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Research Article

In Vitro Antioxidant Activity, Mineral Composition, and *In Silico* Identification of Potential Human COX-2 Inhibitors from *Azadirachta Indica*: A Case Study of Rheumatoid Arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disorder marked by synovial inflammation and joint destruction, with Cyclooxygenase-2 (COX-2) significantly contributing to its pathogenesis. Traditional management includes NSAIDs and COX-2 inhibitors, which pose gastrointestinal and cardiovascular risks. *Azadirachta indica* (neem), known for its anti-inflammatory properties, was studied for its antioxidant activity, mineral content, and potential COX-2 inhibitors, emphasizing its relevance to RA treatment.

Fresh leaves of *neem* were extracted using n-butanol and evaluated for total phenolic content (TPC), total flavonoid content (TFC), and ferric reducing antioxidant power (FRAP) using standard protocols. Mineral content was determined by atomic absorption spectroscopy, and bioactive compounds were identified using high-performance liquid chromatography (HPLC). Identified phytochemicals were subjected to *in silico* analyses against human COX-2.

The fraction exhibited high TPC and TFC and demonstrated strong FRAP activity. HPLC analysis identified seven polyphenolic compounds, and mineral analysis revealed the presence of essential macro- and microelements, all within FAO/WHO permissible limits. E-pharmacophore screening, molecular docking, ADMET predictions, and AutoQSAR analysis identified ferulic acid as a promising COX-2 inhibitor, with ferulic acid showing the strongest binding affinity (−6.899 kcal/mol) and favorable MM-GBSA binding free energy (−34.71 kcal/mol).

This study demonstrates that *Azadirachta indica* leaf possesses significant antioxidant activity, a beneficial mineral profile, and bioactive compounds with promising COX-2 inhibitory potential. Ferulic acid stands out for its strong binding affinity, pharmacokinetic properties, and safety profile. The combined in vitro and in silico results highlight neem's potential as a natural anti-inflammatory agent for RA, necessitating further *in vivo* and clinical validation.

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease affecting the synovial and joint membranes, leading to cartilage destruction, pain, and bone deformation. Inflammatory mediators are crucial in causing inflammation, stiffness, and disability associated with RA [1]. Rheumatoid arthritis (RA) affects 0.5% of the global population annually, with a higher prevalence in women, who outnumber men threefold [2]. Several proinflammatory cytokines, including TNF-α (tumor necrosis factor), interleukin-1β, and others like enzymes of lysyl oxidase (LOX), cyclooxygenase II (COX-II), reactive oxygen species (ROS), prostaglandin-endoperoxide synthase (PTGS), prostaglandins, H_2O_2, TGF (transforming growth factor), and MCSF (macrophage colony-stimulating factor), play a significant role in the disease [3]. This study elucidates the role of cyclooxygenase-2 (COX-2) in rheumatoid arthritis progression while identifying potential COX-2 inhibitors from plant sources for therapeutic intervention.

Cyclooxygenase-2 (COX-2), also known as prostaglandin G/H synthase 2 (PGHS-2), is vital in the inflammatory response, working alongside cyclooxygenase-1 (COX-1) to regulate the synthesis pathway of prostaglandins and thromboxanes, collectively termed prostanoids [4]. Prostanoids play crucial roles in inflammation, fever, pain, renal function, and cardiovascular homeostasis [5-7]. Notably, COX-2 activities extend beyond inflammation, with increased COX-2 expression linked to several conditions such as cancers, neurodegenerative disorders, and nephropathies [5,8,9]. In rheumatoid arthritis (RA) patients, synovial tissues show upregulated COX-2 expression, which is induced rapidly by inflammatory stimuli. Anti-inflammatory agents like glucocorticoids can inhibit this up-regulation. Pro-inflammatory cytokines IL-1 and TNF-α drive the increased COX-2 expression by activating transcription factors such as NF-κB and c/EBP [10].

Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently utilized for their anti-inflammatory, anti-pyretic, analgesic, and anti-rheumatic properties [11]. Traditional NSAIDs are associated with significant gastrointestinal issues due to their inhibition of COX-1, prompting the creation of selective COX-2 inhibitors intended to mitigate these side effects [7,11]. COX-2 selective inhibitors increase the risk of myocardial infarction, stroke, and cardiovascular complications by reducing vascular prostacyclin (PGI2) production, disrupting homeostasis [7,12]. This imbalance between COX-2-derived PGI2, which promotes vasodilation and inhibits platelet aggregation, and thromboxane A2 (TXA2), which promotes thrombosis, elevates the risk of thrombotic cardiovascular events [12,13]. These adverse effects accentuate the need for safer substitutes as well as interest in natural compounds as possible COX-2 inhibitors.

Neem (*Azadirachta indica A. Juss*), which belongs to the Meliaceae family, is a non-deciduous tree indigenous to the Indian subcontinent. It is the most adaptable and productive medicinal plant discovered to date. Every part of this plant is loaded with bioactive substances, which have been traditionally used to cure a variety of ailments, including those caused by infections. Bioactive compounds like nimbolide, azarirachtin, and gedunin present in neem are reported to effectively control many biological activities both in vitro and *in vivo*. [14]. The therapeutic uses have been described especially for neem leaf. Neem leaf and its components exhibit anti-inflammatory, antihyperglycaemic, antioxidant, antimutagenic, and anticarcinogenic properties [15]. Ayurvedic traditions leverage neem for arthritic swellings, supported by its multi-target anti-inflammatory effects [14]. The present study profiles the in vitro antioxidant activity, bioactive compounds, mineral composition, and in silico identification of potential COX-2 inhibitors for rheumatoid arthritis from the n-butanol fraction of *Azadirachta indica*. Outcomes could advance neem as adjunctive RA therapy

Materials and methods

Collection and identification of plant samples

The leaves of *Azadirachta indica* were obtained from Akungba Akoko, Ondo state, Nigeria. The plant was subsequently identified and authenticated in the herbarium of the Plant Science and Biotechnology Department, Adekunle Ajasin University, Akungba Akoko, and voucher specimens were deposited for further reference.

