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ASSESSMENT OF ANTIOXIDANT, ANTHELMINTIC, AND CYTOTOXIC ACTIVITIES OF *ZIZYPHUS OENOPLIA* (L.) LEAVES AND IDENTIFICATION OF POTENTIAL LEAD COMPOUNDS THROUGH MOLECULAR DOCKING ANALYSIS

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Abstract

In this study, we examined the antioxidant, anthelmintic, and cytotoxic activities of methanol extract of *Zizyphus oenoplia* leaves (MEZO) in both experimental and computational models. Here, the computational study (*in silico* molecular docking) was carried out to identify the potential lead compounds of this plant for antioxidant and anthelmintic activities. Quantitative phytochemical analysis of MEZO was performed by established methods. Then, *in vitro* antioxidant activity was evaluated by using three different experimental models like DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity, ferric reducing power, and total antioxidant capacity assays. Additionally, anthelmintic activity was determined using aquarium worm (*Tubifex tubifex*), and cytotoxicity was assessed by brine shrimp lethality bioassay, whereas molecular docking study was performed by Schrödinger Maestro 10.1. Our phytochemical study exhibited that the plant extract contains a substantial amount of phenols (57.33 mg), flavonoids (116.19 mg), flavonol (59.77 mg), and condensed tannins (287.85 mg) contents. In antioxidant assays, the methanol extract showed strong antioxidant activity in all models. Moreover, the extract exhibited significant anthelmintic potentials against aquarium worm (*Tubifex tubifex*), while low cytotoxic activities were observed for the plant extract. The present study confirmed that MEZO possesses significant antioxidant, anthelmintic, and cytotoxic properties, which might be due to the presence of high polyphenols contents and one bioactive phytocompound (Zizyphine F) was found to be most effective in the molecular docking study.

Keywords: *Zizyphus oenoplia, antioxidant, anthelmintic, cytotoxic, molecular docking*

Introduction

Zizyphus oenoplia (L.) Mill., belongs to the Rhamnaceae family, is a climbing, thorny shrub, grows along roadside, forests, and thickets. It is commonly known as 'Siyakul' in Bengali, 'Burgi' in Konkani and Marathi, 'Makkay' in Hindi, 'Curia' in Tamil, and 'Boksi bayar' in Nepalese. It is widely distributed in the Indian subcontinent through southern China and Southeast Asia to northern Australia [1]. The plant (*Zizyphus oenoplia*) has several parts, such as leaf, stem, root, and fruits. Different parts of this plant have extensive traditional uses for the treatment of various diseases. For instance, the Konkani peoples of Maharashtra use leaves as a dressing for wounds. The stem bark is used as a mouthwash for sore throats, dysentery, and inflammation of the uterus (In Burma). A decoction of the root bark is used to increase the healing of wounds. Besides, roots were used against helminths and also used in hyperacidity. The fruits are edible and mainly used for stomachache. Fruits are also used in coryza, aphrodisiac, tonic, and fevers [1,2].

The preliminary phytochemical analysis exposed that the plant contains alkaloids, flavonoids, terpenoids, and phenols. It contains three flavones namely Cglucosides-6"sinapoylspinosin, 6"-feruloylspinisin, and 6-"p-coumaroylspinosin [2]. Also, several phytochemicals have been isolated from this plant like cyclopeptide alkaloids such as zizyphine A–F, amphibine-B, Amphibine-F, abyssinines A and B, frangufoline, and Mauritine-D [1–3]. Several pharmacological activities of this plant (leaf, root, and fruit) have been reported previously. Shukla et al. stated that the hydroalcoholic extract of *Z. oenoplia* leaves has analgesic and anti-nociceptive activity [4]. He also stated that the plant has antimicrobial, hepatoprotective, and hypolipidemic properties [2]. Venkanna et al. stated that the leaf part of this plant has antibacterial activity and anthelmintic property against Indian earthworm *Pheretima posthuma* [1]. Cichewitz et al. stated that [5] the plant has anticancer activity and Majumder et al. demonstrated the anthelmintic activity of *Z. oenoplia* Mill root extract. Kuppast et al. reported [6] the wound healing activity of aqueous and alcoholic extracts of fruits of *Z. oenoplia*. Jadhav et al. reported [7] the anti-ulcer activity of *Z. oenoplia* roots in rats.

