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Antioxidant and acetylcholinesterase inhibitory activities, in silico analyses, and anti-Alzheimer's disease potential of leaf extracts of three Nigerian endemic medicinal plants (*Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*)

Aanuoluwapo Ruth Adetuyi^{1,2} , Michael E. Ayenero² , Mary T. Olaleye², Afolabi A. Akindahunsi² and Afolabi C. Akinmoladun^{2*}

Abstract

Background The evaluation of the correlations between antioxidant and anti-acetylcholinesterase activities of methanol leaf extracts of three Nigerian endemic plants, *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*, was carried out. Their constituent phytochemicals were identified by HPLC–DAD fingerprinting. The antioxidant activity as typified by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenthiazoline-6-sulfonic acid (ABTS⁺) and nitric oxide (NO) scavenging activities were evaluated. The acetylcholinesterase (AChE) inhibitory activity of the extracts was also determined.

Results The extracts contained appreciable amounts of the flavonoids, quercetin and kaempferol. The extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* showed concentration-dependent inhibitory activities against DPPH and ABTS⁺ with IC₅₀ of 43.29 ± 0.443 µg/mL, 59.27 ± 0.644 µg/mL and 80.20 ± 0.414 µg/mL; 25.43 ± 0.325 (µg/mL), 39.84 ± 0.163 µg/mL and 59.02 ± 0.376 (µg/mL), respectively. The IC₅₀ for the NO scavenging activities of the *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* extracts were 41.99 ± 0.217 µg/mL, 50.44 ± 0.281 µg/mL and 60.12 ± 0.512 µg/mL, respectively. The IC₅₀ for the inhibitory effects on AChE was 53.24 ± 0.327 µg/mL, 60.95 ± 0.290 µg/mL and 70.5 ± 0.426 µg/mL, respectively. The effectiveness of the plant in all the experimental tests was in the following order: *S. mombin* > *C. papaya* > *K. crenata*. The total flavonoid and total phenolic contents have extremely significant positive correlations with the antioxidant activities and AChE inhibitory activity. The correlation coefficients (*r*²) of DPPH scavenging activity and NO scavenging activity with the AChE inhibitory activity were 0.8295 µg/mL and 0.7337 µg/mL, respectively (*P* < 0.0001). The molecular docking and pharmacokinetic analyses on some constituent phytochemicals showed that quercetin, kaempferol, ferulic acid, leucocyanidin, gallic acid and isorhamnetin fulfilled the requirements for an anti-Alzheimer drug.

*Correspondence:

Afolabi C. Akinmoladun
acakinmoladun@futa.edu.ng; akinmoladun_afolabi@yahoo.co.uk
Full list of author information is available at the end of the article



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Conclusions The results suggest that the plant species provide a significant source of secondary metabolites that can act as natural antioxidants and acetylcholinesterase inhibitors, which will be helpful in the treatment of Alzheimer's disease.

Keywords Acetylcholinesterase inhibition, Alzheimer's disease, Antioxidant activity, Flavonoids

Background

Discovering improved disease modifying therapies against dementias remains a major challenge. Dementias are characterized by the gradual onset and continuing decline of higher cognitive functioning as exemplified by Alzheimer's disease [1, 25]. Alzheimer's disease (AD) patients present a gradual decrease of acetylcholine levels, which arises from loss of the cholinergic neurons in the hippocampus and cortex of the brain. Other defects that occur include accumulation of decrepit plaques and neurofibrillary tangles [31]. Consequently, inhibition of acetylcholinesterase, that enhances the build-up of acetylcholine at the synapse, will improve the cholinergic shortage, which is a remedial target for the development of drug for AD ("cholinergic hypothesis"). Galantamine, rivastigmine and donepezil are drugs for AD, all are acetylcholinesterase inhibitors [14].

Synthetic drugs show unwanted serious side effects accompanied by insufficient response rates. They mainly get rid of symptoms of AD rather than curbing the progression of the disease so the problem of how to treat the disease still persists [19]. Therefore, there remains an urgent need for new, safe and effective drugs. This opens an avenue for the exploration of medicinal plants. Medicinal plant products have proved to be favorable sources of acetylcholinesterase inhibitors [48]. AChE inhibition is also considered as a promising remedial strategy for other types of dementia, myasthenia gravis, glaucoma and Parkinson's disease in addition to AD [32].

The formation of reactive oxygen species, which leads to oxidative stress, is another significant neurotoxic pathway in AD. Oxidative stress is produced by the disturbance of equilibrium between free radicals and antioxidants. Damage of biomolecules such as lipids and proteins in relation to increased free radical levels leads to oxidative damage of cells and consequently, to overexpression of oncogenes, formation of mutagens, induction of atherogenic activity, or inflammation [47]. Oxidative stress is suggested to play a crucial role in the pathogenesis of numerous neurodegenerative diseases like AD, myasthenia gravis, glaucoma and Parkinson's disease [50].

As of late, there has been an upsurge of interest in plant-derived antioxidants because of their ability to break the chain reactions of free radicals [56].

Numerous constituents of herbal extracts have inherent antioxidant properties. Along these lines, reestablishing oxidative equilibrium might be one of the fundamental mechanisms by which therapeutic plants can protect against ageing and cognitive decline. The antioxidant activity of plants might be because of the presence of polyphenolic compounds, for example, phenolic acids and flavonoids [17, 39]. Medicinal plants with remarkable antioxidant and AChE inhibitory properties could therefore offer benefits in the therapy of neurodegenerative diseases.

Nigeria, one of the most important countries in West Africa, is richly blessed with an incredible variety of medicinal plants. Notable among them are *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*. The plants are known by various names but among the Yorubas, *Spondias mombin* is known as Iyeye, *Carica papaya* is known as ibepe and *Kalanchoe crenata* is known as Odundun. They are used independently or in combination with other herbs for the management of neurodegenerative diseases in Nigeria [41, 55]. Industrially, *Spondias mombin* fruit is commercialized as frozen pulp in Brazil where it is utilized for the production of juices, popsicles, ice creams, yogurts and jams [6, 58]. By-products of *Carica papaya* such as pectin and papain are used in the food industry [15]. *Kalanchoe* is a popular genus, typically produced for the floriculture industry. The new variety is suitable for both indoor and outdoor ornamental uses [13, 46].

Spondias mombin has antioxidant, antimicrobial, cardioprotective, antiepileptic and antipsychotic properties [2, 4, 5, 9, 42]. On the other hand, *Carica papaya* leaves have anticancer and muscle relaxant activities [11, 51]. *Kalanchoe crenata* leaves have been reported to demonstrate antioxidant and anticonvulsant effects [8, 40]. However, there is a dearth of information in the literature on the acetylcholinesterase inhibitory and anti-Alzheimer's disease activities of these plants, which could shed more light on their therapeutic potentials against neurodegenerative diseases. Therefore, this study evaluated the antioxidant and anticholinesterase properties of methanol extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* and ascertained the strength and direction of the correlation between these properties.

Methods

Chemicals

Thiobarbituric acid (TBA), trichloroacetic acid (TCA), Ellman's reagent (DTNB), N-(1-Naphthyl) ethylenediamine dihydrochloride, neocuproine, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), acetylthiocholine iodide and quercetin were obtained from Sigma-Aldrich, USA. Methanol was obtained from Merck (Darmstadt, Germany). The remaining chemicals and reagents used for this study were obtained from other standard sources.