Preparation of plant extract

The plant extract was prepared following the method described by Shodehinde et al. [16] as reported by Bello et al. [17]. Fresh *Azadirachta indica* leaves underwent washing, were shade-dried for 3 weeks, and pulverized, and then 100 g powder was soaked in 750 mL n-butanol for 3 days (72 hours). The filtrate was evaporated over a period of 2 weeks to obtain dried extract, which was then used for antioxidant assays, High-Performance Liquid Chromatography (HPLC), and Atomic absorption spectroscopy (AAS) analysis.

In vitro assay determination

Determination of total phenolic content

The total phenolic content was determined according to the method of Singleton et al. [18]. Briefly, appropriate dilutions of the extracts were oxidized with 2.5 mL of 10% Folin-Ciocalteu's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C, and the absorbance was measured at 765 nm. The total phenolic content was subsequently calculated as gallic acid equivalent (GAE).

Determination of flavonoids content

The total flavonoid content of the extract was determined using a slightly modified method reported by Meda et al. [19]. Briefly, 0.5 mL of appropriately diluted sample was mixed with 0.5 mL methanol, 50 µL of 10% $AlCl_3$, 50 µL of 1M potassium acetate, and 1.4 mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the

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reaction mixture was subsequently measured at 415 nm. The total flavonoids were calculated as quercetin equivalent (QE).

Determination of ferric reducing antioxidant property

The reducing property of the extracts will be determined by assessing the ability of the extract to reduce FeCl3 solution as described by Oyaizu [20]. A 2.5 mL aliquot was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then 2.5 mL of 10% trichloroacetic acid was added. The mixture was centrifuged at 650 rpm for 10 min. 5 mL of the supernatant was measured with an equal volume of water and 1 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric-reducing antioxidant property was subsequently calculated as ascorbic acid equivalent (AAE).

Atomic absorption spectroscopy

The Principle of operation of an atomic absorption spectrometer using a flame ionization detector (FID) requires a liquid (digested) sample to be aspirated, aerosolized, and mixed with combustible gases, such as acetylene and air or acetylene and nitrous oxide (this test utilizes acetylene and air). The mixture is ignited in a flame whose temperature ranges from 2100 to 2800°C. During combustion, atoms of the element of interest in the sample are reduced to free, unexcited ground-state atoms, which absorb light at characteristic wavelengths. To provide element-specific wavelengths, a light beam from a lamp whose cathode is made of the element being determined is passed through the flame. A photomultiplier detects the amount of reduction of the light intensity due to absorption, and this is directly related to the amount of the element in the sample. A series of standard solutions for each metal ion was prepared using deionized distilled water and stock solutions (1000 ppm): 0.00, 0.20, 0.50, 0.60, and 1.00. To obtain accurate quantitative data, the regression coefficient of the standard calibration curve for each element was greater than 0.9960. The Buck Scientific Atomic Absorption Spectrometer Model 210 VGP was used for this analysis.

Digestion procedure

The digestion of the sample was carried out according to the method described by Shodehinde et al. [16] as reported by Bello et al. [17]. One gram (1 g) of the sample was taken into A 250 mL conical flask, 10 mL of conc. Nitric acid was added, and the mixture was placed on a hot plate for about 35 min until the brown fumes started forming and the fume changed gradually to a whitish color, which shows that the samples have been completely digested. The digested samples were allowed to cool and later made up to the 25 mL mark with purified water. The mixture was filtered through a micro glass filter or clean filter paper, and the samples were ready for AAS analysis. Some measures of QA/QC that were put in place in the course of the analysis include replicate determination of samples to ascertain the degree of reproducibility of the data, blank determination was carried out at intervals to prevent carryover of the samples, and a known standard concentration was analyzed like a sample to check the accuracy of the machine.

HPLC analysis

The High-Performance Liquid Chromatography (HPLC) analysis of the n-butanol fraction of *Azadirachta indica* was performed using a NIMR 1260LC instrument equipped with a Poroshell 120 EC C18 column (4 µm, 150×4.6 mm). The mobile phase consisted of acetonitrile (ACN) and 0.1% formic acid in a 70:30 ratio. The flow rate was maintained at 0.700 mL/min, while the column temperature was set to 28°C. The detection was carried out at a wavelength of 257 nm using a diode array detector (DAD). For each injection, a sample volume of 20 µL was used [16,17]. Standard compounds such as ferulic acid, tannic acid, maleic acid, salicylic acid, rutin, saponin, and p-coumaric acid were identified based on their retention times. The data was analyzed using the ChemStation software, and the retention times were compared against reference standards to confirm the identity of the compounds present in the sample.

In Silico Study

Bioactive compounds that were identified from HPLC analysis of the n-butanol fraction of *Azadirachta indica* were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). The protein (human cyclooxegenase 2 [5F1A]) with co-crystallized ligand (salicylate) was retrieved from http://rcsb.org. The protein and compounds, including the co-crystallized molecule extracted from the protein, were prepared using the protein and ligand preparation module of Maestro Schrödinger 12.8, respectively.

