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Assessment of Anthelmintic Activity of Cinnamic Acid and Its Three Analogs: a Combined *in Vitro* and in *Silico* Study

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ABSTRACT

Concern over anthelmintic resistance is a global issue, and researchers are exploring plant-derived compounds for their effectiveness against gastrointestinal nematodes. Cinnamic acid and its primary hydroxy derivatives, including p-coumaric, ferulic, and caffeic acids, are phenolic compounds that are abundant in plants and possess numerous therapeutic properties. This investigation aimed to determine the anthelmintic properties of these compounds. The anthelmintic potential of cinnamic acid and its analogs were investigated on the Indian earthworm species, Pheretima Posthuma. The duration required for paralysis and subsequent death of earthworms was recorded in minutes. The selected compounds exhibited a more potent anthelmintic effect than the conventional medication albendazole. The structural characteristics of these potential compounds were explored using in silico techniques. Molecular docking results displayed that cinnamic acid and its hydroxy derivatives were efficiently binding with tubular proteins, implying that they possess significant anthelmintic activity.

Keywords: *Anthelmintic, Docking, ADME, Cinnamic acid analogs, in vitro, in silico*
Abbreviations: *CIA: Cinnamic acid, PCA: p-Coumaric acid, CAA: Caffeic acid, FEA: Ferulic acid, RO5: Rule of 5.*

INTRODUCTION

Parasitic worms, known as helminths, are a significant source of public health concern due to their widespread occurrence in both human and animal populations, resulting in a high incidence of infection. Helminth infections are estimated to affect approximately a quarter of the world's population. Helminths are unique among parasites because they multiply inside the definitive host and can evade host immune defenses [1]. Infections caused by helminths can result in deficiency diseases such as malnutrition and anemia, as well as weakening of the immune system. The treatment of helminthic diseases presents a major challenge due to the resistance of gastrointestinal helminths to currently available anthelmintic drugs. Conventional anthelmintic medications frequently exhibit adverse effects and lack cost-effectiveness. Albendazole, a broad-spectrum anthelmintic drug, may induce side effects, such as emesis, vertigo, nausea, and gastrointestinal irritation in certain patients. [2]. Hence, the demand for natural anthelmintics is increasing. Phytomedicine employs natural substances sourced from medicinal plants, which possess potent bioactive properties and offer a broad spectrum of applications for tackling both chronic and infectious ailments [3]. The importance of phytochemicals like alkaloids, phenolics, tannins, terpenoids, flavonoids, and glycosides with anthelmintic activity was reported earlier [4, 5].

Phenolic acids are natural compounds found in plants that primarily protect plants from ultraviolet radiation and pathogen invasion. They constitute a diverse category of secondary metabolites that exhibit a broad spectrum of biological activity [6–9]. Phenolic acids are conventionally categorized into two distinct groups: hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids are an important group of bioactive compounds found in edible plants. In contrast, hydroxycinnamic acids have been the subject of extensive research and are widely distributed in various plant-based foods, especially vegetables, fruits, and seeds [10, 11].

Cinnamic acid can provide three main reactions in its 3-phenyl acrylic acid functionality: substitution at the phenyl ring, addition at α, β-unsaturation, and reactions of the carboxylic acid functionality. These chemical properties have made cinnamic acid derivatives a focus of interest in medicinal research. Studies have reported that cinnamic acid exhibits antimicrobial [12], antioxidant, neuroprotective, anti-inflammatory, anticancer [13] and antidiabetic properties [14]. The nature and position of the substituents influence the biological activities of various cinnamic acid derivatives. The rise in drug resistance and the absence of effective treatments with minimal side effects for managing diseases, microbial proliferation, neurological disorders, and associated conditions have led to the development of therapeutic agents derived from cinnamic acid [15].

Major cinnamic acid derivatives reported from plants include ferulic acid [16], caffeic acid, p-coumaric acid [17], and chlorogenic acids [18]. Caffeic acid (3, 4-dihydroxy cinnamic acid) is a phenolic compound having pharmacological properties that are mostly present in medicinal plants [18]. The role of caffeic acid as an antiviral, antihypertension, antioxidant and anti-tumor agent was well established [19].

