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Investigation of the Cytotoxic Potential of Standardized Ziziphus mauritiana Lam. seed extract Against Cell Lines

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ABSTRACT: Cancer remains a significant health concern, necessitating the development of safer and more efficient treatment alternatives, particularly those derived from natural sources. In this study, we explored the cytotoxic potential of a standardized ethanolic seed extract of Ziziphus mauritiana Lam., a plant known for its traditional medicinal uses. Extracts were prepared by successive solvent extraction and the resulting extracts were tested for the presence of various phytochemicals. The total phenolic content and total flavonoid content of the extract were determined using colorimetric methods. The DPPH radical scavenging assay was performed to assess antioxidant activity. Further, the ethanolic extract was subjected to GC-MS analysis and LC-MS profiling. The in vitro cytotoxic potential was then tested on Neuroblastoma (N2a) cancer cell line using MTT assay. Phytochemical screening revealed the presence of flavonoids, phenolics, and saponins in aqueous and alcoholic extracts. The total phenolic and flavonoid contents were estimated for both aqueous and ethanolic extracts, revealing higher levels in the ethanolic extract compared to the aqueous extract. Ethanolic extract exhibited significant antioxidant potential. The ethanolic extract was analyzed by GC–MS and LC–MS techniques to identify and phytochemicals, which revealed various bioactive compounds such as fatty acids, sterols, Phenolic compounds, flavonoids and triterpenoids. The extract showed mild, dose-dependent cytotoxicity with 11.05% inhibition at 100 µg/mL, slightly higher than 10 µM Rotenone (7.73%). The IC_{50} value was 493.73 µg/mL, indicating low toxicity. These findings, along with its rich phytochemical content, suggest potential for safe use in neuroprotective or supportive cancer therapies.

INTRODUCTION

Cancer remains a global concern despite recent advancements in technology and medicine [1]. Cancer remains one of the top causes of death across the globe. In 2022 alone, nearly twenty million people were diagnosed with the disease, and around 9.7 million lives were lost due to cancer-related complications [2]. Neuroblastoma (NB) is a childhood cancer that starts in the peripheral sympathetic nervous system during early development. Neuroblastoma (NB) is an embryonal tumor that originates from primitive cells. It arises from neural crest cells, which are supposed to become nerve cells, but due to genetic and epigenetic problems, they fail to mature properly. This cancer can develop in various parts of the body where these cells travel and settle, most commonly in the adrenal medulla (the inner part of the adrenal glands) or in nerve tissues alongside the spine, called paraspinal ganglia, in 2021, there were an estimated 5,560 cases of neuroblastoma in children worldwide, with about 1,977 deaths. Among all countries, India reported the highest number of neuroblastoma cases that year, with approximately 685 cases [3].

Medicinal plants offer several benefits compared to synthetic chemicals, as the natural compounds they contain are generally gentler and less toxic to healthy human cells [4]. over 60% of the anticancer drugs approved for clinical use are either derived from or inspired by compounds found in medicinal plants**,** highlighting their vital role in modern cancer treatment [5]. *Ziziphus mauritiana* is a hard-evergreen species that grows either as a shrub or a small tree, reaching heights of up to 15 meters [6]. This plant has long been valued in traditional medicine for its wide range of healing uses. The seeds are especially versatile—they're believed to improve eye health, treat leukorrhea, and serve as a tonic that strengthens the heart and brain. They're also used to relieve thirst and have a calming, sleep-inducing effect, making them helpful for insomnia, pain, weakness, and rheumatic problems [7]. This study aimed to explore the potential of *Ziziphus mauritiana* Lam. seeds for its cytotoxic activity against specific cell lines to evaluate its possible use in therapeutic applications.

MATERIALS AND METHODS

The seeds of *Ziziphus mauritiana* Lam. were collected in November 2024 from Doddajalodu village, Pavagada Taluk, Tumakuru district, Karnataka, India. The botanical identity of the plant material was confirmed by Dr. Noorunnisa Begum,Taxonomist, Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, and it was assigned the accession number FRLH 6709.

Preparation of Extracts

Powered *Z. mauritiana* Lam. was subjected to successive solvent extraction using solvents in increasing order of polarity. Initially, it was defatted with *n*-hexane, followed by extraction using chloroform, ethyl acetate, acetone, and ethanol. After each solvent extraction, the material was air-dried at temperatures below 50 °C before proceeding to the next solvent. After the final extraction, the remaining marc was macerated with chloroform water for 24 hrs to obtain the aqueous extract. Each extract was concentrated by removing the solvent through distillation and then evaporated to dryness using a water bath. The dried extracts were weighed, and the percentage yield was calculated based on the air-dried weight of the starting plant material [8]. The consistency and colour of each extract were also recorded. The extracts obtained upon successive solvent extraction will be subjected for preliminary qualitative screening of phytochemicals for the identification of phytoconstituents [9].

Total phenolic content

The Phenolic content was determined by Folin - Ciocalteu method. Absorbance for test and standard solutions were measured against the blank at 725 nm with an UV-Visible spectrophotometer. The amount of Phenolic content in the plant extracts was expressed in mg Gallic acid equivalents (GAE) per g dry weight of the plant extract. For the estimation of total phenolic content (TPC), both ethanolic and aqueous extracts of *Ziziphus mauritiana* Lam. were analyzed at concentrations of 500 and 1000 µg/mL, with three independent samples tested for each concentration. Gallic acid was used to construct a standard calibration curve with concentrations of 0, 25, 50, 75, 100, 125, and 150 µg/mL, prepared in duplicate. The reaction mixtures were then prepared as per the previously described method, and absorbance was measured at the predetermined wavelength of 760 nm after a 30-minute incubation. All measurements were carried out in triplicate [10].

