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Original Research Article

HPLC Analysis, *In Vitro* and *In Silico* Evaluation of Antioxidant Activity of Methanol Stem Bark Extract of *Picralima nitida* (Apocynaceae)

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ABSTRACT

Antioxidant compounds are effective in mitigating or preventing radical-induced damages either by neutralizing the free radicals or activating pro-antioxidant proteins (such as NRF2). *Picralima nitida* has been shown to possess numerous biological activities. This study aimed to investigate the phytochemical constituents, antioxidant activity, and predict the safety and efficacy of compounds in *Picralima nitida* stem bark. Quantitative evaluation of phenols and flavonoids contents were performed using spectrophotometric methods. The antioxidant activity was evaluated *in vitro* using ABTS, DPPH, and FRAP assays. Analytical High-performance Liquid Chromatography (HPLC) was used for the compounds identification. Furthermore, the antioxidant potential of the identified compounds were assessed *in silico* via molecular docking with nuclear factor erythroid 2-related factor 2 (NRF2), followed by pharmacokinetics, and toxicity assessment using computational tools. The results showed that the total phenol, and flavonoid contents of *Picralima nitida* stem bark extract were 95.11 mgGAE/g extract, and 12.22 mgQE/g extract, respectively. *In vitro* antioxidant activity evaluation showed that the extract possesses strong antioxidant activity with IC_{50} values of 7.38 µg/mL, and 27.40 µg/mL for ABTS, and DPPH radical scavenging activity, respectively, and a FRAP value of 145.37 mM $FeSO_4$ equivalents/g. HPLC analysis identified 11 compounds with docking scores between -4.315 and -12.603 Kcal/mol. Comparison of the pharmacokinetic properties of these compounds revealed rutin, naringin, and catechin as the most promising candidates for further studies. This study underscores the use of *Picralima nitida* in the treatment of different ailments in traditional medicine while providing insights for future drug developments from the plant.

Keywords: Antioxidants, Molecular docking, Methanol extract, *Picralima nitida.*

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Introduction

Natural products, particularlly those obtained from medinal plants are of immense interest as sources of drugs and drug candidates especially for chronic diseases such as cancer, diabetes, cardiovascular diseases, and some infectious diseases.[1] Medinal plants produce secondary metabolites such as alkaloids, flavonoids, terpenoids, saponins, and phenolics which play pivotal roles in their therapeutic applications.[2] Remedies derived from these medicinal plants have been found to be more affordable, easily accessible, and are associated with fewer side effects than their synthetic counterparts. *Picralima nitida* commonly known as "Akuamma or abeere" is one of such plants that has been reported to possess various pharmacological properties making it a plant of interest in the search for antioxidants due to its reported antioxidant properties using *in vitro* and *in vivo* techniques. This plant is native to tropical Africa and predominantly contains alkaloids which are contained in the seeds, barks and leaves. Key alkaloids found in the plant include indole alkaloids; akuammine, pseudoakuammine, akuammigine, pseudoakuammigine, akuammicine, akuammidine, akuammenine, and akuammiline. Other isolated alkaloids include picraphylline, picraline, picratidine, burnamine, and pericine.[3]

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Other secondary metabolites identified in the plant are terpenoids, phenolics, and flavonoids, which are considered to contribute to its antioxidant activity and potential use in managing oxidative stress related disorders.[4] These compounds exhibit a wide range of biological activities, including antimicrobial, anti-inflammatory and analgesic properties.[4] Studies have shown the stem barks of *P nitida* to possess good free radical scavenging activities using animal models resulting in significant reductions of oxidative stress markers; malondialdehyde and hydrogen peroxide while increasing catalase activity.[5] *In vitro* antioxidant screening using the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging method also revealed good antioxidant activity.[5]

Oxidative stress occurs when there is an imbalance between the antioxidant systems and the free radicals resulting in overwhelming of the antioxidant system, and subsequently damage to proteins, lipids, and DNA. Oxidative stress plays a central role in multiple non-communicable diseases (NCDs), which account for approximately 60% of global deaths with the largest burden occurring in Low- and Middle-Income Countries (LMICs).[6] This is due to damage at cellular level which has been linked to the onset and progression of various diseases, including; cardiovascular diseases, diabetes, neurodegenerative diseases, and various types of cancers.[7] It has also been suggested that chronic oxidative stress could result in accelerated aging and increase in other inflammatory processes.[8–10]

Internal metabolic processes and external inducement in aerobic animals give rise to highly reactive species known as free radicals or oxidants.[9,10] These free radicals include reactive oxygen species (ROS), e.g., singlet oxygen, superoxide, and hydroxy radicals; reactive nitrogen species (RNS), e.g., nitric oxide and peroxynitrite; reactive sulfur species (RSS), e.g., thiyl radicals, disulfide radical; and others.[11] Oxidants produced or released in response to physiological factors act as important signaling molecules to mediate such processes as inflammation, cell division, autophagy, immune function, and stress response.[10] The body has its own system for eliminating these highly

reactive agents known as the body's intricate antioxidant systems comprising mainly of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and the non-enzymatic antioxidants such as glutathione, thioredoxin, and peroxiredoxins.[7,12]
Antioxidants play a vital role in disease prevention by mopping up these free radicals. They include ascorbic acid, tocopherols, and polyphenolic compounds.[7,12] They bring about this effect by directly binding and terminating the free radicals, and also by activating pathways in the body that mediates the production of pro-antioxidant enzymes at the molecular level.[12] Antioxidants work to maintain redox homeostasis, protect cellular structures, and modulate signaling pathways linked to inflammation and apoptosis.[7,12]
One very important pro-antioxidant system in the body is the nuclear factor erythroid 2-related factor 2 (NRF2). The cytoplasm of the cell contains nuclear factor erythroid 2-related factor 2 (NRF2), a member of the cap'n'collar basic leucine zipper family.[13] The NRF2 binds to the Kelch-like ECH-associated protein 1 (KEAP1) through two motifs: Aspartic acid–Leucine–Glycine (DLG) and Glutamic acid–Threonine–Glycine–Glutamic acid (ETGE). SER (363, 508, 555 & 602), ARG (380, 415 & 483), ASN 382, GLN 530, and TYR 525 are the amino acid residues that the DLG motif of NRF2 binds to, while the ETGE motif binds to the KEAP1 protein's SER (363, 508, 555 & 602), ARG (380, 415 & 483), ASN 382, GLN 530, and TYR 525, respectively.[14] Under normal conditions, NRF2 stays bound by KEAP1, and gets polyubiquitinated by the KEAP1/NRF2 ubiquitin ligase which triggers degradation by proteasomes with a half-life of approximately 20 minutes.[15] The connection between the KEAP1 and the ETGE and DLG motif of NRF2 is broken under high stress conditions releasing free NRF2 which translocate to the nucleus where it is bound and activated by the antioxidant response element which affects gene transcription.[16,17] The susceptibility of cells to oxidative stress is reduced by initiating the transcription of the genes involved in the synthesis of phase-II enzymes and antioxidant proteins, such as glutathione-S-transferases and heme-oxygenase-1.[15]
Recently, studies have been conducted to find compounds that can disrupt the KEAP1-NRF2 protein-protein interaction, releasing NRF2 for oxidative stress defense. Natural products possess significant antioxidant activity and studies have shown that some of the antioxidant activities are mediated through NRF2 activation.[18,19]
Current strategies and modern medicine are shifting away from the use of whole plant extract and are tilting towards single-component-based medicine.[20] While the former offers the benefit of synergistic interaction of the multiple components and the living system, separation and isolation offers better synthetic applicability as it has been proven that often times, not all components of the extract has the desired effect even though it is possible to extract all the components.[21]
Advances in analytical techniques like gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) have enhanced the identification of novel compounds from plants. These compounds are typically characterized by significant scaffold diversity and structural complexity.[1] Based on the use in ethnomedicine and previous scientific findings on its antioxidant activity, this study seeks to investigate the phytochemicals present in the stem barks of *P. nitida* using total phenol, total flavonoids, ABTS, DPPH and FRAP assays, identify key compounds present using HPLC analysis, evaluate NRF2 activation potential of the identified compounds and assess their ADMET properties using *in-silico* tools.

