChE % Inhibition		Conc. (µg/ml)	% Inhibition (Fluorometric)		% Inhibition (Spectrophotometric)	
	Kaempferol	Apigenin	Kaempferol	Apigenin		Apigenin	Kaempferol	Apigenin	Kaempferol
100	86.06 ± 0.32	72.85 ± 1.42	89.21 ± 0.11	79.57 ± 0.06					
50	57.39 ± 1.77	51.05 ± 1.43	78.53 ± 0.44	70.12 ± 0.01	20	26.26 ± 0.34	32.77 ± 0.32	18.19 ± 0.08	19.37 ± 0.32
25	30.46 ± 2.15	22.21 ± 0.75	66.29 ± 0.08	60.70 ± 0.31	16	16.52 ± 0.03	25.17 ± 0.71	14.10 ± 0.29	16.32 ± 0.95
12.5	17.85 ± 1.70	20.55 ± 1.41	48.83 ± 0.14	31.55 ± 0.14	12	11.51 ± 0.44	14.34 ± 0.19	8.15 ± 0.08	6.61 ± 0.10
6.25	6.38 ± 1.13	17.72 ± 0.65	31.26 ± 0.07	25.81 ± 0.08	8	10.41 ± 0.33	9.18 ± 0.59	4.09 ± 0.16	4.31 ± 0.06
3.125	3.99 ± 1.61	12.08 ± 0.78	21.32 ± 0.01	13.97 ± 0.07	4	9.01 ± 0.15	9.03 ± 0.37	2.80 ± 0.27	1.48 ± 0.07
IC_{50} (µg/ml)	42.67 ± 0.76*	50.03 ± 1.58	15.74 ± 0.04*	20.10 ± 0.08	IC_{50} (µg/ml	46.76 ± 0.61	32.19 ± 0.76*	51.75 ± 0.11	43.30 ± 1.36*

Conc. Is concentration * Significant difference when compared with Apigenin at $p < 0.05$

Furthermore, the BACE1 inhibitory assay was conducted using fluorometric and spectrometric methods. Table 5 shows the percentage inhibition of fluorescein thiocarbamoyl (FITC) derivative formation, as caused by apigenin, Kaempferol, and quercetin respectively. The test compounds inhibited the proteolysis of Casein-FITC in a dose-dependent manner, with maximum inhibition occurring at 20 µM. In this inhibitory assay, Apigenin and

Kaempferol produced an IC50 of 46.76 ± 0.61 and 32.19 ± 0.76 µM respectively. In addition, the spectrophotometric assay was performed with D,L-BAPNA, a chromogenic substrate for serine protease enzymes. D,L-BAPNA was tested as another potential alternative substrate for the BACE1 enzyme. The substrate (D,L-BAPNA) was cleaved enzymatically, producing N-α-benzoyl-D,L-arginine, and the intensity of the resulting yellow anion p-nitroaniline (pNA) was monitored at a visible absorbance band of 405 nm. In this inhibitory assay, Apigenin and Kaempferol gave an IC50 value of 51.75 ± 0.11 and 43.30 ± 1.36 µM respectively. Again, Kaempferol showed significantly ($p < 0.05$) better inhibitory activity than Apigenin.

