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Original Research Article

In vitro and *In silico* Evaluation of the Oestrogenic Activities of Some Edible Vegetables in Nigeria

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ABSTRACT

Breast cancer is the most common malignancy in women worldwide with an estimated 2.3 million new cases and 1,380,000 deaths. The steroid hormone; 17-β estradiol plays a significant role in the growth and development of the breast epithelium, and is implicated in the aetiology and progression to breast cancer. The phytoestrogens are plant-derived compounds that mimic endogenous oestrogens, however, reports on their beneficial and adverse health effects remain inconclusive. Therefore, this study aimed to evaluate the oestrogenic activities of some edible vegetables using *in vitro* and *in silico* assays. The leaves of *Gnetum africanum (GA), Lasianthera Africana (LA),* and *Vernonia amygdalina (VA)* were extracted by cold maceration with ethanol. The Yeast Estrogen Screen (YES) which employed Recombinant *Saccharomyces cerevisiae* W303 expressing oestrogen receptors (ERs; α and β) was used to assess the oestrogenic potency of the extracts. The *in silico* investigations involved molecular docking of selected phytochemicals on ERs and prediction of ADMET properties using SwissADME webserver. The YES revealed the following Estradiol Equivalents (EEQs); ERα: LA: 154.05µg/g; VA: 130.20µg/g; GA: 109.40µg/g, and ERβ: LA: 9.26µg/g; VA: 6.22µg/g, GA: 5.73µg/g. Molecular docking studies showed negative binding affinities ranging from -3.3 to -9.0 Kcal/mol, while the SwissADME predictions revealed four compounds with good ADMET properties, interacting with ERα binding pocket similarly to 17-β estradiol. Knowledge of the oestrogenic potencies of edible vegetables is vital as this would help promote safer consumption habits and inform preventive strategies against ER-positive breast cancer.

Keywords: Breast Cancer, 17-β Estradiol, Yeast Estrogen Screen, Phytochemicals, Molecular Docking

Introduction

The recent global cancer statistics report ranked breast cancer as the second most diagnosed cancer with an estimated 2.3 million new cases and 1,380,000 deaths.[1] When compared to the report of 2020,[2] breast cancer mortality rate doubled. In Nigeria, the burden of breast cancer has been on the rise, with Nigeria recording the highest age-standardized breast cancer mortality rates in Africa.[3]

An important risk factor for breast cancer is hormonal exposure i.e. long-term stimulation by both endogenous and exogenous oestrogens.[4]The steroid hormone oestrogen (E2; 17-β estradiol) plays a vital role in the normal growth and development of the breast epithelium, and the dysregulation of oestrogen signaling results in the aetiology and progression of breast cancer.[4] Approximately 70% of breast cancer cases are hormone-dependent with tumour cells, luminal A and B expressing oestrogen receptors (ERs α and β). ERα is the receptor predominantly implicated in breast cancer development,[5] regulating the transcription of genes involved in cell cycles, proliferation, and apoptosis.

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Physiologically, E2 binds to its receptor through the ligand binding domain (LBD) resulting in receptor dimerization. The receptor homodimer translocates to the nucleus, undergoing a conformational change that recruits co-factor proteins; co-activators for agonist effect, or co-repressors for antagonist effect. The receptor dimer-co-factor protein complex binds to DNA directly or indirectly through oestrogen response elements (ERE) on the target genes' promoter region to regulate gene transcription.[6]Ligand bound ERα can also interact with DNA through transcription activators such as activator protein 1 (AP1) and specific protein 1 (SP1) which bind DNA through serum-responsive elements to regulate the expression of genes lacking EREs in their promoter region. In addition to its nuclear translocation, a fraction of ERα stays cytosolic or remains membrane-bound where it exerts a fast and short-lived non-genomic activity through second messenger production (cyclic adenosine monophosphate; cAMP), and interaction with cytoplasmic proteins such as Src to activate growth factor receptors, PI3K/AKT and Ras/MAPK pathways.[4]

The phytoestrogens (PEs) are plant-derived bioactive compounds that mimic endogenous oestrogens. Several studies on the effects of phytoestrogens have reported both beneficial and adverse health effects[7-10]. In addition, there have been reports suggesting that the effects of phytoestrogens depend on the phytoestrogen type, concentration, hormone levels related to age and sex,[11] and interindividual variations in metabolism,[12]thus recommending more in-depth research on the health effects of PEs, and the use of PEs with caution since the beneficial and adverse health effects are inconclusive.[13] A study, Udoh *et al.*[14] revealed that *Gnetum africanum* (African jointfir; Efik/Ibibio: Afang; Igbo: Okazi), a commonly consumed vegetable in Nigeria was oestrogenic as repeated administration of the ethanolic and water extracts of the leaves increased the uterine muscle mass of adult and immature female rats.

These findings suggest that the health benefits of PEs may be overestimated, and by their oestrogenic properties, they could be contributing to an increased risk for ER-positive breast cancer, especially in post-menopausal women where ERα expression is increased, and women with a family history of breast cancer.

By employing the *in vitro* Yeast Estrogen Screen (YES) and computational techniques, this study aims to evaluate the oestrogenic activity of three edible vegetables, *Gnetum africanum* (GA; African jointfir), *Lasianthera africana* (LA), and *Vernonia amygdalina* (VA; Bitter leaf). The YES is a bioassay widely used to quantify ligands that mimic 17-β estradiol in diverse substances including water, plant tissues, consumer products, and foods.[15,16] It is a low-cost *in vitro* alternative to *in vivo* methods such as the rodent uterotrophic assays. The computational methods employed were molecular docking of selected phytochemicals against ERs, and ADMET analysis using SwissADME webserver. Molecular docking is a computational technique that predicts the preferred orientation and binding affinity of a ligand bound to the active site of a target protein, and SwissADME predicts the 'drug-likeness' and pharmacokinetic properties of compounds based on various molecular descriptors such as electronic, steric, spatial and hydrophobic features. Computational methods are less time consuming and allow researchers to screen compound libraries at a fraction of the cost required for wet lab experiments.[17] GA is one of the superior vegetables to most people in the Niger Delta region of Nigeria. In addition to its medicinal value, it has been used in the preparation of soupy meals for very important visitors and during traditional ceremonies by cultures in most states of the Niger Delta region of Nigeria. .[14] LA is a highly nutritional vegetable known for its therapeutic use in managing human ailments such as diabetes, hypertension, and neurodegeneration.[18] VA is a common shrub or small tree that grows in tropical Africa. The leaves are green in colour with a characteristic odour and bitter taste[19,20] associated with saponins, alkaloids, tannins, and glycosides.

