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Phytochemical Profiling and Anthelmintic Activity of *Manilkara zapota* L. (Chiku) Extracts: An Integrated In-vitro and In-silico Approaches

Sourajyoti Goswami [1], Parshant [2], Vivek Singh Rajpoot [3], Madan Singh [4], Ravindra Shukla [2], Akhilesh Tiwari [1,*]

[1] Laboratory of Pharmaceutical Chemistry, Department of Pharmacy, Indira Gandhi National Tribal University, Amarkantak, Annupur District-484887, India
[2] Laboratory of Bioresource Technology, Department of Botany, Indira Gandhi National Tribal University, Amarkantak, Annupur District-484887, India
[3] Laboratory of Pharmacognosy and Phytochemistry, Department of Pharmacy, Indira Gandhi National Tribal University, Amarkantak, Annupur District-484887, India
[4] Laboratory of Advance Pharmaceutical Chemistry, Department of Pharmacy, Indira Gandhi National Tribal University, Amarkantak, Annupur District-484887, India
* Correspondence: pharmaakhilesh@gmail.com;

Scopus Author ID 57216626433
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Abstract: *Manilkara zapota* is an evergreen tree known for its medicinal value against several diseases. The current investigation was designed to assess the phytoconstituents present in hydro-ethanolic and petroleum ether extracts derived from a combination of seeds and leaves of *M. zapota*. Additionally, the study aimed to explore the potential free radical scavenging and the anthelmintic activities exhibited by these extracts. Phytochemical screening showed the presence of alkaloids, flavonoids, and phenolic compounds. The hydro-ethanolic extract exhibited higher concentrations of total phenolic and flavonoid contents. Specifically, the total flavonoid content in the hydro-ethanolic extract derived from a mixture of leaves and seeds was determined to be 433.6±6.62 mg of quercetin equivalent per gram of extract. In contrast, the total phenolic content was measured at 170.3±5.55 mg of gallic acid equivalent per gram of extract. In assessing antioxidant activity using the DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging assay), the hydro-ethanolic extract demonstrated notable antioxidant efficacy, surpassing the activity observed in the petroleum ether extract. Conversely, the petroleum ether extract displayed no significant antioxidant activity. The obtained hydro-ethanolic extract demonstrated notable anthelmintic properties against adult earthworm *Pheretima posthuma*, exhibiting a mortality rate that was highly promising when compared to the standard drug Albendazole. In parallel, a docking study was conducted, elucidating the binding interactions of all optimized compounds with various amino acid residues situated in the active site of 4MS3, identified as a GABA (gamma-aminobutyric acid) receptor in humans. Remarkably, compounds exhibiting favorable docking scores with 4MS3 demonstrated experimental anthelmintic activity comparable to their in-silico predictions. ProTox calculated and examined the toxicity prediction of the most favorable compound.

Keywords: anthelmintic activity; *Manilkara zapota*; medicinal plants; phytochemical profiling.

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1. Introduction

Manilkara zapota, formerly sapodilla, is an enduring tree native to the Caribbean, Central America, and Southern Mexico. It is extensively cultivated in India, Pakistan, and

Mexico [1]. The fruit it produces is distinguished by its unique, sweet flavor reminiscent of caramel or candied pear. When unripe, the fruit is hard and contains saponin, which can impart an astringent taste. Sapodilla trees yield twice yearly; some produce fruit year-round [2]. Various phytochemicals are abundantly present in numerous medicinal plants at varying levels. Traditional medicinal plants have been revered for their efficacy in addressing diverse health issues, encompassing concerns related to oxidative stress and bacterial or viral infections. Rigorous research has substantiated these botanical remedies' antioxidant properties and antimicrobial activity [3,4].

Oxidative stress, which is primarily caused by the presence of free radicals, plays a crucial role in the initiation of different pathological conditions such as inflammation, rheumatoid arthritis, cancer, neurodegenerative disorders, and the aging process [5]. Free radicals, reactive oxygen species (ROS), chemical reactions, and various redox processes involving different compounds can lead to the oxidation of proteins, DNA damage, and lipid peroxidation within living cells [6]. Incorporating antioxidants into food products acts as radical scavengers, reducing radical chain reactions associated with oxidation. This mechanism helps hinder or inhibit the oxidation process, thereby prolonging the shelf life of these products by slowing down lipid peroxidation [7]. Numerous biochemical and pharmacological studies consistently highlight the health benefits of polyphenols in various herbs. The bioactive components within polyphenol-rich plants have been utilized to develop functional foods or supplements [8].

Helminth infections affect approximately three million people globally, particularly in developing country villages [9]. These infections are increasingly acknowledged as significant contributors to acute and chronic illnesses in humans and cattle [10]. A considerable portion of the world's population is susceptible to various infections, and many cattle are also afflicted by worm infections [11–13]. Parasitic helminths thrive within living hosts, disrupting nutrient absorption, weakening their hosts, and inducing human and animal diseases. These infections can result in anorexia and severe iron-deficiency anemia, particularly in heavy hookworm infections, where the parasites feed on their host's blood [14,15]. Moreover, helminth infections are associated with malnutrition and cognitive impairments. It is imperative to find effective treatments for these infections. However, the high cost and the development of resistance to synthetic anthelmintic drugs have prompted research into medicinal plants as potential solutions. Alternative drugs are also under consideration to prevent the emergence of resistant strains and reduce the costs associated with controlling helminthic diseases. Phytochemicals and plant-based agents have demonstrated promise as botanical anthelmintics [16]. Various medicinal plants have been documented to possess anthelmintic properties by expelling or incapacitating parasitic worms. While there is limited available data on the phytochemistry and pharmacological attributes of a combination of *Manilkara zapota* seed and leaf extracts, there is a noticeable absence of systematic documentation in the scientific literature regarding its anthelmintic potential [16–20]. This research aimed to assess the anthelmintic and antioxidant characteristics of petroleum ether and hydro-ethanolic extracts derived from *Manilkara zapota* leaves and seeds mixer, employing *Pheretima posthuma* as an experimental helminth model. Additionally, an in-silico approach involving the protein 4MS3 was utilized to investigate these properties further [21].

2. Materials and Methods

2.1. Plant materials.

Fresh leaves and seeds of *Manilkara zapota* utilized in this study were plucked from the Department of Pharmacy, Indira Gandhi National Tribal University campus (IGNTU), Madhya Pradesh, India, during March 2023, specifically at the flowering stage. The authentication of the plant specimen was done by the Department of Botany at IGNTU-Amarantak (M. P.) India. A voucher specimen (Accession No: MZ/01/Bot/Sap/2023) was meticulously deposited for future reference. The leaves and seeds were washed with distilled water. Subsequently, the plant parts were cut into small pieces, sun-dried over several days, and then bulb-dried for 24 hours to facilitate optimal grinding. A fine powder was made and kept in an air-tight container for the experiment.