Extraction of plant leaves

Spondias mombin, *Carica papaya* and *Kalanchoe crenata* leaves were obtained from farmlands (Latitude 7° 18' 15.372" N and longitude 5° 8' 13.247" E; Latitude 7° 18' 37.076" N and longitude 5° 15' 28.789" E; Latitude 7° 16' 57.698" N and longitude 5° 13' 39.065" E, respectively) in Akure, Southwest Nigeria, in July 2019. Authentication was carried out at The Federal University of Technology, Akure, Nigeria, and voucher specimens were deposited at the University's herbarium. The leaves were air-dried at 25–30 °C for 2 weeks with relative humidity ranging between 56 and 57%. The dried plant materials were pulverized, and 200 g of each powdered sample was extracted by maceration in 800 mL of 80% methanol for 48 h. The mixtures were filtered, using Whatman (No. 1) filter paper, concentrated and lyophilized to obtain the dry extracts of the plants. The percentage yields were *Spondias mombin* 10.5%, *Carica papaya* 8.5% and *Kalanchoe crenata* 7.0%.

Qualitative and quantitative phytochemical screening

Qualitative and quantitative phytochemical screening were carried out to detect and quantify phytochemicals present in the plant extracts.

Qualitative phytochemical

The preliminary phytochemical studies were performed to identify diverse classes of chemical compounds present in the plant extracts using standard procedures. Test for tannins, alkaloids, anthraquinones, saponins [59], test for flavonoids [54] and test for steroids [23] were performed as previously described.

Determination of total phenolic content (TPC)

Deionized water (0.5 mL) and 125 µL of Folin–Ciocalteu reagent were added to 125 µL of extract (1 mg/mL), mixed and then allowed to stand for 6 min before 1.25 mL of a 7% (w/v) Na₂CO₃ solution was added. The reaction mixture was then allowed to stand for an additional 90 min before the absorbance was taken at 760 nm. Various concentrations of gallic acid solutions (6.25, 12.5,

25, 50, 75, 100, 200 µg/mL) were prepared and used to create a standard curve. The amount of total phenolics was expressed as gallic acid equivalents (GAE, mg gallic acid/g sample).

Determination of total flavonoid content (TFC)

The total flavonoid content was determined using a colorimetric method described by [16]. Extracts (1.0 mg/mL), 75 µL of 5% (w/v) NaNO₂ solution, 0.150 mL of freshly prepared 10% (w/v) AlCl₃ and 0.5 mL of 1 M NaOH solution were added. The final volume was then adjusted to 2.5 mL with deionized water. The mixture was allowed to stand for 5 min, and the absorbance was measured at 510 nm. Various concentrations of quercetin solutions (6.25, 12.5, 25, 50, 75, 100, 200 µg/mL) were prepared and used to create the standard curve. The amount of total flavonoids was expressed as quercetin equivalents (QE, mg quercetin/g sample).

Determination of tannin content

Tannin content of extracts was determined by the Folin–Ciocalteu method [28]. Sample (0.1 mL) was added to a 10-mL volumetric flask containing 7.5 mL of distilled water, 0.5 mL of Folin–Ciocalteu phenol reagent, and 1 mL of 35% sodium carbonate solution and diluted to 10 mL with distilled water. The mixture was thoroughly shaken and kept at room temperature for 30 min. A standard curve was prepared with graded concentrations of tannic acid (6.25, 12.5, 25, 50, 75, 100, 200 µg/mL). The absorbance was measured at 700 nm and tannin content was expressed in terms of mg of tannic acid equivalent/ g of dried sample.

HPLC–DAD fingerprinting

High-performance liquid chromatography (HPLC) was used to identify the presence of phytochemicals in methanolic leaf extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*. The samples were dissolved in aqueous acetonitrile (10 mg/20 mL) and mixed vigorously for 30 min. After mixing, the aqueous end was run off while the organic solvent end was collected into a 25-mL standard flask. The analysis was performed on a Shimadzu (NexeraMX) HPLC system fitted with uBONDAPAK C18 column (length 100 mm, diameter 4.6 mm, and thickness 7 µm). The mobile phase consisted of a mixture of an aqueous acetonitrile (acetonitrile/water, 80:20). The flow rate of the sample was 2 mL/min. Compounds were detected by a UV detector (Diode Array Detector, DAD) at 254 nm. The retention times of the identified compounds of interest were measured by standard solution at a concentration of 15.69 mg/g. The extract was injected into the high-performance liquid chromatographic machine to obtain a curve providing

peak area and retention time in a chromatogram. The peak area of the sample was compared with that of the standard relative to the concentration of the standard to obtain the concentration of the sample.

Evaluation of antioxidant and radical scavenging potentials

DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity

The ability of the extracts to scavenge DPPH radical was determined according to the method described by [33]. One mL of 0.3 mM DPPH methanol solution was added to individual extracts and quercetin (6.25–200 µg/mL, 2.5 mL) and allowed to react at room temperature for 30 min in the dark. The absorbance of the resulting mixture was measured at 517 nm and converted to percentage antioxidant activity.

Superoxide radical scavenging activity

The superoxide radical scavenging capacity was determined according to the method of [26]. Tris–HCl buffer (50 mM, pH 8.2, 4.5 mL), 25 mM pyrogallol solution (0.4 mL), sample (1 mL) were mixed together and incubated at 25 °C for 5 min. Then, 1 mL of 8 mM HCl solution was dripped into the mixture promptly to terminate the reaction. The absorbance was measured at 420 nm. Quercetin was used as the reference standard. The superoxide radical scavenging capacity was calculated using the formula:

$$\text{Scavenging capacity (\%)} = \left[\frac{A_0 - A_1}{A_0} \right] * 100$$

where A_0 is the absorbance of the control, A_1 is the absorbance of the sample.

Nitric oxide (NO) scavenging activity

NO scavenging activity was determined as previously described [10]. The reaction mixture (3 mL) containing sodium nitroprusside (10 mM) in phosphate-buffered saline and the extract were incubated at 25 °C for 150 min. Then, 0.5 mL of the reaction mixture was removed, and 0.5 mL of Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. The results were expressed as percentage radical scavenging activity.

Hydroxyl radical scavenging activity

A mixture containing FeCl_3 (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), deoxyribose (28 mM), EDTA (1 mM) and different concentrations of test samples in 500 µL phosphate-buffered saline (PBS, 20 mM, pH 7.4) was incubated for 30 min at 37 °C. After adding 1 mL of trichloroacetic acid (10%, w/v) and 1 mL thiobarbituric

acid (2.8% w/v; in 25 mM NaOH), the reaction mixture was boiled for 15 min. After cooling, the extent of oxidation was measured at 532 nm and the scavenging activity of test sample was expressed as the percentage inhibition of the deoxyribose degradation to malondialdehyde [22].

2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The $\text{ABTS}^{+\cdot}$ stock solution was prepared by mixing the two stock solutions (7 mM ABTS solution and 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ solution) in equal quantities and allowing them to react for 16 h at room temperature in the dark. The working solution was then prepared by mixing 5 mL $\text{ABTS}^{+\cdot}$ solution with 145 mL of distilled water to obtain an absorbance of 0.076 ± 0.001 units at 734 nm. Extracts (1 mL) at various concentrations (6.25–200 µg/mL) were allowed to react with 1 mL of $\text{ABTS}^{+\cdot}$ solution, and the absorbance was measured at 734 nm after 30 min using a spectrophotometer [45]. The percentage scavenging activity was calculated using the formula: scavenging

$$\text{Activity (\%)} = \left[\frac{A_c - A_s}{A_c} \right] * 100$$

where A_c is the absorbance of control and A_s the absorbance of the extract.

Fe^{2+} chelating ability

The principle of the assay is based on disruption of O-phenanthroline- Fe^{2+} complex in the presence of a chelating agent. The Fe^{2+} chelating ability of the extracts was assayed according to a previously described method [36]. FeSO_4 (500 µL, 500 µM) and 200 µL of extract were incubated for 5 min at room temperature, and 500 µL of 1,10-phenanthroline (0.5 mM) was added. The absorbance of the orange-colored solution was read at 510 nm with a spectrophotometer. The in vitro Fe^{2+} chelating ability of the sample is calculated using the formula:

$$\text{Chelating ability (\%)} = \left[\frac{A_c - A_s}{A_c} \right] * 100$$

where A_c is the absorbance of control and A_s the absorbance of the extract.