Even though the plant (*Zizyphus oenoplia*) has a number of significant medicinal properties, up to now, no studies have been performed to determine its antioxidant, anthelmintic, and cytotoxic properties. As a result, this study aimed to examine the antioxidant, anthelmintic, and cytotoxic activities of the methanol extract of *Z. oenoplia* leaves (MEZO) in various experimental models and *in silico* molecular docking study was also performed to identify the potential lead compounds of this plant for antioxidant and anthelmintic activity.

Methods

Plant material collection and identification

Leaves of *Zizyphus oenoplia* (L.) were collected from Bhatiary, Chittagong, Bangladesh in November 2014 and identified by Prof. Dr. Shaikh Bokhtear, botanist, Department of Botany, University of Chittagong, Chittagong 4331, Bangladesh. A voucher specimen with a reference number (CTGUH S1711) has been deposited in the Herbarium of the University of Chittagong for future reference.

Preparation of extract

Approximately 500 g of the powdered materials of the plant was soaked in 950 ml of methanol at room temperature for 14 days with occasional stirring and shaking using a shaker machine. The resultant solution was filtered through a cotton plug followed by filter paper (Whatman No.1). Finally, the filtrate solution evaporated to yield the methanol extract of *Zizyphus oenoplia* leaves (MEZO), which has been stored in a refrigerator at 4°C for further experimental analysis.

Chemical and reagents

Gallic acid, Folin-Ciocalteau reagent (FCR), and aluminum chloride were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Methanol, ethanol, acetic acid, potassium acetate, sodium acetate, sodium carbonate, and formalin were purchased from Merck (Darmstadt, Germany). Vincristine sulfate was obtained from Techno Drugs Ltd., Dhaka, Bangladesh, and Levamisole procured from ACI Limited, Bangladesh, whereas absorbance was taken by using UV-Vis spectrophotometer

(UVmini-1240, Shimadzu, Japan). Quercetin, catechin, and ascorbic acid were obtained from BDH Chemicals Ltd. Poole, UK. All drugs and chemicals used in this experimental works were of analytical grade.

Determination of polyphenols content

Determination of total phenolic and flavonoid content

The total phenolic content was measured by the Folin-Ciocalteu method [8] and expressed as mg GAE/g dried plant extract, while total flavonoid content was estimated by aluminum chloride ($AlCl_3$) assay [9] and expressed as mg QE/g dried plant extract. This study was carried out in triplicates (n = 3), and the results were presented as mean ± SEM.

Determination of total flavonols and condensed tannins contents

The total flavonol content was determined by Kumaran and Karunakaran method [10] and expressed as mg QE/g dried plant extract, whereas total condensed tannins content was measured by using the method described by Sun et al. [11] and expressed as mg CE/g dried plant extract. This study was carried out in triplicates (n = 3), and the results were presented as mean ± SEM.

Antioxidant activity

The DPPH (1,1 Diphenyl-1-picrylhydrazyl) free radical scavenging activity of the MEZO was determined as we described previously [12,13], and results were expressed as µg/ml in compared to reference standard ascorbic acid. Then, the reducing power assay of the extract was determined based on the previously reported method [13,14], using ferric ion reducing antioxidant power assay. Additionally, the total antioxidant activity of the extract was determined by the phosphomolybdate method [13,15], while ascorbic acid used as a reference standard. All the experiments were conducted in triplicates (n = 3), and all the antioxidant procedures were explained with details in our previous article [13].

Anthelmintic activity

Anthelmintic activity of the MEZO was investigated by using the method described by Ajaiyeoba et al., whereas an aquarium worm (*Tubifex tubifex*) was used to perform the anthelmintic study because of its anatomical and physiological resemblance with an intestinal worm. The whole procedure was explained with details in our previous article [13,16,17].

Cytotoxicity screening

Cytotoxic activity of the MEZO was determined by using the method of brine shrimp lethality bioassay as we described with details in our previous article [13,18], and the result was expressed based on the LC50 value in compared to reference standard vincristine sulfate.

Selection of compounds for molecular docking study

Zizyphine A (PubChem CID: 6324833) and Zizyphine F (PubChem CID: 11953941) were selected based on the literature review [1–3] and structures of the chemical compounds were obtained from the PubChem compound repository (https://pubchem.ncbi.nlm.nih.gov/).