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Ferulic acid, also known as 4-hydroxy-3-methoxycinnamic acid, is a naturally occurring organic compound widely found in fruits and vegetables [20] and is well known for its antioxidant and antitumor activities [21, 22]. The most common isomer of cinnamic acid, p-coumaric acid (4-hydroxy cinnamic acid), is found in various edible plants, including tomatoes, peanuts, and carrots. p-Coumaric acid has been demonstrated to exhibit antitumor and antimutagenic properties [23,24].

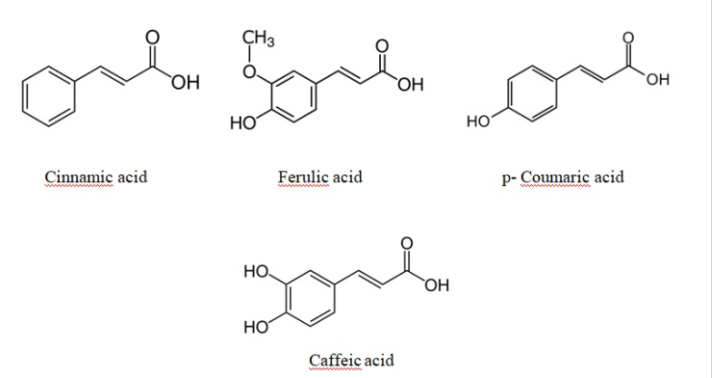


Fig.1. 2D structures of selected compounds.

Cinnamic acid derivatives (Fig.1) are characterized by the presence of hydroxyl groups on their phenyl rings, which can be either unsubstituted or methoxylated. Hydroxycinnamic acids feature a phenylpropanoid C6-C3 framework as their main chemical structure and are identified by hydroxyl groups on the aromatic ring along with a carboxyl group in the side chain [25, 26].

Tubulin is a widely recognized target for anticancer and anthelmintic drugs. Investigating tubulin inhibitors could pave the way for the creation of novel anthelmintic medications. Additionally, tubulin is a significant component of microtubules, and the inhibitors bind selectively with β-tubulin present in nematodes, cestodes, and flukes, disrupting the structure and function of microtubules [27-29]. Microtubules are involved in various cellular processes. Microtubule destruction eventually results in the organism's death. Albendazole is a widely utilized oral anthelmintic agent that demonstrates efficacy against a broad spectrum of intestinal parasites. The mechanism of action involves the inhibition of microtubule formation in nematodes, consequently impeding glucose absorption. This results in the parasites either becoming immobile or dying gradually [30].

Various computational methods like molecular docking, drug-likeness prediction, molecular dynamics simulations, and in silico ADMET studies are frequently used to screen potential molecules from diverse databases and libraries. The application of computational methods in drug discovery and development has increased in significance and is considered to enhance the efficiency of the pharmaceutical industry. Molecular docking represents an expeditious and cost-effective approach for investigating the binding mode and affinity between a chemical compound and its target, with a specified level of confidence. Computational docking can be employed to predict the bound conformations and free energies of the binding of small-molecule ligands to macromolecular targets [31].

The process of ligand binding is crucial in enzymatic reactions and subsequently, inhibiting them. Understanding the interactions between small molecules and proteins is crucial for devising rational drug design strategies that aim to develop compounds to address a wide array of important health challenges [32-36]. The present study aimed to evaluate the anthelmintic efficacy of cinnamic acid and its three prevalent hydroxyl derivatives, p-coumaric acid, ferulic acid, and caffeic acid, using both *in vitro* and *in silico* methodologies. The 2D structures of compounds selected for this study are given in Fig. 1. The adult Indian earthworm, *Pheretima Posthuma (P. Posthuma)* was taken during *in vitro* studies due to its anatomical and physiological similarities to human intestinal roundworm parasites and its ease of availability.

2. Materials and Methods

2.1 Chemicals

All chemicals utilized in this study were of analytical grade obtained from Sigma Aldrich, Bangalore.

