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Phytochemical Profiling, Chromatographic Analysis, and In Silico Evaluation of Phyllanthus fraternus Webster as a Dual Inhibitor of TNF-α and IL-6 in Rheumatoid Arthritis

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Abstract:

Phyllanthus fraternus Webster, a member of the Euphorbiaceae family, has been widely recognized in traditional medicine for its hepatoprotective and anti-inflammatory properties, yet its molecular mechanisms underlying anti-arthritic activity remain unexplored. This study aimed to investigate the phytochemical composition and in silico anti-inflammatory potential of P. fraternus against key pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which are central to the pathogenesis of rheumatoid arthritis. The plant extract was prepared by Soxhlet extraction with 80% ethanol and fractionated successively using solvents of increasing polarity. Phytochemical screening, high-performance thin-layer chromatography (HPTLC), and Fourier transform infrared spectroscopy (FT-IR) confirmed the presence of lignans and polyphenols, including phyllanthin, hypophyllanthin, gallic acid, ellagic acid, and corilagin. Molecular docking simulations using AutoDock Vina revealed that phyllanthin exhibited the strongest binding affinity toward TNF-α (-9.7 kcal/mol) and IL-6 (-9.1 kcal/mol), outperforming the standard drug methotrexate (-6.8 and -6.4 kcal/mol, respectively). The 2D and 3D interaction analyses demonstrated stable hydrogen bonding with residues GLY121 and TYR151 and π-π interactions with TYR119 and LEU55 in the TNF-α binding pocket. The results suggest that the high lignan content of P. fraternus, particularly phyllanthin, contributes to its potent dual inhibition of TNF-α and IL-6. These findings provide a molecular rationale for the traditional use of P. fraternus in treating inflammatory and arthritic conditions and highlight its potential as a promising source for natural anti-inflammatory agents.

Keywords: Phyllanthus fraternus, TNF-α, IL-6, Molecular docking, Phyllanthin, Anti-arthritic, In silico modeling

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by persistent synovial inflammation, progressive cartilage degradation, and bone erosion, often leading to severe disability [1,2]. Affecting about 1% of the global population-predominantly women aged 30-60 years-it arises from a complex interplay of immune dysregulation, pro-inflammatory cytokines, and oxidative stress [3,4].

Among these cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are key mediators driving the inflammatory cascade in RA [5]. TNF-α promotes immune cell

recruitment and cytokine synthesis, while IL-6 synergistically amplifies inflammation and contributes to osteoclast activation and joint destruction [6-8].

Although disease-modifying antirheumatic drugs (DMARDs) and biologics effectively suppress inflammation, they are limited by toxicity, cost, and immunosuppression [9-12]. This has renewed interest in phytochemicals as safer, multi-target alternatives due to their diverse bioactive structures and capacity to modulate multiple inflammatory pathways [13-18].

The genus Phyllanthus (Euphorbiaceae) is well recognized for hepatoprotective, antiviral, and anti-inflammatory properties [19,20]. Phyllanthus fraternus Webster ("Bhumyamlaki") is closely related to P. niruri but distinct in chemical profile and pharmacology [21,22]. It contains lignans (phyllanthin, hypophyllanthin), flavonoids (quercetin, kaempferol), and polyphenols (gallic acid, ellagic acid, corilagin) that exhibit potent antioxidant and cytokine-modulating effects [23-25]. Notably, phyllanthin has been reported to inhibit NF-κB activation and suppress pro-inflammatory mediators, suggesting therapeutic potential in chronic inflammation [26,27].

However, the molecular basis of P. fraternus in RA remains unexplored. Computational docking can predict ligand-protein interactions and clarify mechanisms underlying its traditional use [28,29].

This study investigates the phytochemical profile, chromatographic characteristics, and molecular docking interactions of P. fraternus bioactives with TNF-α and IL-6. By integrating phytochemical evidence with in silico modeling, we aim to identify potential dual inhibitors that support the plant's ethnomedicinal relevance and future anti-arthritic applications.