An e-pharmacophore was generated and used to screen seven compounds identified from *Azadirachta indica* and reference drugs (Celecoxib and Etoricoxib) in search of potential lead compounds that have the appropriate pharmacophoric characteristics. The phase module from Maestro Schrödinger was used for pharmacophore modelling, and the receptor-ligand complex was used as input to create a pharmacophore model. The model featured two distinct characteristics. Strict screening of criteria (2 out of 2 features) was applied to filter the compounds, resulting in two compounds passing the screening. The scoring coordinates of the human cyclooxigenase-2 binding pocket were determined based on the co-crystallized ligand using the receptor grid generation module of Maestro Schrödinger 12.8. The x, y, and z grids are 41.81, 23.96, and 240.14, respectively.

Compounds that fit with the expected pharmacophore theory were subjected to HTVS (High Throughput Virtual Screening), followed by SP (standard precision) and XP (extra precision) docking to correct false-positive results. The docking protocol was validated by re-docking the prepared co-crystallized ligand extracted from the protein into the binding site of human cyclooxygenase-2. The calculated root mean square deviation (RMSD) of 0.8643 Å confirms the reliability and reproducibility of the docking approach. The molecular mechanics generalized Born surface (MM-GBSA) tool integrated with Prime of the Maestro Schrödinger 12.8 was employed to calculate the binding free energy of the docked complexes. The relative free energy of the docked complexes was computed using the OPLS4 force field and VSGB solvent.

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This analysis aimed to determine whether the interactions between the ligands and the target protein were sufficiently strong to potentially induce a biological response [21].

ΔGbind = ΔGcomplex – (ΔGligand + ΔGprotein)

The ADMET study was carried out to evaluate the pharmacokinetic profile of the docked compounds, including drug-likeness properties, Lipinski's rule of five violations, Lipophilicity, and water solubility using SwissADME (https://www.swissadme.ch/). Organ toxicity and toxicity endpoints were predicted using ProTox 3.0 (https://tox.charite.de/protox3/).

Experimental data regarding the activity of human cyclooxygenase 2 inhibitors were sourced from the CHEMBL database. To initiate the search for antagonists, the protein's FASTA sequence extracted from the PDB was employed to query the CHEMBL database (https://www.ebi.ac.uk/chembl/). Subsequently, the resulting list of inhibitors was transformed into an ".SDF" format through the utilization of DataWarrior software [21]. These prepared ligands were then used for the creation of a QSAR model using the AutoQSAR module. Among the generated models, the best model was determined based on ranking, leading to the selection of the kpls_molprint2D_40 model. This chosen model was subsequently applied to predict the bioactivity of the top compounds highlighted in the research.

Statistical analysis

Antioxidant assays were conducted in triplicate. Data are presented as mean ± standard deviation (SD) and visualized using GraphPad Prism. Given the nature of the antioxidant study with only two samples, descriptive statistics are reported, and no formal statistical comparisons were performed.

Results and discussion

In Vitro Antioxidant

In recent years, the prevention of oxidative stress and exploration of medicinal plants' potential to combat it have driven extensive research. Reactive oxygen species (ROS), or free radicals, arise from external chemicals and metabolic processes. Excessive ROS leads to oxidative stress, disrupting the balance of oxidants and antioxidants. This imbalance can damage biomolecules such as nucleic acids, proteins, lipids, and DNA, potentially causing cancer, cardiovascular diseases, muscular degeneration, neurological disorders, and inflammation [17]. Maintaining this balance is crucial for biological health, and external antioxidants can alleviate oxidative stress by acting as free radical scavengers and reducing agents, thereby minimizing associated damage [22,23]. Investigation of the *in vitro* Total Phenol Content (TPC), Total Flavonoids Content (TFC), and ferric reducing antioxidant power (FRAP) revealed that n-butanol extract of *Azadirachta indica* leaf (Figures 1-3) possessed an appreciable amount of TPC, TFC, and potential to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Total phenol content is a measure of the number of phenolic compounds in a sample, and phenolic compounds, known for their electron-donating properties, are key contributors to the neutralization

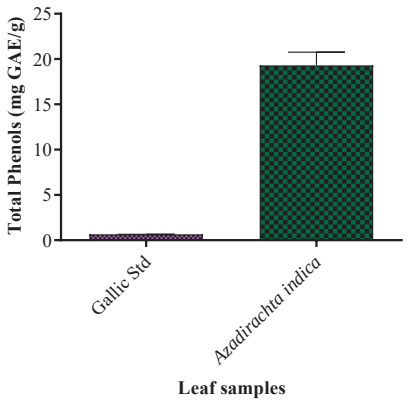

Figure 1: Total phenol content of n-butanol extract of *Azadirachta indica* leaf.

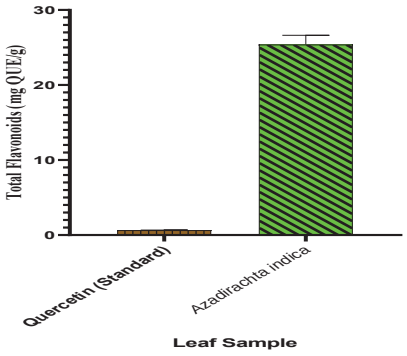

Figure 2: Total flavonoid content of n-butanol extract of *Azadirachta indica* leaf.