2.2 *In vitro* anthelmintic activity

The Indian earthworm *Pheretima Posthuma* utilized in this investigation was procured from the Kerala Agricultural University in Vellayani, Thiruvananthapuram, and washed thoroughly with water. Six earthworms of roughly comparable dimensions were subsequently placed in test solutions containing three distinct concentrations (5, 10, and 20 mg/ mL in DMSO). Albendazole solution (10 mg/mL) was employed as the standard, while distilled water served as the control [37]. The assessment of anthelmintic efficacy encompasses two critical phases: the onset of paralysis and death in worms. Paralysis onset was identified as the point at which the worms' natural motility ceased, whereas death was confirmed when the worms exhibited no response to vigorous agitation or immersion in hot water.

2.3 *In silico* Studies

2.3.1 ADMET calculations

The physicochemical and pharmacokinetics properties of the cinnamic acid and its selected hydroxy derivatives were studied through the SwissADME online server [38]. Lipinski's rule of five [39] was used to estimate the drug-likeness properties of selected compounds. Compounds that satisfy the Lipinski rule are assumed to be ideal drug candidates. (Molecular weight not exceeding 500; H-bond donors ≤ 5; H-bond acceptor ≤ 10; and Lipophilicity, MlogP < 5).

2.3.2 Geometry optimization of ligand structures

Full geometry optimization of the selected molecules was conducted using density functional theory (DFT) with hybrid functional B3LYP5 and basis set 6–31G (d,p) with the Firefly 8.2.0 software, [40] which is based on the GAMESS (US) source code.

2.3.3 Molecular Docking Analysis

The 3D coordinates of the crystal structure of tubulin (PDB: 1Oj0) were downloaded from the RCSB protein data bank. The preparation of proteins for molecular docking involved the elimination of water molecules and extraneous heteromolecules from their original crystalline structures. This process was accomplished utilizing version 1.1 of the PyMol software suite. The two-dimensional structures of selected molecules were retrieved from the PubChem database. The geometry-optimized structures of selected molecules were used in docking (Fig.6). The conversion of ligand molecules into the Protein Data Bank (PDB) format was accomplished using Open Babel software (Open Babel Package, version 2.3.1; http://openbabel.org). To conduct docking simulations, PDBQT files of both the protein and ligand structures were created using the AutoDock tool. This format is required to run the Vina command.

The generation of configuration files for proteins involves the establishment of appropriate Cartesian coordinates, enabling the creation of a grid box. For protein 1Oj0, the parameters of the grid box are X =81.02, Y = 68.11, & Z = 1.59, and grid box dimensions were set at 25x25x25 Aº which covers all the amino acids in the active site [41].
The automated docking software AutoDock Vina 4.2 [42] was used to assess the binding affinity of the ligands. Docking energy was evaluated using empirical-free energy functions and the Lamarckian genetic algorithm. These computational methods calculate the binding free energy (δG) based on various electrostatic, van der Waals, hydrogen bonding, and desolvation effects.

2.4 Statistical analysis

The results were represented as Mean ± SD. Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by a Student's t-test. A p-value of less than 0.05 was considered to indicate statistical significance.

3. Results and Discussion

3.1 *In vitro* anthelmintic analysis

Table 1. Anthelmintic activity of cinnamic acid and its three analogs.

Sample	Concentration of sample (mg/mL)		Time taken for paralysis (minutes)	Time taken for Death (minutes)
-ve Control	-		-	-
Control Drug (Albendazole)	10		32.44± 0.7	49.46± 0.7
FEA	5		4.16 ± 0.7	26.32 ± 0.2
	10		2.50 ± 1.5	20.16 ± 0.4
	20		1.13 ± 0.8	16.32 ± 0.2
PCA	5		5.42 ± 0.7	20.57 ± 0.1
	10		2.16 ± 1.8	15.20 ± 0.6
	20		3.11 ± 0.5	11.34± 1.4
CIA	5		7.10 ± 0.9	21.07 ± 0.5
	10		4.47 ± 1.6	16.58 ± 1.3
	20		3.26 ± 0.7	9.33 ± 0.7
	CAA	5	11.10 ± 0.2	30.54± 1.6
		10	6.08 ± 2.9	22.57 ± 0.8
		20	4.57 ± 1.5	12.50 ± 0.4