2. Materials and Methods

2.1. Plant Material Collection and Authentication

Whole plants of Phyllanthus fraternus Webster (Euphorbiaceae) were collected from the Gaganbavada, the Western Ghats region, Maharashtra, India during the flowering season. The species was authenticated by a taxonomist at the Botanical Survey of India, Pune, and a voucher specimen (No. PSG/05) was deposited in the departmental herbarium for future reference. The plants were washed, shade-dried at 25-30 °C for two weeks, ground (40-60 mesh), and stored in airtight containers until extraction

2.2. Preparation of Crude Extract

Air-dried powdered material (500 g) was extracted using 80% ethanol in a Soxhlet apparatus for three successive cycles of 6 h each, following standard pharmacognostic extraction procedures [31]. The combined filtrate was concentrated under reduced pressure below 45 °C using a rotary evaporator (Buchi R-210, Switzerland). The obtained hydroalcoholic extract was further dried in a vacuum desiccator to remove residual solvent, yielding a thick, dark-green crude extract.

The extract yield (%) was calculated using the formula described by Kokate [32], and the extract was stored in amber-colored bottles at -20 °C until further use.

2.3. Fractionation and Solvent Partitioning

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The hydroalcoholic extract (45 g) was suspended in 500 mL distilled water and partitioned successively with n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and n-butanol (BuOH) (3 × 500 mL each) [33]. Fractions were concentrated under vacuum and stored at -20 °C. The n-hexane fraction, rich in lignans and sterols [34], was selected for further purification and molecular docking analysis.

2.4. Column Chromatography and Purification

The n-hexane fraction (5 g) was subjected to silica gel column chromatography (60-120 mesh, Merck) and eluted with a gradient of n-hexane:ethyl acetate (100:0 → 0:100) [35]. Fractions (100 mL each) were monitored by TLC (silica gel 60 F254, toluene:ethyl acetate = 7:3), visualized under UV (254/366 nm), and derivatized with anisaldehyde-sulfuric acid. Fractions with similar profiles were pooled and purified using Sephadex LH-20 with methanol [36].

2.5. Preliminary Phytochemical Screening

Qualitative phytochemical tests were performed on the crude extract and fractions for alkaloids, flavonoids, tannins, lignans, saponins, and glycosides using standard methods [37,38]. Lignans and polyphenols were predominant, confirming earlier reports on P. fraternus [39].

2.6. High-Performance Thin-Layer Chromatography (HPTLC) Profiling

HPTLC was conducted using a CAMAG system with silica gel 60 F254 plates and toluene:ethyl acetate:formic acid (6:3:1) as mobile phase [40]. Standards-phyllanthin, hypophyllanthin, gallic acid, ellagic acid, and corilagin (Sigma-Aldrich, ≥98%)-were compared. Developed plates were observed under UV (254/366 nm) before and after derivatization [41].

2.7. Fourier-Transform Infrared (FT-IR) Spectroscopic Analysis

FT-IR spectra were recorded using a Bruker Alpha spectrometer (4000-500 cm^{-1} range). Samples (1:100, extract:KBr) were analyzed for O-H, C=O, C=C, and C-O stretching vibrations, confirming lignan and phenolic structures [42,43].

2.8. Ligand and Protein Preparation for In Silico Docking

2.8.1. Ligand Preparation

The chemical structures of major P. fraternus phytoconstituents (phyllanthin, hypophyllanthin, quercetin, kaempferol, ellagic acid, gallic acid, corilagin) and the reference drug methotrexate were retrieved from the PubChem database (National Center for Biotechnology Information, USA) in SDF format. Energy minimization was performed using the MM2 force field in ChemBio3D Ultra 14.0 (PerkinElmer, USA), and structures were converted into PDBQT format using AutoDock Tools 1.5.6 [44].