2.2. Chemicals.

The following reagents and substances were purchased from Merck, India, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ascorbic acid, quercetin, Folin-Ciocalteu's reagent, aluminum chloride, sodium nitrate, potassium acetate, petroleum ether, ethanol, acetone, sodium hydroxide, ethyl acetate, and more. Additionally, Albendazole (1 g) was sourced from carbanion.

2.3. Soxhlet extraction of the plant material.

The dried powder derived from the combination of leaves and seeds underwent sequential extraction using a Soxhlet apparatus. Various solvents, including petroleum ether and hydro-ethanol, were employed based on their polarities [22]. Petroleum ether served the purpose of defatting the extract. A rotary evaporator was used to prepare crude from the extract. Prepared crudes were kept in storage vials within a refrigerator set at 4°C until they were utilized for further analyses (40°C) [23–26].

2.4. Phytochemical screening.

The hydro-ethanolic and petroleum ether extract underwent comprehensive preliminary phytochemical screening to identify various phytoconstituents. This analysis included the detection of flavonoids, tannins, phenolic compounds, terpenoids, alkaloids, steroids, glycosides, saponins, proteins, amino acids, and carbohydrates, among other constituents [27,28].

2.5. Quantitative estimation of total phenolic content (TPC).

Initially, a standard solution of Gallic acid was prepared by dissolving 10 µg of Gallic acid in 10 mL of a hydro-ethanolic solution, resulting in a concentration of 1 mg/mL (1000 µg/mL). A sub-stock solution was created by removing 1 mL from the stock and adjusting the volume to 10 mL. A calibration standard solution was then established through a series of dilutions, resulting in concentrations of 2.5, 5, 10, 15, and 20 µg/mL of Gallic acid solutions, with each dilution prepared in triplicate. For each solution, 0.5 mL of Folin–Ciocalteu reagent was added, and the mixture was incubated for 1 minute at 37°C. After that, 2 mL of 20% Na_2CO_3 was added to each solution, and their absorbance was measured at a wavelength of

740 nm using UV spectroscopy. Similarly, the sample preparation involved adding 1 mL of the sample, 0.5 mL of Folin–Ciocalteu reagent, and 2 mL of 20% Na_2CO_3 simultaneously in triplicate. The absorbance of the sample was then determined at the specific wavelength of 740 nm using UV spectroscopy. The total phenolic content was expressed as milligrams of Gallic acid equivalent per gram of crude extract (mg GAE/g crude extract) [29–32].

2.6. Quantification of total flavonoid content (TFC).

To quantify the total flavonoid content, a standard solution was created by dissolving 10 mg of quercetin in 5 mL of hydro-ethanol. The volume was then adjusted to 10 mL in a volumetric flask, resulting in a concentration of 1 mg/mL (1000 µg/mL). Calibration standard solutions were prepared by diluting a sub-stock solution obtained from the primary stock. Serial dilutions were made by taking 0.5 mL of the solution, adding 0.5 mL of sodium nitrate, waiting for 5 minutes, and then introducing 0.3 mL of aluminum chloride. After 6 minutes, 2 mL of sodium hydroxide was added. Solutions with 20, 40, 60, 80, and 100 µl volumes were prepared, and their absorbance was measured at a wavelength of 510 nm using UV spectroscopy. Similarly, sample solutions were prepared by taking 1 mL of each sample, followed by adding 0.5 mL of aluminum chloride after 5 minutes. Subsequently, 0.3 mL of sodium nitrate was added after 6 minutes, and 2 mL of sodium hydroxide was introduced. The absorbance of these sample solutions was also measured at the wavelength of 510 nm [2,17,33–35].

2.7. Antioxidant assays.

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay is a widely employed method for evaluating the antioxidant activity of substances. This assay assesses a substance's ability to neutralize initially purple DPPH radicals, converting them into a non-radical form, resulting in a visible color change and enabling the quantification of its antioxidant potential. In the experimental procedure, Ascorbic acid was used as a standard. Initially, 2 mg of the crude was mixed with 2 mL of methanol. Subsequently, various concentrations were prepared, ranging from 20 µL to 100 µL. A DPPH solution was created by dissolving 3.94 mg of DPPH in 100 mL of methanol, followed by a 10-minute incubation period in the dark, after which the absorbance was measured at 517 nm. Following this, 300 µL from each concentration vial was transferred to another vial, and 2000 µL of the DPPH solution was mixed with each concentration, initiating the assessment of antioxidant activity [36–39].

$$\text{Inhibition}\ (\%) = (A0 - A1/A0)\ \times 100$$

Where A_0= control absorbance, A_1= sample absorbance.

2.8. TLC autography.

Thin-layer chromatography (TLC) autography is a method utilized to separate small amounts of compounds and characteristic components of compounds from the prepared crude; for TLC only hydro-ethanolic extract was selected because petroleum ether extract showed no effective values in the case of quantitative analysis and antioxidant activity [40,41].

2.9. LC-MS analysis.

Based upon the primary screening of phytochemical analysis, quantitative and antioxidant screening hydro-ethanolic crude was used for LC-MS analysis, utilizing a Micro mass Q-Tof Micro instrument. The mass range of this instrument is 4000 amu in the quadrupole and 20000 amu in Time of Flight since it is outfitted with both atmospheric pressure chemical ionization (APcI) and electrospray ionization (ESI) sources. An MS/MS mode equipment is used to facilitate structural investigations by creating fragmentation using a hexapole collision cell placed between the two mass analyzers [42–44].

2.10. In-vitro anthelmintic activity.

2.10.1. Experimental model for the experimental setup.

Adult Indian earthworms, specifically *Pheretima posthuman*, were utilized to evaluate anthelmintic activity. The selected model exhibited anatomical and physiological resemblances to the intestinal roundworm parasites present in humans. Procured from damp soil, they underwent a normal saline cleansing process to eliminate fecal matter [19,21].

