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Phenolics of *Abelmoschus esculentus* Pods: HPLC Identification and *In Silico* Studies to Identify Potential Anti-inflammatory Agents

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ABSTRACT

Numerous medical disorders are impacted by inflammation. In this study, we aim to evaluate the anti-inflammatory (*in silico*) activities of selected phenolic compounds identified by HPLC analysis of methanol extracts of *Abelmoschus esculentus* pods. Cyclooxygenases are recognised to be the primary mediators of prostaglandin production, which are inflammatory indicators and are hence the focus of anti-inflammatory therapy. Numerous crucial physiological processes, including inflammation, immunological responses, cellular development, apoptosis, and the expression of certain viral growth factors, are regulated by nuclear factor kappa B (NF-κB) transcription factors. Thus, it seems possible to treat inflammatory and cancerous disorders by blocking NF-κB induction. In this study, (NF-κB) and (COX-2) receptors are targets for ligands; caffeic acid, vanillic acid and ferulic acid. PyRx was used for the docking using Autodock Vina embedded in MGL Tools 1.5.6. A Drug-likeness test was performed using ADME tools while ProTox II was used to predict toxicity and LD_{50} of the ligands. The bioactivities were predicted using the prediction of activity spectra for substances (PASS). According to molecular docking, the phytocompounds gave good binding energies. All identified compounds conformed to Lipinski's Rule of Five (RO5). This showed that the identified *A. esculentus* compounds will have lower attrition rates during clinical trials and will have a high chance of making it to the market. The current findings suggest that the identified phytocompounds could be developed as a novel anti-inflammatory medication.

Keywords: In silico, Molecular docking, Drug-likeness, Inflammation, *Abelmoschus esculentus*

Introduction

Inflammation is a helpful recovery process that cells utilize to stop the progression of harm or injury to tissues caused by foreign invaders and start the healing process. It's a complicated process involving white blood cells, macrophages, and inflammatory cytokines such as prostaglandins, TNF- (Tumor Necrosis Factor), interleukin IL-6, and IL-8, to mention a few. The mobilization of arachidonic acid for prostaglandin production is a hallmark of inflammation. Cyclooxygenases such as COX-1 and COX-2 enzymes convert arachidonic acid to prostaglandins. COX-1 is required for the body's homeostatic activities, such as platelet synthesis for blood, kidney development and function, gastric mucosa maintenance, and so on. Increased inflammation, angiogenesis, metastatic and proliferative invasion, decreased apoptosis, and the establishment of an immunosuppressive microenvironment are all linked to COX-2-derived prostaglandin PGE2.[1] Non-steroidal anti-inflammatory medications (NSAIDs) work as COX inhibitors and are a useful tool for treating inflammation, but they have drawbacks and adverse effects.

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Hence, organic COX-2 inhibitors should be investigated. Deregulation of the inflammatory response is an issue, and chronic inflammation has been linked to cancer, diabetes, Alzheimer's and other diseases.[1] Inflammation regulation is a complicated process that can be simplified by focusing on the causes of inflammation. COX-II and NF-κB are two genes that have been linked to cancer, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, atherosclerosis, systemic lupus erythematosus, type I diabetes, chronic obstructive pulmonary disease, and asthma.[2] NSAIDS such as ibuprofen, a currently available therapeutic agent, have several drawbacks, including ulcerative perforations of the stomach lining, [3] severe stomach cramps, and hepatotoxicity. [4] Targeting inflammation is currently a therapeutic means to cure ailments. The use of topical corticosteroids, hydrocortisone, and prednisone to treat eczema is a good example.[5] Tumor necrosis factor (TNF) inhibitors are also commonly used in the treatment of rheumatoid arthritis.[6] As a result, safer and more natural ways of reducing inflammation are required.

Abelmoschus esculentus L. (Moench), commonly known as okra, is a flowering plant belonging to the Malvaceae family which produces tasty green pods with a slimy inside filled with seeds arranged unevenly and is native to Africa's tropics.[7]*A. esculentus* has been demonstrated to contain a wide range of photochemical and nutritional value, which explains its widespread use in traditional medicine. The seeds and pods are high in minerals and vitamins, all of which contribute to the health advantages.[7] Syphilis is treated with an infusion made from the roots.[8] In Nigeria, the root juice is applied externally to cure cuts, wounds, and boils.[8] Catarrhal infections, dysuria, and gonorrhoea are all treated with it.[9]The roasted seed infusion has sudorific effects. [9]*A. esculentus* is used to treat dysentery, catarrhal infections, plasma replacement, and gonorrhea. spermatorrhoea, bronchitis, pneumonia, diarrhoea, acute inflammation and irritation of the stomach and intestines.[10] Several