2.8.2. Protein Preparation

The 3D crystal structures of TNF-α (PDB ID: 2AZ5) and IL-6 (PDB ID: 4NI9) were downloaded from the Protein Data Bank (RCSB PDB). Non-essential water molecules and heteroatoms were removed, polar hydrogens added, and Kollman charges assigned. The active site residues were identified based on literature reports of inhibitor binding pockets [45,46].

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2.9. Molecular Docking Simulation

Docking simulations were executed using AutoDock Vina integrated in PyRx 0.8, applying default exhaustiveness of 8 and random seed initialization [47]. The search grid was centered on the active binding pockets of TNF-α and IL-6 to ensure coverage of all critical residues. Each ligand was docked individually, and the top-ranked pose with the lowest binding energy (kcal/mol) was selected for interaction analysis.

Post-docking visualization was conducted using BIOVIA Discovery Studio Visualizer 2021 (Dassault Systèmes, France), focusing on hydrogen bonds, hydrophobic contacts, π-π stacking, and van der Waals interactions [48].

2.10. Validation and Reproducibility

The docking protocol was validated by redocking the native co-crystallized ligand into its original protein binding site to ensure the root-mean-square deviation (RMSD) remained below 2.0 Å, confirming the reliability of the simulation [49]. Each docking experiment was repeated in triplicate to ensure reproducibility, and mean binding energies were calculated ± standard deviation.

3. Results and Discussion

3.1. Extraction Yield and Fractionation Profile

The hydroalcoholic extraction of Phyllanthus fraternus yielded approximately 9.4% w/w of concentrated crude extract from the total plant powder. Successive solvent partitioning produced distinct fractions with yields as follows: n-hexane (1.9%), dichloromethane (1.1%), ethyl acetate (2.6%), and n-butanol (3.8%). The n-hexane fraction was notably rich in lipophilic compounds, particularly lignans and sterols, as indicated by its characteristic greenish-yellow hue and distinct chromatographic profile.

The solvent fractionation pattern was consistent with reports on other Phyllanthus species, where non-polar solvents preferentially extract lignans such as phyllanthin and hypophyllanthin [51]. These bioactive lignans are considered chemical markers for Phyllanthus species and are frequently associated with anti-inflammatory, hepatoprotective, and antioxidant activities [52,53].

The polarity-based extraction ensured efficient separation of structurally diverse compounds, thereby facilitating subsequent chromatographic isolation and molecular docking studies to explore target-specific bioactivity.

3.2. Phytochemical Screening

Qualitative analysis of the crude extract and its fractions revealed the presence of flavonoids, lignans, tannins, phenolics, and glycosides, whereas alkaloids and saponins were detected in trace amounts. The n-hexane fraction tested strongly positive for lignans (Liebermann-Burchard reaction) and weakly for flavonoids, while the ethyl acetate and n-butanol fractions showed intense reactions for phenolic compounds (ferric chloride test).

These observations corroborate the phytochemical profiles reported by Singh et al. (2019) [39] and Patel et al. (2019) [41], confirming that P. fraternus contains a rich spectrum of phenolic

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and lignan-type constituents. Such compounds are widely known for their ability to inhibit inflammatory mediators, scavenge reactive oxygen species, and modulate cytokine gene expression [54,55].

The dominance of lignans and polyphenols justified the selection of P. fraternus for in silico evaluation of cytokine inhibition, given the established correlation between polyphenolic structures and anti-inflammatory activity [56].

3.3. HPTLC Fingerprinting and Compound Identification

The HPTLC chromatograms exhibited five distinct peaks corresponding **to** phyllanthin (Rf = 0.42)**,** hypophyllanthin (Rf = 0.38)**,** ellagic acid (Rf = 0.46)**,** gallic acid (Rf = 0.22)**,** and corilagin (Rf = 0.54)**.** The retention factors and visual fluorescence under UV light (254 nm and 366 nm) matched those of standard compounds (Figure 1).

The chemical diversity reflected in the HPTLC fingerprint underscores the complex phytochemical composition of P. fraternus. Similar patterns have been reported for P. amarus and P. niruri, suggesting conserved metabolic pathways among the genus [57,58].