Vegetables are important to health due to their rich phytochemical content providing essential antioxidants, vitamins, and minerals. Preclinical studies have shown that these vast phytochemical constituents may impact drug therapy.[21,22,23] To our knowledge, no prior research employing recombinant *Saccharomyces cerevisiae* to screen for oestrogenic activity in the study plants has been published. This study would enhance understanding and raise awareness of the potential risks of phytoestrogens in vegetables for oestrogen receptor-positive breast cancer. Its goal is to promote safer consumption habits and develop preventive strategies that may help reduce the global incidence and breast cancer burden.

Materials and Methods

Collection, Identification, and Authentication of plant samples

Gnetum africanum, Lasianthera africana, and *Vernonia amygdalina* leaves were purchased in March 2023 from a local market in Calabar, lat 4.9589, long 8.3270, Nigeria. They were identified and received their voucher numbers, Bot/Herb/UCC/032, Bot/Herb/UCC/041, Bot/Herb/UCC/188, respectively.

Drugs, Chemicals, and Reagents

Glucose, Galactose, Yeast extract, Peptone, Chlorophenol-red galactopyranoside (CPRG), Absolute Ethanol, Sodium carbonate, Sodium phosphate monobasic, Sodium phosphate dibasic, Magnesium chloride, Potassium chloride, N-lauroyl sarcosine sodium salt, Dithiothreitol, deionized water were analytical grade reagents sourced from Sigma Chemical Company (Sigma-Aldrich Laborchemikalien GmbH. D-30926, Seelze, Germany). Conjugated oestrogen (17-β estradiol) tablets USP (Primarin 0.625mg) from Pfizer Ireland Pharmaceuticals, Little Connell, Newbridge, Co. Kildare, Ireland. Recombinant *Saccharomyces cerevisiae* W303 was received as a kind gift from Professor Thea Edwards of the Columbia Environmental Research Center, U.S.A, originally designed by Dr. Charles Miller at Tulane University.

Equipment

Spectrovis Plus spectrophotometer (range: 380-950 nm; resolution: ~2.5 nm optical resolution) with Logger Pro Software (Vernier International, 5026 Calle Minorga, Sarasota, FL 34242 U.S.A.) running on an Intel Pentium PC; 6 Bucket Centrifuge (Microfield Instrument England Model SM800D); Electric Heated Portable Autoclave Sterilizer Machine Wt280A (Wincom Company Ltd, Shanghai, China), Labnet I5110-230 Incubator(Netherlands); Chemical balance (Danwer Scales Private Limited, New Delhi, India); Laboratory Bunsen burner (EISCO labs, NY, U.S.A); Simple Plastic Sample bottles (Winlabs and Store, Watford, England); 15ml Screw Cap Centrifuge Tube Polypropylene (The Consumables Company Ltd, Oakworth Road, Keighley, UK); Micropipettes 10-100µL (Eppendorf Research Plus Adjustable volume, Zantek Medical, MD, U.S.A); Agary Disposable Syringes (Genral Drug Centre, Ilorin, Nigeria); Petri dishes with Polystyrene (PS) Aseptic (The Consumables Company Ltd, Oakworth Road, Keighley, UK).

Preparation of Reagents

Glucose and galactose media, LacZ buffer, sodium carbonate, and dithiothreitol were prepared according to standard procedure. A 1mg/ml stock solution of 17-β estradiol was prepared and diluted to the appropriate working concentrations as described by Edwards *et al*. [24]

Extraction procedure

As described by Edwards *et al*.,[24]1g of powdered sample, in triplicates, was combined with 10mL of anhydrous ethanol in 25mL beakers and homogenized for 30 min by a combination of manual shaking and vortex mixing. They were centrifuged at the maximal allowable speed for 10 min, and the supernatant was decanted into 25mL beakers and left open in a ventilated hood for one week to allow ethanol to evaporate completely. The beakers were protected from light to avoid degradation of light-sensitive constituents and reconstituted in 1.0 mL of 50% ethanol. A negative extraction control of 11mL ethanol in a 25 mL beaker was prepared in triplicates.

Yeast Cells, Media, and Products

The yeast estrogen screen (YES) was performed using the method of Edwards *et al*.[24] with slight modifications. Yeast cells employed in this assay were Recombinant *Saccharomyces cerevisiae* W303. Yeast transformed with these plasmids constitutively express human ERα or ERβ when grown in galactose media for ERα, and glucose or galactose media for ERβ.[25] If the media contains oestrogenic ligands, they bind to ERs, forming a ligand-ER complex that activates the production of β-galactosidase enzyme at a level directly proportional to the concentration of oestrogenic ligands. Yeast cells are then lysed to release the β-galactosidase and the lysis buffer containing CPRG is cleaved by β-galactosidase to release the red colour (LacZ). The colour produced is quantified by measuring the absorbance, and its intensity is proportional to the concentration of oestrogenic ligands in the media.[24,25]

Culturing and Subculturing Yeast

Using sterile techniques, Recombinant *Saccharomyces cerevisiae* W303 were grown on agar plates; and maintained as active yeast cultures by incubating in filter-sterilized glucose media at 30°C for 48h before preparing the Yeast Estrogen Screen YES plates.[24]

Preparation of YES plates (Day 1)

17-β Estradiol Standard Curve

After the 48h incubation, using sterile techniques, yeast media was diluted to an optical density, OD610nm of 0.195 ±0.015 with filter-sterilized galactose media. 960µL of the resultant media was placed into sample bottles A1-3, while 360µL was placed into B1-3 through rows G1-3. 15µL of 17-β estradiol was added to A1-3 to get the first concentration and serially diluted until G1-3 by removing 615µL from each row such that all bottles contained 360µL. 15µL of vehicle (50% ethanol) was added to vehicle controls H1-3 containing 960µL of yeast

suspension, and the volume was adjusted to 360µL after mixing. Next, 360µL of filter sterilized galactose media was added to I1-3 to serve as media controls to account for absorbance contributed by the media alone.[24]

Plant Sample Assay
15µL of test sample extracts were added to appropriately labeled sample bottles containing 960µL of yeast suspension, and 15µL of test sample extracts were added to corresponding sample bottles containing 960µL of galactose media to account for the absorbance contributed by the extracts. All samples were prepared in triplicates.[24]
After mixing with a pipette, volumes were adjusted to 360µL. The bottles were sealed and incubated for 17h at 30 °C without shaking.[24]

Preparation of YES plates (Day 2)
After the 17h incubation, a pipette was used to mix the contents of each sample bottle and transfer 150µL to new bottles. New pipette tips were used for each row to avoid cross-contamination of samples. 600µL of LacZ buffer containing 1mM DTT and CPRG was added to all sample bottles, and the optical density, OD_{610} was measured immediately using the Spectrovis Plus spectrophotometer. Samples containing yeast expressing ERα were incubated at 30°C for 3h, and yeast expressing ERβ were incubated at 30°C for 4h. After incubation, 300µL of sodium carbonate was added to raise the pH and halt the β-galactosidase reaction. The optical density, OD_{574} was measured and recorded using the spectrovis plus spectrophotometer.[24]