studies on the antioxidant activity of various portions of the plant have been undertaken.[11] *In vitro* antioxidant assay of methanol extract of okra fruits was reported by Atawodi and colleagues.[11] They used the xanthine oxidase and 2-deoxyguanosine techniques to demonstrate antioxidant and radical scavenging activity, with 50 percent inhibitory concentration values of 25 and 43 mg/ml. Khomsug and his colleagues discovered that procycanidin B_2 was the most common phenolic compound.[12]Procycanidin B_2, epicatechin, and rutin have been found in pulped seeds.[13] Pre-treatment (soaking and blanching) boosted nutritional composition but decreased antioxidant activity, whereas roasting (1600 °C for 10-60 minutes) increased nutrient composition but decreased antioxidant activity.[14] Ansari and colleagues found okra extract to be a non-enzymatic inhibitor of lipid peroxidation in liposomes.[15] Total phenolics, total flavonoids, and antioxidant activity of different parts (flower, fruit, leaf, and seed) and different enrichment fractions of water extracts of the *A. esculentus* plant were compared by Liao and co-workers.[16] They found total phenolics and total flavonoids, both of which are antioxidants, in all of the plant extracts, though the percentages differed. The maximum levels of total phenolics and total flavonoids were identified in the okra leaves. [17]

Molecular docking, an *in silico* method, is extremely beneficial since it lowers the cost of wet-lab research, saves animals, time, and resources, and properly guides medication selection and production. The goal of molecular docking simulation is to anticipate a ligand's binding affinity with a protein and the most stable complex; the lower or more negative the binding affinity, the better. Molecular docking simulation has simplified and verified *in vivo* and *in vitro* studies, as well as drug modeling and design for pharmaceutical researchers.[18] Drug-likeness is a qualitative assessment of a molecule's potential as an oral drug in terms of bioavailability. It was determined through structural or physicochemical inspections of research compounds that they had progressed enough to be deemed oral drug candidates, using five separate rule-based filters with varying ranges of attributes within which the molecule is defined as drug-like. These filters include; Lipinski set of 5, [19]Ghose, [20]Veber, [21] Egan,[22] and Muegge[23] methods. Several researchers have used chromatographic methods to uncover phytocompounds in plants.[24-34] Only a few *A. esculentus* pod reports have been documented. The structural formula of bioactive chemicals found in *A. esculentus* pods has not been adequately characterized by HPLC and molecular docking studies of its bioactive phytocompounds. To the best of our knowledge, this is the first study of *A. esculentus* pod compounds using HPLC analysis and *in silico* molecular docking methods. The work aims to use HPLC and molecular docking to uncover possible anti-inflammatory inhibitors in *A. esculentus* pods.

Materials and Methods

Sample processing
A. esculentus pods were harvested in Umudike, Abia State, Nigeria on January 2022. The plant was identified and was assigned the herbarium number ICA DALZ 2094 by the Michael Okpara University of Agriculture, Umudike (MOUAU) Forestry Department's Taxonomy division. Using a blender (Tsk 949: Westpoint, France), the chopped, air-dried pods were ground to powder and weighed. Exactly 100g of homogenized *A. esculentus* flesh samples were measured in separate beakers. Then, 500 mL of methanol was added to the weighed samples and left to stand for seven (7) days before filtration and concentrations at 40ºC using a rotary evaporator at lower pressure.

Fractionation of A. esculentus extracts using column chromatography
For separation, a silica-gel column (60-120 mesh) was utilized. Petroleum ether was used to prepare the column bed (which was uniformly distributed). *A. esculentus* methanol extract (7 g) was loaded into the silica-gel column after being impregnated with silica-gel. The column had a capacity of 500 mL, 32 mm outside diameter, 26 mm inside diameter and 203 mm length.
The column was eluted with ratios of solvent mixtures as shown in Table 1.

High-performance liquid chromatography (HPLC)
A Waters 2695 Alliance HPLC system (Waters Inc., Milford, CT, USA) equipped with a UV-Vis DAD was used to analyze the phenolic chemicals. A Waters SunfireTM C18 reverse-phase chromatography column with a length of 250 mm, a width of 4.6 mm, and a particle size of 5 m was used to separate the samples. An autoinjector was used to inject the phenolic standard solutions and combinations into the apparatus. To discover a viable separation method for the standards, numerous isocratic and gradient mobile phases were evaluated at various flow rates and column temperatures. Following a series of preliminary studies, a mixture of acetonitrile (mobile phase A, HPLC grade 99.9%; Honeywell Seelze, Germany) and phosphoric acid (mobile phase B) was subsequently chosen as the gradient method. The procedure took 60 minutes to complete; and the concentration gradient was changed as follows: a) 5 percent A and 95 percent B at the start, b) 15 minutes of 35 percent A and 65 percent B, c) 20 minutes of 35 percent A and 65 percent B, d) 30 minutes of 40 percent A and 60 percent B, e) 35 minutes of 40 percent A and 60 percent B, f) 40 minutes of 50 percent A and 50 percent B, g) 52 minutes of 70 percent A and 30 percent B, and h) 60 minutes of 5 percent A and 95 percent B. The flow rate was kept constant at 0.5 mL/min and the temperature was maintained at 5 °C. The wavelength of 254 nm was chosen for examination in this experiment utilizing the HPLC-DAD after analysing the UV-Vis spectra of the different phenolic standards. Caffeic acid, vanillic acid, and ferulic acid standards were used to identify the compounds. The peaks were identified by comparing the retention time (RT) of standard chemicals to the RT of various peaks obtained from HPLC analysis of extracts.