Discussion

In this study, the Liquid Chromatography/Mass Spectroscopy (LCMS) analysis of polyphenolic extract of *F. benjamina* leaves established the presence of some polyphenolic compounds, including apigenin-7-xylosyl-(1-6)-glucoside, blumenol-C-o-[apiosyl-(1-6)-glucoside], salicylaldehyde, kaempferol-3-neohesperidoside, 2(1H)-Quinolinone and 7-hydroxy-4-methylphthalide-o-[arabinosyl-(1-6)-glucoside] (Table 1). Our result was consistent with previous studies where various polyphenolic compounds have been identified and isolated from *F. benjamina* plant extract as well as other Ficus species (Hassan *et al.,* 2003; Taha *et al.,* 2011; Ritiel *et al.,* 2012). There is compelling evidence in support of the application of polyphenols as antioxidant and neuroprotective agents in neurodegenerative diseases (Pandey & Rizvi, 2009; Azadeh & Hermann, 2012; Figueira *et al.,* 2017). For instance, several derivatives of salicylaldehyde have shown antioxidant, ion-chelation, cytotoxic, and antibacterial properties (Li *et al.,* 2011; Potůčková *et al.,* 2014; Caro *et al.,* 2015). Also, quinolinone derivatives are potent antioxidants (Vats *et al.,* 2014). In addition, the ability of Apigenin to mitigate β-amyloid neurotoxicity has been linked to antioxidation, mitochondrion protection, and inactivation of MAPK signal (Zhao *et al.,* 2013). Also, Kaempferol has been shown to possess antioxidant, anti-inflammatory, and neuroprotective activities possibly by preventing loss of tyrosine hydroxylase (Hussein *et al.,* 2018; Pan *et al.,* 2020; Silva dos Santos *et al.,* 2021). Therefore, the combination of phytoconstituents with antioxidant, neuroprotection, and anti-inflammatory effects, may help to explain the potential usefulness of *F. benjamina* in neurodegenerative diseases like AD.

Absorption, distribution, metabolism, excretion, and toxicity (ADMET) constitute the pharmacokinetic profile of a drug candidate and are essential in evaluating its pharmacodynamic activities (Chaluveelaveedu *et al.,* 2016). In this study, ADMET analysis together with binding affinity established Apigenin and Kaempferol as drug-able compounds (Table 2). With predicted better human intestinal absorption (HIA) score and enhanced blood-brain barrier (BBB) penetrations, Apigenin and Kaempferol could be better absorbed from the intestinal tract upon oral administration, and effectively cross BBB. However, as non-inhibitors of CYP2D6 and CYP3A4, Apigenin and Kaempferol are predicted not to be able to alter or interfere with the metabolism of commonly used drugs, but their predicted ability to serve as substrates of P-glycoprotein (P-gp), may influence their ability to alter the drug absorption or uptake, especially across the BBB (Li *et al.,* 2010). Nevertheless, their combined neuroprotective, anti-inflammatory, and antioxidant activities, as well as their ability to mitigate neurotoxicity associated with β-amyloid formation (Zhao *et al.,* 2013 Hussein *et al.,* 2018; Pan *et al.,* 2020; Silva dos Santos *et al.,* 2021) make them suitable candidates in the management of AD.

Three enzymes that have been implicated in the pathology of AD were selected as targets for the evaluation of the potential functional roles of selected polyphenols (Apigenin and Kaempferol) in the management of AD. They are beta-amyloid cleaving enzyme 1 (BACE1), acetylcholinesterase (AChE), and butyrylcholinesterase (BUChE). BACE1, a critical enzyme in Alzheimer's disease (AD) pathology, is responsible for the generation of beta-amyloid (Aβ), forming senile plaques, with eventual degeneration into cognitive dysfunction in AD (Sathya *et al.*, 2012; Hampel *et al.*, 2021; Wen *et al.*, 2022). Also, the cholinergic deficit is a common feature in AD, and available evidence has implicated AChE in the exacerbation of neurotoxicity of amyloid components, possibly due to its ability to form a stable complex with senile plaque components (Talesa, 2001; García-Ayllón *et al.*, 2011). In addition, BUChE activity is known to increase progressively in AD, potentially due to compensatory upregulation of BUChE in the face of declining AChE. Thus, while BUChE has become an important target for the amelioration of cholinergic deficit, dual inhibition of BUChE and AChE is gaining increase attention (; Greig *et al.*, 2002; Nordberg *et al.*, 2013; Reid & Darvesh, 2015; Gabr & Abdel-Raziq, 2018). Therefore, current research efforts have focused on searching for inhibitors of these enzymes (BACE1, AChE, BUChE), making them essential targets of drug action in AD (Greig *et al.*, 2002; Ghosh & Osswald, 2015; Zhe Ying Ha *et al.*, 2020; Marucci *et al.*, 2021). In this study, the analysis of molecular docking, protein-ligand interactions, and molecular dynamic simulation of the docked complexes of Apigenin and Kaempferol with selected enzymes relevant to the pathology of AD (BACE1, AChE, and BUChE) provided insights into the potential functional activities of these polyphenols. Not only did the analysis of molecular docking reveal stronger binding of Apigenin and Kaempferol to the catalytic sites of BACE1, AChE, and BUChE, but the molecular dynamic simulation confirmed the stability of the interactions. However, from the analysis of the molecular dynamic trajectory, it can be inferred that Apigenin is well favoured for more stable interaction with the selected enzyme targets. In addition, the interaction analysis revealed that the two flavonoids directly interacted with core BACE1, AChE, and BUChE catalytic residues, forming critical hydrogen bonds, with Kaempferol showing