Cupric ion-reducing antioxidant capacity (CUPRAC)

Determination of the cupric ion (Cu^{2+})-reducing ability of the individual extracts was based on a previously described method [7]. CuCl_2 solution (0.01 M), 1.0 M ammonium acetate buffer solution and 7.5 mM of ethanol neocuproine solution were added to each test tube containing different concentrations of standard antioxidant (Trolox) or extracts. Finally, the total volume was adjusted to 2 mL with distilled water and incubated for

30 min at room temperature. Absorbance was measured at 450 nm against a reagent blank.

Ferric-reducing antioxidant power (FRAP)

The assay involved the rapid reduction of ferric-tripyridyltriazine (Fe^{3+} -TPTZ) to ferrous-tripyridyltriazine (Fe^{2+} -TPTZ), a blue-colored product by antioxidants present in sample [12]. FRAP reagent comprising 300 mM acetate buffer (pH 3.6), 100 mM TPTZ in 40 mM HCL solution, and 20 mM ferric chloride (10:1:1) was prepared, and 0.2 mL of each sample was mixed with 1.3 mL of the FRAP stock solution. Absorbance was measured at 620 nm, and FRAP value was extrapolated from a standard curve of Fe^{2+} solution.

Lipid peroxidation inhibitory activity

Brains were obtained from albino rats and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4). The resulting homogenate was centrifuged at 3000 rpm for 10 min to obtain the supernatant. Aliquot (0.5 mL) of the supernatant was added to 0.2 mL extracts of various concentrations (6.25–200 $\mu\text{g}/\text{mL}$), and the volume was made up to 1 mL with distilled water. Then, 0.05 mL of 0.07 mM FeSO_4 was added, and the mixture was incubated at 37 °C for 30 min and 1.5 mL of acetic acid (pH 3.5, 20%) was added. Thereafter, 1.5 mL of 0.8% (w/v) TBA in sodium dodecyl sulfate 1.1% (w/v) was added. The mixture was heated at 95 °C for 60 min. Then, the samples were cooled and centrifuged at 3000 rpm for 10 min. The intensity of the pink-colored complex was measured at 532 nm and converted to percentage inhibition of lipid peroxidation [49].

Acetylcholinesterase (AChE) inhibitory activity

AChE inhibitory activity was measured by the colorimetric method of [18]. Rats were decapitated; the brains quickly removed and placed on an ice-cold plate. The brain was weighed and homogenized in cold 10 mM Tris-HCl buffer, pH 7.2, containing 160 mM sucrose. The homogenates were centrifuged at 10,000 $\times g$ for 10 min at 4 °C, and the resulting clear supernatants were used as enzyme sources. Briefly, enzyme in 20 mM phosphate buffer (pH 7.4) was incubated in the presence of 10 mM DTNB solution with different concentrations of each extract. The enzyme reaction was initiated by the addition of 75 mM acetylthiocholine iodide after the pre-incubation times of 0, 1, 2 and 3 min. Substrate hydrolysis was monitored by the formation of a yellow anion of 5-thio-2-nitrobenzoic acid at 415 nm. Enzyme activity was estimated through differences in absorbance/min and the percentage inhibition of AChE.

Prediction of pharmacokinetic properties

Pharmacokinetic properties of natural compounds such as MW (molecular weight), LogP, HBD (number of hydrogen bond donors), HBA (number of hydrogen bond acceptors), TPSA (topological polar surface area), nrtB (number of rotatable bonds), nViolation (violations of Lipinski's rule of five) were predicted using SwissDock Online server (<http://www.swissadme.ch/>) and Molinspiration Online tool (<http://www.molinspiration.com/>). The percentage of absorption (% ABS) was calculated using the Zhao et al. formula:

$$\% \text{ABS} = [109 - (0.3345 \times \text{TPSA})]$$

Molecular docking

The molecular docking study of compounds was performed to evaluate the binding interaction mode in the active site of the AChE enzyme (4EY5) that was obtained from the Protein Data Bank. The binding pocket of the receptor was predicted using DogSite platform of the protein-plus webserver (<http://proteinsplus.zbh.uni-hamburg.de>). The protein was prepared by removing co-crystallized ligands and additional water molecules using Pymol 2.5.1. The 3D sdf file of the compounds (Quercetin (CID: 5280343), Kaempferol (CID: 5280863), Ferulic acid (CID: 445858), Lycopenene (CID: 446925), Leucocyanidin (CID: 3705436), Gallic acid (CID: 370), Isorhamnetin (CID: 5281654)) were obtained from PubChem database and OpenBabel 2.4.1 was used to convert to the pdb format. AutoDock Vina version 1.1.2 was used for molecular docking process. Docking analysis was carried out with the grid size set as 60 \times 60 \times 60 with 1.0 Angstrom spacing and Centres x , y and z to be -2.857 , -40.075 and 30.865 , respectively. The exhaustiveness that determines how comprehensive the software search for the best binding mode was set to the default value of 8 Angstrom. Biovia Discovery Studio 2021 was used for visualization and analyzing of the docking results.

Inhibition constant (Ki)

The inhibition constant (K_i) of all the compounds against AChE was calculated from docking energy using the following equation:

$$K_i (\text{nM}) = \exp(\Delta G \times 1000) / RT$$

where $T = 298.15$ K, $R = 1.987$.

Correlation analyses

The strength and direction of the relationship between the antioxidant properties and AChE inhibitory activities of the extracts were evaluated statistically.

Statistical analyses

All statistical analyses were performed using the GraphPad version 6 software. Results were expressed as mean \pm SEM ($n=3$). One-way analysis of variance was used for data analysis. Significant differences between groups were detected in the analysis of variance using Duncan's multiple range test at $P<0.05$. Statistical differences between mean values of individual tests were detected using independent-sample t test. The correlation analyses the GraphPad software.

Results

The phytochemical screening of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* leaves methanolic extract showed the presence of alkaloids, flavonoids, and tannins (Table 1).

The total phenolic content (TPC) of the extracts calculated using the gallic acid regression equation of a calibrated linear curve ($y=0.0117x+0.1241$; $R^2=0.9916$) is shown in Table 2. The highest TPC value was observed in *S. mombin*, followed by *C. papaya*. Total flavonoid content (TFC) of the extracts was calculated from the regression equation of the calibration curve ($Y=0.0071x-0.0901$; $R^2=0.9903$) and showed a similar trend to that of TPC. *Spondias mombin* has the highest

value (43.86 ± 0.905 mg QE/g) and *K. crenata* the lowest (22.89 ± 0.586 mg QE/g) (Table 2). The TTC of *S. mombin*, *C. papaya* and *K. crenata* was 89.52 ± 1.360 , 38.21 ± 0.136 and 18.48 ± 0.156 mg TAE/g of plant extract, respectively, as shown in Table 2.