Molecular docking study

Ligand and enzyme preparations

Ligand was prepared by using the LigPrep tool, which embedded in Schrödinger suite-Maestro v 10.1, where the following parameters were used as follows: neutralized at pH 7.0 ± 2.0 using Epik 2.2 and the OPLS_2005 force field was used for minimization. Conversely, 3D crystallographic structures of enzymes were obtained from the Protein Data Bank RCSB PDB [19]: xanthine oxidoreductase enzyme (PDB: 1R4U) [20] and tubulin (PDB ID: 1SA0) in complex with colchicine [21]. The enzymes were prepared for the docking study using Protein Preparation Wizard, which embedded in Schrödinger suite-Maestro v 10.1, as we described previously [22,23].

Glide docking

Molecular docking study was performed to explain the possible mechanism of action of the selected compounds behind the pharmacological activities (antioxidant and anthelmintic) of the plant against the respective enzymes. Docking experiments were performed using Glide standard precision docking, which was embedded in

Schrödinger suite-Maestro v 10.1, as we described previously [22–25].

Statistical analysis

The values were presented as mean ± SEM (standard error of mean) while SPSS (v 20) was used for data analysis, and all comparisons were made by using one-way ANOVA followed by Dunnett's multiple comparison test. The value is considered as statistically significant when p-value less than 0.001.

Results

Polyphenols content

The total phenol, flavonoid, and flavonol contents of MEZO were 57.33 ± 3.18 mg Gallic acid/g, 116.19 ± 4.29 mg Quercetin/g, and 59.77 ± 0.15 mg Quercetin/g dried extract, respectively (**Table 1**). On the other hand, the total condensed tannins content of MEZO was expressed in catechin equivalents (CE), and the result was shown in **Table 1**, where the total condensed tannins content was 287.85 ± 0.54 mg CE/g dried plant extract.

Antioxidant activity

DPPH free radical scavenging activity

Free radical scavenging activity of MEZO was measured using the DPPH method, as shown in **Figure 1**. The percentage of scavenging activity was plotted against concentration, and from the graph, IC50 value was calculated using linear regression analysis. The extract (MEZO) exhibited mild free radical scavenging effects compared to reference standard ascorbic acid. Where the IC50 value of ascorbic acid was 6.76 µg/ml, and MEZO was 738.82 µg/ml.

Ferric reducing antioxidant power assay

The ferric reducing power of a compound is related to its electron transferability and may, therefore, serve as an indicator of its potential antioxidant activity. Data for the reducing power of MEZO was shown in **Figure 2**, and a dose-dependent reducing capability was observed in reducing power assay.

Total antioxidant activity

The total antioxidant capacity of MEZO was expressed in ascorbic acid (AA) equivalents, and the result was represented in **Table 1**, where the total antioxidant capacity was 166.90 ± 1.31 mg ascorbic acid/g dried plant extract.

Anthelmintic activity

Following the procedure of Ajaiyeoba, the anthelmintic activity of MEZO was determined on an aquarium worm (*Tubifex tubifex*). The degree of anthelmintic activity shown by the extracts (MEZO) was found to be directly proportional to the concentration of the MEZO (methanol extract), ranging from the lowest concentration (5 mg/ml) to the highest concentration (10 mg/ml). At the concentration of 5 and 8 mg/ml, the extract showed significantly ($P < 0.001$) paralysis time of 31.66 ± 1.45, 11.35 ± 0.64 min and significant death time of 63.33 ± 2.60, 22.11 ± 1.06 min respectively. In contrast, for 10 mg/ml, the extract showed paralysis time of 5.67 ± 0.23 min and death time of 10.66 ± 1.20 min (**Table 3**). These results were compared to that of the standard drug of Levamisole at 1 mg/ml concentration showed paralysis time 3.22 ± 0.05 min, and death time 6.19 ± 0.35 min.

Cytotoxicity assay

In vitro brine shrimps lethality bioassay was used to check the cytotoxic effect of the MEZO in different concentrations, as shown in **Figure 3**. The positive control (vincristine sulfate) and negative control (using DMSO and Seawater) were also used to compare the toxic activities of the extract. The LC50 value for the methanol extract of *Zizyphus oenoplia* leaf was found to be 1252.61 µg/ml, and that of vincristine sulfate was 0.89 µg/ml. However, no mortality was obtained for the negative control group.