potentially higher binding affinity than Apigenin. For instance, Apigenin formed hydrogen interaction with BACE1 via Ile126 and Thr231, with AChE via Tyr341, and with BUChE via Ser198, Leu286, and His438. Also, Kaempferol formed hydrogen bond interaction with BACE1 via Ile126, Arg128 and Thr231, with AChE via Phe295, and with BUChE via Thr120, Tyr128, Ala328 and His438 (Figures 2 – 4). Interestingly, both Apigenin and Kaempferol form hydrogen bonds with the catalytic triad His438 of BUChE, suggesting a possible mechanism for their potential inhibitory activities (Dvir *et al.*, 2010; Lushchekina *et al.*, 2016). While these hydrogen bond interactions may be important to enhance the inhibitory activity of Apigenin and Kaempferol and confer stability on the formed complexes, the observed relatively higher number of hydrogen bond forming interacting residues in all the Kaempferol – protein complexes (Figures 2 – 4), and their similarities to those formed by co-crystalized ligands, further confirmed the potential for a better functional relationship with Kaempferol.

Free radicals are known to play critical roles in the pathology of Alzheimer's disease (Azadeh & Hermann, 2012). Oxidative stress is a critical factor in AD-related metabolic dysfunction, apoptosis of neurons, as well as BACE1 expression and activity (Tabner *et al.,* 2010; Budimir, 2011). Therefore, any propose solution with the added advantage of being able to effectively protect the body against the effect of free radical-mediated oxidative stress will be of benefit to the AD patient. Natural antioxidants, including medicinal plants derived polyphenols, are a good choice to abrogating oxidative stress (Scalbert *et al.,* 2005; Pandey & Rizvi, 2009). Therefore, in this study, our evaluation of the antioxidant potential of the two selected polyphenols, Apigenin and Kaempferol, using total antioxidant activity (TAC), metal chelating activity, ferric reducing antioxidant power (FRAP), and copper reducing antioxidant capacity (CUPRAC) assays, confirmed their antioxidant capacity (Figure 6). The antioxidant activities of Apigenin and Kaempferol may be attributed to their ability to scavenge radicals, and chelate transition metal ions (e.g. iron and copper), thereby inhibiting their catalytic activities in the formation of reactive oxygen species (Fenton process) (Budimir, 2011). The roles of metals in the pathophysiology of AD include the induction of Aβ aggregation, leading to the generation of harmful reactive oxygen species (ROS). Also, ROS can be generated using the bound transition metal ions (Cu(I) or Fe(II)) found on Aβ oligomers, by reducing molecular oxygen to hydrogen peroxide. Therefore, the stability of pro-oxidative metal ions in living systems by chelation or complex formation is an essential approach to reducing the deleterious effects of metals-induced ROS generation (Abhishek *et al.,* 2013). Also, reduction or amelioration of neurotoxicity associated with amyloid aggregation and free radical generation can be achieved by metal chelation and radical scavenging approaches (Tabner *et al.,* 2010; Budimir, 2011). In fact, the neuroprotective activity of polyphenols is associated with their ability to chelate transition metal ions, thereby preventing free radical formation (Figueira *et al.,* 2019; Callizot *et al.,* 2021). In this study, Apigenin and Kaempferol showed strong inhibitory activities against AChE, BUChE, and BACE1 (Table 5). Their ability to interact effectively with these enzymes further confirmed their potential usefulness in the management of AD. However, Kaempferol generally showed much better *in vitro* activities than Apigenin.