Spondias mombin leaf extract HPLC–DAD fingerprinting (Additional file 1: Fig. S1) revealed the presence of phenolic acids (chlorogenic acid, ellagic acid and gallic acid), flavonoids (rhamnetin, isorhamnetin, isoquercetin, quercetin, kaempferol, rutin, isoquercitrin and astragalinalin), terpenoids (humulene, lupeol and cadinene), and other compounds as shown in Table 3. For *C. papaya* leaf extract, the phytochemicals obtained from the analysis of the chromatogram (Additional file 1: Fig. S2) include carotenoids (β -carotene, lycopene), terpenoid (linalool), sterols (papayaglyceride, glucopaclin and β -sitosterol), flavonoids (quercetin, kaempferol), and alkaloids (carpaine, sinigrin) (Table 3). The analysis of the HPLC chromatogram (Additional file 1: Fig. S3) of the *K. crenata* leaf extract showed the presence of phenolic acids such as caffeic acid, *p*-coumaric acid, *p*-hydroxycinnamic acid, protocatechuic acid and ferlic acid; flavonoids such as luteolin, quercetin, kaempferol, rutin and leucocyanidin (Table 3). The major chemical class identified in *S. mombin*, *C. papaya* and *K. crenata* leaf extracts was flavonoids with quercetin as predominant compound.

Figure 1 shows the antioxidant activity of the extracts. A clear comparison of the performance of the extracts and the reference compounds as revealed by their IC_{50} values (obtained using inverse logarithmic method) in the different tests is depicted in Table 4. The scavenging activities, metal chelating ability, reducing power and inhibition of lipid peroxidation were in a concentration-dependent manner. *Spondias mombin* was the most effective extract followed by *C. papaya* and then *K. crenata*. The CUPRAC assay shows the effectiveness of *S. mombin* (IC_{50} 24.29 ± 0.165 μ g/mL) where its value was clearly superior to that of the reference standard, trolox (IC_{50} 34.77 ± 0.242) and other samples.

The order of AChE inhibitory activity of the extracts followed the pattern established in the antioxidant activity assays. The AChE inhibitory activity of *S. mombin* (53.24 ± 0.327) was the highest, while that of *K. crenata* (70.5 ± 0.426) was the least. The AChE inhibitory

Table 1 Phytochemicals detected in methanol extracts of the plants

Test	<i>Spondias mombin</i>	<i>Carica papaya</i>	<i>Kalanchoe crenata</i>
Tannins	+	+	+
Alkaloids	+	+	+
Flavonoids	+	+	+
Anthraquinones	+	+	+
Steroids	+	–	+
Saponins	+	–	+

Key: + indicates present; – indicates absent

Table 2 Quantitative estimates of constituent phytochemicals in the plant extracts

Test	<i>Spondias mombin</i>	<i>Carica papaya</i>	<i>Kalanchoe crenata</i>
Total phenols (mg GAE/g)	91.30 ± 0.794^a	34.15 ± 0.242^b	18.60 ± 0.786^c
Total flavonoids (mg QE/g)	43.86 ± 0.905^a	39.97 ± 0.666^b	22.89 ± 0.586^c
Total tannins (mg TAE/g)	89.52 ± 1.360^a	38.21 ± 0.136^b	18.48 ± 0.156^c

Data are expressed as mean \pm SEM ($n=3$).

GAE gallic acid equivalent, QE quercetin equivalent, TAE tannic acid equivalent. Values with the same superscript letter in a row are not significantly different ($P>0.05$)

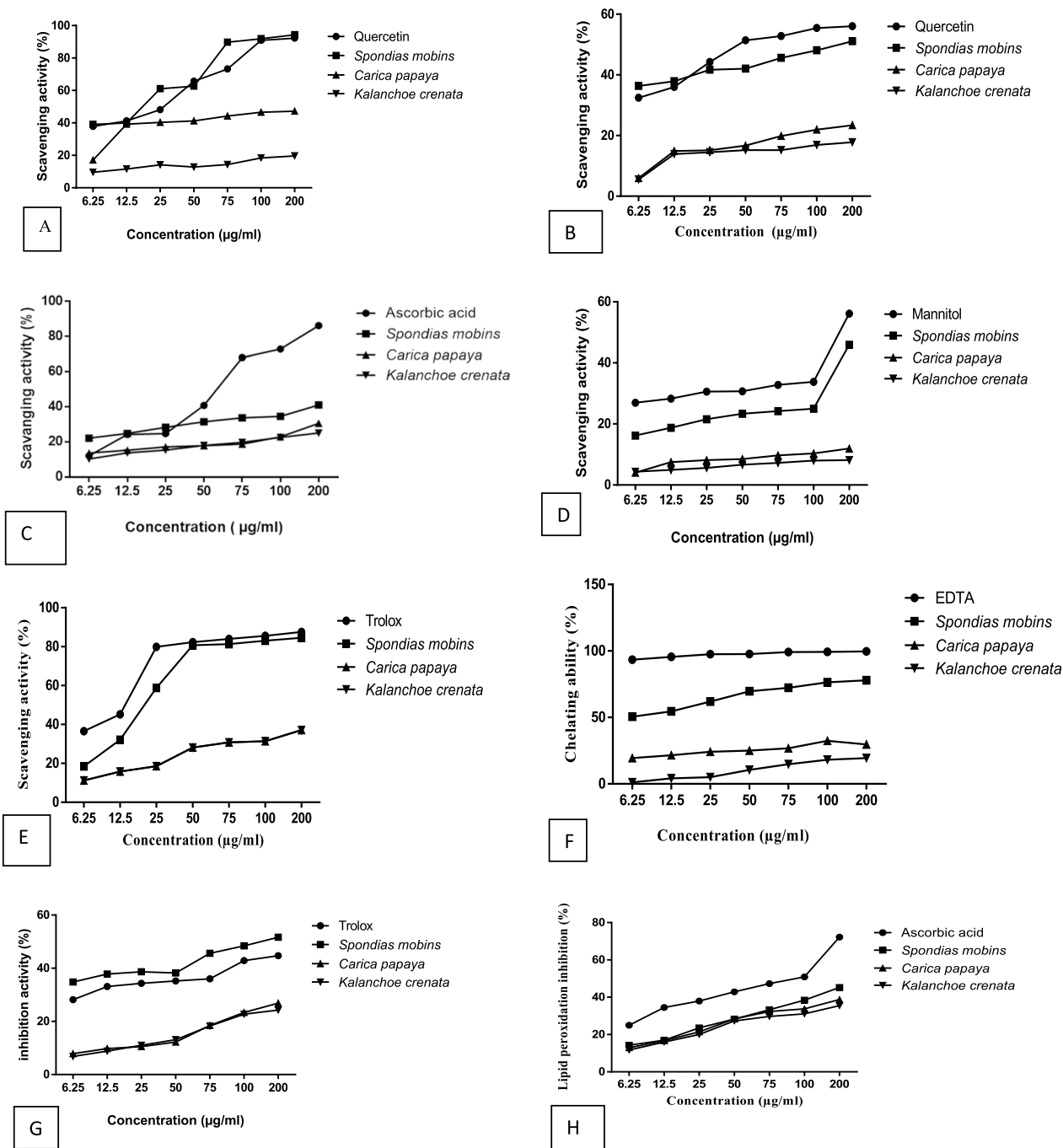


Fig. 1 The comparative antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* leaf extracts **A** 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity **B** Superoxide radical scavenging activity **C** Nitric oxide (NO) radical scavenging activity **D** Hydroxyl radical scavenging activity **D** 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) radical (ABTS⁺) scavenging activity **E** Iron chelating activity **F** Cupric ion reducing antioxidant capacity (CUPRAC) **G** Lipid peroxidation inhibitory activity

activities of the extracts were lower than that of the reference standard, quercetin (20.52 ± 0.112) as shown in Fig. 2.