Materials and Methods

Reagents and Equipment
Methanol (99.8%) (Loba Chemie®, India), distilled water, hydrochloric acid (Loba Chemie®, India), glacial acetic acid (GHTECH®, China), sodium acetate (Merck®), sodium chloride (Loba Chemie®, India), ferric chloride hexahydrate (Xi'an tian mao chemicals®, China), ascorbic acid (Sigma Aldrich®, Germany), TPTZ (2,4,6-tripyridyl-s-triazine) (Molychem®, India), potassium persulfate, DPPH (2,2-diphenyl-1-picrylhydrazyl) (Molychem®, India), Quercetin (Sigma Aldrich®, Germany), ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) (Molychem®, India), ferrous sulphate heptahydrate (Kermel®, India).
The equipment used include; Rotary evaporator coupled to temperature-controlled water bath (Bibby Scientific Limited®, UK), UV-Visible spectrophotometer (PG Instruments LTD®, England), weighing balance (Ohaus®, USA) and 8GB RAM core i5 laptop (HP®).

Software
Schrödinger software suite (version 2021) was used in this study. Databases used were RCSB Protein Data Bank, PubChem, and ADMETLAB 3.0

Plant collection, identification and preparation
Stem barks of *Picralima nitida* Durand and Hook were harvested from a forest in Agekpanor Village, Ovia North-East Local Government Area, Edo State, Nigeria (GPS: 6°25'26" N, 5°30'59" E). The plant material was identified at the Department of Plant Biology and Biotechnology, University of Benin, Benin City, and assigned a boucher number: UBH-P424. It was dried away from direct sunlight for 10 days; the dried stembark was subsequently ground into a fine powder. The powder was stored in an air-tight container and labelled properly until further analysis.

Extraction of plant sample
The powdered plant sample (600 g) was macerated in methanol (2 L) at room temperature for 48 h with stirring every 12 h. the extract was filtered, and the marc was re-extracted twice with methanol (2 L) for another 48 h. The combined extract was concentrated *in vacuo* using a rotary evaporator at 40ºC, and subsequently air-dried at room temperature for 8 days to obtain the solid crude methanol extract.

Determination of total phenol content
The total phenol content of the extract was evaluated using the method described by Kim *et al*. (2003).[22] briefly, 4.5 mL of deionized distilled water was mixed with 0.5 mL of Folin Ciocalteu's reagent (which had been diluted with water 1:10, v/v) and then added to 0.5 mL of 1000 µg/mL. extract solution. The tubes were shaken and allowed to stand at room temperature for 5 minutes, then 5 mL of 7% sodium carbonate and 2 mL of deionized distilled water were added. The samples were mixed and then allowed to stand at room temperature for 90 minutes. The absorbance of the reaction mixture was measured at 750 nm using a spectrophotometer. Gallic acid in six different strengths (12.5, 25, 50, 75, 100 and 150 mg/L) was used to create a standard curve and triplicate readings were taken.
The total phenolic content was represented as milligrams of gallic acid equivalent (GAE) per gram of extract (mg GAE/g extract).

Determination of total flavonoid content
The method described by Ebrahimzadeh *et al*. (2008)[22] was used to estimate the total flavonoid content. Briefly, 1.5 mL of methanol and 0.5 mL of extract solution (1 mg/mL) were mixed together and then 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added sequentially. The mixture was incubated for 30 minutes at room temperature, and a spectrophotometer was used to measure the absorbance at 415 nm. Quercetin in six distinct concentrations (12.5, 25, 50, 75, 100, and 150 mg/L) were used to create the standard curve, and all readings were done in triplicates. The total flavonoid content was expressed as milligrams quercetin equivalent (QE) per gram of extract (mg QE/g extract).

High performance liquid chromatography (HPLC)
Sample preparation
Extract (0.2 g) was weighed and transferred in a test tube and 15 mL ethanol and 10 mL of 50% potassium hydroxide was added. The test tube was allowed to react in a water bath at 60ºC for 3 h. After the reaction time, the reaction product contained in the test tube was transferred to a separatory funnel. The tube was washed successfully with 20 mL of ethanol, 10 mL of cold water, 10 mL of hot water and 3 mL of n-hexane, which were all transferred to the funnel. These extracts were combined and washed three times with 10 mL of 10% v/v ethanol. The ethanol solvent was evaporated. The sample was solubilized in 1000 µL of pyridine of which 200 µL was transferred to a vial for analysis.

HPLC analysis

High performance liquid chromatography (HPLC) analysis was performed using Shimadzu LC-10AD dual binary pumps, Shimadzu CTO-10AS column oven, and Shimadzu Prominence SPD-20A UV/Vis detector. The analysis was performed using a C-12 normal phase column (Phenomenex, Gemini 5 µ, 200 mm length × 4.8 mm internal diameter). The mobile phase consisted of acetic acid-acidified deionized water (pH 2.8) as solvent A and acetonitrile as solvent B at a flow rate of 0.8 mL/min. The column was equilibrated with 5% solvent B for 20 min after each injection of samples. The column temperature was set to 38°C and the injection volume was 20 µL. The wavelengths were set to 280 nm for the detection of phenolics. Phenolic compounds identification and quantification were performed by comparing respective retention times and peak areas with pure standard compounds utilizing the method of external standards to construct calibration curve. Gradient elution was executed as follows: 0-5 min, 5-9% solvent B; 5-15 min, 9% solvent B; 15-22 min, 9-11% solvent B; 22-38 min, 11-18% solvent B; 38-43 min, 18-23% solvent B; 43-44 min 23-90% solvent B; 44-45 min, 90-80%, solvent B; 45-55 min.[23]

In vitro antioxidant activity tests

The antioxidant activity of the crude methanol extract of *Picralima nitida* was evaluated using three different models; ABTS, DPPH, and FRAP assays.