2.10.2. Anthelmintic activity of earthworms.

In-vitro anthelmintic bioassay of the hydro-ethanolic extract, adult Indian earthworms measuring 4-5 cm in length and 0.1-0.2 cm in width were utilized. The earthworms were divided into groups, each comprising six individuals. Hydro-ethanolic extract were prepared at various concentrations (25, 50, and 100 mg/mL), and a standard drug, Albendazole (at 10 mg/mL), was dissolved in 20 mL of water. The earthworms were exposed to these solutions, and their anthelmintic activity was observed [45]. Paralysis was considered to have occurred when the worms failed to recover even when placed in normal saline. Death was confirmed when the worms lost mobility and their body color faded. Each Petri dish contained six worms of approximately the same size. The worms were observed for their natural movement as well as their reactions to external stimuli. Noteworthy observations were recorded regarding the duration it took for the worms to become paralyzed and eventually die.

2.11. In-silico docking study.

Molecular docking analysis was done by using Autodock-vina software. The protein selected for investigation was 4MS3, functioning as a GABA (gamma-aminobutyric acid) receptor in humans. Due to the unavailability of the nematode GABA protein crystal structure, the human GABA receptor was retrieved from the Protein Data Bank (https://www.rcsb.org/). The GABA receptor is a recognized target for piperazine, a compound with weak GABA-mimetic properties, inducing reversible paralysis of body wall muscles, and the active site was predicted by Dogsitescorer (https://proteins.plus/help/dogsite). The molecular structures of these compounds were visualized using Discovery Studio Visualizer and subsequently converted into a three-dimensional (3D) format. The molecules were saved in PDB format using Open Babel Gui (https://sourceforge.net/projects/openbabel/). The internal ligand was initially removed to identify the most active molecule, and the docking process was carried out in a manner analogous to that of a typical ligand [21,46].

2.12. Toxicity prediction of leading ligand.

Toxicity prediction was calculated by using ProTox. ProTox is a significant tool for the prediction of toxicity of small molecules. After the docking analysis, the ligand showed the best result for predicting the toxicological studies [47].

3. Results and Discussion

3.1. Determine the total extractive value.

The extraction process is crucial in recovering antioxidant phytochemicals from plant samples. Among the various factors influencing the process, the choice of solvents and the chemical properties of the samples are two of the most critical elements, even when time and temperature conditions are consistent. Numerous studies have highlighted how the extractive yield can vary with different solvents [41,48]. The extractive values indicate the extract's overall nutritional or therapeutic potential, which can be used for further experiments. The extractive values of *M. zapota* in petroleum ether and hydro-ethanolic extract are 13.124 gm and 12.157 gm, respectively, as mentioned in Table 1.

Table 1. Total extractive value *M. zapota.*

S. No	Plant extract	Total extract value
1.	Hydro-ethanolic extract	12.157 gm
2.	Petroleum ether	13.124 gm

3.2. Qualitative phytochemical investigations of M. zapota.

Based on existing information, the pharmacological action of *M. zapota* is due to various secondary metabolites, including alkaloids, terpenoids, lignans, steroids, and other phytoconstituents. Hence, the present study confirmed the presence of different phytochemicals, as shown by the qualitative analysis of both extracts given in Table 2.

Table 2. The qualitative analysis of *M. zapota.*

S. No.	Test names	Observations	
		Petroleum ether extract	Hydro-ethanolic extract
1.	Alkaloids	+ve	-ve
2.	Carbohydrates	+ve	+ve
3.	Glycosides	-ve	+ve
4.	Anthocyanins	-ve	+ve
5.	Phenolic compounds	-ve	+ve
6.	Flavonoids	-ve	+ve

3.3. Quantification of total phenol (TPC) and flavonoid contents (TFC).

The bioactive components of phenols and flavonoids are the most prominent in medicinal plants. Thus, the results of TPC and TFC of hydro-alcoholic extracts of *M. zapota* are shown in Table 3. In the current study, the hydro-ethanolic extracts exhibited higher levels of both TPC and TFC, while the petroleum ether extract did not have any positive results in these studies. The widely accepted notion is that plants with higher phenolic and flavonoid content demonstrate superior antioxidant activity, indicating a direct correlation between total phenol content, total flavonoid content, and antioxidant effectiveness [48].

Table 3. Total phenolic contents (TPC) and total flavonoid content (TFC) of *M. zapota*.

S. No.	Extract	TPC (mgGA/g)	TFC (mgQ/g)
1.	Hydro-ethanolic	170.3±5.55	419.56±6.62
2.	Petroleum Ether	-	-

3.4. DPPH antioxidant activity.

The antioxidant activity depends on the context, and the particulars of the system might have an enormous impact on the analysis's result. Therefore, relying solely on a single assay may not adequately represent the antioxidant potential of plant extracts. The effect of antioxidants on DPPH radical scavenging is attributed to their capacity to donate hydrogen atoms or participate in radical scavenging. When a solution of DPPH comes into contact with a substance capable of donating a hydrogen atom, it reduces DPPH to its non-radical form, diphenyl picrylhydrazine, resulting in the loss of its violet color [36]. A lower IC_{50} value suggests strong antioxidant activity. Therefore, the hydroethanolic extract exhibited remarkable antioxidant activity. Numerous studies have shown significant scavenging effects of phytochemicals against DPPH free radicals [38,48]. Thus, Table 4 and Figure 1 mentioned that only the hydro-ethanolic extract exhibited an IC_{50} value higher than the petroleum ether extract. The IC_{50} value for the hydro-ethanolic extract was 204.92 µg/mL, while the standard ascorbic acid showed a 44.83 µg/mL value.

Table 4. IC_{50} value of *M. zapota plant* extracts with the standard.

S. No.	Extracts	IC_{50} values (µg/mL)
1.	Hydro-ethanolic	204.92
2.	Petroleum-ether	-
3.	Ascorbic acid	44.83

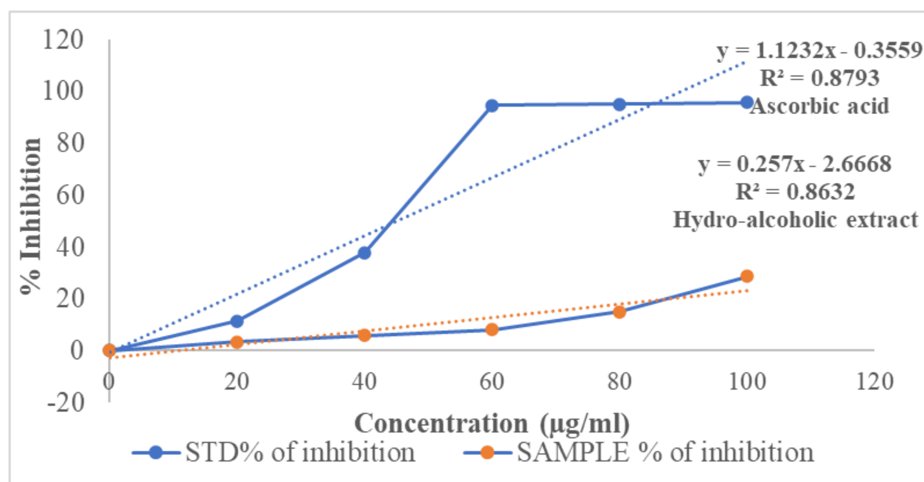

Figure 1. DPPH free radicle activity of hydro-ethanolic extract of *M. zapota*.