Table 5 shows the correlation analyses of the total flavonoid contents, total phenolic contents, antioxidant

activities and AChE inhibitory activities of the extracts, which confirmed a positive association between antioxidant activity and AChE inhibitory activity. The *r*² values have been used to show the relationship between the phytochemical constituents, antioxidant activities and AChE

Table 4 IC₅₀ of methanol-leaf extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* in antioxidant tests

Antioxidant test	<i>Spondias mombin</i> IC ₅₀ (µg/mL)	<i>Carica papaya</i> IC ₅₀ (µg/mL)	<i>Kalanchoe crenata</i> IC ₅₀ (µg/mL)	Standard	IC ₅₀ (µg/mL)
DPPH [•] scavenging activity	43.29 ± 0.443 ^b	59.27 ± 0.644 ^c	80.20 ± 0.414 ^d	Quercetin	38.43 ± 0.440 ^a
Superoxide radical scavenging activity	50.83 ± 0.23 ^b	77.84 ± 0.926 ^c	84.19 ± 0.119 ^d	Quercetin	48.73 ± 0.468 ^a
Nitric oxide radical scavenging activity	41.99 ± 0.217 ^b	50.44 ± 0.281 ^c	60.12 ± 0.512 ^d	Ascorbic Acid	35.73 ± 0.490 ^a
Hydroxyl radical scavenging activity	10.73 ± 0.681 ^b	38.22 ± 0.960 ^c	67.74 ± 0.684 ^d	Mannitol	8.39 ± 0.609 ^a
ABTS ^{•+} scavenging activity	25.43 ± 0.325 ^b	39.84 ± 0.163 ^c	59.02 ± 0.376 ^d	Trolox	20.46 ± 0.221 ^a
Iron chelating	30.49 ± 0.212 ^b	46.90 ± 0.291 ^c	53.14 ± 0.401 ^d	EDTA	13.73 ± 0.163 ^a
CUPRAC	24.29 ± 0.165 ^b	68.21 ± 0.882 ^c	73.04 ± 0.956 ^d	Trolox	34.77 ± 0.242 ^a
Lipid peroxidation inhibitory activity	59.24 ± 0.694 ^b	62.34 ± 0.942 ^c	69.95 ± 0.256 ^d	Ascorbic Acid	32.51 ± 0.242 ^a

Values are presented as mean ± SD (n = 3). Values having the same superscript letters are not significantly different (P < 0.001)

DPPH 1,1-diphenyl-2-picrylhydrazyl, ABTS 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate), CUPRAC Cupric ion reducing antioxidant capacity

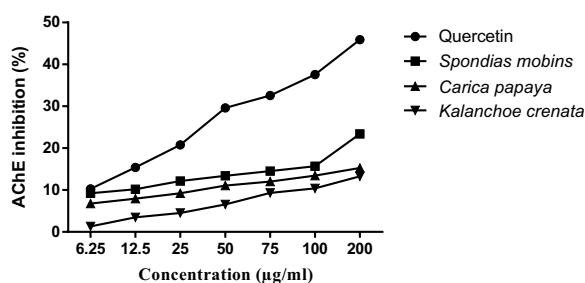


Fig. 2 Acetylcholinesterase inhibition activity of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* leaf extracts

inhibitory activities of the extracts of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*. The total flavonoid and total phenolic contents have extremely significant correlations with the antioxidant activities and AChE inhibitory activities. Table 5 shows that DPPH scavenging activity, NO scavenging activity and lipid

peroxidation inhibitory activity all have extremely significant positive correlations with AChE inhibitory activity ($r^2 = 0.8295, 0.7337, 0.7214$, respectively, $P < 0.0001$). Superoxide radical scavenging activity, hydroxyl radical scavenging activity, ABTS radical scavenging activity and iron chelating ability also have above 50% correlation with AChE inhibitory activity.

The results of the predicted pharmacokinetic properties for selected phytochemicals (quercetin, kaempferol, ferulic acid, lycopene, leucocyanidin, gallic acid and isorhamnetin), the TPSA and %ABS are presented in Table 6. According to the Lipinski's rule of five, drug compounds must have molecular weight < 500, hydrogen-bond donors < 5, hydrogen-bond acceptors < 10, partition coefficient (log *P*) value not greater than 5 and not more than one rule can be violated by an orally active drug. Among the selected phytochemicals, only lycopene violated the rule. With the exception of lycopene, ferulic acid has the lowest TPSA (66.76 Å) while

Table 5 Correlation coefficients of the total flavonoids, total phenolics, total tannins and acetylcholinesterase inhibitory activity with antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*

Antioxidant test	R ²			
	AChE	Total flavonoid	Total phenolic	Total tannin
DPPH [•] scavenging activity	0.8295	0.6457	0.6177	0.6316
Superoxide radical scavenging activity	0.5485	0.3684	0.9046	0.8654
Nitric oxide radical scavenging activity	0.7337	0.5463	0.8705	0.9074
Hydroxyl radical scavenging activity	0.6595	0.4387	0.9489	0.9074
ABTS ^{•+} scavenging activity	0.6214	0.1754	0.4855	0.4715
Iron chelating	0.5876	0.7011	0.9580	0.9699
CUPRAC	0.6295	0.3880	0.9580	0.9294
Lipid peroxidation inhibitory activity	0.7214	0.0529	0.0689	0.06281
Acetylcholinesterase inhibitory activity	–	0.8306	0.6699	0.7012

Correlation is significant at the 0.05 level (one-tailed)

DPPH 1,1-diphenyl-2-picrylhydrazyl, ABTS 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate), CUPRAC Cupric ion reducing antioxidant capacity. R² coefficient of determination

Table 6 Predicted pharmacokinetic properties of selected compounds

Compound	Formular	Molecular weight (g/mol)	Log P	HBA	HBD	TPSA	%ABS	nAtom	nRB	nViolation
Quercetin	C ₁₅ H ₁₀ O ₇	302.24	1.23	7	5	131.36	65.06	22	1	0
Kaempferol	C ₁₅ H ₁₀ O ₆	286.24	1.58	6	4	111.13	71.83	21	1	0
Ferulic acid	C ₁₀ H ₁₀ O ₄	194.18	1.36	4	2	66.76	86.69	14	3	0
Lycopene	C ₄₀ H ₅₆	536.89	9.98	0	0	0	109.00	40	16	2
Leucocyanidin	C ₁₅ H ₁₄ O ₇	306.27	0.11	7	6	130.61	65.31	22	1	1
Gallic acid	C ₇ H ₆ O ₅	170.12	0.21	5	4	97.98	76.23	12	1	0
Isorhamnetin	C ₁₆ H ₁₂ O ₇	316.26	1.65	7	4	120.36	68.74	23	2	0
Rivastigmine	C ₁₄ H ₂₂ N ₂ O ₂	250.34	2.34	3	0	32.78	98.03	18	6	0

Log P Octanol/water partition coefficient, *HBA* hydrogen bond acceptor, *HBD* hydrogen bond donor, *TPSA* topological surface area, *%ABS* % absorption, *nAtom* number of atoms, *nrb* number of rotatable bonds, *nViolation* number of violation. There was no violation of the Lipinski's rule

the highest TPSA is observed in quercetin (131.36 Å). Quercetin, kaempferol, ferulic acid, lycopene, leucocyanidin, gallic acid and isorhamnetin also show similar pharmacokinetic properties when compared to rivastigmine commonly prescribed for AD patients.