(i) ABTS assay

In this assay, 5 mL each of 14 mM ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) solution and 4.9 mM potassium persulphate solution were mixed and kept in the dark for 16 hours at room temperature to produce the $ABTS^{\cdot+}$ cation radicals. A 1 in 60 dilution was made with methanol to prepare the ABTS working solution and adjusted to attain an absorbance of 0.700 ± 0.020 at 745 nm in a spectrophotometer. Thereafter, 100 µL of extract dilution in methanol was combined with 3 mL of ABTS working solution, thoroughly mixed, and allowed to stand at room temperature for 5 minutes. Absorbance was determined using a spectrophotometer at 745 nm in triplicates. The standard used was ascorbic acid and the percentage radical scavenging activity (RSA) was calculated for different concentrations of standard and extract using the formula below (equation 1);

$$ABTS\ radical\ scavenging\ activity\ (\%) = [\frac{(A0-A1)}{A0}] \times 100 \ldots (1)$$

Where; A0 is the absorbance of the control at 5 min and A1 is the absorbance of the sample at 5 min. The concentration of extract at which 50% inhibition was observed (IC_{50}) was calculated in µg/mL.[24]

(ii) DPPH assay

The scavenging activity of the crude methanol extract on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was evaluated using the method described by Jain *et al*. (2008).[25] Briefly, 3.0 mL of extract in methanol containing 0.01 to 0.2 mg/mL of the extract was mixed with 1.0 mL of a solution of 0.1 mM DPPH in methanol. The mixture was thoroughly shaken and was allowed to stand in the dark at room temperature for 30 minutes. Using a spectrophotometer, the absorbance of the mixture was determined at 517 nm. The reference standard was ascorbic acid. The ability to scavenge DPPH radical was calculated by the following equation 2:

$$DPPH\ radical\ scavenging\ activity\ (\%) = [\frac{(A0-A1)}{A0}] \times 100 \ldots (2)$$

Where; A0 is the absorbance of DPPH radical + methanol, A1 is the absorbance of DPPH radical + sample extract /standard.

The 50% inhibitory concentration (IC_{50}) is the effective concentration of the sample that can scavenge 50% of the DPPH free radical.

(iii) FRAP assay

The ferric reducing antioxidant power (FRAP) assay was done according to Benzie and Strain (1996) method with some modifications.[26] Stock solutions containing 20 mM $FeCl_3.6H_2O$ solution, 10 mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40 mM HCl, 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$) were prepared, and adjusted to pH 3.6. Acetate buffer (100 mL), 10 mL of TPTZ solution, and 10 mL of $FeCl_3.6H_2O$ solution were combined to create the working solution, which was then heated to 37°C before use. Then 1.5 mL of 0.1 mg/mL of the crude extract was measured into a test tube and 3 mL of FRAP working solution was added, it was incubated in the dark at room temperature for 30 minutes, and the absorbance of the reaction mixture was measured at 593 nm. For construction of the calibration curve, five concentrations of $FeSO_4.7H_2O$ (0.1, 0.4, 0.8, 1, 1.2, 1.5 mM) were used and the absorbance values were measured as for sample solutions and triplicate readings were taken.

In silico study

The *in-silico* study primarily involved protein and ligand retrieval, docking, MMGBSA, Pharmacophore screening and ADMET.

Protein and ligand retrieval

Crystallized 3D version of the nuclear factor erythroid 2-related factor 2 (NRF2) in complex with the co-crystallized ligand: 1VX, PDB ID: 4L7D was retrieved from the RCSB Protein Data Bank (RCSBPDB: https://www.rcsb.org/). The ligands reported to be present in the extract based on literature review were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in SDF formats.

Protein preparation

The protein was included into the workspace and the protein preparation module was opened and the preparation process was initiated by filling of missing loops, hydrogens and chains, it was refined and water molecules were eliminated. PROPKA pH 7.5 was applied and the protein was optimized, followed by removal of water beyond 5 Å radius and subsequent minimization. The OPLS4 force field was applied and the protein was minimized to achieve a lower energy state.[27]

Ligand preparation

The phytochemicals were prepared using the LigPrep module at OPLS4 force field.[28] The stereoisomer generation was restricted to a single isomer per ligand and the tautomer generation was excluded and the ligands prepared and formatted in the maestro output format. The reference ligand: Bardoxolone methyl, PubChem ID: 400769 was also retrieved from PubChem database and the similar procedure was applied.

Receptor grid generation

The minimized protein was included and the receptor grid was generated using the glide module to determine the active site of the receptor.[27] The ligand was selected on the workspace from the minimized protein to define the docking pocket. The grid was developed with specific parameters: default settings for site constraints, rotatable groups, Van der Waals radius scaling factor of 1.0, a partial charge cut off of 0.25 and excluded volumes. The coordinates of the receptor grid were: X (-22.81), Y (39.07) & Z (-36.63)

Molecular docking

After the ligand preparation, the compounds were docked using XP and the 2D interactions were retrieved from the panel and reported. The root mean square deviation RMSD was also determined using the co-crystallized ligand as a means of validation of the docking protocol. This was determined using the RMSD Superposition module with the structure for superimposition defined by ligand.

MMGBSA

The Prime module of Maestro was used to calculate the MMGBSA for the top ranked compounds using the same glide grid file previously generated. The free binding energy was determined using the formula below (equation 3):

$$[\Delta G]\ Bind = G^{compex}\ X - G^{protein} + G^{ligand} \ldots\ldots\ldots\ldots (3)$$

Where; ΔGbind is the complex's binding free energy. $G^{complex}X$ is the free energy of the complex, $G^{protein}$ is the free energy of the protein without the ligand, and G^{Ligand} is the free energy of the ligand without the protein.

ADMET prediction
The test compounds as well as the reference compound (Bardoxolone methyl) were analyzed using the ADMETLAB webserver (https://admetlab3.scbdd.com/).

Statistical analysis
Microsoft Excel 2019 (Microsoft Corp., USA, 2019) was used for plotting all the curves. All assays were conducted in triplicate (n = 3) to ensure the reliability and consistency of the results. The mean was calculated and the values reported as the mean ± standard deviation (Mean ± SD).