3.5. TLC autography.

A densitometric TLC approach was attempted to evaluate *M. zapota* extracts using the different solvent systems as tabulated in Table 5 and Figure 2. To confirm different bioactive metabolites in *M. zapota*, the plant extracts were carefully carried out with their TLC autography using different solvent systems. The correct dilution of both extracts was determined using a TLC densitometer.

Table 5. Mobile phases for different phytochemical tests.

S. No.	Metabolites	Mobile phase	Spot identified	Retardation factor (R_f)	Reference
1.	Alkaloids	Chloroform: methanol: water (70:30:4)	A) Hydro-ethanolic- 1 (Figure 2A)	0.18	[49]
		Chloroform: methanol: 10% ammonia water (80:20:1.5)	A) Hydro-ethanolic- 1 (Figure 2B)	0.91	
2.	Phenols	Hexane: ethyl acetate: glacial acetic acid (5:3:1)	A) Hydro-ethanolic- 1 (Figure 2C)	0.28	[50]
		Methanol: water (50:50)	A) Hydro-ethanolic- 1 (Figure 2D)	0.87	
3.	Flavonoids	Acetic acid: methanol: water (5:36:59)	A) Hydro-ethanolic- 3 (Figure 2E)	0.85,0.61 and 0.25	[50]
4.	Cardiac glycosides	Hexane: ethyl acetate (10:1)	A) Hydro-ethanolic- 1 (Figure 2F)	0.75	[51]
		DCM: methanol (100:1)	A) Hydro-ethanolic- 3 (Figure 2G)	0.64,0.30 and 0.20	
5.	Saponins	Hexane: Ethyl acetate (5:5)	A) Hydro-ethanolic- 2 B) Petroleum ether- 2 (Figure 2H)	A) 0.61 and 0.53 B) 0.64 and 0.38	[52]
6.	Tannins	Methanol: chloroform (70:30)	A) Hydro-ethanolic- 1 B) Petroleum ether- 1 (Figure 2I)	A) 0.26 B) 0.25	[52]
		Ethyl acetate: hexane (20:80)	A) Hydro-ethanolic-1 B) Petroleum ether- 4 (Figure 2J)	A) 0.31 B)0.74, 0.45, 0.36, and 0.12	[40]

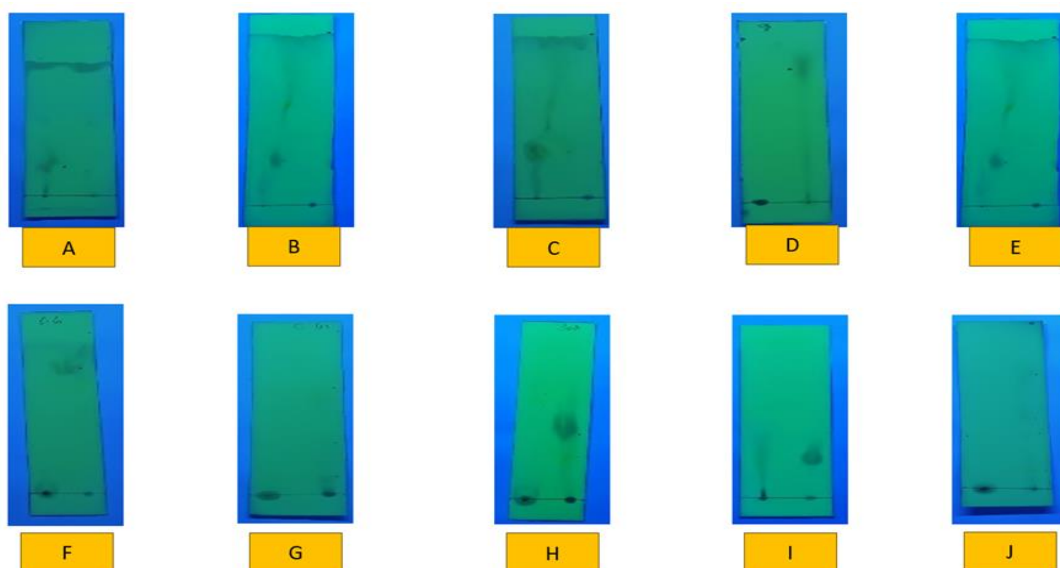

Figure 2. TLC autography plates showing the presence of phytochemicals.

3.6. LC-MS analysis of hydro-ethanolic extract.

The bioactive hydro-ethanolic extract of *M. zapota* underwent analysis by LC-MS spectra, revealing three significant compounds: (1Z,2E)-1-({[(2R,3S,4S,5R,6S)-6-{[2-(3,4-dihydroxyphenyl)-7-hydroxy-5-oxo-5H-chromen-3-yl]oxy}-3,4-dihydroxy-5 {[(2S,3R,4S,5R)-3,4,5 trihydroxy tetrahydro-2H-pyran (HP), having a retention time of 9.23 with a mass of m/z 768.2105, α-Tocotrienol at 21.40162 retention time with a mass of m/z 389.3209, benzyl β-primeveroside at 9.84886 retention time with a mass of m/z 403.1606, δ-Tocotrienol at 19.17 retention time with a mass of m/z 419.2922 as mentioned in Figure 3 and Table 6. The outcomes suggest that the two compounds are the main active ingredients in the

hydro-ethanolic extract and are mostly in charge of the plant's anthelmintic action. Also, Icariside, having a retention time of 9.84 with a mass of m/z 403.1606, Justidine b, Geranyl, Europetin, and Molybdopterin are primarily responsible for antimicrobial and antioxidant activity (Table 6).