Cinnamic acid and its analogs displayed greater efficacy in inhibiting helminths compared to the standard drug albendazole. All selected compounds exhibited concentration-dependent activity, and significant activity was observed at 20 mg/mL. Ferulic acid demonstrated the best activity at this concentration, with paralysis occurring within 1.13 minutes and death occurring within 16.32 minutes. For PCA, the time to paralysis was 2.16 minutes at a concentration of 10 mg/ mL, and the time to death was 11.34 minutes at a concentration of 20 mg/mL. Significant activity was observed for CIA and CAA at a concentration of 20 mg/mL.

Previous studies have suggested that tannins and phenolics might hinder energy production in helminth parasites by either disrupting oxidative phosphorylation or binding to free proteins in the gastrointestinal tract of worms, ultimately causing their death [43]. Additionally, flavonoids and polyphenolic compounds were believed to contribute to anthelmintic activity. Alkaloids can cause paralysis by binding to the central nervous system (CNS). The anthelmintic activity of saponins is due to their tendency to permeate membranes [44, 45]

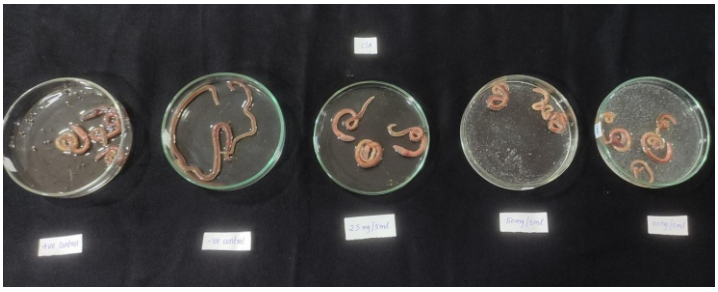


Fig.2. Anthelmintic activity of CIA on Pheretima Posthuma

Fig.3. Anthelmintic activity of FEA on Pheretima Posthuma

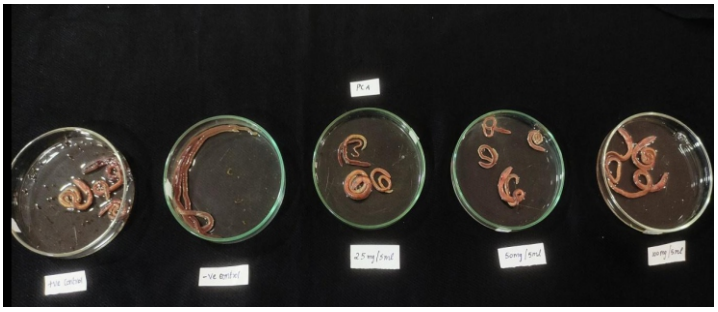


Fig.4. Anthelmintic activity of PCA on Pheretima Posthuma

Fig.5. Anthelmintic activity of CAA on Pheretima Posthuma

3.2 Computational studies

3.2.1 *In silico* ADME Profile

Table 2. Calculation of molecular properties of selected ligands- Lipinski's Ro5

Ligand	Mol. Wt. (<500)	No. of HBD (<5)	No. of HBA(<10)	MlogP(<5)
CiA	148.16	1	2	1.90
*p*CA	164.16	2	3	1.28
FA	194.18	2	4	1.00
CaA	180.16	3	4	0.70

Table 3. Calculation of ADME properties of selected ligands

Ligands	Bioavailability score	GI (Gastro intestinal) Absorption	BBB (Blood Brain Barrier penetration)	P-gp substrate (P-glycoprotein substrate)	Water solubility	Number of rotatable bonds	Log P (−0.7 - +5.0)
CiA	0.85	High	Yes	No	Soluble	2	2.13
*p*CA	0.85	High	Yes	No	Soluble	2	1.46
FA	0.85	High	Yes	No	Soluble	3	1.51
CaA	0.56	High	No	No	Soluble	2	1.15

To identify the most promising compounds and minimize the risk of drug attrition in the later stages, the ADME parameters of the selected compounds were thoroughly examined. The molecular characteristics of selected molecules are listed in Tables 2 and 3. The selected compounds, adhering to Lipinski's rule, demonstrated favorable oral absorption properties and exhibited promising bioavailability. These characteristics indicate a strong potential for effective oral administration of these substances.