DogSite platform of the protein-plus server was used to predict the active site of AD target (4EY5). The predicted druggable pocket of the co-crystallized ligand in 4EY5 are Trp86, Tyr337, Tyr133, Tyr337 and Gly121. These amino acids were selected for the binding of all the selected phytochemicals. Figure 3 and Table 7 show the top docked binding pose of the selected phytochemicals; quercetin (−9.1 kcal/mol), kaempferol (−8.6 kcal/mol), ferulic acid (−7.3 kcal/mol), lycopene (−15.1 kcal/mol), leucocyanidin (−7.8 kcal/mol), gallic acid (−7.2 kcal/mol) and isorhamnetin (−8.2 kcal/mol). Although lycopene showed very good binding affinity, it was not considered for further docking analysis because it does not comply with the Lipinski's rule of five. The binding of rivastigmine to the predicted site on 4EY5 shows a binding affinity of −6.9 kcal/mol (Table 7). This implies that the selected phytochemicals showed high affinity toward 4EY5 as compared to rivastigmine commonly prescribed for AD patients. The 2D interactions by Biovia Discovery Studio 2021 are presented in Fig. 4, and parameters such as hydrogen bond, distance, hydrophobic interactions, π -interactions and inhibitory constant are presented in Table 7. The orientation of each of these phytochemicals resembles that of the native ligand. The selected compounds and rivastigmine (Fig. 5) showed similar binding interactions with the amino acids on analysis. The interactions were prominently observed in the region of Tyr337 and Trp86 amino acid residues due to the pronounced existence of the pi-cation interaction at the catalytic anionic site.

Discussion

Plants as a potential source of drugs for the management of clinical disorders have been extensively studied over the past few years. The limitations and side effects of drugs in current use for the management of AD and other dementias warrant search for more effective therapeutic agents [19]. A gradual decrease of acetylcholine levels, arising from the loss of the cholinergic synapses and reactive oxygen species production, play an important role in the pathogenesis of AD [20]. Therefore, plants and phytochemicals with antioxidant activity and the ability to balance acetylcholine levels will be potentially useful in the management of AD. These considerations necessitated the phytochemical investigation, and evaluation of the antioxidant and anticholinesterase activities of the three medicinal plants popularly used in traditional herbal medicine in Nigeria for the treatment of brain-related disorders.

Phytochemicals such as tannins, alkaloids, flavonoids, anthraquinones, steroids and saponins detected in the extracts are bioactive agents. These phytochemicals have demonstrated activities such as inhibition of neuroinflammation and oxidative stress, maintenance of neurotransmitter balance, antiapoptosis and mitochondrial stabilization in the brain [61].

The analysis of the chromatograms obtained from the three plant validates the presence of various phytochemicals like phenolic acids, flavonoids and terpenoids. Flavonoids were the preponderant phytochemicals in the extracts. Flavonoids, for example, quercetin and kaempferol, are viewed as having powerful cell reinforcement property that are helpful in the avoidance of different oxidative stress-related diseases including neurodegenerative disorders, for example, AD [34].

The presence of polyphenolics (which includes phenolic acids, flavonoids and tannins) in plants has a direct

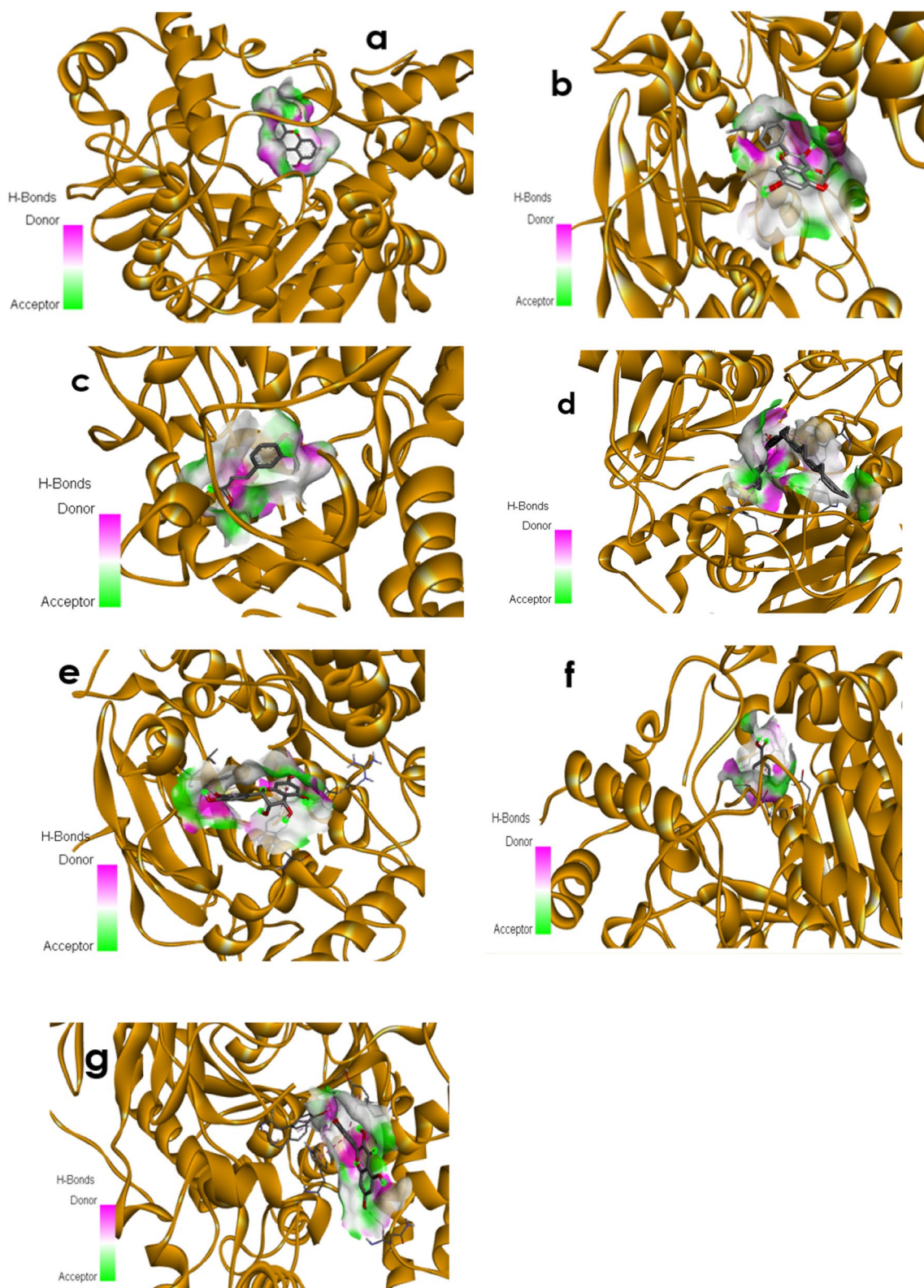


Fig. 3 3D Binding interaction of 4EY5 with **a** Quercetin **b** Kaempferol **c** Ferulic acid **d** Lycopene **e** Leucocyanidin **f** Gallic acid and **g** Isorhamnetin

Table 7 Summary of docking results

Compound	Binding affinity (kcal/mol)	Inhibition constant (Ki) (nM)	No. of H bond formed	No. of hydrogen bond interaction residues	Distance	Hydrophobic interaction	Residues forming π -interactions
Quercetin	-9.7	7.76E-08	2	Gln71, Tyr133	2.36 and 2.76	Ser125, An87, Pro88, Leu130, Tyr337, Gly126, Glu202, Val73, Gly120, Gly121 and Asp74	Trp86, Tyr337 and Asn87
Kaempferol	-8.6	4.97E-07	1	Arg296,	2.30	Tyr337, Val294, Phe338, Tyr124, Phe295, Phe297, Leu289, Glu292 and Gln291	Tyr341 and Trp286
Ferulic acid	-7.3	4.46E-06	3	His-447, Ser-203 and Glu-202	2.59, 1.86 and 2.97	Gly448, Tyr72, Gly120, Asp74 Gly122, Gly121, Ser125, Tyr124, and Asn87,	Phe338, Phe297, Trp86 and Tyr337
Lycopene	-15.1	-	0	Violated the Lipinski's rule of five			
Leucocyanidin	-7.8	1.92E-06	1	Asp74	2.58	Leu289, Thr75, Phe297, Tyr341, Phe338, Val294, Ser293, Tyr72, and Phe295	Leu76 and Trp286
Gallic acid	-7.2	5.28E-06	4	Trp86, Tyr133, Glu202 and Gly120	2.84, 1.92, 2.21 and 2.94	Gly121, Ser125, Gly126, Ile451, Ser203, Leu130, Tyr 119, Tyr337, His447 and Tyr124	Trp86 and Gly120
Isorhamnetin	-8.2	9.76E-07	1	Gln291	2.11	Tyr337, Phe297, Val294, Ser293, Phe338, Arg296, Phe295, Glu292 and Leu289	Tyr124, Tyr72, Tyr341, and Trp286
Rivastigmine	-6.9	8.75E-06	1	Trp286	3.75	Tyr337, Val294, Phe297, Arg296, Ser293, Phe295, Try124, Phe338, Try341, Try72, Leu289	Trp286