Calculation of LacZ Values
The LacZ value represents the degree of colour change produced by the yeast estrogen screen (YES) in direct proportion to the concentration of oestrogenic ligands.[24]

$$LacZ\ Value\ (\mu L^{-1}\ h^{-1}) = \frac{(OD_{574} - MeanOD_{574}vehicle\ control) \times 1{,}000}{(OD_{610} - MeanOD_{610}media\ control)\ vt}$$

…… Equation 1

Where; OD_{574} = Optical density of sample after incubation with LacZ buffer. $MeanOD_{574}^{vehichle\ control}$ = Mean optical density of vehicle controls after incubation with LacZ buffer. OD_{610} = Optical density (610nm) of sample after 17h incubation with 17-β estradiol (Standard), or plant extracts. $MeanOD_{610}^{media\ control}$ = Mean optical density (610nm) of media controls (galactose blanks). v = volume of sample incubated with LacZ buffer. t = Incubation time with LacZ buffer; 3h for yeast expressing ERα, and 4h for yeast expressing ERβ.[24]

Statistical analysis
The mean of triplicate LacZ values from rows A through G were plotted against the log concentration to produce 17-β estradiol standard curve. This was fitted to a five-parameter logistic regression model using GraphPad Prism and the model parameters; growth rate, inflection point, lower asymptote, and upper asymptote were obtained. The concentration of the test samples was determined by interpolating their LacZ values on the standard curve.[24] All statistical tests were carried out using GraphPad Prism (GraphPad Prism Five for Windows, version 5.01. GraphPad Software Inc.)

Standardizing oestrogen equivalents (EEQs) of Test sample mass.
EEQs (µg/g) of test samples were calculated by multiplying the concentration obtained from interpolation of LacZ values by the total volume pipetted into sample bottles (975 µL), and then dividing by the volume of extracted sample added (15 µL).[24]

$$Estradiol\ Equivalent\ (EEQ) of\ sample = \frac{C \times V1}{V2} \ldots\ldots..$$

Equation 2

Where; C = concentration determined from interpolation of LacZ value on 17-β estradiol standard curve. *V1* = Volume pipetted into sample bottles (975µL). *V2* = Volume of test sample (15µL)[24]

Molecular docking simulations and determination of ADMET properties.
Protein preparation
The 3D crystal structures of ERα: 5WGD; and ERβ: 3OLS were downloaded from the Protein Data Bank, www.rcsb.org. They were considered good targets based on the R-value free, R-value work, R-value observed, receptor resolution, and the Ligand Structure Quality Assessment. Both receptors were prepared for docking using Discovery Studio Visualizer (DSV) and AutoDock tools. The dimensions of the receptor binding pocket, x, y, z, were obtained using DSV 'define and edit binding site'. The pdb file was presented in AutoDock tools, water molecules were removed, polar hydrogens were added, charges were assigned, and the pdbqt format was generated.

Ligand Preparation
The ligands used in this study were 20 compounds each from previous characterization studies of *Gnetum africanum* (GA; compounds 1-20),[26,27] *Lasianthera africana* (LA; compounds 21-40),[28,29] and *Vernonia amygdalina* (VA; compounds 41-60).[30,31] They were downloaded in structure data format (SDF) from the NCBI PubChem database (https://pubchem.ncbi.nlm.nih.gov./), and subjected to energy minimization and geometry optimization using Molecular Mechanics (MM+) and Semi-empirical-PM3 (SE-PM3) methods of HyperChem 8.0 software, and saved as pdb files. The pdb files were presented in AutoDock tools, torsions were chosen, charges assigned, and the pdbqt generated.

Molecular Docking and ADMET Analysis
The ligands (compounds 1-60) were docked individually against 5WGD and 3OLS using AutoDock Vina,[32] following the interactive docking protocol. The binding affinities and root mean square deviation (RMSD)/Lower bound were obtained, and the receptor-ligand complexes were analyzed using Discovery Studio Visualizer (DSV 2021). The ADMET properties were analysed was using SwissADME webserver.

Results and Discussion

17-β Estradiol Standard Curve
Figure 1 shows 17-β Estradiol standard curve; a plot of the mean of triplicate LacZ values from the serial dilution against the log concentration. The standard curve was fitted to a five-parameter logistic regression model using GraphPad Prism (version 5.01.) to give the model parameters: Growth rate, Inflection point, Lower asymptote, Upper Asymptote, and Asymmetry parameter (Table 1). The concentrations of the test samples were determined by interpolating their LacZ values on the standard curve. The LacZ values represent the colour change produced by the media in direct proportion to the concentration of oestrogenic ligands (Equation 1),[24] quantified by measuring the absorbance. The linear portion of the standard curve defines the assay detection range and is the only portion of the standard curve that can be used to interpolate sample LacZ values.[24]

Standardization of Estradiol Equivalents (EEQs)
The Yeast Estrogen Screen (YES) assay measured the net oestrogenicity, i.e. the difference between the total oestrogenic and anti-oestrogenic ligands in the media, expressed as Estradiol Equivalents (EEQs)[24]. Figure 2 shows the Estradiol Equivalents (EEQs) of the extracts calculated using Equation 2 as follows; LA: 154.05µg/g; VA: 130.20µg/g; GA: 109.40 µg/g, for yeast expressing ERα, and LA: 9.26µg/g; VA: 6.22µg/g, GA: 5.73µg/g, for yeast expressing ERβ. The EEQs from the assay containing yeast expressing ERβ were much lower than those for yeast expressing ERα. These findings were consistent with the report of Miller *et al*.[25] who engineered the cells used for this study, noting that yeast expressing ERβ were 30 times more sensitive to 17-β estradiol than yeast expressing ERα,[25] thus highlighting the viability of the cells used in this study. Furthermore, the low EEQs obtained from the assay for yeast expressing ERβ is in line with studies on the binding affinity of phytoestrogens for ERs, where lower IC_{50} values have been reported for ERβ compared to ERα, thus indicating a

higher binding preference for ERβ.[33] Our results suggest that *Gnetum africanum*, *Lasianthera africana*, and *Vernonia amygdalina* contain phytoestrogens with a higher binding preference for ERβ.