Software
For this study, the Python programming language was downloaded from www.python.com. The Molecular Graphics Laboratory (MGL) tools software was downloaded from http://mgltools.scripps.edu; Pyrx version 0.8 was downloaded from https://pyrx.sourceforge.io/, and BIOVIA Discovery Studio visualizer, version 2021, was downloaded from http://accelrys.com.

Protein preparation
Human Cyclooxygenase-2 bound to Vioxx (PDB ID: 1PXX) (Figure 1.1) and NF-κB (PDB ID: 1SVC) (Figure 1.2) were retrieved from www.rcsb.org. Water molecules and ions were removed, and polar hydrogens were added, using Biovia Discovery 2021. Using the PyRx virtual screening tool [35], the file was transformed to PDBQT format.

Ligand preparation
Selected phenolic compounds identified from HPLC analysis of *A. esculentus* pods were retrieved from www.pubchem.ncbi.nlm.nih.gov. The ligands include the following; Cinnamic acid (PubChem ID 444539), p-Coumaric acid (PubChem ID 689043), Vanillin (PubChem ID 1183), while Vioxx (PubChem ID 5090) and Ibuprofen (PubChem ID 3672) were used as the positive controls.

Table 1: Solvent ratios used in column chromatography

| Fraction No	Solvent ratios
F1	Petroleum ether 5: chloroform 1
F2	Petroleum ether 4: chloroform 1
F3	Petroleum ether 3: chloroform 1
F4	Petroleum ether 2: chloroform 1
F5	Petroleum ether 1: chlorofoam 1
F6	Diethyl ether 4: methanol 1
F7	Diethyl ether 3: methanol 1
F8	Diethyl ether 2: methanol 1
F9	Diethyl ether 1: methanol 1
F10	Water 100 %
F11	Water 2: methanol 1
F12	Water 1: methanol 1
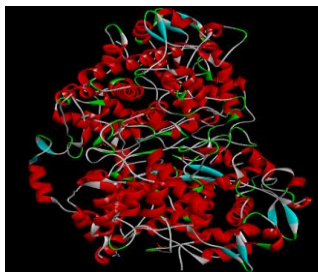
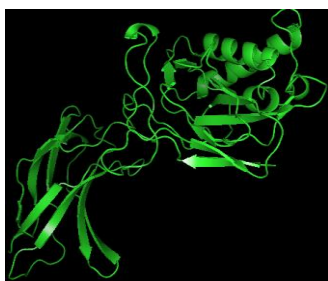**Figure 1.1:** Human Cyclooxygenase-2 (PDB ID: 1PXX)

Figure 1.2: 3D structure of NF-κB (PDB ID: 1SVC)

Docking protocol
Ferulic acid (PubChem ID 445858), caffeic acid (PubChem ID 637542), vanillin (PubChem ID 8468), vioxx (PubChem ID 5090) and ibuprofen (PubChem ID 3672) were loaded onto Pyrx. The energies were minimised and converted to PDBQT format with Open Babel software v 3.1.1.[36] Ligands screened in the second round were energy minimised and converted to PDBQT format using the PyRx virtual screening tool.[37] The binding conformation of the ligands complexed with proteins was visualised using Biovia Discovery 2021. Residues involved in hydrogen-bonding and hydrophobic interactions were analysed and plotted using the LigPlot⁺ software.[38]

Drug-Likeness Analysis
The drug-like features of the identified compounds were assessed using the commercially available software SwissADME.[39] A list of SMILES codes for the selected ligands from HPLC identification of methanol extracts of *A. esculentus* were prepared and inputed into the software to compute drug-likeness (e.g., molecular weight, hydrogen bond donors, hydrogen bond acceptors, lipophilicity and molecular refractivity). Finally, the calculated parameters were checked for each compound according to Lipinski and drug similarity criterion.