relation with their antioxidant and radical scavenging properties [29]. The antioxidant activity of polyphenols is influenced by the presence of free hydroxyl groups. Several mechanisms have been demonstrated for the antioxidant activity of polyphenols. These include free radical scavenging, inhibition of lipid peroxide formation, metal chelation, and reductive ability. The notable activities of the extracts under consideration in the in vitro tests which covered different mechanisms of antioxidant protection are an allusion to their potential therapeutic efficacy.

The oxidation of pyrogallol forms superoxide anions (a purple solution). It reacts with proton in solution and form hydrogen peroxide. Hydrogen peroxide is an important substrate that produce singlet oxygen and hydroxyl radicals. Superoxide anion is a major reactive oxygen species that leads to the oxidation of cells and tissues [21, 53]. *Spondias mombin*, *C. papaya* and *K. crenata* leaves extracts inhibited pyrogallol autoxidation in a dose-dependent manner, thereby reducing the probability of peroxide formation. Also, a number of physiological processes need nitric oxide during metabolism.

Abnormalities in NO production, that is high concentration of NO, has been linked to different diseases [57]. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxy-nitrite anion (ONOO⁻) [62]. The NO scavenging activity of the extracts buttresses their potential to limit oxidant-mediated damage.

Hydroxyl radical is the leading cause of oxidative biological damage such as protein disulfide bond breakage or denaturation, which results in unfolding and refolding of proteins into abnormal spatial configurations, and it is observed in neurological disorders [44, 52]. The results of the present study showed that *S. mombin*, *C. papaya* and *K. crenata* leaf extracts are effective hydroxyl radical scavengers, indicating that they can prevent or mitigate brain oxidative biological damage.

Lipid peroxidation is a chief feature of many pathologies including AD. Many studies have demonstrated increased lipid peroxidation in brain of patients with AD [38, 43]. Damage to cell membrane generates a number of degraded products which is associated with lipid peroxidation. A major degraded product of lipid peroxidation is

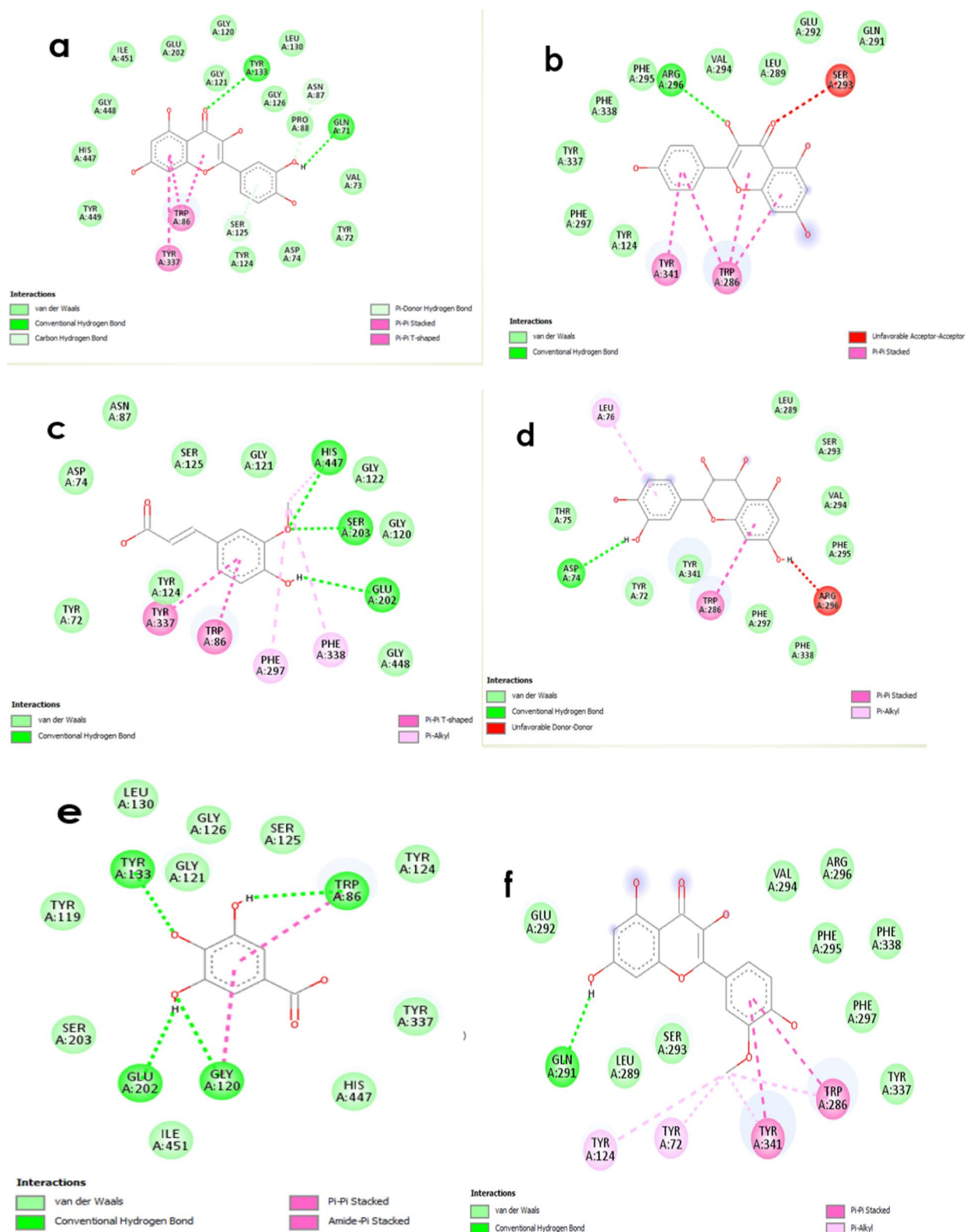


Fig. 4 2D Binding interaction of 4EY5 with **a** Quercetin **b** Kaempferol **c** Ferulic acid **d** Leucocyanidin **e** Gallic acid and **f** Isorhamnetin using Biovia Discovery Studio 2021