The YES assay specifically detects ligands that bind to nuclear ERs to regulate gene expression and promote cell proliferation through oestrogen response elements (EREs).[25] It does not detect ligands that require biotransformation to become oestrogenic, or ligands that act through non-nuclear mechanisms such as membrane ERs, or mechanisms that involve additional proteins such as activator protein 1 (AP-1) transcription factors. Following the EEQs obtained, our YES assay suggests that the study plants contain phytochemicals that can elicit the classical genomic effects of ER activation, i.e. to regulate gene expression and enhance cell proliferation, especially through long-term exposure at high concentrations. Therefore, the consistent consumption of these vegetables in large quantities, especially among women at risk of ER-positive breast cancer is not advisable.

In the YES assay, we employed chlorophenol red galactopyranoside (CPRG) as the substrate in the lysis buffer due to reports of its being 10 times more sensitive than the alternative substrate, ortho-nitrophenyl galactopyranoside (ONPG), thus could be used to detect very low concentrations of β-galactosidase,[34,35] and the colouration of ONPG could interfere with absorbance readings leading to erroneously high values due to the pigmentation of plant extracts.

Table 1: Model Parameters for 17-β estradiol standard curve for yeast expressing ER α and β

Assay Conditions		**Model Parameters**					
Receptor	Substrate	Model R^2	Growth Rate LacZ Unit (ng/ml)\	Infection Point (ng/ml)	Lower Asymptote (LacZ Units)	Upper Asymptote (LacZ Units)	Asymmetry Parameter (S)
ERα	CPRG	0.9999	8.517 ±6.8448	1644	0.3162 ± 0.05831	2.927 ± 0.09445	0.1771
ERβ	CPRG	0.8448	0.7267 ±6.935	23.65	0.05144 ± 4.760	0.5140 ± 1.147	3.301

E2 working standards were serially diluted and tested using CPRG after a 17h incubation in yeast media expressing ER α and β was performed. LacZ values; the colour change in direct proportion to the concentration of oestrogenic ligands in the sample were calculated and plotted against the log concentration to produce 17-β estradiol standard curve which was fitted into a five-parameter logistic regression to obtained the model parameters.

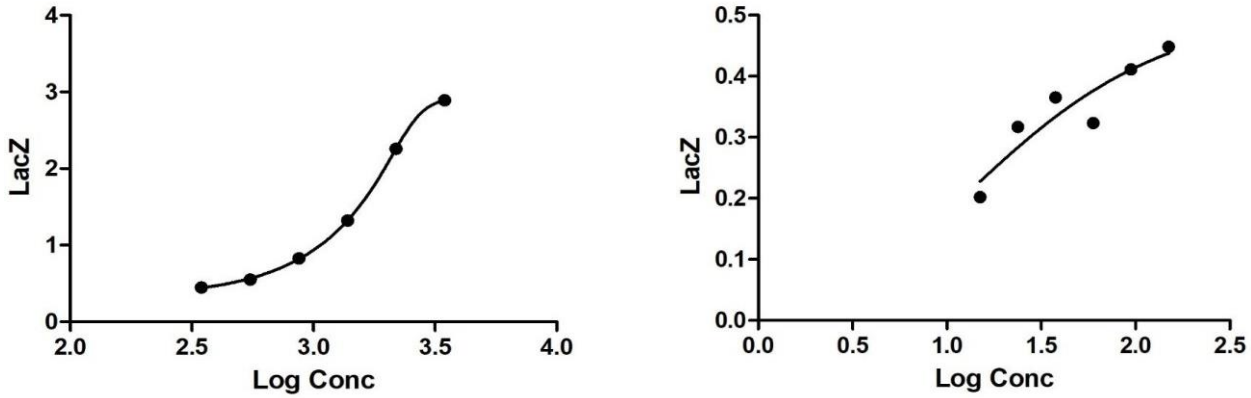


Figure 1: Standard Curves for the YES Assay fitted to a 5-Parameter Logistic Regression model. Yeast expressing human oestrogen receptors, ERα and ERβ in separate culture media were incubated for 17h in the presence of 17β-estradiol. The substrate CPRG was added after incubation. All E2 standards, media and vehicle controls were tested in triplicate. Values on the y-axis (LacZ) represent means of triplicate LacZ values which measure the degree of colour change induced by β-galactosidase. The Logistic regression model parameters are listed in Table 1. CPRG = chlorophenol red-β-D- galactopyranoside.

Molecular Docking Simulation

The docking method was validated by redocking the native ligand, 17-β Estradiol (E2) to ERα: 5WGD (-8.7 Kcal/mol) and ERβ: 3OLS (-11.4 Kcal/mol), with a root mean square deviation (RMSD)/Lower bound less than two. The docking of GA, LA, and VA phytochemicals against 5WGD and 3OLS gave negative binding affinities ranging from -3.5 to -8.6 Kcal/mol for 5WGD, and -3.3 to -9.0 Kcal/mol for 3OLS (Tables 2-5). The negative binding affinities suggest that the selected phytochemicals possess oestrogenic activity. Since ERα is the receptor predominantly implicated in breast cancer pathogenesis, further analysis of the receptor-ligand complexes focused on the ligands docked against 5WGD from which we identified four compounds: Tetradecanoic acid (10), Eugenol (27), Bergenin (40), and vernodalol (45) with good pharmacokinetic properties; having comparable binding affinities with the native ligand (17-β estradiol; -8.7 Kcal/mol), and interacting similarly to E2 at ERα active site. It has been established that E2 binds to ERα to elicit its cellular effects by at least one of three interactions: (i) a ring hydroxyl group, hydrogen bonding with Arg394 and Glu353; (ii) a 17β-OH hydrogen bonding with His524; and (iii) a hydrophobic interaction of the planar hydrophobic skeleton of oestrogen to the receptor hydrophobic pocket.[36,37] Also, Masand *et al*.[38] reported that the ERα active site is predominantly hydrophobic with H-bond capable regions at Arg394, His524, Glu353, and Leu387. Analysis of the receptor-ligand complexes of E2 and compounds 10, 27, 40, and 45 showed that they interacted with ERα active site through hydrophobic interactions with amino acid residues, and H-bonds with Arg394, Glu353, and His524.