Results and Discussion

Total phenolic and total flavonoid contents
The total phenol and total flavonoid contents of *Picralima nitida* stem bark extract were determined using colorimetric methods. These tests were carefully selected since these classes of compounds are typically known for their antioxidant properties.[29] The total phenol was determined using gallic acid as standard represented on the gallic acid calibration curve (Figure 1). Extrapolation of the absorbance of the sample using the calibration curve gave the total phenolic content of 95.11 mg GAE/g extract. This indicates that 1 g of the crude extract contains as much phenolic compounds as would be found in 95.11 mg of gallic acid. This confirms and quantifies the presence of phenolic compounds in the sample, a strong marker for antioxidant activity. The total flavonoid content was determined to be 12.22 mg QE/g extract using quercetin calibration curve as a standard (Figure 2). These tests were used to establish the presence of phenolic compounds and flavonoids in the extract, a strong marker for antioxidant activity. Phenolic compounds are important constituents of plants due to their ability to mop up free radicals using their free hydroxyl groups. Flavonoids are considered the most widespread and diverse phenolics. Findings from this study showed that the stem bark of *P nitida* is rich in flavonoids and other phenolic compounds which can account for the antioxidant activity. The fruit pulp, root barks and leaves have been shown to contain significant quantities of phenols and flavonoids.[30–32]

High performance liquid chromatography
Eighteen (18) peaks were identified from the HPLC chromatogram, however, only 11 gave distinct compounds. Seven flavonoid compounds (Naringin, Naringenin, Kaempferol, Rutin, Catechin, Epicatechin, Flavan-3-ol, Flavanones, and Anthocyanin), two alkaloids (Ribalinidine and Sparteine) and two antinutrients (Phytate and Oxalate) were identified (Figure 3 and Table 1). The compounds were identified from the extract by matching their retention times against those of standards with the peak assignment confirmed by injection of standards. However, it was not possible to compare the findings of this study with those found in other literature, primarily because this study is the first to conduct preliminary identification and quantification of stembarks of the plant and secondly because the gradient elution program, solvent content, and extract matrix all affect retention time. The 11 distinct compounds identified (Figure 4) formed the basis for the *in-silico* study using computational tools.

Table 1: Compounds identified from the HPLC analysis of *Picralima nitida* stem bark

S/N	Component	Retention	Molecular Formula	Molecular Weight (g/mol)	% Area
1.	Lunamarin	0.196	$C_{18}H_{15}NO_{14}$	469.311	3.17%
2.	Naringin	1.583	$C_{27}H_{32}O_{14}$	580.54	3.32%
3.	Cardiac glycoside	2.633	N/A	N/A	8.58%
4.	Anthocyanin	3.550	$C_{41}H_{44}O_{22}$	888.80	2.69%
5.	Flavan-3-ol	4.400	$C_{15}H_{14}O_2$	226.27	7.20%
6.	Ribalinidine	12.623	$C_{15}H_{17}NO_4$	275.30	4.53%
7.	Naringenin	13.003	$C_{15}H_{12}O_5$	272.25	5.64%
8.	Sparteine	13.233	$C_{15}H_{26}N_2$	234.38	3.21%
9.	Cyanogenic glycoside	13.973	N/A	N/A	2.55%
10.	Rutin	15.620	$C_{27}H_{30}O_{16}$	610.50	3.77%
11.	Flavonones	18.950	N/A	N/A	4.49%
12.	Steroids	22.456	N/A	N/A	6.03%
13.	Kaempferol	25.563	$C_{15}H_{10}O_6$	286.24	3.44%
14.	Epicatechin	27.913	$C_{15}H_{14}O_6$	290.27	9.87%
15.	Phytate	28.273	$C_6H_{18}O_{24}P_6$	660.04	6.29%
16.	Oxalate	35.650	$C_2O_4^{-2}$	88.02	12.27%
17.	Catechin	36.526	$C_{15}H_{14}O_6$	290.27	3.63%
18.	Sapogenin	42.706	$C_{27}H_{44}O_2$	400.60	9.34%
	Total				

Key: N/A = Not Applicable

In vitro antioxidant activity

The antioxidant activity of the crude methanol extract of *Picralima nitida* stem bark was evaluated using three different *in vitro* assays (ABTS, DPPH, and FRAP) elucidating the two main mechanisms involved in redox antioxidation; ABTS and DPPH assays were used to demonstrate the ability of the extract to release hydrogen and participate in redox reactions while the FRAP assay was used to determine the ability of the extract to donate electrons to ferric ion thus reducing it to ferrous ion.

In the ABTS assay, the reduction of the ABTS radical cation ABTS-H by the extract resulted in a colour change from blue-green to colourless. The extent of the reduction shown by the absorbance has an inverse relationship with the radical scavenging activity (RSA) of the extract.[24] The extract was found to have a concentration-dependent inhibitory activity demonstrated by the calculated RSA values plotted against the concentration. The IC_{50} which is the concentration of the extract required to scavenge 50% of the free radicals with a lower value indicating higher activity was determined to be 7.38 µg/mL which is 5-times higher than the calculated value for the standard - ascorbic acid (IC_{50} = 1.57 µg/mL) (Table 2).

Table 2: *In vitro* antioxidant activity of *Picralima nitida* stem bark extract

Sample	IC_{50} (µg/mL)		FRAP (mM $FeSO_4$ Eq/g)
	ABTS	DPPH	
P. nitida extract	7.38	27.40	145.37
Ascorbic acid	1.57	1.75	240.91

DPPH assay involves the transfer of hydrogen ions, the reduction of DPPH radicals by the extracts to DPPH-H resulted in a colour change from violet to yellow. The absorbance and RSA have similar relationship as with ABTS.[33] Similarly, the extract was found to possess a concentration-dependent inhibitory activity against DPPH radical. The IC_{50} was determined to be 27.40 µg/mL which is approximately 16 times higher than the determined IC_{50} for ascorbic acid (IC_{50} = 1.75 µg/mL) (Table 2). Similar findings of the DPPH radical scavenging activity of the root bark, seeds, leaves, fruit bark and pulp have been reported.[31,33–35]