In Silico toxicity prediction study
For the identified compounds, ProTox-II was used to predict the toxicity and lethal dose (LD₅₀).[40]

In Silico Prediction of activity spectra for Studied substances
The internet program, PASS,[41] which estimates the bioactivities of 3750 compounds based on a chemical structural study, was used to assess the potential bioactivities of docked compounds. Pa (potential activity) and Pi (potential inactivity) values ranging from 0.000 to 1.000 were used to report the test results. To define a molecule's bioactivity, we used Pa > Pi and Pa > 0.700 values.[42]

Results and Discussion

The HPLC chromatogram of the *A. esculentus* pod fractions (F1-F12) is presented in Figure 2. The active component was identified by comparing the retention time (Rt) and chromatographic peaks of *A. esculentus* pods samples with their respective active components (standards): ferulic acid, caffeic acid, and vanillin acid. The HPLC fingerprint profiles of F4, F5, F8 and F11 fractions of *A. esculentus* pods showed a major peak at the retention time of 1.66 minutes, whereas, the pure standard solution of ferulic acid showed a major peak at the retention time of 1.66 minutes. These indicated the presence of ferulic acid on column fractions F4, F5, F8, and F11. The HPLC fingerprint profiles of F1, F2, F3, F6 and F7 fractions of *A. esculentus* pods showed a major peak at the retention time of 1.67 minutes, whereas, the pure standard solution of caffeic acid showed a major peak at the retention time of 1.67 minutes. These indicated the presence of caffeic acid in column fractions F1, F2, F3, F6 and F7. The HPLC fingerprint profiles of F9, F10, and F12 fractions of *A. esculentus* pods showed major a peak at the retention time of 1.65 minutes, whereas, the pure standard solution of vanillic acid showed a major peak at the retention time of 1.65 minute. These indicated the presence of vanillic acid on column fractions F9, F10, and F12. The structures of the identified phenolics in the fractions of *A. esculentus* pods are presented in Figure 3. The docking studies for COX-2 showed good binding scores. The results are listed in Table 2. and that for NF-κB showed good binding scores as listed in Table 3. 2D interactions of the identified phenolics, ibuprofen and vioxx with COX-2 are depicted in Figure 4. 2D interactions of the identified phenolics, ibuprofen, and vioxx with NFκB are shown in Figure 5. Drug-likeness analyses for the ligands identified in *A. esculentus* pod are presented in Table 4. Predicted toxicity for the ligands identified in *A. esculentus* pod is listed in Table 5. Bioactivity predictions of the ligands using PASS are listed in Table 6.