17-β estradiol (E2) interacted with 5WGD binding pocket with a binding affinity of -8.7 Kcal/mol, and one conventional hydrogen bond with Arg394 (2.27 Å) and Leu525 (2.77 Å); including hydrophobic interactions with Leu387, Leu346, Phe404, Leu391, Ala350, and Leu349 (Figure 4A). Tetradecanoic acid (Compound 10) had a binding affinity of -6.1 Kcal/mol forming one conventional hydrogen bond with His524 (2.14 Å), and hydrophobic interactions with Leu387, Leu346, Phe404, Leu391, Ala350, Leu349 (Figure 4B). Eugenol (Compound 27) had a binding affinity of -6.2 Kcal/mol, and the complex interacted with the receptor binding pocket through two conventional hydrogen bonds with Arg394 (2.45 Å; 3.01 Å), and one with Pro325 (2.04 Å), including hydrophobic interactions (Figure 4C). Bergenin (Compound

40) had a binding affinity of -6.5 Kcal/mol. Its atoms interacted with the receptor binding pocket through one conventional hydrogen bond with His524 (2.04 Å) and Gly521 (2.15 Å), including hydrophobic interactions (Figure 4D). Vernodalol (Compound 45) interacted with 5WGD binding pocket with a binding affinity of -7.3 Kcal/mol, and three conventional hydrogen bonds with Glu353 (1.86 Å), Arg394 (2.11 Å), and His524 (2.08); including hydrophobic interactions with Leu525 and Ala350 (Figure 4E). These findings suggest that compounds 10, 27, 40, and 45 are potential phytoestrogens capable of inducing ERα genomic effects which include regulation of gene transcription and promoting cell proliferation. Thus, their long-term exposure at high concentrations may increase the risk of ER-positive breast cancer. This awareness is important for women at risk of ER-positive breast cancer i.e post-menopausal women and women with a family history of breast cancer. Such information could be extended to other vegetables containing these phytochemicals.

The comparable binding affinities of compounds 10, 27, 40, and 45 with the reference standard, E2 and presence of H-bond interactions in their receptor-ligand complexes suggest that they formed strong bonds with ERα active site. A molecule is considered to form a stronger bond if its binding affinity is more negative. Also, the number of H-bonds in protein-ligand complexes determines the strength and stability of the binding interaction as H-bonds ensure specific and directed interactions between ligands and proteins, allowing ligands to be precisely positioned and stabilized in the active site.[39,40] The results from the molecular docking simulation of compounds with good ADMET properties suggests that vernodalol had the strongest binding interactions with ERα, having the highest binding affinity and number of H-bonds. A Root Mean Square Deviation (RMSD)/Lower bound value of less than 2Å implied that the conformations of the test ligands were close to that of the native ligand.[41,42] From this study, all the selected phytochemicals had a RMSD/Lower bound value less than 2Å; hence, they met the docking requirements.

It is worth stating that the YES assay and *in silico* investigations for *Gnetum africanum* oestrogenic activity, in this study, were in agreement with the findings of Udoh *et al*. who reported that *Gnetum africanum* was oestrogenic following their *in vivo* rodent uterotrophic assay.[14]

Tetradecanoic acid (Myristic Acid), a compound from *Gnetum africanum*, is a common saturated fatty acid, and one of the most abundant fatty acids in milk fat. It is also obtained from plant sources such as oil palm and coconut. Commercially, Tetradecanoic acid esters and salts are used in soaps, detergents, beauty and care products.[43] Eugenol, an essential oil from *Lasianthera africana* is a highly potent antimicrobial agent with a wide range of pharmacological activities. Also, it has been extracted from various plants such as cloves, lemon grass, tulsi, *Ocimum basilicum*, and Cinnamon.[44] Bergenin, a compound from *Lasianthera africana*, is an isocoumarin with a wide range of pharmacological actions, including anti-infective, anti-cancer, anti-diabetic, neuroprotective, hepatoprotective, anti-urolithiatic, anti-hyperuricemic, and anti-bradykinin properties. It is a key constituent of *Bergenia crassifolia* (Saxifragaceae)[45] Vernodalol is a sesquiterpene lactone isolated from the roots and leaves of VA and other Vernonia species. It is renowned for its medicinal properties including anti-leishmaniasis, insecticidal, and antimicrobial properties against bacteria and fungi.[46] Furthermore, antiproliferative activity has been reported in various cell lines, such as acute promyelocytic leukemia (APL), by inducing apoptosis through the mitochondrial pathway and causing cell cycle arrest in the G2/M phase.[47]

We also analysed the binding affinities of the selected phytochemicals from *Gnetum africanum*, *Lasianthera africana*, and *Vernonia amygdalina* against ERβ: 3OLS. The role of ERβ in cell proliferation is still remains under investigation. Activation of ERβ generally results in inhibition of proliferation and induction of apoptosis,[48] however, some studies indicate that ERβ and its isoforms including certain coactivators such as AIB1, NF-kB, and TIF-2 tend to coregulate breast cancer cell proliferation and progression.[49,50] Mal *et al*.[48] reported that ERβ is a ligand-activated tumor suppressor, however, the presence of multiple isoforms of ERβ may have distinct impacts on breast cancer prognosis. Therefore, its full potential would rely on the development of selective ERβ agonists devoid of ERα activity. These findings indicate that ERβ inhibition of cell proliferation may be an unreliable mechanism.

Table 2: Molecular Docking Simulation of Compounds from *Gnetum africanum*.

Receptor	Compound	Binding Affinity (Kcal/mol)	Interactions		
			Type	Amino acid Residues	H-Bonding Distance (Å)
5WGD	1	-7.0	Hydrophobic		
	2	-7.2	Hydrophobic		
	3	-6.4	Hydrophobic		
	4	-4.9	H-bonding	Arg503	2.40; 2.65
				Met437	2.91
	5	-7.9	Hydrophobic		
	6	-5.3	H-bonding	Arg515	2.30
				Asn455	2.31
				Glu385	1.98
	7	-6.0	Hydrophobic		
	8	-4.9	H-bonding	Lys499	2.60; 2.74
	9	-6.3	Hydrophobic		
	10	-6.1	H-bonding	His524	2.14
	11	-3.9	H-bonding	Leu346	2.42
	12	-5.8	H-bonding	His524	2.31
	13	-5.2	H-bonding	Glu353	2.20; 2.54
				Ile386	2.32
				Arg394	1.97;2.59; 2.80
	14	-6.4	Hydrophobic		
	15	-4.1	Hydrophobic		
	16	-5.0	H-bonding	Gln499	1.93
				Gln506	2.24
	17	-4.8	H-bonding	Ser527	2.25
	18	-4.8	Hydrophobic		
	19	-5.1	Hydrophobic		
	20	-4.8	H-bonding	Arg515	2.55; 2.94

(E)-α-ionone (1), (E)-β-ionone (2), Estragole (3), n-hexadecanoic acid (4), β-selinene (5), Linoelaidic acid (6), 2-methoxy-4-vinylphenol (7), 2-cyclopenten-1-2-hydroxy (8), 1,3 Benzodioxole, 4-methoxy-6-(2-propenyl) (9), Tetradecanoic acid (10), Hexyl amine (11), Caffiene (12), 3-P-methyl-d-glucose (13), n-Hexadacanotic acid (14), Phytol (15), Linoelaidic acid (16), Cyclopentaneundecanoic acid (17), Cis, cis, cis-7-10-13-Hexadecatrienal (18), Dendrolasin (19), 6,10,14-Trimethyl-2-pentadecanone (20) ((Edet *et al*., 2013). (Ezekwe *et al*., 2020). Hydrophobic interactions comprised of alkyl, Pi-alkyl, and Pi-sigma bonds. Compounds interacting with 5WGD active site through H-bonding also had hydrophobic interactions.