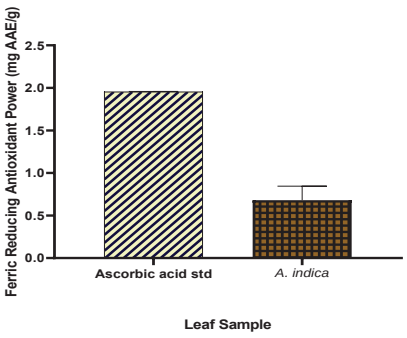

Figure 3: Ferric Reducing Antioxidant Power of n-butanol extract of *Azadirachta indica* leaf.

of free radicals and oxidative stress [17]. Flavonoids are recognized for their diverse biological activities, including antioxidant, anti-inflammatory, and anticancer properties, and play an essential role in safeguarding cells from oxidative damage [24]. The TPC and TFC of the n-butanol extract of *Azadirachta indica* were significantly high compared to the known standard gallic acid and quercetin, respectively, with a remarkable demonstration of ability to reduce (Fe^{3+}) to ferrous ions (Fe^{2+}). These might be associated with the presence of polyphenols, as evident in the HPLC analysis (Figure 4).

HPLC analysis of the n-butanol fraction of Azadirachta indica leaf

The results of the HPLC study of *Azadirachta indica* n-butanol

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fraction showed the presence of seven polyphenols in the increasing concentration order of p-coumaric acid > Ferulic acid > Maleic acid > Salicylic acid > Tannic acid > Saponin > Rutin (Figure 4, Table 1). Notably, p-coumaric acid, ferulic acid, salicylic acid, saponin, rutin, and tannic acid have previously shown potential anti-arthritis activity. The p-coumaric acid, a hydroxylated derivative of cinnamic acid, helps prevent the oxidation of low-density lipoproteins and decreases the risk of stomach cancer [25]. In an *in vivo* study using a rat model of adjuvant-induced arthritis, p-coumaric acid intake significantly reduced the expression of TNF-α [26]. Through preventing ECM degradation, suppressing inflammatory responses, and inhibiting oxidative stress via modulation of the Sirt1/AMPK/PGC-1α signaling pathway, ferulic acid has potent protective effects on IL-1β-induced osteoarthritis degeneration [27]. Maleic acid is a versatile chemical used to make resins (polyesters, coatings), agricultural chemicals, and pharmaceuticals, but it's also vital for creating fumaric acid, converting into the widely known food/cosmetic additive malic acid, and serving as a polymer additive, water treatment agent, and pH stabilizer in various products, from adhesives to orally disintegrating tablets, showcasing its role in enhancing material properties and product stability. Salicylic acid, the precursor to aspirin and related salicylates, is one of the first effective treatments for rheumatoid arthritis and other inflammatory arthritides. Its derivatives, including 5-aminosalicylic acid and sulfasalazine, alleviate joint pain and swelling primarily by inhibiting prostaglandin production and modulating inflammatory cytokines [28,29]. Tannic acid (TA) directly binds interleukin-1β (IL-1β) and prevents IL-1β–IL-1 receptor 1 interaction, suppressing downstream MAPK and NF-κB signalling. In rodent arthritis and osteoarthritis models, TA reduces pain, cartilage destruction, and expression of inflammatory mediators, suggesting utility against IL-1β-driven joint diseases such as RA [30]. Saponin-rich fractions from *Achyranthes aspera* reduce paw swelling, enhance pain thresholds, and improve oxidative stress in rats with adjuvant-induced arthritis. These benefits correlate with decreased TNF-α and pro-inflammatory cytokines, as well as restored antioxidant enzymes, suggesting their potential as anti-arthritic agents [31]. Rutin, a flavonol glycoside, attenuates CFA-induced RA in rats, reducing paw edema, oxidative stress, TNF-α levels, and nociceptive behaviours while improving antioxidant enzyme activity [32] [Table 1].

Mineral analysis (mg/kg)

Table 2 shows the mineral content of the n-butanol fraction of *Azadirachta indica*. Calcium is essential for the health of bones, teeth, the nervous system, and muscles, making it crucial for children's nutrition. Additionally, sodium, a common element, regulates body fluid levels [33, 34]. Magnesium has an important role in the functioning of the immune system, muscles, heart health, and nerves [35]. Potassium is a crucial mineral that supports muscle balance, nerve function, electrolyte balance, and cardiovascular health. Potassium, in addition to magnesium and other macro elements, including calcium and sodium are required by the

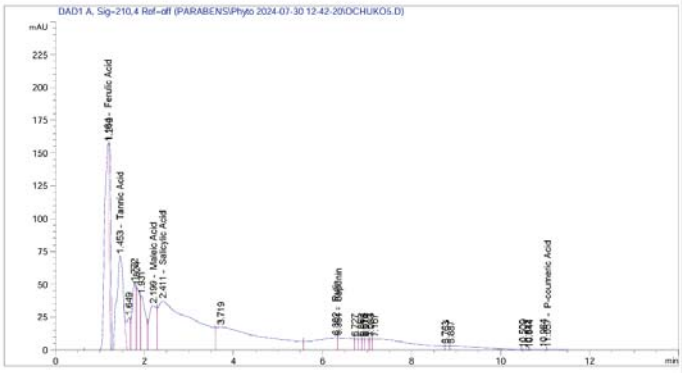

Figure 4: Chromatogram of HPLC compounds identified from n-butanol extract of *Azadirachta indica* leaf.

Table 1: Concentration of polyphenolic contents in n-butanol fraction of *Azadirachta indica* leaf.

Compounds	Concentration (mg/L)
Ferulic acid	1.82171
Tannic acid	0.725113
Maleic acid	0.879928
Salicylic acid	0.734058
Rutin	0.360900
Saponin	0.702130
p-coumaric acid	2.56258

Table 2: Showing the concentration of minerals in *Azadirachta indica*.