The 2D interaction between COX-2 and ferulic acid showed conventional hydrogen bonds between protein residues ARG311A, THR561A, LYS2253C, LEU2246C, and oxygen of the acid moiety. The binding affinity was -5.1 kcal/mol. The 2D interaction between COX-2 and caffeic acid showed a conventional hydrogen bonds between protein residues ARG311A, ASN570A, ASP2268C and oxygen of the acid moiety of caffeic acid. The binding affinity was -5.2 kcal/mol. The 2D interaction between Cox-2 and vanillic acid showed conventional hydrogen bonds between protein residues ASN570A, ILE558A, ARG311A, and oxygen of the acid moiety of vanillic acid. An unfavourable donor-donor bond was observed between protein residue LYS 557A and the hydrogen of the acid moiety. A π-anion bond was observed between ARG311A and the delocalized electron of the benzene ring. The binding affinity was -4.7 kcal/mol. The 2D interaction between NFκB and ferulic acid showed a conventional hydrogen bond between protein residues GLN204P, HIS108P, and oxygen of the acid moiety. An unfavourable donor-donor bond was observed between protein residue MET208P and the hydrogen of the acid moiety. The π-anion bond was observed with protein residue ASP209P and the delocalized electrons of the benzene ring. An amide-π stacked bond was observed between ASN103P and the delocalized electrons of the benzene ring. Van der Waals force was observed in protein residue GLY104P. The binding affinity was -5.6 kcal/mol. The 2D interaction between NFκB and caffeic acid showed a conventional hydrogen bond between protein residues GLN204P and hydrogen of the acid moiety. An amide-π stacked bond was observed between ASN103P and the delocalized electrons of the benzene ring. Van der Waals forces were observed in protein residue GLY104P. The binding affinity was -5.4 Kcal/mol. The 2D interaction between NFκB and vanillic acid showed a conventional hydrogen bond between protein residue HIS108P and oxygen of the acid moiety. An unfavorable acceptor-acceptor bond was observed between protein residue GLN204P and the oxygen of the acid moiety. A Carbon hydrogen bond was observed between protein residue GLU207P and methoxyl group. The binding affinity was -5.0 Kcal/mol. Generally, the binding affinity and the interacting amino acids of the phenolics compared well with the standard drugs.
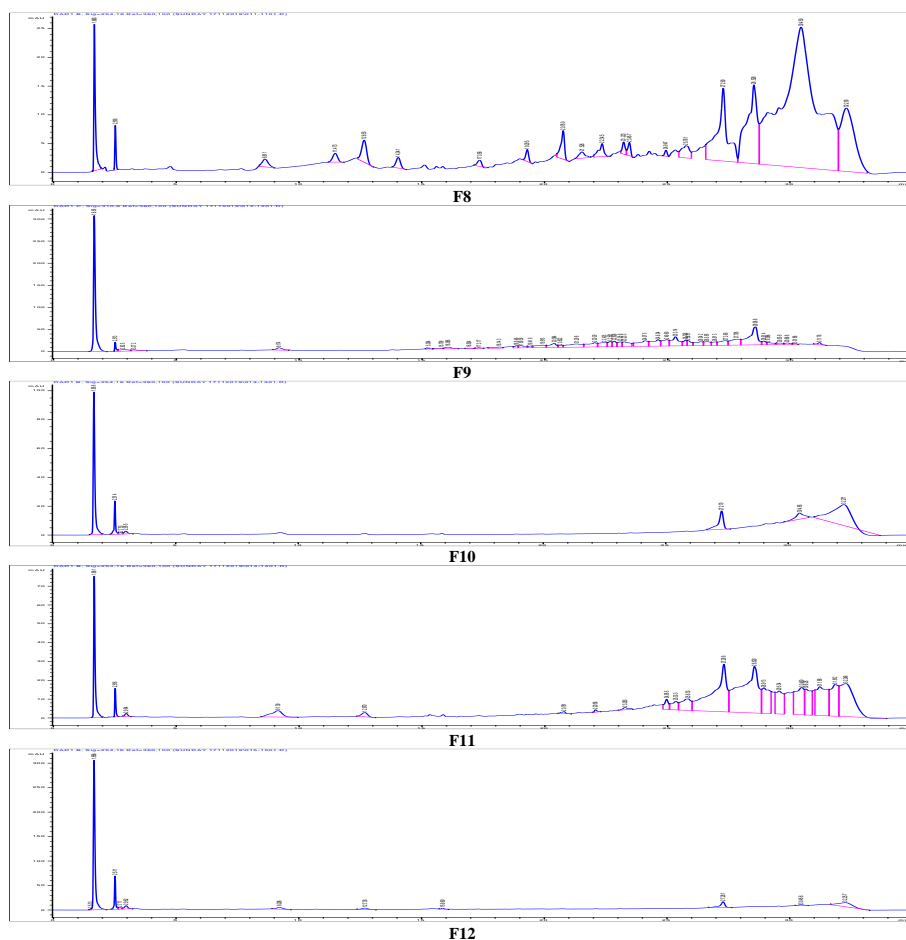
F1