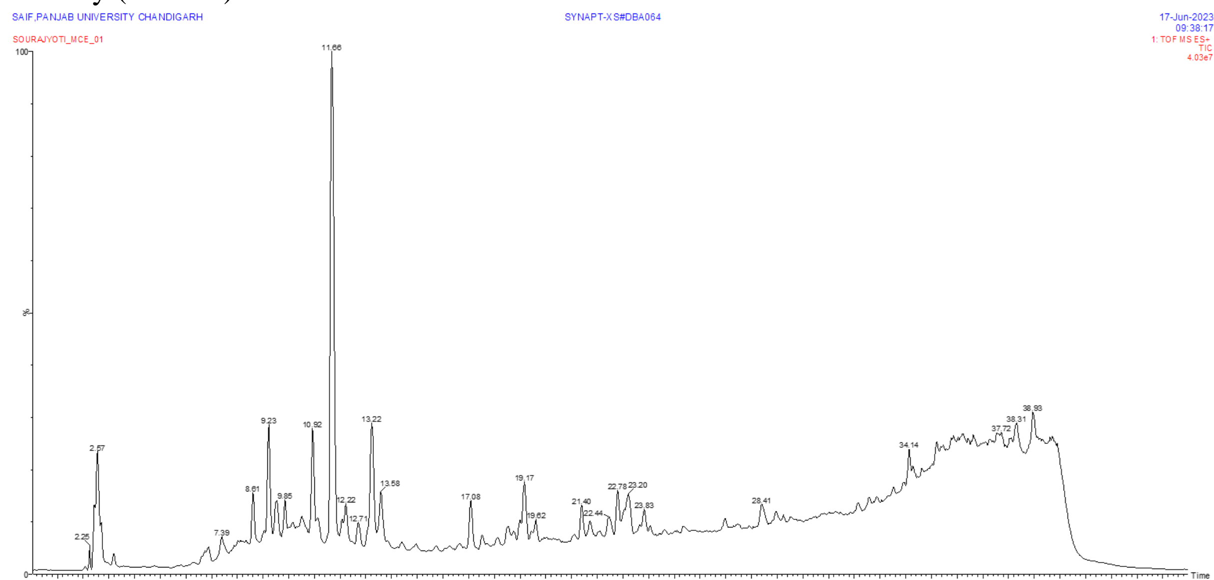

Figure 3. Liquid chromatography-mass spectroscopy (LC-MS) analysis of hydro-ethanolic extract.

Table 6. Bioactive compounds detected in the LC-MS analysis of *M. zapota*

S. No.	Compound	Formula	Score	Fragments	Description	m/z	Retention time
1	7.39	$C_{21}H_{20}O_8$	38.1	0	(-)-4'-demethylepipodophyllotoxin	365.1009	7.39
2	11.66	$C_{33}H_{40}O_{20}$	34.8	0	(1S)-1,5-anhydro-1-[7-(alpha-D-galactopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-6-yl]-2-O-beta-D-glucopyranosyl-D-glucitol	779.2049	11.66
3	9.23	$C_{35}H_{34}O_{17}$	33.1	4.38	(1Z,2E)-1-({[(2R,3S,4S,5R,6S)-6-{[2-(3,4-dihydroxyphenyl)-7-hydroxy-5-oxo-5H-chromen-3-yl]oxy}-3,4-dihydroxy-5-{[(2S,3R,4S,5R)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl]oxy}tetrahydro-2H-pyran-2-yl]methyl}oxonio)-3-(4-hydroxyphenyl)-2-proper-1-plate	768.2105	9.23
4	13.22	$C_6H_{13}O_8PS$	40	18.8	(2R,3S)-2,3,5-trihydroxy-5-(methylsulfanyl)-4-oxopentyl dihydrogen phosphate (non-preferred name)	318.0393	13.22
5	21.40	$C_{19}H_{36}O_5$	35.6	0	(2S)-3-hydroxy-1,2-propanediyl bis(2-propylpentanoate)	383.2187	21.40
6	11.66	$C_{18}H_{12}N_2O_{8-2}$	38.5	0.584	(4E)-4-[(2E)-2-(2-carboxylato-5,6-dioxo-2,3,5,6-tetrahydro-1H-indolium-1-ylidene)ethylidene]-1,2,3,4-tetrahydro-2,6-pyridinedicarboxylate	385.0677	11.66
7	9.85	$C_{19}H_{18}O_3$	37.4	0	(4E,6E)-1-(3,4-dihydroxyphenyl)-7-phenyl-4,6-heptadien-3-one	317.116	9.84
8	11.66	$C_{16}H_{24}O_4$	35.9	0	(5S)-1-(3,4-dihydroxyphenyl)-5-hydroxy-3-decanone	319.1292	11.66
9	11.66	$C_{16}H_{24}O_4$	35.9	0	1-(3,4-dihydroxyphenyl)-5-hydroxy-3-decanone	319.1292	11.66