Mineral contents	Calcium	Magnesium	Iron	Copper	Manganese	Potassium	Sodium	Zinc
Azadirachta indica	95.06	21.951	1.25	1.06	6.58	38.06	25.59	10.62

body in higher amounts and is more important than any other minerals [16, 36]. However, manganese, in addition to copper, iron, and zinc are regarded as a microelement that is required in trace amounts. As shown in table 2, the amount of manganese, copper, iron and zinc present in *Azadirachta indica* are 6.58 mg/kg, 1.06 mg/kg, 1.25 mg/kg and 10.62 mg/kg respectively which do not violates the Food and Agriculture Organisation of the United Nations together with the World Health Organisation FAO/WHO, (2011) guideline for metals in food and vegetables.

Manganese is an essential trace element involved in blood clotting and hemostasis, working alongside vitamin K. It is naturally present in many foods and can be taken as a dietary supplement. Manganese functions as a cofactor for various enzymes, including manganese superoxide dismutase, arginase, and pyruvate carboxylase [37, 38]. Copper is an essential mineral found in various foods and available as a dietary supplement. It serves as a vital trace element in humans, acting as a cofactor in electron transfer reactions, especially in the brain, where metal demand is particularly high [39]. The role of iron in the body includes making hemoglobin, a protein in red blood cells that carries oxygen throughout the body [16]. Zinc is an essential trace element vital for numerous physiological processes, including cell growth, metabolism, cognitive function, reproduction, and immune system health.

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Its importance in human health has prompted extensive research into its beneficial properties relative to other essential metal ions [40].

E-pharmacophore modeling and screening

The receptor-ligand complex was used as input to create a pharmacophore model. The model featured two distinct characteristics; however, among all compounds, including ferulic acid, tannic acid, maleic acid, salicylic acid, rutin, saponin, and p-coumaric acid identified from the n-butanol fraction of *Azadirachta indica* and standard drugs (Celecoxib and Etoricoxib), only ferulic acid and salicylic acid have the appropriate pharmacophoric characteristics.

Rheumatoid arthritis involves chronic synovial inflammation, primarily driven by cyclooxygenase-2 (COX-2), which converts arachidonic acid to pro-inflammatory prostaglandins, causing pain, swelling, and joint damage. The overexpression of COX-2 in affected tissues promotes persistent inflammation and joint destruction. Consequently, selective COX-2 inhibition is a key pharmacological strategy to reduce inflammatory prostaglandins while minimizing gastrointestinal side effects from non-selective NSAIDs [41, 28]. In this study, we utilized the PHASE module of Maestro Schrödinger to develop a pharmacophore hypothesis for e-pharmacophore modeling and based on the co-crystallized ligand with the human cyclooxegenase 2 active site, which resulted in two pharmacophoric features afterward (Figure 5), we screened nine compounds from the Pubchem database against these two features and two compounds (Ferulic acid and Salicyclic acid) matched all the features. To further validate this screening and investigate the interaction between COX-2 and the screened compounds, we utilized the Glide docking tool to conduct molecular docking of the selected compounds and to re-dock COX-2 co-crystalized ligand, where docking scores (Table 3) analysis and detailed investigation of the 2D and 3D interactions between COX-2 and the top two compounds and the reference molecule was carried out using the graphical depiction of the Schrodinger ligand interaction diagram, as seen in Figure 6 and Supplementary Figure 1.

Molecular docking and MMGBSA analysis of identified compounds from *Azadirachta indica* against human cyclooxygenase-2.

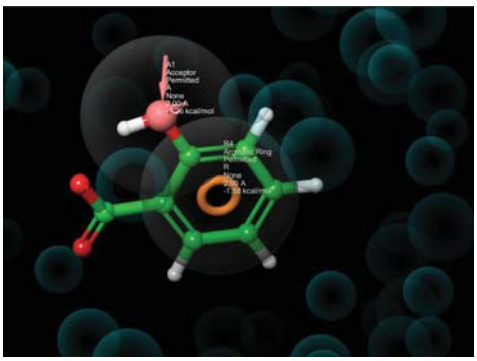

Figure 5: A pharmacophore model used to screen for potential active compounds against the human cycooxegenase-2 target.

Table 3: Docking score and MMGBSA analysis.

Compounds	Docking score (kcal/mol)	MMGBSA dG bind (kcal/mol)
Ferulic acid	-6.899	-34.71
Co-crystallized ligand	-6.559	-30.92
Salicylic acid	-6.527	-29.28