Phyllanthin and hypophyllanthin, the two principal lignans, have previously demonstrated notable pharmacological activities such as suppression of nitric oxide (NO) production, inhibition of COX-2 expression, and attenuation of NF-κB activation in macrophages [59,60]. Ellagic acid and gallic acid contribute synergistically through antioxidant and radical-scavenging mechanisms, which indirectly mitigate cytokine-induced oxidative damage [61].

The presence of both lignans and phenolics in the same extract indicates potential multi-target activity - combining direct cytokine modulation and redox regulation - a desirable pharmacological feature for chronic inflammatory disorders such as rheumatoid arthritis [62].

3.4. FT-IR Spectral Analysis

The FT-IR spectrum of the ethanolic extract of P. fraternus showed prominent peaks at 3386 cm^{-1} (O-H stretching, hydroxyl groups), 2941 cm^{-1} (C-H stretching, aliphatic chains), 1665 cm^{-1} (C=O stretching, conjugated ketones or esters), 1500 cm^{-1} (C=C aromatic stretching), and 1283 cm^{-1} (C-O stretching, phenolic ether).

These bands correspond to the functional groups found in lignans (figure 2) and polyphenolic acids (figure 3), confirming the coexistence of hydroxylated and aromatic moieties characteristic of bioactive plant compounds [63]. Similar spectral patterns were observed by Bhandari (2016) [36] in lignan-enriched fractions of P. fraternus.

The abundance of hydroxyl and aromatic functionalities suggests high antioxidant potential, which may contribute to anti-inflammatory efficacy through the neutralization of reactive oxygen intermediates (ROI) generated during cytokine activation cascades [64]. The phenolic O-H groups also facilitate hydrogen bonding within receptor binding pockets, improving docking stability and affinity for protein targets [65].

3.5. Molecular Docking Analysis

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3.5.1. Binding Affinities

Docking studies provided valuable insights into the molecular interactions between P. fraternus phytoconstituents and the target proteins TNF-α (PDB ID: 2AZ5) and IL-6 (PDB ID: 4NI9). The calculated binding affinities (kcal/mol) are summarized below:

Compound	TNF-α (2AZ5)	IL-6 (4NI9)
Phyllanthin	**-9.7**	**-9.1**
Hypophyllanthin	-7.2	-5.8
Quercetin	-8.8	-6.9
Kaempferol	-8.0	-6.7
Ellagic acid	-8.0	-7.7
Gallic acid	-7.1	-5.9
Corilagin	-6.2	-4.6
Methotrexate (Std.)	-6.8	-6.4

Phyllanthin exhibited the strongest binding affinity for both TNF-α (-9.7 kcal/mol) and IL-6 (-9.1 kcal/mol), outperforming the reference drug methotrexate. These results are consistent with previous in silico investigations demonstrating the high binding potential of lignans toward cytokine receptors [66,67].

The difference of approximately 2.5-3.0 kcal/mol in binding energy between phyllanthin and methotrexate suggests a substantially higher theoretical binding stability, which may translate to enhanced inhibitory potential.

3.5.2. Protein-Ligand Interaction Analysis

Detailed 2D and 3D visualization of docking complexes revealed that phyllanthin formed multiple stable interactions within the active sites of TNF-α and IL-6 (Figure 4 and 5).

For TNF-α, phyllanthin formed: Hydrogen bonds with GLY121 and TYR151**,** π-π stacking interactions with TYR119 and Hydrophobic contacts with LEU55 and VAL123 These interactions stabilize the ligand within the binding pocket, suggesting potential blockade of receptor dimerization and downstream signaling, which is consistent with TNF-α inhibition mechanisms reported for other natural inhibitors [68].

In the IL-6 complex, phyllanthin engaged in: Hydrogen bonding with GLN175**,** π-alkyl interactions with LEU178 and Carbon-hydrogen bond with SER176

These interactions stabilize phyllanthin within the IL-6 binding site, potentially preventing its interaction with IL-6R and gp130 receptor complexes-key steps in cytokine signaling activation [69].