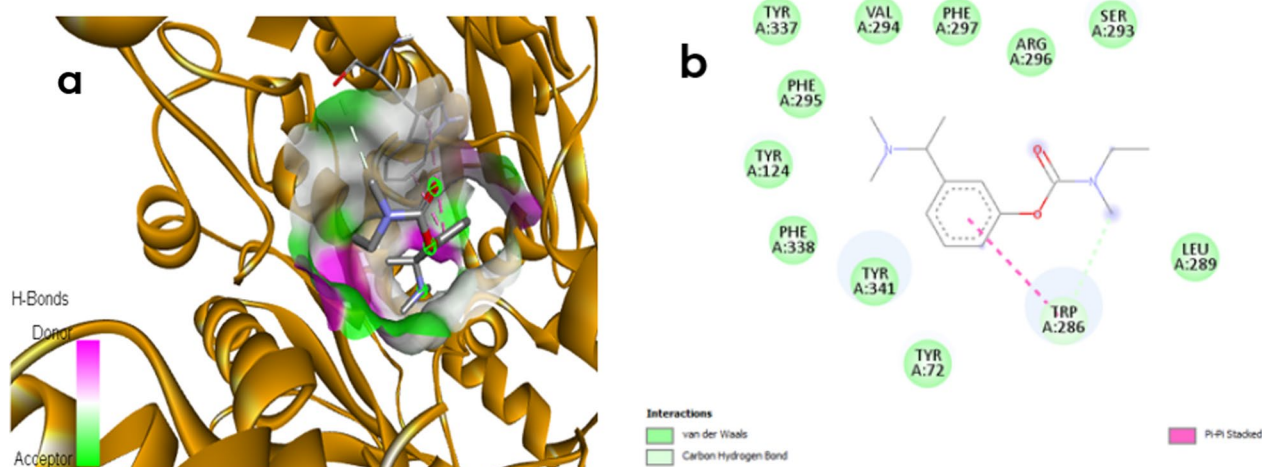


Fig. 5 Molecular docking of 4EY5 with Rivastigmine. **a** 3D Binding pose of rivastigmine after docking experiment with 4EY5 and **b** 2D Binding interaction of Rivastigmine and 4EY5 with using Biovia Discovery Studio 2021

malondialdehyde [24]. The significant lipid peroxidation inhibitory activity of *S. mombin*, *C. papaya* and *K. crenata* leaf extracts is a further reflection of their medicinal potential.

A promising management of neurological and neurodegenerative disorders such as AD and senile dementia is linked to acetylcholinesterase enzyme inhibition [19]. The enzyme is important in the breakdown of acetylcholine, and inhibition of the enzyme leads to increase in the concentration of acetylcholine and increase in communication between the brain nerve cells [62]. The anti-acetylcholinesterase activity shown by the extracts in this study suggests that the plants are potential sources of effective compounds that can stimulate an increase in acetylcholine level in AD and other dementias. Plants that possess high phenolic content have also been reported to inhibit AChE activity [3]. Therefore, the inhibitory effect of the extracts on AChE activity may be linked to their phenolic components. The Pearson correlation coefficients of the total phenolic and flavonoid contents, and the antioxidant activities obtained in this study buttresses this point. The Pearson correlation coefficients obtained in the present study also show that the polyphenolic contents and antioxidant activities of the extracts have strong positive correlations with the AChE inhibitory activity.

Alzheimer's disease (AD) is the most prevailing neurodegenerative disease in the ageing population. Two major factors involved in the pathogenesis of AD are oxidative stress and reduction in brain acetylcholine level. *Spondias mombin*, *C. papaya* and *K. crenata* leaf extracts demonstrated positive correlation between their remarkable antioxidant and anticholinesterase inhibitory effects supporting their traditional use in managing

brain-related disorders and indicating their potential usefulness in the treatment of AD. However, further investigations are necessary in in vivo and clinical settings to establish the promising in vitro effects.

Lipinski's rule of five assists in evaluating the pharmacokinetic properties and bioavailability of oral drugs. According to the rule, a violation of more than one rule is an indication of poor bioavailability. The TPSA reflects the phytochemicals' hydrophilicity and is important in protein–ligand interaction. Generally, druggable compounds with TPSA less than 140 Å and the number of rotatable bonds less than 10 have good oral bioavailability [60].

Molecular docking is the process by which 2 molecules fit together in 3-dimensional space; it is a key tool in structural biology and computer-aided drug design [30]. The best pose of each compound is always selected based on their best conformation that allows the lowest free binding energy and analyzed for further interaction of the docked structure [27]. Quercetin, kaempferol, ferulic acid, leucocyanidin, gallic acid and isorhamnetin fit into the active site of 4EY5, and the binding affinity between these phytochemicals and AChE (4EY5) is stabilized by non-covalent bonds, which includes hydrogen bond, hydrophobic bond and pi-interactions. Hydrogen bonds play a crucial role in enzyme catalysis, protein–substrate and protein–inhibitor complexes, and the structural stability of various biological molecules [37]. Pi–pi stacking observed in most of the interactions is formed between the phenyl ring of the phytochemical and the amino acid residues. Pi–pi interactions are a type of non-covalent interaction pivotal to biological events such as protein–ligand recognition by providing a significant amount of

binding enthalpy [35]. The results of the molecular docking and pharmacokinetic studies showed that quercetin, kaempferol, ferulic acid, leucocyanidin, gallic acid and isorhamnetin fulfill the requirements for an anti-Alzheimer's disease drug, such as ADMET, non-toxicity, binding affinity, inhibition constants, antioxidant and neuroprotective inhibitory properties and good interaction with Alzheimer's disease-associated target. Thus, these six phytochemicals from *S. mombin*, *C. papaya* and *K. crenata* leaf extracts with antioxidant activity, inhibitory and neuroprotective activities may be considered an anti-Alzheimer's disease drug agents.

Conclusions

This study reveals that *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata* methanol leaf extracts provide a significant source of secondary metabolites that act as natural antioxidants and acetylcholinesterase inhibitors, which will be helpful in the treatment of Alzheimer's disease.

Abbreviations

ABTS	2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate)
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ATCI	Acetylthiocholine iodide
ROS	Reactive oxygen species
FRAP	Ferric-reducing antioxidant power
NO	Nitric oxide
NMDA	N-Methyl-D-aspartate
DPPH	1,1-Diphenyl-2-picrylhydrazyl
HPLC-DAD	High-performance liquid chromatography-diode-array detector
CUPRAC	Cupric ion reducing antioxidant capacity

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43094-023-00578-x>.

Additional file 1: Figure S1. HPLC-DAD chromatogram of *Spondias mombin* leaf extract. **Figure S2.** HPLC-DAD chromatogram of *Carica papaya* leaf extract. **Figure S3.** HPLC-DAD chromatogram of *Kalanchoe crenata* leaf extract. **Figure S4.** Relationship between AChE inhibition activity (%) versus (a) DPPH (b) Superoxide radical (c) Nitric oxide (d) CUPRAC (e) ABTS (f) Iron chelating and (g) Lipid peroxidation antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*. **Figure S5.** Relationship between Total phenols (mg GAE/g) versus (a) DPPH (b) Superoxide radical (c) Nitric oxide (d) Hydroxyl radical (e) CUPRAC (f) ABTS (g) Iron chelating and (h) Lipid peroxidation antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*. **Figure S6.** Relationship between Total flavonoids (mg QE/g) versus (a) DPPH (b) Superoxide radical (c) Nitric oxide (d) Hydroxyl radical (e) CUPRAC (f) ABTS (g) Iron chelating and (h) Lipid peroxidation antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*. **Figure S7.** Relationship between total tannins (mg TAE/g) versus (a) DPPH (b) Superoxide radical (c) Nitric oxide (d) Hydroxyl radical (e) CUPRAC (f) ABTS (g) Iron chelating and (h) Lipid peroxidation antioxidant activities of *Spondias mombin*, *Carica papaya* and *Kalanchoe crenata*.

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Author contributions

ARA did investigation, data curation, formal analysis, writing—original draft; MEA carried out formal analysis and writing; MTO contributed to project administration and supervision. AAA performed project administration and contributed resources. ACA contributed to conceptualization, supervision, validation, writing—review and editing.

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Raw data were generated and will be provided from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors (ARA, MEA, MTO, AAA, ACA) declare that they have no competing interests.

Author details

¹Department of Medical Biochemistry, School of Basic Medical Sciences, The Federal University of Technology, PMB 704, Akure 340110, Nigeria. ²Department of Biochemistry, School of Life Sciences, The Federal University of Technology, PMB 704, Akure 340110, Nigeria.

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