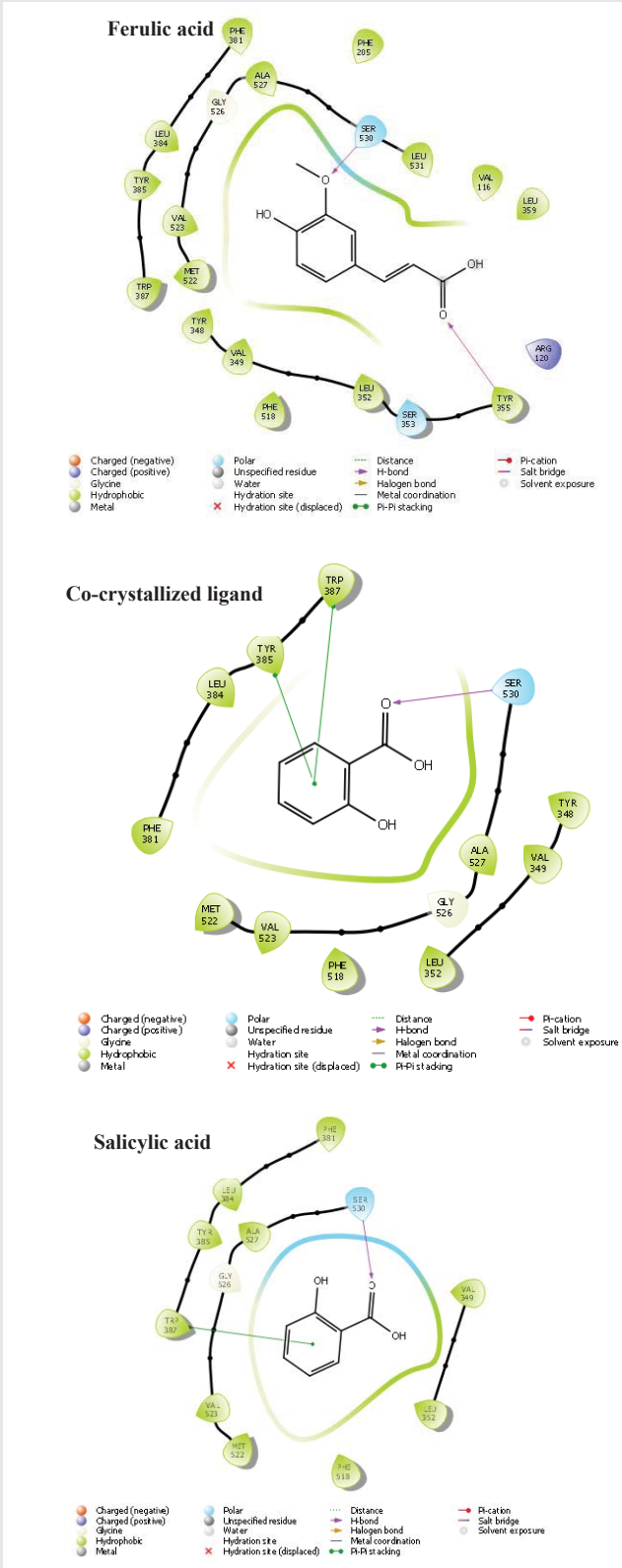

Figure 6: Two-dimensional presentation of interactions of compounds with catalytic pocket of human cyclooxegenase-2 (5F1A).

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One crucial tool for drug design and discovery is molecular docking. It implies how small compounds effectively engage with the specific receptor's binding site [42]. Both Ferulic acid (-6.899 kcal/mol) and Salicylic acid (-6.527 kcal/mol) showed varying degrees of binding affinities for cyclooxygenase 2; Ferulic acid had higher docking scores against the target protein compared to the reference ligand (-6.559 kcal/mol) (Table 3). This is consistent with an earlier report where ferulic acid demonstrated an inhibitory effect as a potential COX-2 enzyme binder in the hydrophobic pocket stabilized mainly by two hydrogen bonds [43]. The ligands interacted with amino acids present in the binding pockets of cyclooxygenase 2 with molecular interactions such as hydrogen bonds and pi-pi stacking (Figure 6). The interaction of small molecules with the amino acid at the binding site of the target is vital for the inhibition of such a protein [44].

Molecular docking was further validated using MMGBSA calculations. Ferulic acid (-34.71 kcal/mol) and Salicylic acid (-29.28 kcal/mol) displayed exceptionally excellent binding affinity. These top two ligands adhere to the two pharmacophore characteristics and ferulic acid, particularly demonstrating stronger binding free energy compared to the reference ligand (-29.28 kcal/mol).

ADMET Profile

Molecule 1; Ferulic acid, Molecule 2; Co-crystallized ligand, Molecule 3; Salicylic acid

The two most promising compounds that interact with the COX-2 protein in this research and the reference ligand were selected for the ADMET study. The ADMET study was done for the lead compounds to probe their physicochemical, pharmacokinetics, drug-likeness, and toxicity properties [45]. From Figure 7, the BOILED-Egg is a method for predicting two key ADME parameters at the same time, namely passive gastrointestinal absorption (HIA) and Blood-Brain Barrier (BBB). The blue dots indicate P-gp substrates (PGP+), and the red dots indicate P-gp non-substrates (PGP-). The yellow portion (yolk) is for a high probability of brain penetration, and the white region is for a high probability of passive absorption by the gastrointestinal tract. Yolk and white areas are not mutually exclusive. The points are coloured blue if the compound is predicted as actively effluxed by P-gp (PGP+) and red if predicted as a non-substrate of P-gp (PGP-). The points outside the egg are predicted as non-absorbent or non-penetrant (outside the egg). All molecules were predicted to be BBB-permeant and non-PGP substrates (Figure 7) [Table 4].

The bioavailability score is a measure of a compound's potential to be absorbed and become bioavailable when administered [46]. The drug-likeness and oral bioavailability of the selected *Azadirachta indica* compounds were evaluated based on Lipinski's Rule of Five and bioavailability scores. Ferulic acid and Salicylic acid showed no violations of Lipinski's rules, each with a bioavailability score of 0.85, indicating good oral absorption potential compared to the co-crystallized (Table 4). This signifies that 85% of each lead compound would be readily bioavailable in the cell and get to the site of action ligand. These results support the suitability of the plant-derived compounds as promising drug-like candidates with favorable pharmacokinetic properties. Table 5 depicts the information about the lipophilicity (Consensus Log Po/w) and water solubility (SILICOS-IT) of compounds. The result indicated that all the compounds have a balanced character, with good solubility in water. Excessively lipophilic compounds may face absorption issues, while those that are overly hydrophilic might have poor distribution in the body. The water solubility of the compounds, classified as "Soluble" by Silicos-IT, suggests they possess good water solubility, which is crucial for bioavailability. High water solubility enhances distribution and therapeutic potential, while poor solubility can reduce effectiveness [47]. The results indicate that all compounds maintain a balanced character with good solubility, positively impacting their prospects as lead candidates in drug development due to enhanced absorption and bioavailability. The drug-likeness and bioavailability predictions for the compounds suggested characteristics that are typically associated with successful oral absorption and bioavailability. Physicochemical analysis of selected *Azadirachta indica* leaf compounds revealed that all tested phytochemicals, including ferulic acid (194.18 g/mol) and Salicylic acid (138.12g/mol), fall within a suitable molecular weight range and are classified as soluble based on Silicos-IT predictions. Their consensus Log P values ranged from 1.36 (ferulic acid) to 1.24 (Salicylic acid), indicating moderate lipophilicity, while Log S values varied from 1.26 to 0.74, supporting their solubility and favorable drug-like properties for the phytochemicals in Table 5.