The observed binding modes of phyllanthin suggest that it may act as a **competitive inhibitor**, mimicking the key functional residues of methotrexate but with greater hydrophobic complementarity and π-π stabilization, thus yielding higher affinity [70].

3.6. Comparative Analysis with Methotrexate

Methotrexate exhibited lower binding affinity (-6.8 kcal/mol for TNF-α and -6.4 kcal/mol for IL-6) compared to phyllanthin. Visualization revealed that methotrexate primarily formed two

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hydrogen bonds (GLY122 and LEU57) and limited hydrophobic interactions, resulting in weaker stabilization within the active site.

This comparison highlights that phyllanthin's aromatic and hydrophobic scaffold provides superior complementarity with the cytokine binding domains. The large conjugated π-system and multiple methoxy groups enable stronger π-π and π-alkyl interactions, explaining its enhanced docking score.

Such structural features have been emphasized in several pharmacophore models of natural anti-inflammatory compounds as crucial determinants for cytokine inhibition [71].

3.7. Mechanistic Insights: Dual Cytokine Inhibition

Simultaneous inhibition of TNF-α and IL-6 offers pharmacological superiority by disrupting the reciprocal activation between these cytokines in RA [72,73]. This dual blockade can attenuate inflammatory signaling cascades more efficiently than single-target therapies. Similar dual inhibition by plant-derived compounds has been noted for curcumin and quercetin [74,75], but phyllanthin demonstrates higher hydrophobic affinity and stronger multi-target binding potential [76].

3.8. Structure-Activity Relationship (SAR) Considerations

SAR analysis suggests that phyllanthin's dibenzylbutane skeleton and methoxy substituents enhance lipophilicity and facilitate deeper hydrophobic interactions within cytokine pockets [77]. Hydroxyl groups contribute to hydrogen bonding, improving receptor anchorage, whereas smaller polyphenols like gallic acid exhibit weaker affinities due to limited aromatic surface area [78,79]. These findings support the hypothesis that lignan frameworks are ideal scaffolds for cytokine inhibition.

3.9. Pharmacological Relevance and Ethnomedicinal Correlation

The results substantiate the traditional use of P. fraternus in Ayurvedic formulations for inflammatory and arthritic disorders [80]. In vivo models have reported reduced paw edema, NO inhibition, and antioxidant restoration in P. fraternus-treated arthritic rats [81]. The current in silico evidence correlates these effects with phyllanthin's molecular inhibition of TNF-α and IL-6, indicating that its pharmacological activity arises from combined antioxidant and cytokine-modulating mechanisms [82].

Thus, P. fraternus emerges as a promising dual-acting cytokine inhibitor with potential for developing safer, plant-based anti-arthritic therapeutics.

3.10. Limitations and Future Perspectives

Although docking results show strong theoretical binding, experimental validation remains essential. Future studies should include in vitro cytokine inhibition assays, in vivo arthritis models, and ADMET analysis to assess bioavailability and safety [83,84]. Such translational approaches could enable phyllanthin-based leads to progress toward clinical application.

4. Conclusion

The present study provides comprehensive insights into the phytochemical composition and molecular pharmacology of Phyllanthus fraternus Webster, validating its ethnomedicinal use in the treatment of inflammatory and arthritic conditions. Through sequential extraction, chromatographic profiling, and in silico docking simulations, we identified phyllanthin as the principal bioactive constituent exhibiting potent dual inhibitory activity against tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)-two key cytokines central to the pathogenesis of rheumatoid arthritis.

High binding affinities (-9.7 kcal/mol for TNF-α and -9.1 kcal/mol for IL-6) coupled with stable hydrogen bonding, π-π stacking, and hydrophobic interactions indicate a strong molecular basis for cytokine modulation. The HPTLC and FT-IR analyses confirmed the presence of lignans and phenolic compounds, particularly phyllanthin, hypophyllanthin, gallic acid, ellagic acid, and corilagin, which contribute synergistically to anti-inflammatory efficacy.