Low molecular weight drug molecules (less than 500) are generally more easily transported, diffused, and absorbed compared to heavier molecules. The therapeutic efficacy of pharmaceutical agents is significantly influenced by their molecular weights. As this parameter surpasses a specific threshold, the resultant increase in molecular size can potentially modulate the drug activity. The tested compounds were found to have hydrogen bond acceptors (N and O atoms) and donors (OH and NH) within Lipinski's range, which is less than 10 and 5, respectively. In rational drug design, the octanol-water partition coefficient (logP) is employed as a crucial indicator of the hydrophobicity and lipophilicity of a molecule. This parameter plays a pivotal role in determining various pharmacological properties including drug absorption, bioavailability, interactions between drugs and receptors, metabolic processes, and potential toxicity. LogP is also a crucial parameter in studies of the environmental dynamics of chemicals.

Lipophilicity (Log P) is a key factor that determines the oral bioavailability of pharmaceutical compounds. These physicochemical properties play crucial roles in determining the potential success of drug molecules intended for oral administration [46]. The highest degree of lipophilicity was observed for all compounds under investigation, indicating favorable lipid solubility, which facilitates drug interactions with membranes. An increase in the number of rotatable bonds enhances molecular flexibility and adaptability, promoting efficient interactions with specific binding pockets. The selected compounds exhibited an adequate number of flexible bonds, thereby enabling effective interactions with the active site of the protein.

Soluble molecules play a crucial role in drug development by making it easier to handle and formulate, especially for oral administration.

For drugs intended for parenteral use, high water solubility is necessary to deliver the active ingredient in a small volume of pharmaceutical dosage. Therefore, solubility is a critical property that influences the success of drug discovery processes. The impact of P-glycoprotein on drug absorption and elimination has been established through *in vitro* and *in vivo* studies. P-glycoprotein appears to have a greater influence on limiting the entry of drugs into the brain and epithelial cells from the bloodstream and intestinal lumen, rather than enhancing the excretion of drugs from hepatocytes and renal tubules. Therefore, it is crucial to determine whether a drug is a substrate or inhibitor of P-glycoprotein to understand its pharmacokinetics. P-gp substrates can be pumped out of cells, leading to reduced absorption [47].

3.2.2 Molecular docking

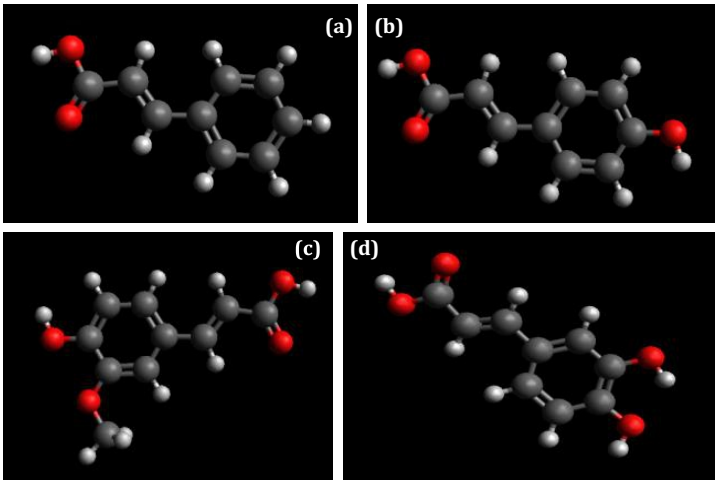


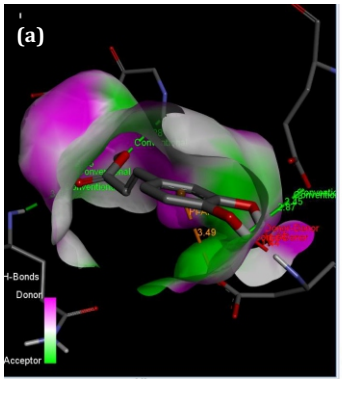
Fig. 6. Optimised geometries a) CiA b) pCA c) FA d) CaA