S. No.	Compound	Formula	Score	Fragments	Description	m/z	Retention time
10	13.58	$C_9H_{11}N_5O_2$	38.8	7.16	1-(4-hydroxy-2-imino-2,4a,7,8-tetrahydro-1H-pyrimido[4,5-b][1,4]diazepin-6-yl)ethenone	186.0778	13.58
11.	12.71	$C_{30}H_{30}O_8$	34.7	0	1',3,6,6',7,7'-hexahydroxy-5',8-diisopropyl-1,3'-dimethyl-2,2'-binaphthalene-5,8'-dicarbaldehyde	271.0947	12.71
12	28.41	$C_{30}H_{48}O_5$	36.7	0.564	10,11-dihydroxy-9-(hydroxymethyl)-2,2,6,6b,9,12a-hexamethyl-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydro-4a(2H)-picenecarboxylic acid	511.3438	28.405
13	22.78	$C_{22}H_{34}O_4$	38.7	5.71	10,14-dihydroxytaxa-4(20),11-dien-5-yl acetate	385.2583	22.78
14	8.61	$C_6H_{11}OS_2+$	36.6	0	1-hydroxy-1,2-di[(1E)-1-propen-1-yl]disulfanium	205.0583	8.61
15	19.17	$C_{27}H_{40}O_2$	38.6	0	2-(geranylgeranyl)-methyl-benzohydroquin	419.2922	19.17
16	17.08	$C_5H_{14}NO_{2+}$	38.3	3.52	2,2-dihydroxy-N, N, N-trimethylethanaminium	143.0917	17.08
17	12.22	$C_{15}H_{17}N_2O_{3-}$	35.7	0	2-[(1H-indol-3-ylacetyl)amino]-3-methylbutanoate	312.0889	12.22
18	17.08	$C_{27}H_{42}O$	36	0	26,27-cyclocholesta-8,24-dien-3-ol	405.3148	17.08
19	9.85	$C_{16}H_{22}O_4$	38	0	2-methyl-1-[2,4,6-trihydroxy-3-(3-methyl-2-buten-1-yl)phenyl]-1-butanone	317.116	9.84
20	13.22	$C_{16}H_{12}O_8$	38.7	3.13	3,3',4',5,7 pentahydroxy-8-methoxyflavone	333.0625	13.22
21	11.66	$C_{33}H_{40}O_{20}$	35.4	3.2	3-[(6-O-hexopyranosylhexopyranosyl)oxy]-5-hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl 6-deoxyhexopyranoside	779.2049	11.66
22	21.40	$C_{19}H_{36}O_5$	35.6	0	3-hydroxy-1,2-propanediyl bis(2-propylpentanoate)	383.2187	21.40
23.	17.08	$C_{30}H_{44}O_{5-2}$	37.5	7.24	3-hydroxyolean-12-ene-23,28-dioate	449.3032	17.08
24	10.92	$C_6H_{10}N_3O_4P$	37.9	0	4-amino-2-methyl-5-phosphooxymethylpyrimidine	261.0752	10.92
25	21.40	$C_{16}H_{26}O_2$	39	0	4-tert-octylphenol monoethoxylate	215.1795	21.40
26	21.40	$C_{22}H_{32}O_4$	35.9	3.54	5-methoxy-3-[(8E,11E)-8,11,14-pentadecatrien-1-yl]-1,2,4-benzenetriol	383.2187	21.40
27	17.08	$C_{27}H_{42}O$	37.4	7.31	7-dehydrodesmosterol	405.3148	17.08
28	13.22	$C_{16}H_{12}O_8$	38.7	3.12	Annulatin	333.0625	13.22
29	9.85	$C_{18}H_{26}O_{10}$	37.3	0	Benzyl ÃŽÂ²-primeveroside	403.1606	9.84
30	9.85	$C_{22}H_{32}O_4$	35.2	0	cannabigerolic acid	383.2187	21.40
31	11.66	$C_{33}H_{40}O_{20}$	34.8	0	DL-cerulenin	262.0849	8.61
32	8.61	$C_{12}H_{17}NO_3$	36.4	0	EUROPETIN	333.0625	13.22
33	13.22	$C_{16}H_{12}O_8$	38.7	3.12	Flavone	245.0582	13.58
34	13.58	$C_{15}H_{10}O_2$	39.3	4.6	Geroquinol	211.1475	17.08
35	17.08	$C_{16}H_{22}O_2$	39.3	0.947	Gibberellin a8-catabolite	327.1211	12.22
36	12.22	$C_{19}H_{22}O_7$	39.4	6	Gossypol	271.0947	12.71
37	12.71	$C_{30}H_{30}O_8$	34.7	0	Icariside F2	403.1606	9.84
38	9.85	$C_{18}H_{26}O_{10}$	37.3	0.0446	Isoflavone	245.0582	13.58
39	13.58	$C_{15}H_{10}O_2$	38.6	1.42	Justicidin B	365.1009	7.39
40	7.39	$C_{21}H_{16}O_6$	38.1	0	Juvenile hormone III	231.1743	17.08
41	17.08	$C_{16}H_{26}O_3$	39.4	0	L-(+)-lysine	147.1229	34.13
42	34.14	$C_6H_{14}N_2O_2$	38.4	1.56	Laricitrin	333.0625	13.22

S. No.	Compound	Formula	Score	Fragments	Description	m/z	Retention time
43	13.22	$C_{16}H_{12}O_8$	38.6	2.94	methyl farnesoate	215.1795	21.40
44	21.40	$C_{16}H_{26}O_2$	39	0	Mevalonic acid-5P	193.0251	11.66
45	11.66	$C_6H_{13}O_7P$	35.8	0	MFCD11045308 α-Tocotrienol	389.3209	21.40
46	21.40	$C_{29}H_{44}O_2$	34.4	1.39	MFCD11045308	425.344	28.40
47	19.17	$C_{29}H_{44}O_2$	36.8	0	MFCD11045309 δ-Tocotrienol	419.2922	19.17
48	13.58	$C_{27}H_{40}O_2$	38.6	0	N-hydroxy-8-(methylsulfanyl) octane thioamide	186.0778	13.58
49	13.22	$C_{16}H_{12}O_8$	38.7	3.13	Patuletin	333.0625	13.22
50	10.92	$C_5H_{10}N_2O_4$	37.4	0	ser-gly	185.0541	10.92
51	13.58	$C_6H_{13}O_7PS$	37.2	0	S-methyl-5thio-D-ribulose 1-phosphate	142.0046	13.58
52	17.08	$C_{27}H_{42}O$	36.7	3.81	zymosterol intermediate 2	405.3148	17.08

3.7. In-vitro anthelmintic activity.

Helminth infections are widespread in regions characterized by warm, humid equatorial climates and inadequate sanitation facilities. Medicinal plants are known to contain a variety of secondary metabolites recognized for their anthelmintic properties. The plant *M. zapota* was previously reported for its anthelmintic activity [20,21].

Table 7. *In-vitro* anthelmintic activity of hydro-ethanolic extracts of *M. zapota* against *Pheretima posthuman.*

S. No.	Test samples	Concentration (20 mL)	Paralysis time (min)	Death time (min)
1.	Control (Saline water)	25 mg/mL	91.54±4.58	200±3.35
2.	Hydro-ethanolic extract	25 mg/mL	21.23±2.08	35.65±4.38
		50 mg/mL	16.12±5.34	25.43±1.45
		100 mg/mL	10.75±2.06	13.34±1.82
3.	Albendazole	10 mg/mL	26.67±3.45	35.30±2.50