F2

F3

F4

F5

F6

F7
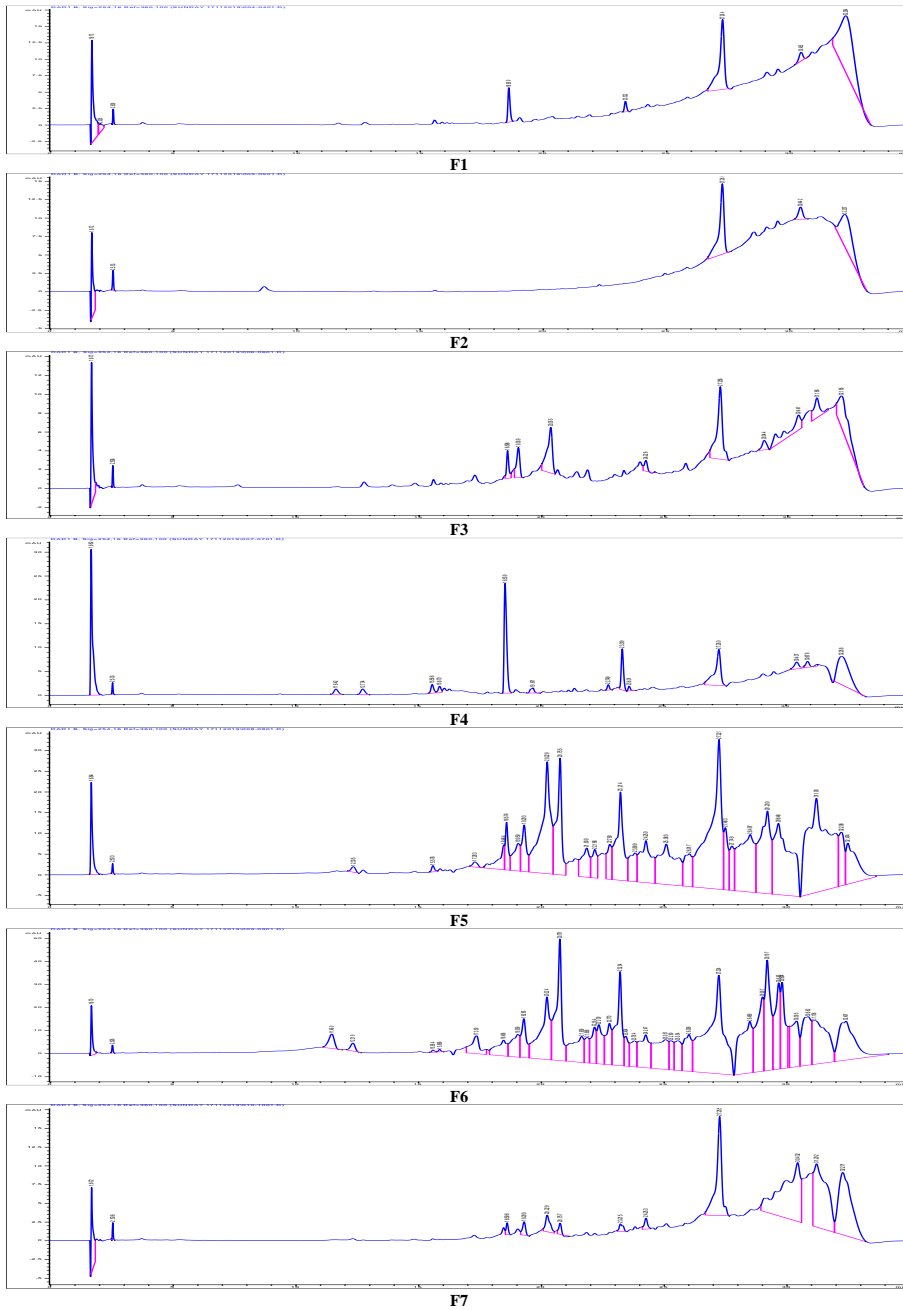
Figure 2: HPLC chromatogram of the *A. esculentus* pod fractions (F1-F12)

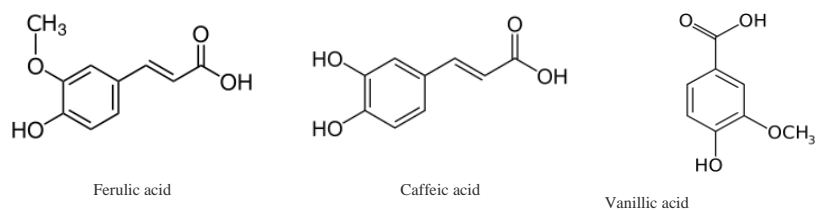Ferulic acid   Caffeic acid   Vanillic acid

Figure 3: The structures of the identified phenolics in the fractions of *A. esculentus* pods

Several authors have also reported molecular docking of identified phytocompounds from chromatographic analysis.[43-46]
Drug-likeness analyses for phytocompounds are listed in Table 3.3.
The RO5 is a thumb rule for identifying whether a compound with specific bioactivity has physical and chemical features that indicate it would be an orally active drug. All of the identified compounds meet the RO5 criteria. As a result, the identified *A. esculentus* pod lead molecules will have reduced attrition rates throughout clinical trials,

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giving them a better chance of commercialization. The docked compounds with their anticipated toxicity class and LD_{50} value are listed in Table 3.4. Toxicity predictions reveal that caffeic acid, may be harmful if consumed ($2000 < LD_{50} \le 5000$ mg/kg); ferulic acid and vanillic acid are harmful if consumed ($300 < LD_{50} \le 2000$ mg/kg). The docked compounds' potential biological activities were assessed using Prediction of Activity Spectra for Substances (PASS), an online structure-based bioactivity prediction tool. The PASS analysis determined the probable targets and biological activities of each chemical. We looked at five biological activities for each molecule based on Pa > Pi and Pa > 0.7 values. The results demonstrated various biological activities with Pa > 0.903, showing that the identified compounds of *A. esculentus* pod have a broader potential (Table 3.5). Hence, ferulic and caffeic acid were predicted as membrane integrity agonists which implied that they prevent inflammatory cell death, hence are anti-inflammatory agents. Vanillic acid was predicted as a chlordecone reductase inhibitor. Chlordecone triggers inflammation of the prostate, hence as an anti-inflammatory agent it induces chlordecone inhibition.