Table 3: Molecular Docking Simulation of Compounds from *Lasianthera africana*

Receptor	**Compound**	**Binding Affinity (Kcal/mol)**	**Interactions**		
			Type	Amino acid Residues	H-Bonding Distance (Å)
5WGD	21	-7.1	H-bonding	Asn439	2.32
				Gln502	2.48
				Gln506	2.18; 2.25
	22	-7.1	H-bonding	Ser456	2.70
				Arg515	2.42;2.72; 2.90
	23	-6.8	H-bonding	Pro325	2.04
	24	-8.4	H-bonding	Asp332	1.81
				Arg335	2.54
	25	-6.9	Hydrophobic		
	26	-6.1	Hydrophobic		
	27	-6.2	H-bonding	Arg394	2.45, 3.10
				Pro325	2.04
	28	-4.8	Hydrophobic		
	29	-4.8	H-bonding	Gln506	2.80
				Gln502	1.87
	30	-5.2	H-bonding	Asn455	2.32
	31	-6.9	Hydrophobic		
	32	-7.3	Hydrophobic		
	33	-4.7	Hydrophobic		
	34	-5.6	Hydrophobic		
	35	-8.6	Hydrophobic		
	36	-8.2	Hydrophobic		
	37	-6.9	Hydrophobic		
	38	-5.3	H-bonding	Glu353	2.70
				Arg394	2.70;3.04
	39	-6.4	Hydrophobic		
	40	-6.3	H-bonding	His524	2.04
				Gly521	2.15

Isorhamnetin (21), Oleanolate-3-O-β-glucoside (22), Quercetin-3-O-rutinoside (Rutin) (23), Soyasapogenol-B-3-O-β-glucuronate (24), (Ekpo *et al*., 2020), Lobeline (25), Coumaric acid (26), Eugenol (27) (Obia *et al*., 2023). 9,12,15-Octadecatrienoic acid, ethyl ester (28), Z,Z-3,13-Octadecedien-1-ol (29), Vitamin E (30), Squalene (31), 2-Methyl-5-(1- methylethyl)-phenol (32), 9,12,15- Octadecatrienoic acid methylester (33), 9,12, Octadecadienoic acid (34), Neocurdione (35), Beta-Caryophyllene (36), Benzylisoquinoline (37), Maltol (38), Anabasine (39), Bergenin (40). Hydrophobic interactions comprised of alkyl, Pi-alkyl, and Pi-sigma bonds. Compounds interacting with 5WGD active site through H-bonding also had hydrophobic interactions.

Table 4: Molecular Docking Simulation of Compounds from *Vernonia amygdalina*

Receptor	**Compound**	**Binding Affinity (Kcal/mol)**	**Interactions**		
			Type	Amino acid Residues	H-Bonding Distance (Å)
5WGD	41	-8.0	H-bonding	Gln506	1.83
				Gln499	2.52
				Arg503	2.75
				Glu444	2.46
	42	-8.0	H-bonding	Gln506	1.92; 2.36
				Arg503	2.99
				Glu444	1.87; 2.29
	43	-6.6	H-bonding	Glu330	2.58
				Asn348	2.61;2.67
				Asp351	2.74
				Ser537	2.83
	44	-6.3	H-bonding	Arg503	2.91
	45	-7.3	H-bonding	Arg394	2.11
				Glu353	1.86
				His524	2.08
	46	-8.1	H-bonding	Leu346	2.09
	47	-6.3	H-bonding	Ser527	2.89

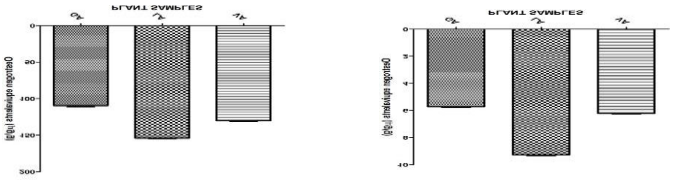
48	-6.2	H-bonding	Glu330	2.63
			Asn348	2.62;2.70; 2.72
49	-4.3	H-bonding	Lys449	2.50; 2.70
50	-4.4	H-bonding	His524	2.07; 2.56
51	-4.1	H-bonding	Leu346	2.92
52	-4.5	H-bonding	Arg394	2.08
			Leu387	2.24
53	-4.9	H-bonding	Leu346	2.68
54	-3.5	Hydrophobic		
55	-5.4	H-bonding	Asp332	2.38
56	-5.5	H-bonding	Arg394	2.07
			Glu353	2.28
			Gly390	2.88
			Lys449	2.58; 2.81
57	-5.9	H-bonding	Ile386	2.69
			Lys449	2.13;2.56; 2.71
58	-3.4	H-bonding	Glu353	2.36; 2.60
			Pro325	2.41
59	-3.7	H-bonding	Arg394	2.86
			Glu353	2.25; 2.56
			Ile386	2.32; 2.66
60	-6.2	Hydrophobic		

Luteolin-7-O-β-glucoside (41), Luteolin-7-O-glucuronide (42), hydroxyvernolide (43), Vernodalin (44), vernodalol (45), vernolepin (46), vernolide (47), vernomygdin (48), (Luo *et al*., 2017) 2,3-pentanedione (49), 2,3-hexanedione (50), 2-pentanol (51), Pentanoic acid (52), 2-methyl-3-hexanol (53), 2-methyl azetidine (54), Thiamine (55), Ascorbic acid (56), Pyridoxine (57), Glycine (58), Cysteine (59), Vernomenin (60). Hydrophobic interactions comprised of alkyl, Pi-alkyl, and Pi-sigma bonds. Compounds interacting with 5WGD active site through H-bonding also had hydrophobic interactions.