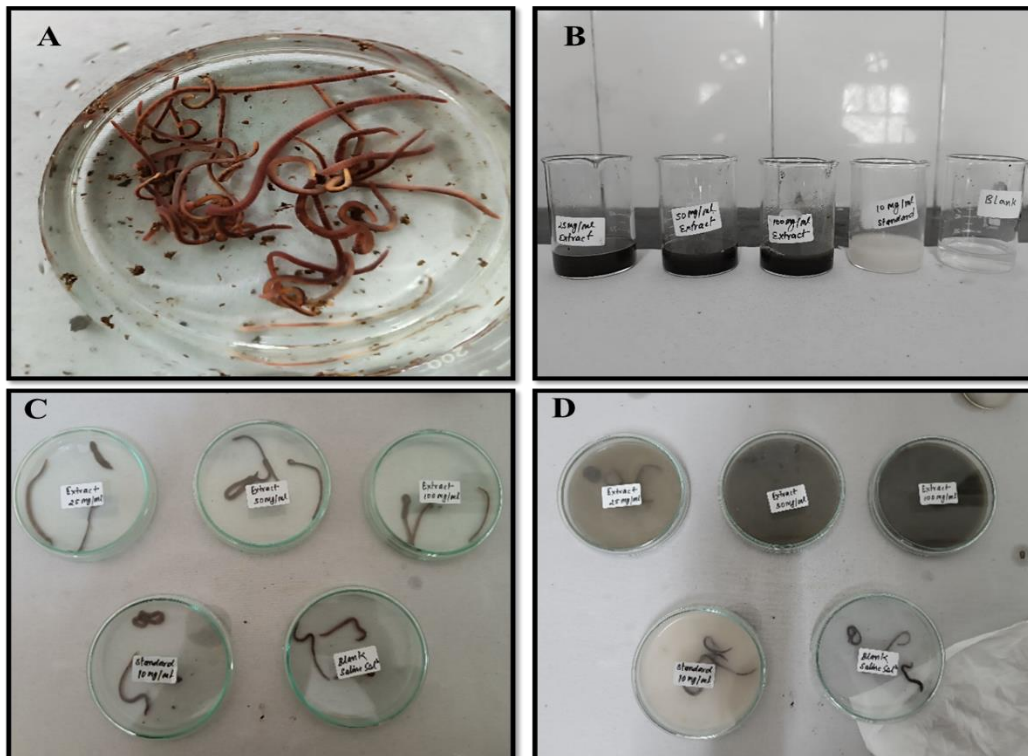

Figure 4. Experimental setup for the in-vitro anthelminthic activity: **(A)** collected earthworms; **(B)** plant extracts with different concentrations; **(C)** paralysis of earthworms; **(D)** death of earthworms.

However, the specific secondary metabolites responsible for this activity and the underlying mechanism have not been thoroughly investigated. In this context, a combination of *M. zapota* seeds and leaves underwent extraction using the Soxhlet technique with various solvents. Preliminary phytochemical screening of the extracts revealed the presence of various secondary metabolites. The petroleum ether and hydro-ethanolic extracts were then assessed for their anthelmintic activity on adult Indian earthworms (*Pheritima posthuma*). Among these two extracts, only hydro-ethanolic extract induced paralysis and mortality in the earthworms and demonstrated potent anthelmintic activity at concentrations ranging from 25 to 100 mg/mL, resulting in paralysis and death of the earthworms as mentioned in Table 7 and Figure 4. Significantly, it surpassed the standard drug Albendazole in its anthelmintic efficacy. The hydro-ethanolic extract displayed highly significant and excellent anthelmintic activity, surpassing the standard drug Albendazole at a concentration of 10 mg/mL. The times for paralysis and death with the hydro-ethanolic extract at a concentration of 25 mg/mL - 100 mg/mL were recorded, respectively. In comparison, it was, respectively, for the standard Albendazole at 10 mg/mL [38,43].

3.8. In-silico anthelmintic activity.

The phytochemicals HP, δ-Tocotrienol, and α-Tocotrienol exhibited significant binding docking scores of -8.8, -8.0, and -8.3 kcal/mol, respectively, compared to the standard drug Albendazole which was -7.7 kcal/mol on the GABA receptor. The docking scores revealed that the compounds from *M. zapota* have a great ability to become an anthelminthic agent. Detailed interactions for HP, δ-Tocotrienol, and α-Tocotrienol are depicted in Figures 5, 6, and 7, respectively, at the GABA receptor binding site, and the presence of conventional hydrogen bonding in all the docked complex makes them more suitable for the anthelminthic activity [45].

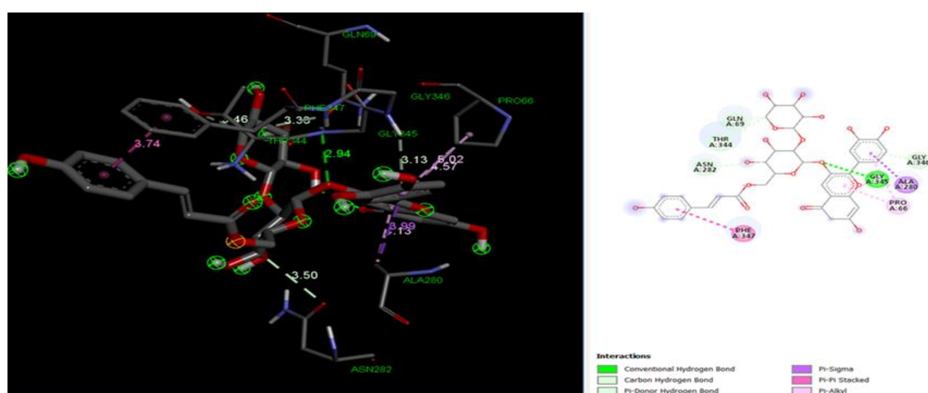

Figure 5. Interaction between HP and the GABA receptor.

Table 8. Binding interactions and distance of bonds (HP and the GABA receptor).
(1Z,2E)-1-({[(2R,3S,4S,5R,6S)-6-{[2-(3,4-Dihydroxyphenyl)-7-hydroxy-5-oxo-5H-chromen-3-yl]oxy}-3,4-dihydroxy-5-{[(2S,3R,4S,5R)-3,4,5-trihydroxytetrahydro-2H-pyran.

Amino acid name and number Docking score:- -8.8	Bond name	Bond distance (Å)
PRO A:66	Alkyl	4.57, 5.02
ALA A:280	Sigma	3.99, 4.13
GLY A:349	Conventional hydrogen bond	2.94
GLY A:346	Cabon hydrogen bond	3.13
PHE A:347	Pi-pi stacked	3.74

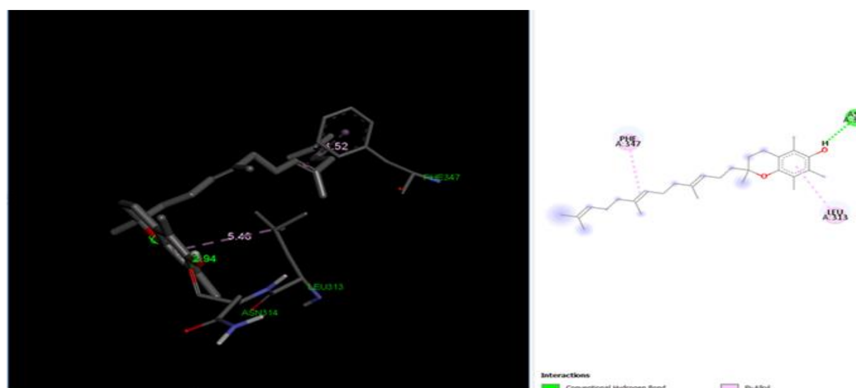

Figure 6. Interaction between δ-Tocotrienol and the GABA receptor.