Table 2: The docking scores of the identified phenolics of *A. esculentus* pods with COX-II

Compound	PubChem ID no	Amino acid	Binding Energy (Kcal/mol)	RMSD(Å)
Ferulic acid	445858	ARG311A, THR561A, LYS2253C, LEU2246C	-5.1	0
Caffeic acid	689043	ARG311A, ASN570A, ASP2268C	-5.2	0
Vanillic acid	8468	ASN570A, ILE558A, ARG311A, LYS557A	-4.7	0
Ibuprofen	3672	MET1048B, PRO 547A, LYS1056B, VAL554A, GLU553A	-5.5	0
Vioxx	5090	ARG109A, LYS360A, TRP 545A, PHE361A	-6.0	0

Table 3: The docking scores of the identified phenolics of *A. esculentus* pods with NF-κB

| Compound	PubChem ID no	Amino acid	Binding Energy (Kcal/mol)	RMSD(Å)
Ferulic acid	445858	GLN204P, HIS108P, MET208P, ASP209P, ASN 103P , GLY104P	-5.6	0
Caffeic acid	689043	GLN204P, GLY104P, ASN 103P	-5.4	0
Vanillic acid	8468	GLN204P,HIS108P,GLU207P	-5.0	0
Ibuprofen	3672	ASN103P, HIS108P, ASP209P, GLY104P	-5.4	0
Vioxx	5090	ARG57P, ARG59P, PRO71P	-5.6	0
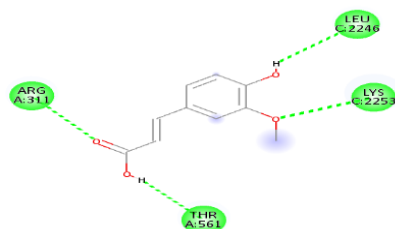
2D interaction of Ferulic acid and COX-II
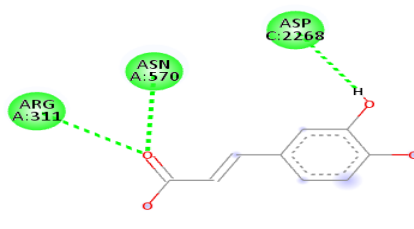
2D interaction of caffeic acid and COX-II
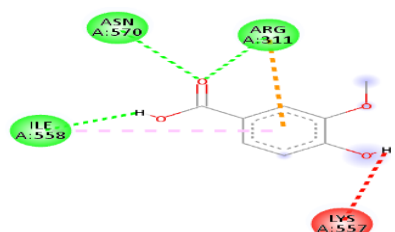
2D interaction of vanillic acid and COX-II
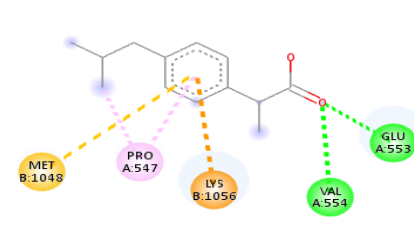
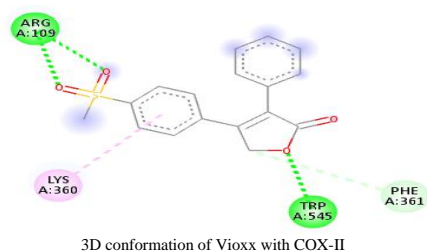
2D interaction Ibuprofen with COX-II

3D conformation of Vioxx with COX-II

Figure 4: 2D interaction of the identified phenolics, ibuprofen and vioxx with COX-II