Molecular docking study for antioxidant and anthelmintic activity

In the present study, the two selected compounds were docked against the Xanthine oxidoreductase (PDB: 1R4U) and the tubulin colchicine enzymes (PDB ID: 1SA0) for antioxidant and anthelmintic activity, respectively. The result of the docking study is shown in **Table 3**, and the docking figure is presented in **Figures 4**. Our result demonstrated that only Zizyphine F showed the binding affinity towards the target enzymes with a

docking score of -2.48 and -3.61 kcal/mol for antioxidant and anthelmintic activity, respectively.

Discussion

The present study was performed to examine the antioxidant, anthelmintic, and cytotoxic activities of the methanol extract of *Z. oenoplia* leaves (MEZO) in both experimental and computational methods. The preliminary phytochemical analysis of this plant revealed that the plant contains various phytochemicals, like alkaloids, flavonoids, terpenoids, and phenols [2]. Moreover, our quantitative phytochemical analysis exhibited that MEZO contains a significant amount of phenols (57.33 ± 3.18 mg gallic acid equivalent/g dried extract), flavonoids (116.19±4.29 mg quercetin equivalent /g dried extract), flavonol (59.77 ± 0.15 mg quercetin equivalent /g dried extract) and condensed tannins (287.85 ± 0.54 mg catechin equivalent /g dried extract).

Free radicals (DPPH, hydrogen peroxide, superoxide anions, and nitric oxide radicals) are known for creating a wide variety of pathological manifestations such as neurodegenerative, cardiovascular and liver diseases, arthritis, atherosclerosis, diabetes mellitus, renal failure, cancer, metabolic disorders, thrombus formation, aging, DNA damage, etc. In this case, antioxidants fight against free radicals and protect us from various diseases [26,27]. For the assessment of the antioxidant activity of MEZO, we began our examination with the DPPH free radical scavenging assay, which is a broadly used method to assess the scavenging activity of plant extract or novel compounds. This assay is primarily happening in the presence of antioxidant compounds of plant extract where the DPPH solution is reduced into non-radical DPPH-H (α, α diphenyl-β-picryl hydrazine) by the reaction. After the reduction of stable DPPH radical, it changes the color from purple to yellow to a varying degree depending on the presence of antioxidant compounds in the plant extract [28,29]. In this study, the crude methanol extract showed a good scavenging effect compared to the reference standard ascorbic acid (**Figure 1**). Secondly, we were carried out the ferric reducing antioxidant power assay to examine the antioxidant activity of MEZO. This method is commonly known as the potassium ferric cyanide reduction method, which is based on the reduction of ferric to ferrous by antioxidant compounds visible in changing the yellow color of the test solution to various shades of green and Prussian blue, depending on the reducing power capacity of the plant extract [27]. In this study, the crude methanol extract showed a dose-dependent ferric reducing capacity (**Figure 2**). Thirdly, the total antioxidant capacity of MEZO was examined by using the phosphomolybdenum method. It is based on the reduction of molybdenum (VI) to molybdenum (V) by the test extract and succeeding formation of a molybdenum (V) complex (green phosphate) at acidic pH [27]. In this study, our result indicated that crude methanol extract showed significant antioxidant activity for phosphomolybdate reduction (**Table 1**). Our quantitative phytochemical study revealed that MEZO contains a substantial amount of phenols (57.33 mg), flavonoids (116.19 mg), flavonol (59.77 mg) and condensed tannins (287.85 mg) contents, and also previously reported preliminary phytochemical study indicated that the plant contains alkaloids, flavonoids, terpenoids, and phenols [2]. It has been reported previously that phenolic compounds are responsible for the free radical scavenging effect of the plant and also could play an essential role in the reducing power of the plant extract [2,30–32]. Moreover, earlier studies have been noticed that flavonoids and polyphenols (such as flavonoids, phenols, and tannins) contribute significantly to the phosphomolybdate scavenging activity of plants [27,33,34]. Therefore, it might be possible that the presence of such phytochemicals could be

responsible for the free radical scavenging activity, ferric reducing power, and total antioxidant capacity of the methanol extract.