Table 5: Binding Affinities of Compounds from *Gnetum africanum*, *Lasianthera africana*, and *Vernonia amygdalina* against ERβ: 3OLS

Compound	ΔG Value (Kcal/mol)	Compound	ΔG Value (Kcal/mol)	Compound	ΔG Value (Kcal/mol)
1	-7.1	21	-7.3	41	-7.4
2	-7.2	22	-7.2	42	-7.2
3	-6.3	23	-7.4	43	-6.5
4	-6.5	24	-7.3	44	-6.3
5	-8.2	25	-7.1	45	-5.7
6	-5.0	26	-6.2	46	-8.2
7	-6.2	27	-6.1	47	-6.6
8	-5.1	28	-5.0	48	-6.3
9	-6.4	29	-4.8	49	-4.5
10	-6.3	30	-5.0	50	-4.5
11	-4.0	31	-5.6	51	-4.0
12	-5.7	32	-7.0	52	-4.4
13	-5.2	33	-6.4	53	-4.8
14	-6.2	34	-5.3	54	-3.4
15	-5.4	35	-8.6	55	-6.8
16	-6.8	36	-8.0	56	-5.9
17	-6.6	37	-8.8	57	-5.8
18	-6.6	38	-5.2	58	-3.3
19	-6.7	39	-6.7	59	-4.0
20	-6.7	40	-7.1	60	-8.8

Gnetum africanum: 1-20, *Lasianthera africana*: 21-40, *Vernonia amygdalina*: 41-60. (E)-α-ionone (1), (E)-β-ionone (2), Estragole (3), n-hexadecanoic acid (4), β-selinene (5), Linoelaidic acid (6), 2-methoxy-4-vinylphenol (7), 2-cyclopenten-1-2-hydroxy (8), 1,3 Benzodioxole, 4-methoxy-6-(2-propenyl) (9), Tetradecanoic acid (10), Hexyl amine (11), Caffiene (12), 3-P-methyl-d-glucose (13), n-Hexadacanotic acid (14), Phytol (15), Linoelaidic acid (16), Cyclopentaneundecanoic acid (17), Cis, cis, cis-7-10-13-Hexadecatrienal (18), Dendrolasin (19), 6,10,14-Trimethyl-2-pentadecanone (20) Isorhamnetin (21), Oleanolate-3-O-β-glucoside (22), Quercetin-3-O-rutinoside (Rutin) (23), Soyasapogenol-B-3-O-β-glucuronate (24), (Ekpo *et al*., 2020), Lobeline (25), Coumaric acid (26), Eugenol (27) (Obia *et al*., 2023). 9,12,15-Octadecatrienoic acid, ethyl ester (28), Z,Z-3,13-Octadecedien-1-ol (29), Vitamin E (30), Squalene (31), 2-Methyl-5-(1- methylethyl)-phenol (32), 9,12,15- Octadecatrienoic acid methylester (33), 9,12, Octadecadienoic acid (34), Neocurdione (35), Beta-Caryophyllene (36), Benzylisoquinoline (37), Maltol (38), Anabasine (39), Bergenin (40) Luteolin-7-O-β-glucoside (41), Luteolin-7-O-glucuronide (42), hydroxyvernolide (43), Vernodalin (44), vernodalol (45), vernolepin (46), vernolide (47), vernomygdin (48), (Luo *et al*., 2017) 2,3-pentanedione (49), 2,3-hexanedione (50), 2-pentanol (51), Pentanoic acid (52), 2-methyl-3-hexanol (53), 2-methyl azetidine (54), Thiamine (55), Ascorbic acid (56), Pyridoxine (57), Glycine (58), Cysteine (59), Vernomenin (60).



(A) (B)

Figure 2: Oestrogen equivalents (EEQs; µg/g) evaluated using the yeast estrogen screen. 15µL aliquots of GA, LA, and VA extracts were incubated for 17h in yeast culture media containing yeast expressing ERα (A) and ERβ (B). After the incubation period, LacZ values (degree of colour change) were calculated. Oestrogen equivalents (EEQs; µg/g) were obtained by interpolating LacZ values on 17-β estradiol standard curve. EEQs were expressed as mean of triplicate values.

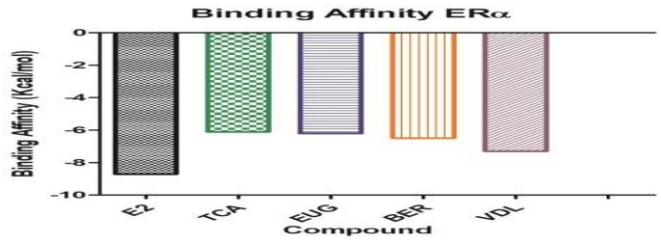


Figure 3: Binding Affinity (Kcal/mol) of compounds on ERα 5WGD. 17-β estradiol (E2), Tetradecanoic acid (TCA; 10), Eugenol (EUG; 27), Bergenin (BER; 40), Vernodalol (VDL; 45).

Physicochemical and ADMET Properties Prediction

The physicochemical and ADMET properties of compounds 10, 27, 40, and 45 were predicted using the SwissADME web tool. Assessment of their drug-likeness was performed based on Lipinski's rule of five; that is, it does not violate more than one of the following: ≤ 5 hydrogen bond donors; ≤ 10 hydrogen bond acceptors; a molecular mass < 500 daltons; and a Log P < 5 for octanol-water partition coefficient.[51] According to the aforementioned criteria, compounds 10, 27, 40, and 45 emerged as potentially druggable with good bioavailability scores that affirm their drug-likeness (Table 6). The concept of Lead-likeness refers to a molecule's potential for optimization.[52] Based on the predicted physicochemical properties only Bergenin (40) met the requirements for lead-likeness. A synthetic accessibility score of less than 6 indicates that a molecule can be synthesized.[53] Compounds 10, 27, 40, and 45 showed favourable synthetic accessibility scores indicating their potential for successful synthesis (Table 6). The number of rotatable bonds is an important physicochemical parameter influencing a molecule's conformation and oral bioavailability. Generally, compounds with less than 10 rotatable bonds are likely to display stable conformations and enhanced oral bioavailability.[54] The results showed that Eugenol, Bergenin, and Vernodalol each had less than ten rotatable bonds, indicating their stable conformations and potential for good oral bioavailability.

Log P, which measures lipophilicity, is a parameter that determines a molecule's solubility, absorption, and distribution properties. For a good balance between solubility and passive diffusion permeability, the Log P value should be less than 5.[55] Our findings showed that compounds 10, 27, 40, and 45 had Log P values less than 5 suggesting good solubility, oral absorption, and distribution (Table 6).