2D interaction between NFκB and ferulic acid

2D interaction between NFκB and Caffeic acid

2D interaction between NFκB and vanillic acid

2D interaction between NFκB and Ibuprofen

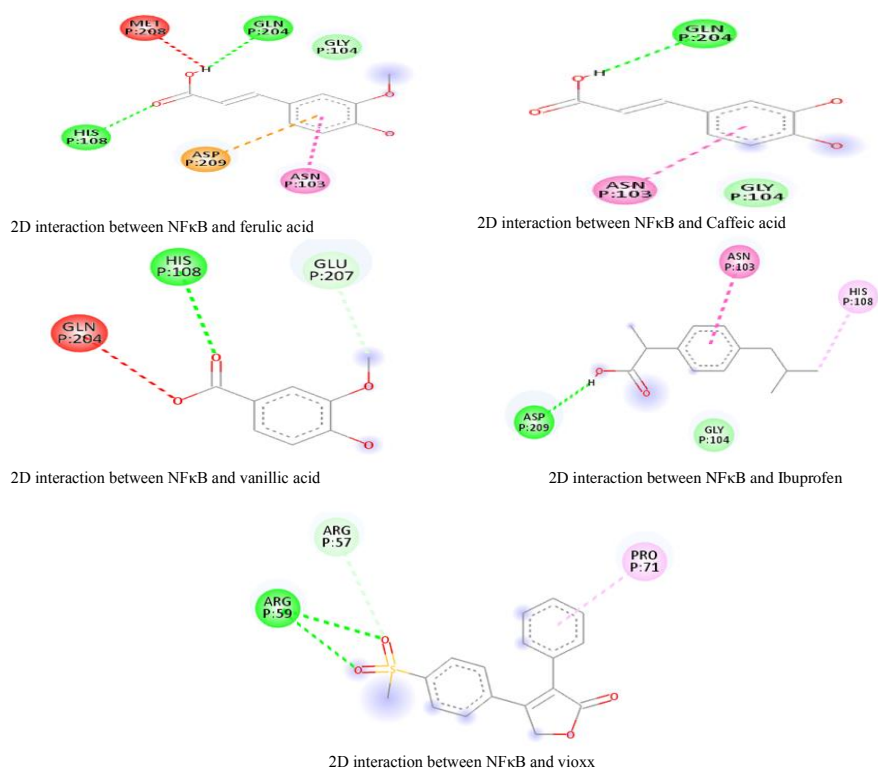2D interaction between NFκB and vioxx

Figure 5: 2D interaction of the identified phenolics, ibuprofen and vioxx with NFκB

Table 4: Drug-likeness analysis of docked compounds identified in the pod of *A. esculentus*

S/N	Compound	Mol. Weight[1](g/mol)	HB Acceptor[2]	HB Donor [3]	Lipophilicity[4]	Molecular Refractivity [5]	Rule of Five [6]
1	Ferulic acid	194.18	4	2	1.36	51.63	0
2	Caffeic acid	180.16	4	3	0.93	47.16	0
3	Vanillic acid	168.15	4	2	1.08	41.92	0

[1] Molecular weight (acceptable range: <500). [2] HB, Hydrogen bond acceptor (acceptable range: ≤10). [3] HB, Hydrogen bond donor (acceptable range: ≤5). [4]Lipophilicity (Log Po/w, acceptable bounds <5). [5] Molar refractivity, acceptable bounds 40-130.[6] RO5: Number of RO5 violations ideal range: 0–4.

Table 5: Predicted toxicity for the docked compounds identified in *A. esculentus* pod

Compound	Predicted LD$_{50}$ (mg/kg)	Predicted Toxicity class	Predicted Toxicity
Ferulic acid	1772	4	Inactive
Caffeic acid	2980	5	Carcinogenic
Vanillic acid	2000	4	Inactive

ProTox (http://tox.charite.de/protox_II, accessed on 1 April 4, 2022 2022) Class 1: deadly if consumed (LD$_{50}$ ≤ 5); Class 2: deadly if consumed(5 < LD50 ≤ 50); Class 3: lethal if consumed (50 < LD$_{50}$ ≤ 300); Class 4: harmful if consumed (300 < LD$_{50}$ ≤ 2000); Class 5: maybe harmful ifconsumed (2000 < LD$_{50}$ ≤ 5000); Class 6: non-lethal (LD$_{50}$> 5000)

Table 6: Bioactivity prediction of the docked compounds identified in *A. esculentus* pod using PASS

S/N	Compound	Pa	Pi	Biological Activity
1	Ferulic acid	0.944	0.004	Membrane integrity agonist
		0.915	0.003	JAK2 expression inhibitor
		0.903	0.001	Aryl sulfotransferase inhibitor
		0.903	0.002	Preneoplastic conditions treatment
		0.906	0.005	Mucomembranous protector
2	Caffeic acid	0.977	0.001	Feruloyl esterase inhibitor
		0.955	0.003	Membrane integrity agonist
		0.945	0.003	Mucomembranous protector
		0.940	0.001	4-Hydroxybenzoate 3-monooxygenase inhibitor
		0.940	0.002	Benzoate 4-monooxygenase inhibitor
3	Vanillic acid	0.964	0.002	Chlordeconereductase inhibitor
		0.937	0.003	Aldehyde oxidase inhibitor
		0.931	0.003	Feruloyl esterase inhibitor
		0.932	0.004	Aspulvinonedimethylallyltransferase inhibitor
		0.924	0.002	Hydroxyquinoline 8-monooxygenase inhibitor

[a]Pa = Possibility of activity; [b]Pi = Possibility of inactivity.

Conclusion

The HPLC analysis of *A. esculentus* pods revealed several biologically active phytoconstituents with excellent binding affinity to COX-2 and NFκB in molecular docking studies. Their drug-like properties were established through ADME and toxicity studies predicted. The predicted toxicity values of the phenolics were not favorable. However, given the positive *in silico* results, more research is needed to extract pure chemicals from this plant.

Conflict of Interest

The authors declare no conflict of interest.

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