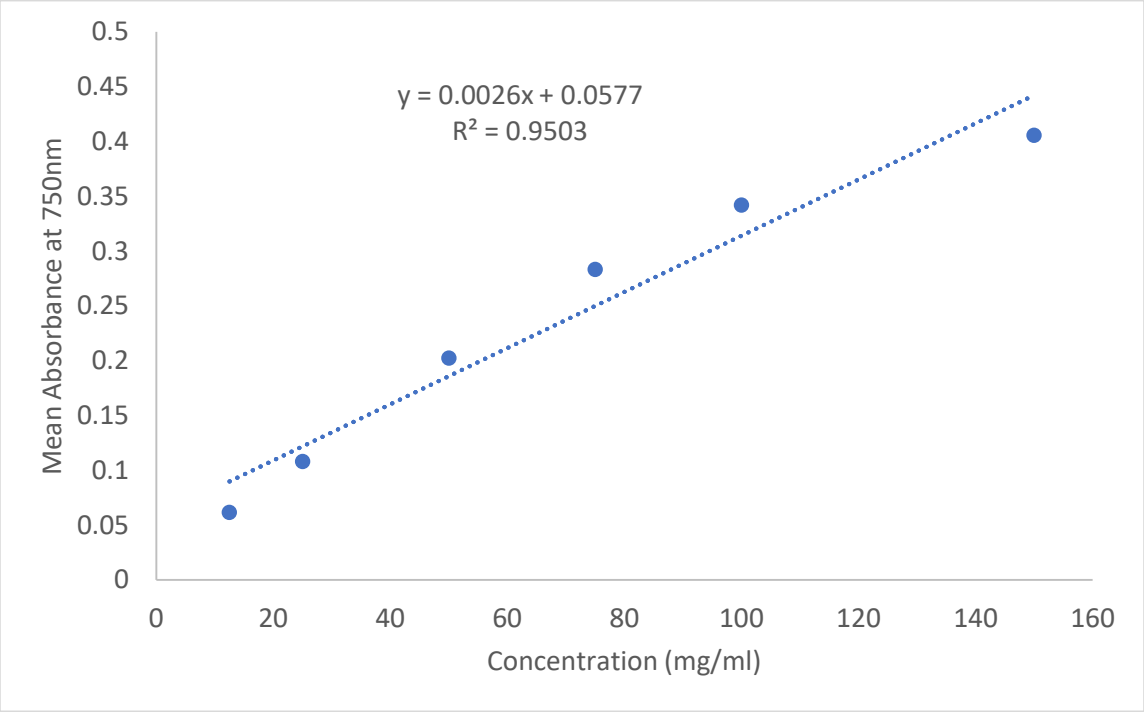

Figure 1: Gallic Acid Calibration Curve

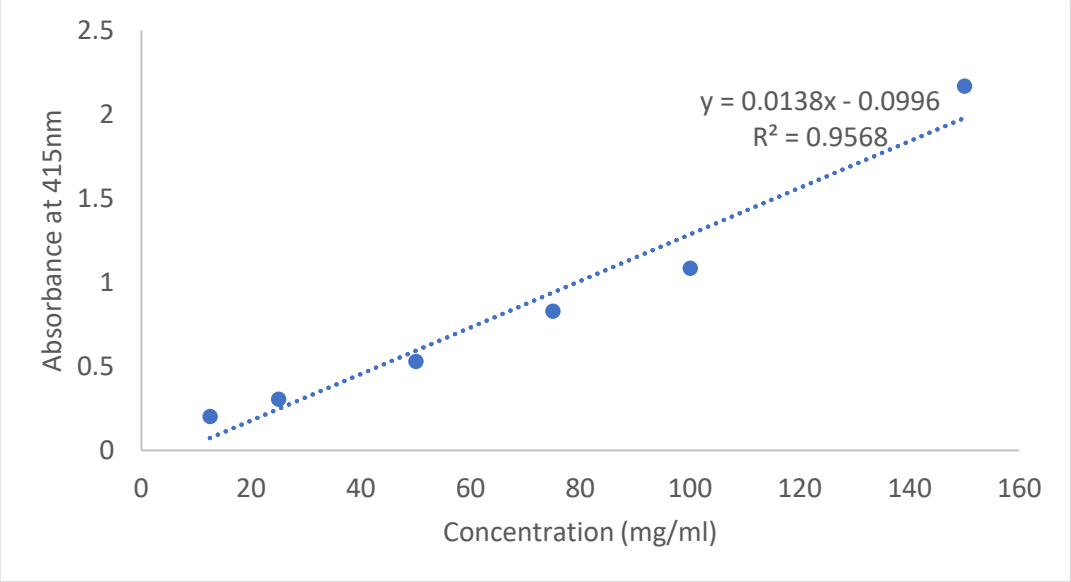

Figure 2: Quercetin Calibration Curve

The FRAP assay uses the ability of the extract to reduce ferric ions to quantify the antioxidant activity. The reducing capacity is a concentration-dependent phenomenon as seen in the calibration curve. However, a direct relationship exists between the absorbance and the FRAP values because the ferrous ions being measured by the spectrophotometer increases with increasing antioxidant concentration while ferric ions decrease.[36] The ferric reducing antioxidant power of the extract was extrapolated to be 145.37 mM $FeSO_4$ equivalents/g which is approximately one and a half times lower than the extrapolated antioxidant power of the standard – ascorbic acid (FRAP = 240.91 mM $FeSO_4$ equivalents/g) (Table 2).

Antioxidants protect the body system by neutralizing free radicals and/or inhibiting chemical processes that produce these free radicals.[7] Natural antioxidants have the potential to prevent or reverse damages associated with antioxidants and oxidative stress.[12] In this study, methanol extracts of *Picralima nitida* stem barks showed good antioxidant activity offering scientific justification for the use in ethnomedicine and possible use in conventional practice.

In silico antioxidant activity

Molecular docking and MMGBSA

Molecular docking allows the estimation of binding affinities and as a reference, Bardoxolone methyl was selected. This molecule has been demonstrated to possess NRF2 activation *in-vitro* and *in-vivo* making it ideal for comparing binding affinities although it failed phase 3 trials due to increase in risk for cardiovascular diseases. Table 3 shows the docking score which revealed that the compounds identified have binding affinities within the range of -4.315 to -12.603 Kcal/mol far outperforming the reference compound with a binding affinity of -3.382 Kcal/mol. The Root Mean Squared Deviation (RMSD) was also determined to be 0.8341. This value represents the difference between poses of the docked ligand and a reference ligand usually obtained by the superimposition of the docked co-crystallized ligand and the native binding pose of the co-crystallized ligand with zero reflecting a perfect alignment and acceptable values being <2Å.[37]

MMGBSA estimates the free energy of binding and provides insight on the affinity and can sometimes be used as supporting data for docking predictions. MMGBSA values ranged from -39.37 to -77.28 ΔGbind against the reference ligand which had ΔGbind of -45.77. Pose ranking between docking scores and MMGBSA may vary because docking utilizes an empirical scoring function which can best be described as "Machine learning" where Van Der Waals, Hydrophobic and solvation components are not considered. MMGBSA on the other hand is a direct physics-based method that takes explicit terms and factors into consideration before estimations are provided. Nonetheless, they are both to be treated as approximations rather than exact determination of binding affinities.