Table 6: Physicochemical parameters of Tetradecanoic acid, Eugenol, Bergenin, and Vernodalol

Physicochemical Property	Tetradecanoic acid	Eugenol	Bergenin	Vernodalol
Molecular weight	228.37g/mol	164.20g/mol	328.27g/mol	392.40g/mol
Num. of Rotatable bonds	12	3	2	8
Num. of H-bond Acceptors	2	2	9	8
Num. of H-bond Donors	1	1	5	2
Consensus Log Po/W	4.45	2.25	-0.80	1.37
Drug Likeness Lipinski Violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation
Bioavailability Score	0.85	0.55	0.55	0.55
Medicinal Chemistry Leadlikeness	No	No	Yes	No
Synthetic Accessibility	2.09	1.58	4.39	4.88

The pharmacokinetic properties prediction indicated that compounds 10, 27, 40, and 45 were not substrates for P-glycoprotein (P-gp) which suggests that they may exhibit high oral bioavailablity and intestinal absorption. Tetradecanoic acid (10) and eugenol (27) were blood-brain-barrier (BBB) permeant. Substances that cross the BBB may pose a health risk based on their potential to release toxic metabolites in the brain and bloodstream. The LogKp measures a molecule's skin permeability where more negative values signify reduced skin permeability.[56] The negative LogKp values observed for compounds 10, 27, 40, and 45 suggests that they are unlikely to penetrate the skin effectively (Table 7). In summary, the physicochemical and pharmacokinetic predictions of teradecanoic acid, eugenol, bergenin, and vernodalol showed that they had good ADMET properties.

Table 7: Pharmacokinetic properties of Tetradecanoic acid, Eugenol, Bergenin, and Vernodalol

Pharmaacokinetic Property	**Tetradecanoic acid**	**Eugenol**	**Bergenin**	**Vernodalol**
GI absorption	High	High	High	High
BBB Permeant	Yes	Yes	No	No
P-gp substrate	No	No	No	No
CYP1A2 Inhibitor	Yes	Yes	No	No
CYPC9 Inhibitor	No	No	No	No
CYPD6 Inhibitor	No	No	No	No
CYPA4 Inhibitor	No	No	No	No
LogKp	-3.35cm/s	-5.63cm/s	-8.99cm/s	-7.86cm/s

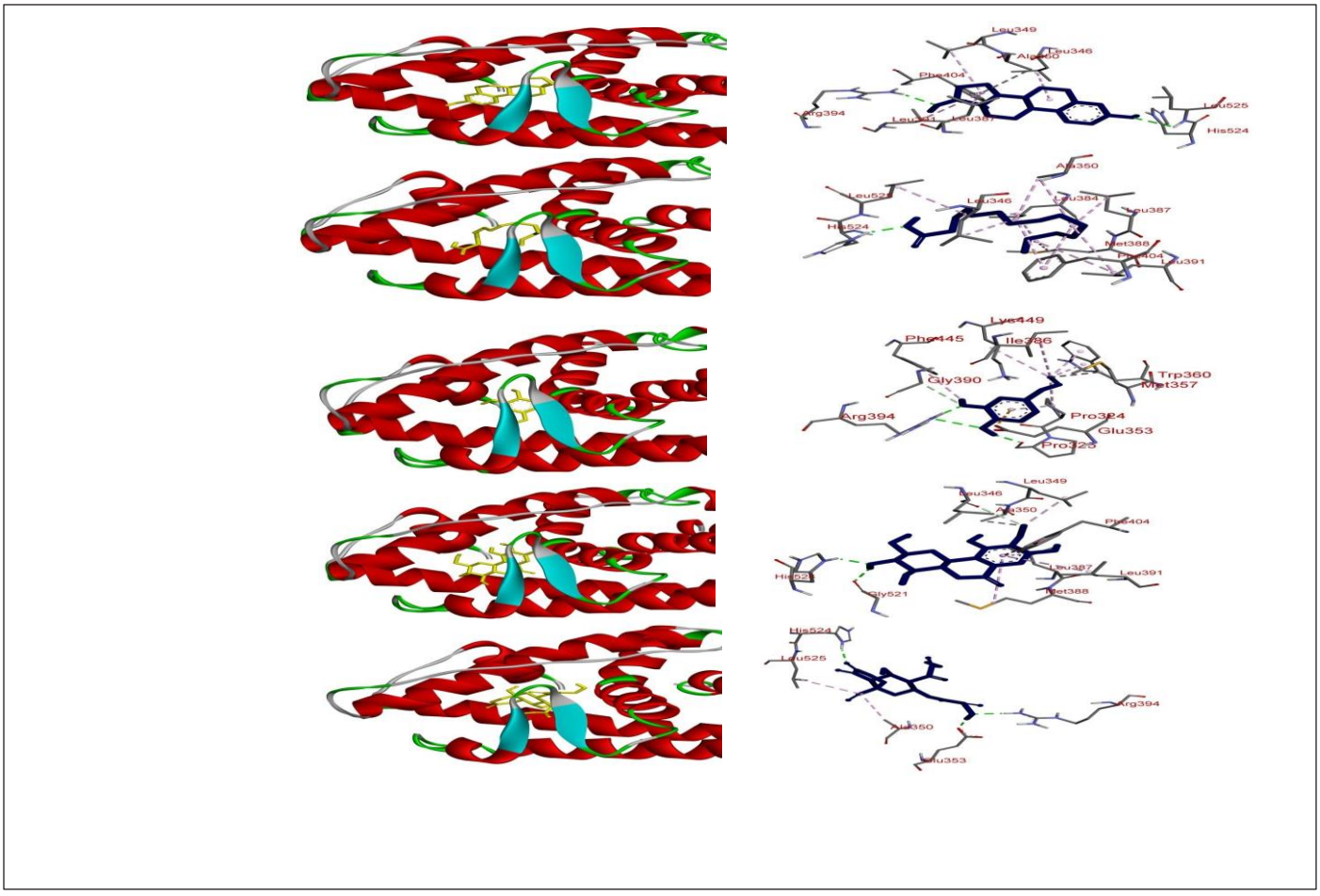


Figure 4: Binding Interactions of Phytochemicals with ERα active site. (A) 17-β estradiol (B) Tetradecanoic acid (C) Eugenol (D) Bergenin (E) Vernodalol

Conclusion

This study detected and quantified oestrogenic activity in three commonly consumed vegetables; *Gnetum africanum*, *Lasianthera africana*, and *Vernonia amygdalina*, utilizing both *in vitro* and *in silico* assays. Potential phytoestrogens identified include Tetradecanoic acid from *Gnetum africanum*; Eugenol and Bergenin form *Lasianthera africana*; and Vernodalol from *Vernonia amygdalina*. In the context of oestrogen signaling, prolonged exposure to high concentrations of these compounds may cause endocrine disruptions that can lead to adverse health including increased risk of ER-positive breast cancer. As a preclinical investigation, this study provides more insights into the potential health risks associated with phytoestrogen consumption. These findings open a new line of investigation, focused on validating the oestrogenic potency of the isolated phytoestrogens through *in vitro* studies and *in vivo* modeling. This would further elucidate the oestrogenic effects of these phytoestrogens and their potential impact on human health. Such information could help promote safer consumption habits and develop preventive strategies against ER-

positive breast cancer thereby reducing the global incidence and health burden of this disease.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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