Infections by helminths are a significant cause of concern for human health and livestock. These infections are more common in tropical countries (especially Asian countries) and develop several clinical manifestations including malnutrition, pneumonia, eosinophilia, anemia, dysentery, dermatological disorders, and loss of appetite and body weight [35]. Moreover, the unregulated use of anthelmintic agents has developed resistance against most helminths, which push us to search novel anthelmintic drug candidates that have fewer side effects and most compatibility with human physiology [36]. In this study, a dose-dependent and significant anthelmintic activity were displayed by the methanol extract (**Table 2**). Our quantitative phytochemical study revealed that the extract contains a substantial amount of phenols (57.33 mg), flavonoids (116.19 mg), flavonol (59.77 mg) and condensed tannins (287.85 mg) contents, and also previously reported preliminary phytochemical study indicated that the plant contains alkaloids [37], flavonoids [38], terpenoids [39], and phenols [38,40]. This activity might be due to the presence of such phytochemicals in the plant extract as an earlier report demonstrated that alkaloids, flavonoids, tannins, terpenoids, and phenols have strong anthelmintic potential.

Brine shrimp lethality bioassay is a rapid, economical, and simple bioassay technique to investigate the cytotoxic activity and considered to be the most important and preliminary choice for the development of new chemotherapeutic agents [27,41]. In this bioassay, the smaller the LC50 value, the more toxic the compound/extract is, and the larger the LC50 value, the lower the toxicity of the extract. Here, a plant extract is considered to be non-toxic at the value of LC50 greater than 1000 µg/ml, weakly toxic at 500–1000 µg/ml, moderately toxic at 100–500 µg/ml and while that with an LC50 less than 100 µg/ml is said to be highly toxic. Our result demonstrated that MEZO has an LC50 value of 1252.61 µg/ml, which is a clear indication for non-toxic plant extract [42]. Besides, a previous acute oral toxicity study of this plant did not show any abnormal behavior, mortality, and neurological changes up to 1000 mg/kg doses, which indicates that the plant extract has a low toxicity profile and safe for a therapeutic dose [4].

Molecular docking is a key computer-assisted drug design (CADD) tool for the drug discovery process, which primarily used to characterize the behavior of small molecules in the binding site of target proteins as well as to understand the mechanism of pharmacological activity [23,43]. Because of that, *in silico* molecular docking study was performed to understand the molecular mechanism behind the pharmacological responses better. In this study, two significant compounds of *Z. oenoplia* were investigated against two target enzymes, i.e., Xanthine oxidoreductase (PDB: 1R4U) and tubulin colchicine enzymes (PDB ID: 1SA0) for antioxidant and anthelmintic activity, respectively, and the docking scores obtained for all compounds have been reported in **Table 3**. Here, our result indicated that only Zizyphine F docked against the target enzymes for both activities. From these results, we can conclude that the studied phytochemical may, in part, be responsible for the antioxidant and anthelmintic activity of MEZO through interaction with these target enzymes.

In summary, results of the present study revealed that the methanol extract of *Z. oenoplia* leaves (MEZO) showed significant antioxidant, anthelmintic and cytotoxic activities. These activities might be attributed to the occurrence of high polyphenol contents and could be due to the synergistic effect of several phytochemicals like alkaloids, flavonoids, phenols, and terpenoids. In addition, our

molecular docking study exhibited that Zizyphine F has a higher binding affinity towards the target enzymes for antioxidant and anthelmintic activity, respectively. Therefore, it can be concluded that this compound could be a good candidate for the development of new antioxidant and anthelmintic agents through further study is still necessary to explain its molecular mechanism of action, safety, and toxicity in animal model.

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Table 1. Total phenols, flavonoids, flavonols, condensed tannins content and total antioxidant capacity of methanol leaf extract of *Zizyphus oenoplia*.

Tested extract	Total phenol content (mg gallic acid /g dried extract)	Total flavonoid content (mg quercetin/g dried extract)	Total flavonols content (mg quercetin /g dried extract)	Total condensed tannins content (mg catechin /g dried extract)	Total antioxidant capacity (mg ascorbic acid/g dried extract)
MEZO	57.33 ± 3.18	116.19 ± 4.29	59.77 ± 0.15	287.85 ± 0.54	166.90 ± 1.31

Each value in the table is represented as mean ± SEM (n = 3). MEZO refers to methanol extract of *Zizyphus oenoplia*.

Table 2. Anthelmintic activity of methanol leaf extract of *Zizyphus oenoplia*

Treatment	Time is taken for paralysis (min)	Time is taken for death (min)
NC (Water)	0.00	0.00
PC (1 mg/ml)	3.22 ± 0.05	6.19 ± 0.35
MEZO (10 mg/ml)	5.67 ± 0.23	10.66 ± 1.20
MEZO (8 mg/ml)	11.35 ± 0.64***	22.11 ± 1.06***
MEZO (5 mg/ml)	31.66 ± 1.45***	63.33 ± 2.60***

Each value in the table is represented as mean ± SEM (n = 3); MEZO denote for methanol extract of *Zizyphus oenoplia*; NC, Negative control; PC, Positive control (Levamisole). ***P < 0.001 compared with positive control group (Dunnett's test).