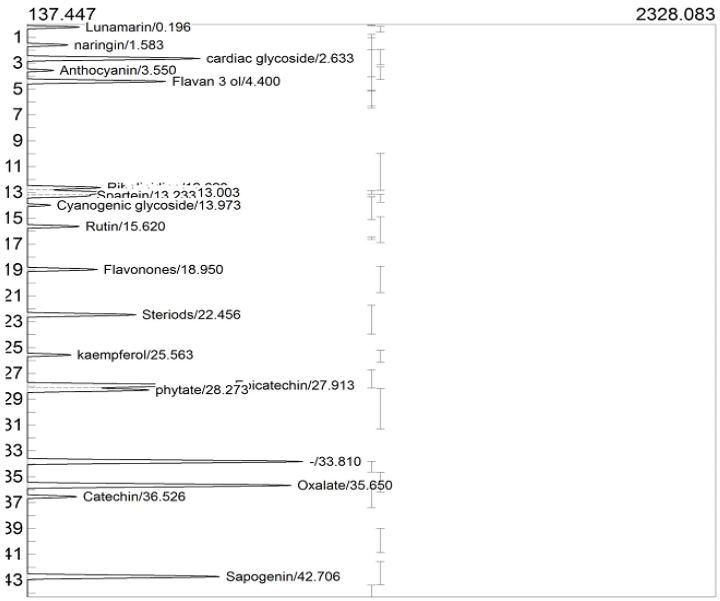

Figure 3: HPLC Chromatogram of *Picralima nitida* stem bark extract

Phytate interacted with amino acid residues ARG415, AEG483, SER508, SER555, SER602, GLN530, and TYR572 via hydrogen bonds. Rutin interacted with ASN382, SER363, SER508, ASN414 via hydrogen Bonds, TYR572 via Pi-Pi Stacking, and ARG415 via Pi Cation. Naringin interacted with TYR334, SER363, ASN414, SER602, and SER555 via hydrogen Bonds, and ARG415 via Pi Cations. Naringenin interacted with SER363, ASN414, and ARG 380 via hydrogen bonds, while catechin interacted with ASN414, and TYR572 via hydrogen bonds and ARG415 via Pi Cation. Epicatechin interacted with SER363, SER555, SER602, and ASN414 via hydrogen bonds, while kaempferol interacted with SER602 and ASN414 via hydrogen bonds, TYR572 via Pi-Pi Stacking, and ARG415 through Pi Cation.

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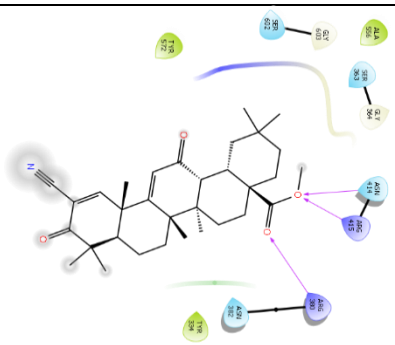
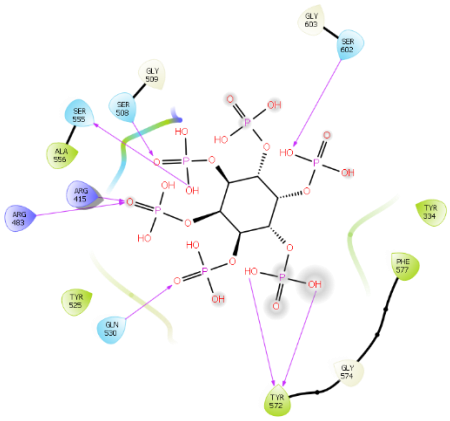
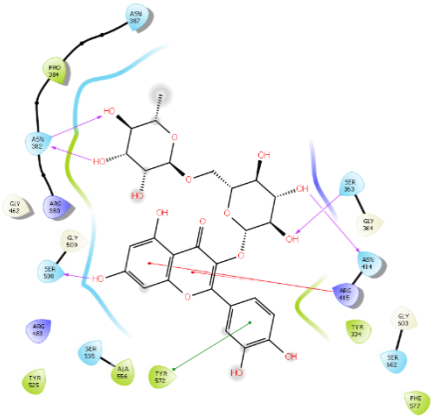
Table 3: Molecular docking results of *Picralima nitida* compounds with NRF2 protein

Compound	Docking Score (Kcal/mol)	MMGBSA (ΔGbind)	2D Visualization	Interactions: Amino acid residue
Bardoxolone methyl	-3.382	-45.77		H-Bonds: ASN414, ARG415, ARG380
Phytate	-12.603	-55.02		H-Bonds: ARG415, AEG483, SER508, SER555, SER602, GLN530, TYR572
Rutin	-11.680	-77.28		H-Bonds: ASN382, SER363, SER508, ASN414 Pi-Pi Stacking: TYR572 Pi Cation: ARG415

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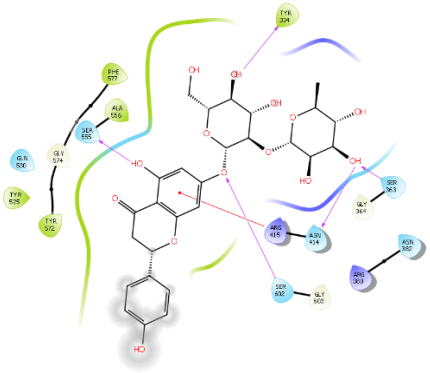
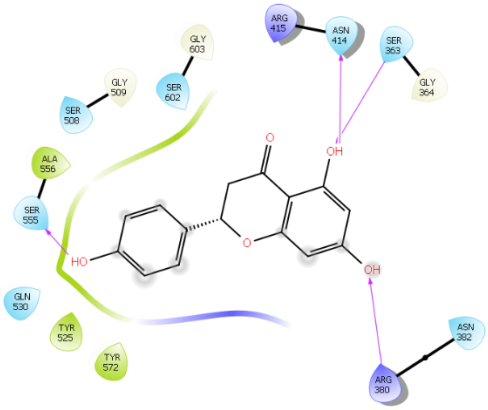
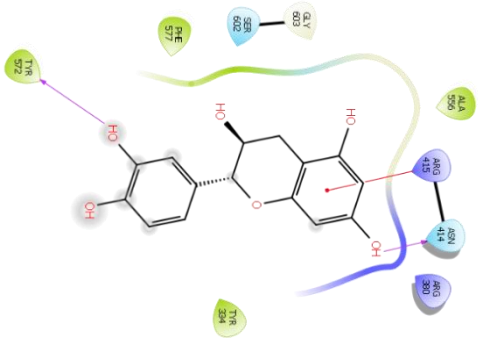
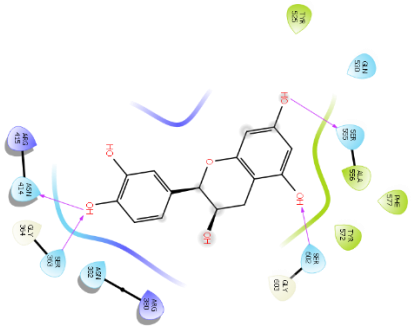
Naringin	-7.902	-55.96		H-Bonds: TYR334, SER363, ASN414, SER602, SER555 Pi Cations: ARG415
Naringenin	-7.138	-39.37		H-Bonds: SER363, ASN414, ARG 380
Catechin	-6.758	-52.71		H-Bonds: ASN414, TYR572 Pi Cation: ARG415
Epicatechin	-6.688	-41.05		H-Bonds: SER363, SER555, SER602, ASN414