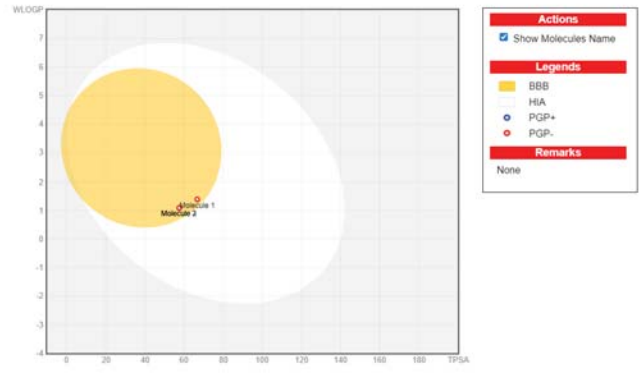

Figure 7: The BOILED-Egg model is used for predicting HIA and BBB.

Table 4: Drug-likeness and bioavailability prediction with SwissADME.

Compounds	Lipinski violation	Bioavailability score
Ferulic acid	0	0.85
Co-crystallized	0	0.85
Salicylic acid	0	0.85

Table 5: Lipophilicity (Consensus Log Po/w) and water solubility (Log S) prediction with SwissADME.

Compounds	Molecular weight (g/mol)	Consensus Log Po/w	Log S (SILICOS-IT)	SILICOS-IT Class
Ferulic acid	194.18	1.36	1.26	Soluble
Co-crystallized	138.12	1.24	0.74	Soluble
Salicylic acid	138.12	1.24	0.74	Soluble

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The ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) property predictions for the compounds (Table 6): Ferulic acid, Co-crystallized, and Salicylic acid. These predictions are essential in evaluating the potential safety and efficacy of these compounds as drug candidates [48]. As for the prediction, all compounds were predicted to have high gastrointestinal (GI) absorption and BBB Permeant (Blood-Brain Barrier Permeability), indicating balanced absorption through the gastrointestinal tract (GIT) and central nervous system (CNS), which can be advantageous for therapeutic applications.

The Pgp is a transporter protein that can influence the absorption and distribution of drugs. All compounds are predicted to be non-P-glycoprotein (Pgp) substrates. Being a Pgp substrate may affect the bioavailability and distribution of these compounds. Ferulic acid and Salicylic acid are not predicted to be Pgp substrates, suggesting that they may not be as strongly influenced by Pgp-mediated efflux mechanisms, thereby leading to improved oral bioavailability. CYP (Cytochrome P450) enzymes are a vast superfamily of heme-containing proteins that play a key role in breaking down drugs, toxins (xenobiotics), and endogenous substances, mainly in the liver and other tissues. None of the compounds in this study were predicted to inhibit Cytochrome p450 enzymes, indicating a reduced chance of increased side effects or toxicity (Tables 6,7).

The LD_{50} (mg/kg): The LD_{50} is a measure of acute toxicity and represents the dose at which 50% of a tested population is expected to die as a result of exposure to a substance [49]. A higher LD50 value indicates lower acute toxicity, while a lower LD_{50} value suggests higher toxicity. Ferulic acid has an LD_{50} value of 1772 mg/kg. This relatively high LD_{50} value suggests low acute toxicity for Ferulic acid. Both the reference ligand and salicylic acid has LD50 of 1034 mg/kg, indicating low acute toxicity. The compounds are assigned a toxicity class, with a higher number indicating higher predicted toxicity. It's important to note that this classification is specific to the ProTox 3.0 model. All compounds belongs toxicity class 4 indicate a lower level of predicted toxicity (Table 7). Ferulic acid was predicted to be nephrotoxic, while the reference ligand and salicylic acid were predicted to be hepatotoxic and nephrotoxic. The ProTox 3.0 toxicity predictions provide insights into the acute toxicity of these compounds. While all compounds, including the co-crystallized ligand, were predicted to have low acute toxicity. The predictions for specific types of toxicity (hepatotoxicity, Nephrotoxicity, cardiotoxicity, carcinogenicity, mutagenicity, cytotoxicity) vary for each compound. Considering these findings, it can be inferred that ferulic acid has more favorable overall ADMET profiles and lower predicted acute toxicity compared to the reference ligand and salicylic acid. Salicylic acid, while demonstrating good ADMET properties, raises concerns due to its higher predicted toxicity. Ferulic acid stands out as a particularly strong candidate, as it combines favorable ADMET properties with low predicted acute toxicity, organ toxicity, and toxicity endpoints. The comprehensive evaluation of the compounds reveals valuable insights into their potential as drug candidates in terms of their ADMET properties and toxicity predictions.