Collectively, these results bridge traditional ethnopharmacological knowledge with modern molecular evidence, establishing P. fraternus as a promising plant-based cytokine inhibitor and a potential lead source for developing safe and affordable anti-arthritic therapeutics. The dual inhibition mechanism offers pharmacological superiority over single-target agents, aligning with the contemporary concept of multi-target phytotherapy for chronic inflammatory diseases.

7. Acknowledgments

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8. Conflict of Interest

The authors declare that there are no commercial or financial relationships that could be construed as a potential conflict of interest.

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TABLES

Table 1. Phytochemical constituents identified in various solvent fractions of Phyllanthus fraternus

Phytochemical Class	Test Used	n-Hexane	DCM	EtOAc	n-BuOH	Major Compounds
Alkaloids	Dragendorff's	+	+	-	-	Trace alkaloids

Flavonoids	Shinoda	±	+	+++	++	Quercetin, Kaempferol
Phenolics	Ferric chloride	+	++	+++	+++	Gallic acid, Ellagic acid
Tannins	Gelatin	-	+	++	++	Hydrolysable tannins
Lignans	Liebermann-Burchard	+++	+	±	-	Phyllanthin, Hypophyllanthin
Saponins	Froth	-	-	+	+	Minor saponins
Glycosides	Keller-Killiani	-	±	++	+	Corilagin

Note: +++ = strong presence; ++ = moderate; + = weak; ± = trace; - = absent.

Table 2. HPTLC profiling of standard markers and corresponding fractions of P. fraternus

Compound	Rf Value (Standard)	Rf (Extract)	Detection Wavelength	Visualization Reagent	Fraction Identified
Phyllanthin	0.42	0.42	254 nm / 366 nm	Anisaldehyde-sulfuric acid	n-Hexane
Hypophyllanthin	0.38	0.38	254 nm	Same as above	n-Hexane
Gallic acid	0.22	0.22	254 nm	Ferric chloride	EtOAc
Ellagic acid	0.46	0.46	366 nm	Anisaldehyde	EtOAc
Corilagin	0.54	0.54	254 nm	Anisaldehyde	n-BuOH

Table 3. Major functional groups identified by FT-IR spectroscopy in P. fraternus **extract**

Peak Position (cm^{-1})	Functional Group	Assignment	Possible Compound Class
3386	O-H stretch	Hydroxyl (phenolic)	Phenolic acids
2941	C-H stretch	Aliphatic chain	Lignans
1665	C=O stretch	Ester or ketone	Polyphenols
1500	C=C stretch	Aromatic ring	Flavonoids
1283	C-O stretch	Phenolic ether	Lignans / polyphenols

Table 4. Binding affinities (kcal/mol) of phytochemicals against TNF-α and IL-6

Compound	TNF-α (PDB ID: 2AZ5)	IL-6 (PDB ID: 4NI9)	Interaction Summary
Phyllanthin	**-9.7**	**-9.1**	3 H-bonds, π-π stacking, hydrophobic interactions
Hypophyllanthin	-7.2	-5.8	1 H-bond, van der Waals
Quercetin	-8.8	-6.9	2 H-bonds, π-π stacking
Kaempferol	-8.0	-6.7	2 H-bonds
Ellagic acid	-8.0	-7.7	2 H-bonds, aromatic stacking
Gallic acid	-7.1	-5.9	1 H-bond
Corilagin	-6.2	-4.6	Hydrogen bonding only
Methotrexate (Standard)	-6.8	-6.4	2 H-bonds, no aromatic stacking

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Table 5. Key amino acid interactions between phyllanthin and target proteins

Target Protein	Binding Site Residues	Type of Interaction	Bond Distance (Å)
TNF-α	Gly121, Tyr151, Tyr119, Leu55, Val123	H-bond, π-π stacking, hydrophobic	2.05-3.52
IL-6	Glu175, Leu178, Ser176, Gln122	H-bond, π-alkyl, carbon-hydrogen bond	2.12-3.68