Table 3. Docking score of the selected compounds with *xanthine oxidoreductase* (PDB: 1R4U) and tubulin colchicine (PDB ID: 1SA0) enzymes for antioxidant and anthelmintic activity respectively

Compounds	Antioxidant activity: *Xanthine oxidoreductase* enzyme (PDB: 1R4U)			Anthelmintic activity: Tubulin colchicine enzyme (PDB ID: 1SA0)		
	Docking score (kcal/mol)	Glide e model (kcal/mol)	Glide energy (kcal/mol)	Docking score (kcal/mol)	Glide e model (kcal/mol)	Glide energy (kcal/mol)
Zizyphine A	-	-	-	-	-	-
Zizyphine F	-2.48	-26.72	-31.90	-3.61	-42.34	-45.41

Figure 1. DPPH free radical scavenging activity of MEZO compared with the reference standard (ascorbic acid) as assessed by spectrophotometric method using DPPH free radicals. MEZO denote for methanol extract of *Zizyphus oenoplia* leaves.

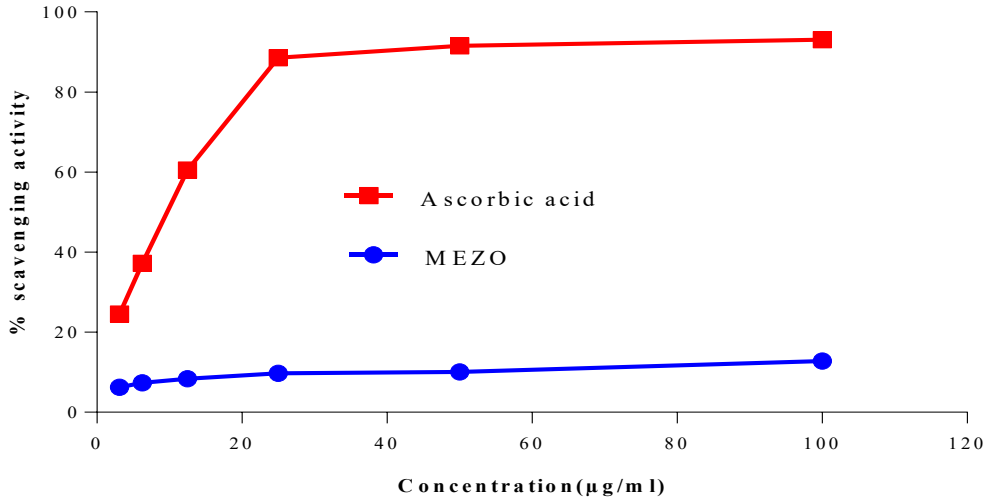

Figure 2. Reducing power capacity of methanol extract of *Zizyphus oenoplia* leaves (MEZO) compared with the reference standard ascorbic acid.