Table 9. Binding interactions and distance of bonds (δ-Tocotrienol and the GABA receptor).

δ-Tocotrienol

Amino acid name and number Doc score:- -8.3	Bond name	Bond distance (Å)
LEU A:313	Alkyl	5.48
PHE A:347	Alkyl	4.52
ASN A:314	Conventional hydrogen bond	2.94

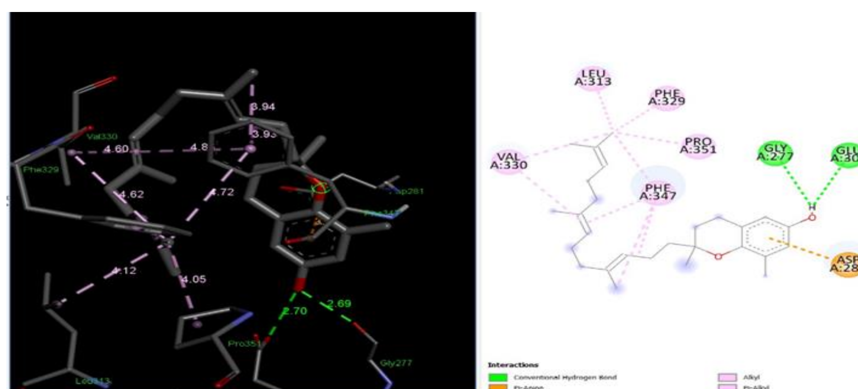

Figure 7. Interaction between α-Tocotrienol and GABA receptor.

Table 10. Binding interactions and distance of bonds (α-Tocotrienol and the GABA receptor).

α-Tocotrienol

Amino acid name and number doc score:- -8	Bond name	Bond distance (Å)
PHE A:347	Pi-Pi stacked	3.72
GLY A:348	Conventional hydrogen bond	2.42
ASP A:281	Conventional hydrogen bond	2.59
GLY A:251	Conventional hydrogen bond	2.30, 2.20
TRP A:65	Carbon hydrogen bond	3.42
TRP A:278	Unfavorable acceptor-acceptor	2.86

Furthermore, LC-MS spectra analysis of the bioactive hydro-ethanolic extract identified four significant compounds, HP, α-Tocotrienol, δ-Tocotrienol, as the primary contributors to the plant's anthelmintic activity. In silico computational studies were conducted to elucidate the mechanism of action of these identified compounds. Remarkably, all three phytochemicals exhibited significantly higher binding affinities than the standard drug Albendazole. Albendazole, a drug with weak GABA-mimetic action, is known for inducing the reversible paralysis of body wall muscles. The four principal compounds identified in *Manilkara zapota* are primarily responsible for its GABA-mimetic action, which leads to the reversible paralysis of the earthworm's body muscles.

3.9. Calculations of toxicity prediction.

The toxicity calculations of the leading ligand or compound were examined, and the network chart showed the connection between the compound and predicted activities. Figure 8 illustrates high predictability against nephrotoxicity, respiratory toxicity, and immunotoxicity. However, there is mild activeness against nutritional toxicity. Toxicity analysis shows the absurdity of dosage and its effects on non-targeted organisms [21,46]. These findings underscore the potential of *M. zapota* as a promising anthelmintic agent.

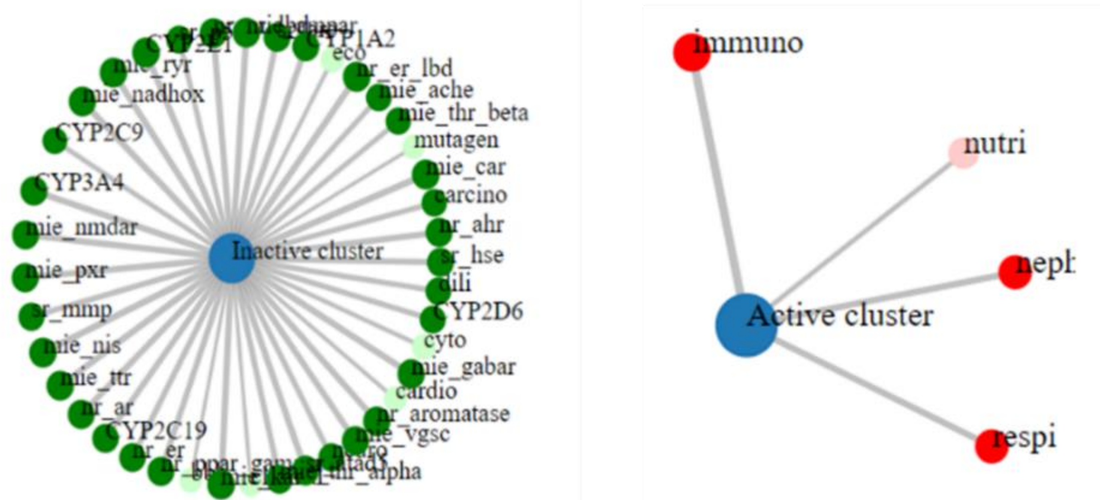

Figure 8. Toxicity prediction of leading ligand/compound.

4. Conclusions

The study reveals that the hydro-ethanolic extract of *Manilkara zapota* possesses substantial antioxidant capabilities and remarkable antimicrobial and anthelmintic properties, indicating its potential to prevent various health issues. *M. zapota* is an accessible and abundant source of natural antioxidants with promising applications in health supplements or the pharmaceutical industries. It is worth noting that all the extracts, excluding the petroleum ether extract, caused paralysis and death in the earthworm. The hydro-ethanolic extract exhibited potent anthelmintic activity at concentrations ranging from 25 to 100 mg/mL, leading to the paralysis and demise of the earthworms, surpassing the efficacy of Albendazole suspension. The hydro-ethanolic extract was found to contain four significant compounds that are primarily responsible for the plant's anthelmintic activity. In silico, the toxicity of the compounds helps reduce the laborious work. Therefore, further research to isolate and identify these bioactive components could pave the way for developing a modern drug derived from this plant. Research in this direction is already underway.

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Conflicts of Interest

The authors declare no conflict of interest.

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