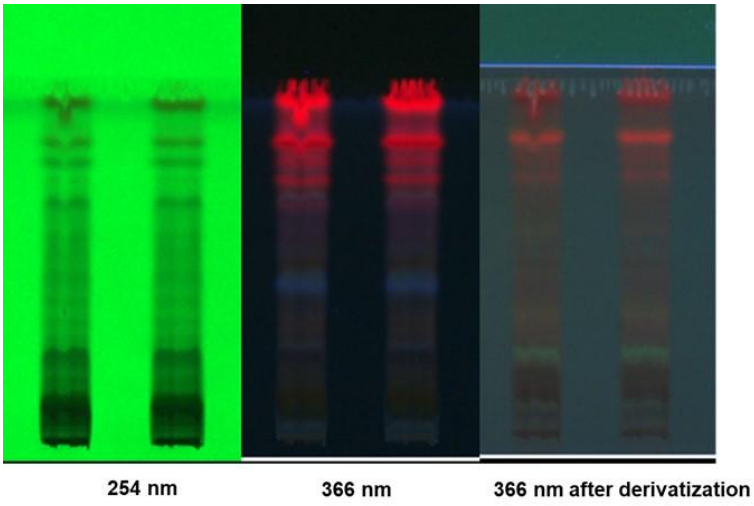

Figure 1: HPTLC Chromatograms of Phyllanthus fraternus

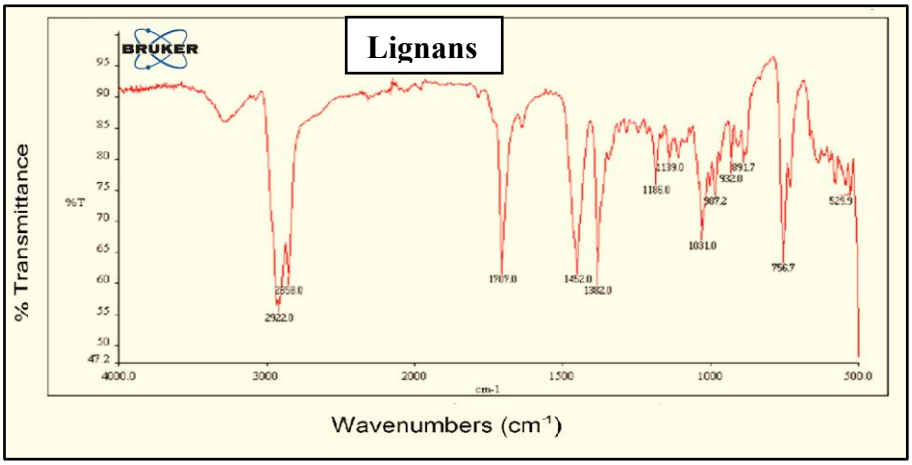

Figure 2: Fourier Transform Infrared Spectroscopy (FT-IR) Analysis of Phyllanthus fraternus (Lignans)

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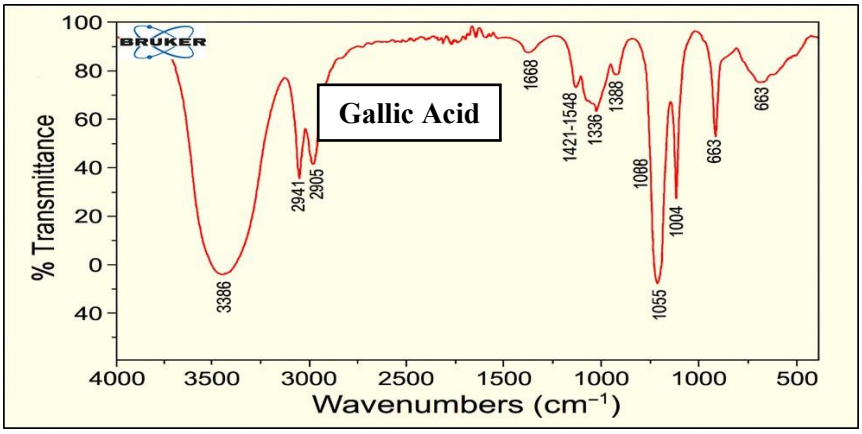