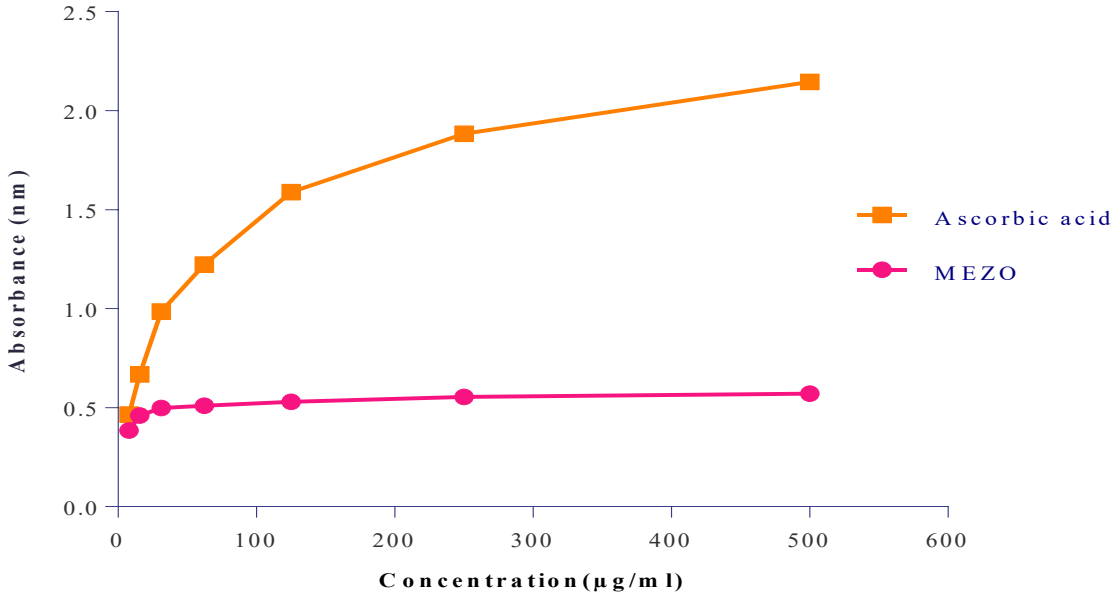

Figure 3. Determination of LC50 value of methanol extract of the *Zizyphus oenoplia* (MEZO) from linear correlation between concentrations versus percentage of mortality

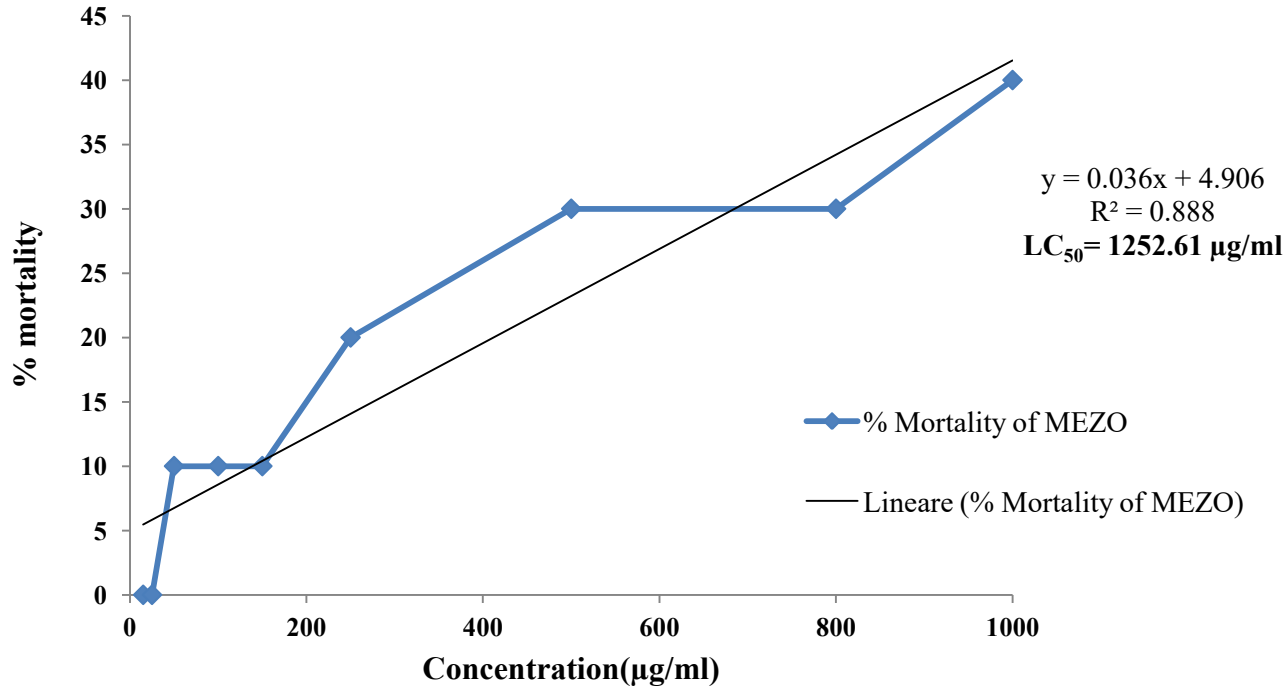

Figure 4. 2D interactions of the (A) Zizyphine F and (B) Zizyphine F with the active site of *xanthine oxidoreductase* (PDB: 1R4U) and tubulin colchicine (PDB ID: 1SA0) enzymes for antioxidant and anthelmintic activity, respectively.