Several reported works enumerating the potential anti-AD properties of Apigenin and Kaempferol are based on the reported antioxidant and anti-inflammatory activities. In addition to confirming these properties, our work provided new evidence for their potential usefulness in AD. For instance, as non-inhibitors of CYP2D6 and CYP3A4 (by in silico analysis), Apigenin and Kaempferol may be considered more credible candidates for improved bioavailability and pharmacokinetic profiles. This will also make their formulation and incorporation into a novel delivery system much easier. In addition, in our view, this is the first report of an attempt to explore the potential molecular basis for the role of Apigenin and Kaempferol in AD. The inhibitory actions of Apigenin and Kaempferol on AChE, BUChE, and BACE1, not only provide an opportunity to address the problem of compensatory upregulation of BUChE in the face of declining AChE, but also an opportunity for a multi-targets approach to addressing the problem of AD. The combined neuroprotective, anti-inflammatory, and antioxidant activities of Apigenin and Kaempferol (Zhao *et al.*, 2013 Hussein *et al.*, 2018; Pan *et al.*, 2020; Silva dos Santos *et al.*, 2021), coupled with their stable interactions with critical residues at the catalytic sites of BACE1, AChE, and BUChE, make them suitable candidates in the management of AD.

CONCLUSION

In this study, our *In silico* investigation has identified and established the potential inhibitory activities of Apigenin and Kaempferol, isolated from the leaves of F. benjamina, against the selected enzymes that are relevant to the pathology of Alzheimer's disease (BACE1, AChE, and BUChE). The metal chelating and radical scavenging activities, as well as the enzyme inhibitory assays, confirmed the potential roles of Apigenin and Kaempferol in addressing problems associated with AD, possibly via inhibition of BACE1, AChE, and BUChE. Therefore, the leaves of F. benjamina and its associated phytoconstituents, especially Apigenin and Kaempferol, could be explored further for potential therapeutic applications in neurodegenerative disorders. While there are limitations to the use of polyphenols in human subjects, the AD-related biological activities (neuroprotective, anti-inflammatory, and antioxidant) of Apigenin and Kaempferol, stability of their interactions with critical residues of BACE1, AChE, and BUChE, and favourable ADMET properties make them suitable candidates for further development as lead

compounds for Alzheimer's therapy. This can be better achieved with a novel delivery system.

Study limitations

This study is largely based on in silico methods and in vitro assays, which may not fully reflect in vivo effects. It may therefore not be directly translated into clinical application without further experimental validations. Also, despite their numerous pharmacological benefits, the clinical applications of polyphenols in human subjects are limited by potential challenges related to their bioavailability and pharmacokinetics profile. Among these challenges are issues relating to the mechanisms of absorption, distribution, metabolism, and excretion of an ingested polyphenol. The design and application of novel delivery systems in the formulation of polyphenols, such as Apigenin and Kaempferol, will go a long way to address these challenges.

Data availability statement

All data supporting the conclusion from this work are supplied in this manuscript and its supporting/supplementary documents. Supplementary files can be downloaded from doi:10.5281/zenodo.14988792 or can be obtained via the corresponding author

AUTHORS' CONTRIBUTIONS

EMO: Conceptualization, Resources, Supervision and Project Administration. MOD: Validation, Software, Supervision and Project Administration. JIO, OAF and GPI: Performed the experiments. JIO and MOD: Data analysis and initial draft of the manuscript. JIO, MOD, OAF, GPI and EMO: Contributed equally to reviewing and editing of the manuscript, and approved the final submission.

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CONFLICT OF INTEREST

The authors declare no competing financial interest

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