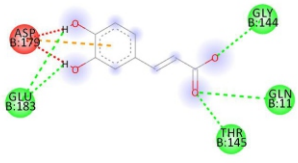
Table 4. Docking results

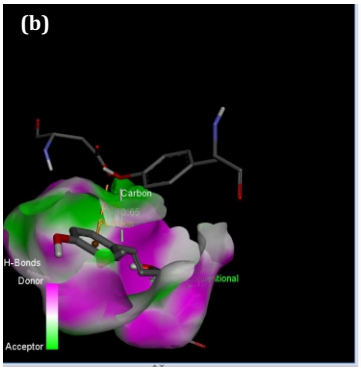
Protein	Ligand	Binding affinity (KCal/mol)	No. of hydrogen bonds	Aminoacid residues involved [Bond distance(A⁰)]
Beta-tubulin **(PDB ID: 1OJ0)**	CaA	-6.4	5	1. GLN B:11(2.35) 2. THR B: 145 (3.03 A⁰) 3. GLY B: 144(2.28 A⁰) 4. GLU B: 183(2.87 A⁰) 5. GLU B: 183(2.45 A⁰)
	FA	-6.1	2	1. THR B: 145(2.19 A⁰) 2. THR B: 145(2. 06 A⁰)
	pCA	-5.7	4	1. TYR B:124(2.66 A⁰) 2. THR B:145(2.17 A⁰) 3. ASN B:101 (2.99 A⁰) 4. GLY B:144(2.20 A⁰)
	CiA	-6.6	4	1. THR B: 145(2.91 A⁰) 2. THR B: 145(1.91 A⁰) 3. GLY B: 144(2.15 A⁰) 4. ASN B:101 (2.85 A⁰)
	Albendazole	-7.4	3	1. GLYB:133 (3.15 A⁰) 2. SER B: 165(2.60 A⁰) 3. SER B: 165(3.09 A⁰)

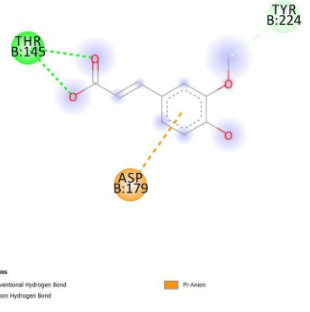
Cinnamic acid and its analogs (Fig.6) are bioactive compounds with many therapeutic properties. The molecular docking analysis of the chosen ligand molecules with β-tubulin revealed favorable interactions with the residues in the active site. Cinnamic, p-coumaric, ferulic, and caffeic acids showed docking scores that were comparable to those of the reference drug albendazole (-6.9 kcal/mol). The amino acids involved in the protein-ligand interaction, along with the binding energy values, are detailed in Table 4. The analysis of amino acid residues involved in forming hydrogen bonds and their strength were analyzed to understand the affinity of the selected molecules with the target protein. Molecular interactions, such as hydrogen bonds and hydrophobic interactions are crucial in giving shape and stabilizing the docking complexes. [48]. The 3D and 2D images of complexes formed after docking are given in Fig.7.

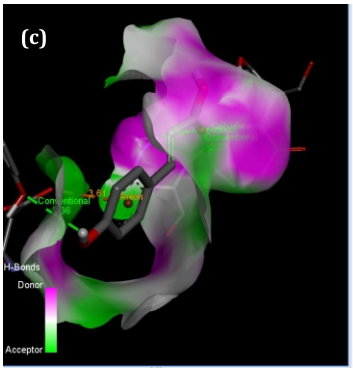
CAA showed the strongest correlation with the protein receptor. The docking results revealed that caffeic acid is embedded in the active cavity of the tubulin receptor by forming hydrogen bonds with GLY B: 144, THR B: 144, and GLN B: 11 residues, and two hydrogen bonds with GLUB: 183 residues. FA showed better interaction with the β-tubulin receptor by forming two hydrogen bonds with THR B: 145 residues. With AutoDock binding energy -5.7 kcal/mol, PCA formed hydrogen bonds with TYR B:124, THR B:145, ASN B:101, and GLY B:144 residues. Cinnamic acid formed a complex with tubular protein with a binding energy of -5.6 kcal/mol. The hydrogen bonding interactions were facilitated by amino acid residues like THR B: 145, GLY B: 144, and ASN B:101. p-CA and CiA formed two hydrogen bonds with the receptor protein.