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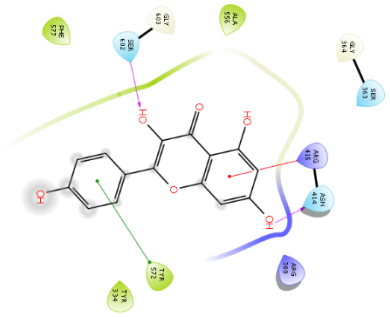
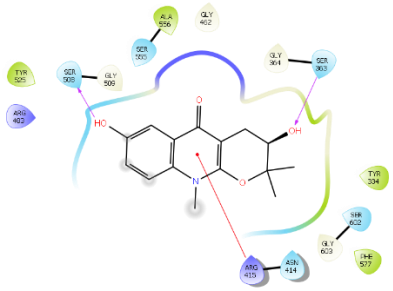
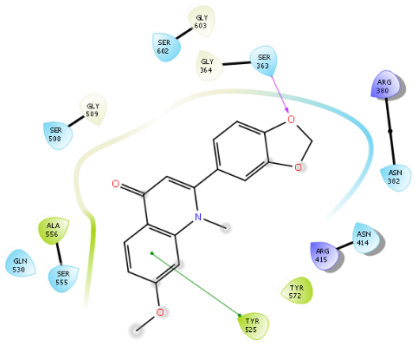
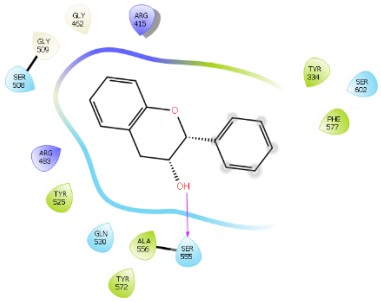
Kaempferol	-6.185	-45.98		H-Bonds: SER602, ASN414 Pi-Pi Stacking: TYR572 Pi Cation: ARG415
Ribalindine	-5.464	-41.72		H-Bonds: SER363, SER508 Pi Cations: ARG 415
Lunamarin	-4.559	-46.83		H-Bonds: SER363 Pi-Pi Stacking: TYR525
Flavan-3-ol	-4.315	-44.59		H-Bonds: SER555

Ribalindine formed hydrogen bonds residues at SER363 and SER508, and Pi Cations at ARG 415. The last compounds had minimal interactions with the docking pocket with lunamarin forming hydrogen bond at SER363 and Pi-Pi Stacking at TYR52 while flavon-3-ol formed only one hydrogen bond at SER555. However, Bardoxolone methyl formed hydrogen bonds at ASN414, ARG415, and ARG380; the more elaborate interaction by most of the compounds with the docking site could account for why they possess stronger binding affinity than the reference compound.

Post docking analysis

Table 4 shows the physicochemical properties of the isolated compounds. The quantitative estimation of drug-likeness (QED) evaluates the features of compounds to determine if they are desirable drug molecules. It operates on a probability range of 0.34 to 0.67 for fairly unattractive compounds with compounds ranked below considered too complex to be drugs while those above are considered attractive drug candidates.[38] The compounds; phytate, rutin, and naringin performed lower than the reference compound, while the other compounds screened achieved a higher QED score with Naringenin, Ribalinidine, Lunamarin, and Flavan-3-ol identified as attractive drug candidates.

Table 4: Physicochemical properties of compounds identified in *Picralima nitida* stem bark

Compound	QED	SAS	Lipinski	logS (-5 to -1)	logP (0 to 5)	logD7.4 (-1.5 to 4.5)
Bardoxolone methyl	0.3860	4.9360	0.0000	-5.8790	3.7493	3.5917
Phytate	0.0920	3.2140	1.0000	2.1543	-6.3882	-4.0463
Rutin	0.1400	4.7830	1.0000	-2.3967	0.9861	1.4544
Naringin	0.2020	4.7380	1.0000	-2.3529	0.4747	1.2515
Naringenin	0.7420	2.8250	0.0000	-4.0207	2.5956	2.6778
Catechin	0.5100	3.3440	0.0000	-2.5814	1.1726	1.5373
Epicatechin	0.5100	3.3440	0.0000	-2.5204	1.0942	1.5323
Kaempferol	0.5460	2.3750	0.0000	-3.6480	1.9653	1.9311
Ribalinidine	0.7600	3.4240	0.0000	-2.9473	1.0645	1.5275
Lunamarin	0.7300	2.2460	0.0000	-4.6023	2.8246	2.9135
Flavan-3-ol	0.8100	2.5880	0.0000	-2.2588	2.2758	2.4058

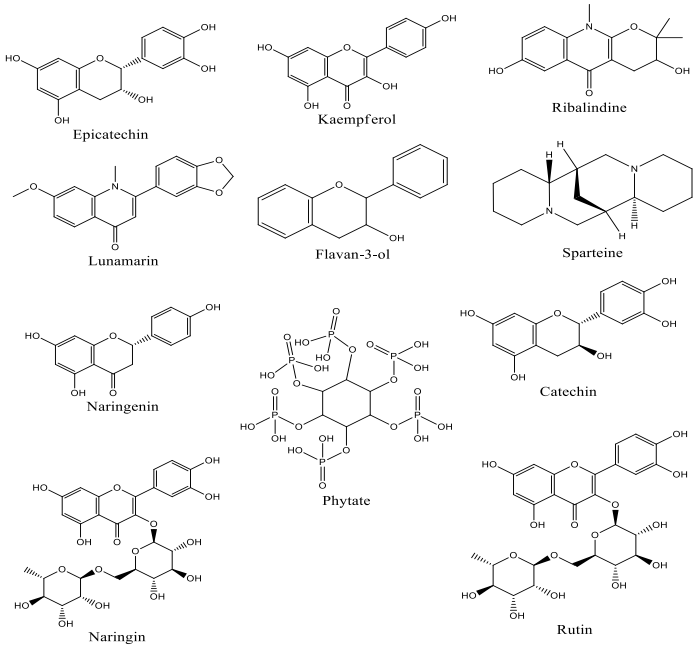


Figure 4: Molecular structures of compounds identified from the HPLC analysis of *Picralima nitida* stem bark extract

The synthetic accessibility score (SAS) uses a similar scale to rate the ease of synthesis of compounds with molecules rated less than 6.0 considered easy to synthesize while those rated above are considered difficult to synthesize.[39] The compounds had SAS ranging from 2.2460 to 4.7830, outperforming the reference compound (SAS = 4.9360). Lipinski's rule is an empirical measure of oral bioavailability, and of all the compounds, phytate and rutin violated more than one of these rules reducing the likelihood of oral availability for both compounds. However, natural products are frequently cited as exclusion to Lipinski's rule because the environment is capable of sustaining low hydrophobicity and intermolecular hydrogen bond donating ability in dealing with high molecular weight bioactive compounds.[40] The solubility assessments (Log S, Log P and LogD7.4 i.e. Log P at pH 7.4) revealed all compounds having solubility values within normal ranges (logS: -5 to -1, logP: 0 to 5, logD7.4: -1.5 to 4.5) and generally comparable to the reference ligand.[41]