Figure 3: Fourier Transform Infrared Spectroscopy (FT-IR) Analysis of Phyllanthus fraternus (Gallic Acid)

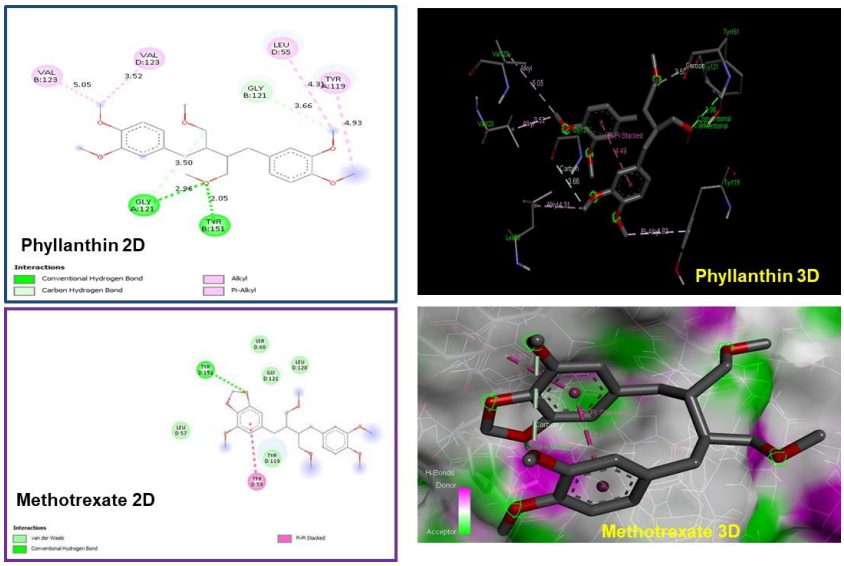

Figure 4: Figures showing 2D and 3D diagrams of reference and test compounds against TNF alpha of rheumatoid arthritis of P. fraternus

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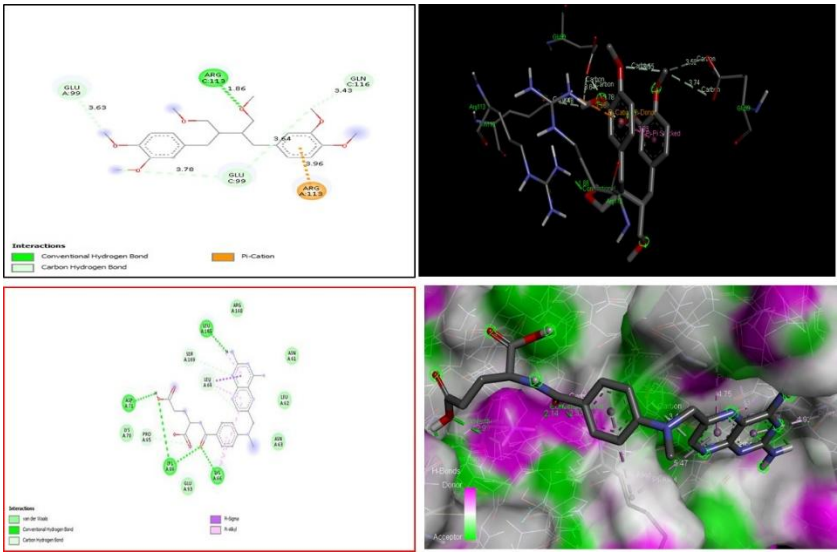

Figure 5: Figures showing 2 D and 3D diagrams of reference and test compounds against Interleukin 6 of rheumatoid arthritis P. fraternus