AutoQSAR

The developed QSAR model accurately replicates the biological activities of the leading compounds and compares them to the standard human cyclooxygenase-2 inhibition. The AutoQSAR module was used in constructing the QSAR model. The best model (kpls_molprint2D_40) for predicting the pIC50 values of the top compounds has an R-squared value of 0.4469 and a Q-squared value of 0.4510. The root mean square error (RMSE) stands at 0.8714, with a standard deviation (S.D) of 0.8870 (Supplementary Table 2). Figure 8 illustrates scatter plots depicting the model's performance. The dataset's predictive and observed values are comprehensively detailed in Supplementary Table 1. In addition, Table 8 highlights that the top compounds exhibit superior pIC50 values compared to the standard [Table 8].

Consequently, this study shows the potential of the identified lead compounds to display remarkable inhibitory properties against the human cyclooxygenase-2 protein. This promising outcome warrants further investigation through *in vivo* studies.

Table 6: Pharmacokinetics prediction with SwissADME.

Compounds	GI absorption	BBB permeant	Pgp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
Ferulic acid	High	Yes	No	No	No	No	No	No
Co-crystallized	High	Yes	No	No	No	No	No	No
Salicylic acid	High	Yes	No	No	No	No	No	No

Table 7: Toxicity prediction by ProTox 3.0.

Compounds	LD_{50} (mg/kg)	Toxicity class	Hepatotoxicity	Nephrotoxicity	Cardiotoxicity	Carcinogenicity	Mutagenicity	Cytotoxicity
Ferulic acid	1772	4	Inactive	Active	Inactive	Inactive	Inactive	Inactive
Co-crystallized	1034	4	Active	Active	Inactive	Inactive	Inactive	Inactive
Salicylic acid	1034	4	Active	Active	Inactive	Inactive	Inactive	Inactive

Active: Compound is predicted to have that type of toxicity
Inactive: Compound is predicted not to have that type of toxicity

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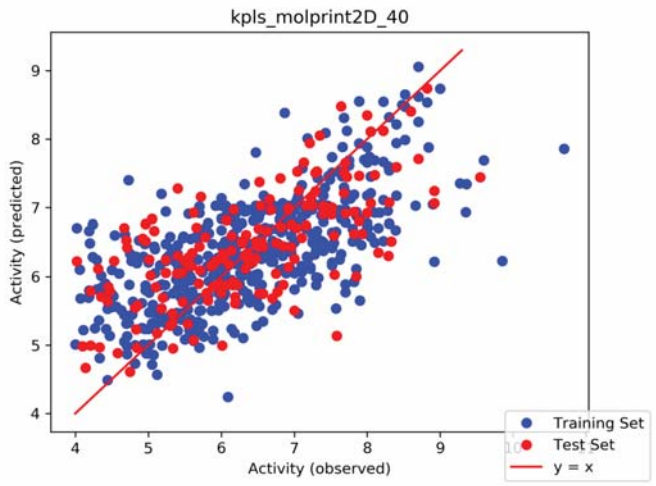

Figure 8: Plot of pIC50 observed vs pIC50 predicted of QSAR model.

Table 8: Predicted of pIC50values of top compounds and co-crystallized ligand.

Compounds	Pred Y
Ferulic acid	5.347
co-crystallized ligand	4.944
Salicylic acid	4.983

Conclusion

This study evaluated the antioxidant potential, mineral composition, and anti-inflammatory properties of the n-butanol fraction of *Azadirachta indica* leaf concerning rheumatoid arthritis. The extract demonstrated significant antioxidant capacity with high levels of phenolic and flavonoid contents and strong ferric reducing antioxidant power. Antioxidant properties are linked to bioactive polyphenols identified by HPLC analysis, including p-coumaric acid, ferulic acid, salicylic acid, tannic acid, saponin, and rutin, which are well-documented modulators of oxidative stress and inflammation pathways related to rheumatoid arthritis. The mineral analysis further revealed that the extract contains essential macro- and microelements within permissible safety limits. Additionally, the mineral analysis indicated a range of essential elements beneficial for immune and inflammatory responses. Molecular docking and pharmacophore-based virtual screening identified ferulic acid and salicylic acid as the most promising cyclooxygenase-2 (COX-2) inhibitors among the compounds detected. Both compounds demonstrated drug-likeness and favorable ADMET profiles, including high oral bioavailability and low acute toxicity. Notably, ferulic acid emerged as the most promising lead candidate, combining strong COX-2 binding, favorable ADMET characteristics, and lower predicted toxicity compared to the reference ligand and salicylic acid.

Furthermore, AutoQSAR modeling supported the predicted inhibitory potential of the lead compounds, with ferulic acid displaying superior predicted pIC_{50} values compared to the reference ligand and salicylic acid. Collectively, these findings provide mechanistic and computational evidence that bioactive constituents of Azadirachta indica, particularly ferulic acid, may contribute to anti-inflammatory activity through selective COX-2 inhibition, antioxidant action, and supportive mineral content. This study highlights Azadirachta indica as a valuable source of bioactive compounds with potential relevance in the management of rheumatoid arthritis. While the findings strongly support its therapeutic promise, further *in vivo* validation and clinical investigations are warranted to substantiate its efficacy and safety in rheumatoid arthritis treatment.

Authors' contributions

Lateef Bello, Sidiqat A. Shodehinde, Olamide V. Awelewa: Writing – original draft, Methodology, and Conceptualization. Lateef Bello, Success O. Olubode, Daniel O. Nwankwo: Formal analysis, Writing – review & editing. Lateef Bello, Olajumoke B. Ademoyegun, Bolanle Adalumo, Dorcas O. Oluwafemi, Oyinkansola E. Adewale, Olamide V. Awelewa: Visualization and Validation. Lateef Bello, Samuel A. Oginni: Data Curation. Lateef Bello: Result analysis. Lateef Bello, Sidiqat A. Shodehinde, Olajumoke B. Ademoyegun: Validation and Supervision.

(**Supplementary-File-1**)

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