Tables 5 – 7 highlight the key ADMET parameters. Caco-2 permeability is a measure of gastrointestinal absorption of the drug and compounds possessing higher values than -5.15 log unit are predicted to have optimal absorption. The reference compound met this criterion along with naringenin, ribalindine, lunamarin, and flavon-3-ol. Organic anion transporters (OATP1B1 & OATP1B3) are found in hepatocytes, and are used for drug transport from the bloodstream into the hepatocytes. Inhibitors of these channels have the potential to interfere with the metabolism of other drugs using these channels; all compounds except ribalindine were predicted to inhibit these channels. The probability of having bioavailability above 50% showed that all compounds except phytate had high probability of having high bioavailability. The tendency to cross the blood-brain barrier (BBB) was also estimated and only flavon-3-ol showed high probability. The plasma protein binding (PBB) predictions showed that some of the compounds (Naringenin, epicatechin, kaempferol, lunamarin, and flavon-3-ol) including the reference compound has plasma protein binding above 90%. However, lower values are required for optimal activity.

The reference compound was predicted to be a substrate and inhibitor of key metabolic enzymes in the liver with a moderate clearance of 7.4350 mL/min/kg and ultra-short half-life of 0.4803 hour. Naringenin, kaempferol, and lunamarin were the only compounds that showed high probability of inhibiting key enzymes, while ribalindine, lunamarin, and flavon-3-ol had high probability of being substrates for key enzymes. The plasma clearance was analyzed with a range of 5 to 15 mL/min/kg as moderate with higher values indicating high clearance and lower values indicating low clearance. Besides phytate, rutin, and naringin, the other compounds had moderate plasma clearance with catechin and epicatechin showing high clearance. Lower clearance values could be indicative of tissue accumulation.

The toxicity profile was evaluated and only 5 compounds had low probability of carcinogenicity; Rutin, naringin, catechin, epicatechin, and flavan-3-ol (Table 7). These 5 compounds had low to moderate probability for causing toxicities in at most 2 different organs with rutin standing out with only risk of ototoxicity. Comparison with the reference compound here may not paint a good picture as the reference compound has been shown to pose serious health risks through *in-vitro* and *in-vivo* studies.

Table 5: Absorption and distribution properties of compounds identified in *Picralima nitida* stem bark

Compound	Caco2 (Log unit)	f50	OATP1B1	OATP1B3	BBB	PPB (%)
Bardoxolone methyl	-5.1479	0.9573	0.9993	0.9825	0.0248	94.7874
Phytate	-5.4470	0.0576	0.9985	1.0000	0.0585	44.7913
Rutin	-6.5465	0.9999	0.9964	0.9999	0.0000	85.0054
Naringin	-6.7645	0.9997	0.9997	1.0000	0.0000	78.1044
Naringenin	-4.9873	0.9985	0.9928	0.9999	0.0002	94.9868
Catechin	-6.1686	0.9930	0.9986	0.9972	0.1353	89.2808
Epicatechin	-6.4723	0.9960	0.9627	0.9868	0.0911	92.6505
Kaempferol	-5.9693	0.9833	0.9188	0.9949	0.0010	97.8808
Ribalinidine	-4.9430	0.7886	0.2242	0.5802	0.0360	69.3792
Lunamarin	-4.8997	0.7188	0.9305	0.9983	0.4209	91.4142
Flavan-3-ol	-4.6445	0.8499	0.8736	0.9742	0.9998	94.7813

Table 6: Metabolism and excretion properties of compounds identified in *Picralima nitida* stem bark

Compounds	CYP2C19 Inhibitor	CYP2C19 Substrate	CYP3A4 Inhibitor	CYP3A4 Substrate	cl-plasma (ml/min/kg)	t0.5 (hour)
Bardoxolone methyl	0.9736	0.9741	0.9996	1.0000	7.4350	0.4803
Phytate	0.0000	0.0000	0.0000	0.0000	-0.3055	2.4520
Rutin	0.0000	0.0000	0.0292	0.0000	1.6107	4.6160
Naringin	0.0003	0.0000	0.0003	0.0000	1.8583	4.3946
Naringenin	0.5261	0.0005	0.9987	0.0000	6.8940	1.3119
Catechin	0.0000	0.0003	0.0002	0.0000	16.4553	2.4427
Epicatechin	0.0001	0.0007	0.0015	0.0011	15.8288	2.2679
Kaempferol	0.1324	0.0005	0.9745	0.0017	5.6944	1.3876
Ribalinidine	0.0095	0.2744	0.0226	0.5670	8.4344	1.2914
Lunamarin	0.6063	0.9983	1.0000	0.0144	5.1116	0.4172
Flavan-3-ol	0.8442	0.5339	0.0060	0.9763	10.1385	2.3010

Table 7: Toxicity profile of compounds identified in *Picralima nitida* stem bark

Compound	Carcinogenicity	H-HT	Neurotoxicity	Ototoxicity	Hematotoxicity	Nephrotoxicity
Bardoxolone methyl	0.7839	0.7973	0.4629	0.6450	0.3777	0.8253
Phytate	0.8652	0.2169	0.0003	0.0269	0.0004	1.0000
Rutin	0.0466	0.4063	0.0004	0.8842	0.0235	0.1477
Naringin	0.0531	0.8949	0.0550	0.9871	0.2461	0.9865
Naringenin	0.5910	0.6728	0.6443	0.2510	0.0753	0.3282
Catechin	0.2261	0.5567	0.0584	0.6742	0.0332	0.0588
Epicatechin	0.2159	0.6110	0.1014	0.6347	0.1329	0.0832
Kaempferol	0.7160	0.3862	0.0387	0.0745	0.0446	0.0186
Ribalinidine	0.6546	0.5614	0.7058	0.6122	0.2621	0.2545
Lunamarin	0.9545	0.7458	0.9764	0.5405	0.8522	0.9562
Flavan-3-ol	0.4001	0.6671	0.7090	0.5485	0.2027	0.4220

Conclusion

The study confirmed antioxidant properties of the methanol extract of *Picralima nitida* stem bark, highlighting its potential therapeutic applications. Africa has a growing burden of these chronic diseases and natural products which are found in abundance on the continent can provide a source of cheap and easily accessible medication and potentially growing to meet global needs. The top three compounds which can form the basis for further research based on decreasing binding affinities and toxicity profiles are; rutin, naringin and catechin. Due to various limitations in the *in vitro* and computational experiments, further study is required to establish these compounds as candidates for lead optimization and subsequent drug candidates for *in-vivo* studies. As such, this study could provide a template for further research into formulating standardized pharmaceutical and nutraceutical products.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

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

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