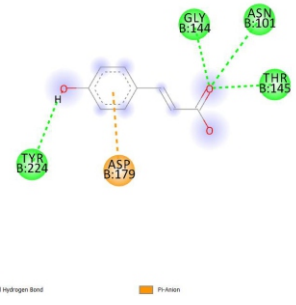


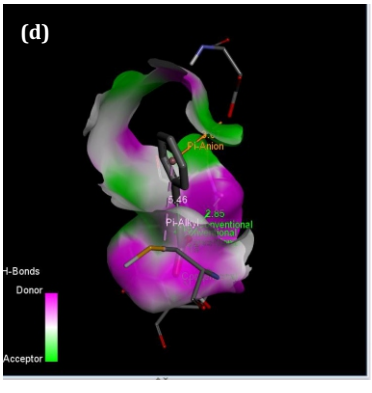


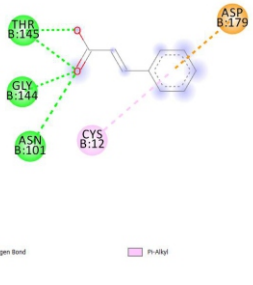












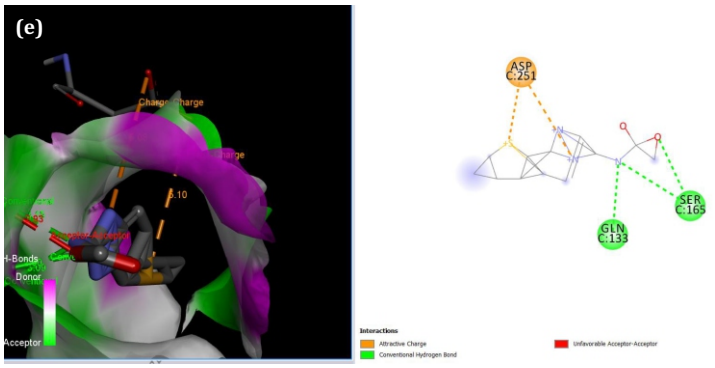


Fig. 7. 3D and 2D images of best docking interaction of selected compounds with 1Oj0 a) CAA b) FEA c) PCA d) CIA e) Albendazole

4. Conclusion

Tubular proteins have emerged as pivotal and highly efficacious targets for inhibiting helminth function. This study analyzed the anthelmintic activity of four phenolic compounds; cinnamic, ferulic, p-coumaric, and caffeic acids. Cinnamic acid and its analogs were found to have significant anthelmintic activity against the Indian earthworm *Pheretima Posthuma,* surpassing the effectiveness of the conventional medication albendazole. Pharmacokinetic and drug-likeness property assessments indicated that cinnamic acid and its analogs might be optimal candidates for use as templates in the design and development of new drugs to treat helminthic infections. After analyzing the interactions between cinnamic acid and its analogs with the tubulin receptor during docking, it was observed that they could effectively bind to the active site of the protein, thereby inhibiting its action. The presence of carboxylic acid, two hydroxyl, and a methoxy group appears to significantly influence the anthelmintic activity of the studied phenolic compounds. Caffeic acid and ferulic acid are highly effective against helminthic infections based on these *in vitro* and *in silico* studies. Further investigations are necessary to use *in vivo* models to establish the pharmacological efficacy of these compounds as potential anthelmintic agents.

Conflict of interest statement

We affirm that we have no conflicts of interest.

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