Total flavonoid content

The plant extracts were first dissolved in methanol and then filtered using Whatman filter paper to estimate total flavonoid content, 300 µL of each extract was taken in a 10 mL test tube. To this, 3.4 mL of 30% methanol, 150 µL of 0.5 M sodium nitrite, and 150 µL of 0.3 M Aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) were added and thoroughly mixed. After incubating the mixture for 5 minutes, 1 mL of 1 M sodium hydroxide (NaOH) was added.The absorbance of the resulting solution was then measured at 510 nm using a spectrophotometer. A standard calibration curve was also prepared for quantifying total flavonoids using rutin as the reference standard. Different concentrations of rutin (ranging from 0 to 100 mg/L) were processed using the same procedure described above [11,12]

***In-vitro* Antioxidant activity**: DPPH assay was adopted to evaluate the antioxidant potential of the extracts. 3 mL of a 0.1 mM DPPH solution in methanol was mixed with 5 mL of the extract at varying concentrations (ranging from 100 µL to 500 µL) in separate test tubes. The mixtures were then incubated at room temperature for 30 minutes. After incubation, the absorbance was measured at 517 nm using ascorbic acid as the reference standard[13].

Percentage Inhibition= $(ABS\ control - ABS\ test)\ ABS\ control \times 100$

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GC-MS Analysis:

The alcoholic extract of *Z. mauritiana Lam* was analyzed using GC-MS to identify its chemical composition. GC-MS analysis was conducted using a Shimadzu GCMS-QP2010 SE system with a GC-2010 gas chromatograph and AOC-20i auto-injector. Separation was achieved using a DB-5 MS capillary column (30 m × 0.32 mm ID × 0.25 µm film). Helium served as the carrier gas at a flow rate of 1.50 mL/min, with the injector operating in split mode (5:1 ratio) at 280 °C. The oven temperature program began at 50 °C (3 min hold), ramped to 160 °C at 5 °C/min, then to 280 °C at 20 °C/min, with a 4 min final hold. The mass spectrometer operated in electron ionization mode (EI) at 220 °C ion source temperature and 280 °C interface temperature, scanning from m/z 35–500 with a scan speed of 1666 amu/sec. Data acquisition was performed between 3.0 and 35.0 minutes, with compound identification based on comparison with NIST14 and Wiley11 libraries

***In - Vitro* Cytotoxicity**

Anticancer activity of the standardized extract was investigated *in- vitro* using N2a (neuroblastoma) cell lines using MTT assay [14]. N2a cell lines were cultured in DMEM-HG Medium supplemented with FBS and antibiotic solution under standard conditions (37 0C, 5% CO2). Seeding cells in 96-well plates at a defined density and allowing them to adhere for 24 hours. Appropriate concentrations of the extract and one predetermined concentration of the standard were prepared using 0.4% DMSO. After cell attachment, cells were treated with different concentration of the extract and the standard. Plates were incubated for till cells were 70-80% confluent. MTT reagent was added after the treatment period and incubated for 4 hours to allow the formation of formazan crystals. The resulting crystals were dissolved using DMSO. Absorbance was at 570 nm using a microplate reader. The percentage of cell viability was calculated, and the IC_{50} value was determined using non-linear regression analysis.

RESULTS AND DISCUSSION:

Preparation of extracts

The dried seeds of *Z. mauritiana Lam* were subjected to Soxhlet extraction using n-Hexane, Toluene, Chloroform, Ethyl acetate, Ethanol and water respectively. Percentage yield and nature of extracts of *Z. mauritiana* Lam. Seed were shown in the Table 1.

Table 1: Percentage yield and nature of extracts of *Z. mauritiana* Lam. seed

Solvent	Colour	Consistency	% yield(w/w)
n-Hexane	Yellowish Brown	oily	4.16
Toluene	Black	Sticky	0.896
Chloroform	Cream	Powder	0.722
Ethyl acetate	Light Brown	Powder	0.02
Ethanol	Brown	Sticky	0.28
Water	Blackish red	Sticky	3.18

Phytochemical Analysis: The presence of phytoconstituents in various extracts were shown in Table 2

Table 2: Phytochemical Screening of extracts

SI No	Test	n-Hexane	Toluene	Chloroform	Ethyl acetate	Ethanol	Water
1	Alkaloids	-	-	-	+	+	+
2	Carbohydrates	-	-	-	-	+	+
3	Glycosides	-	-	-	-	+	+
4	Phenolic compounds	-	-	-	-	++	+

5	Flavonoids	-	-	+	+	++	+
6	Fixed oils	+	+	-	-	-	-
7	Saponins	-	-	-	-	+	+
8	Phytosterols	+	+	-	-	-	-

Total phenolic content: The total Phenol content (TPC) was determined using the Folin-ciocalteu colorimetric method. A standard calibration curve was prepared using gallic acid, and the results were expressed as gallic acid equivalents (RE) per gram of dry extract weight. The total phenol content of ethanolic extract and aqueous extract are tabulated in Table 3 and fig 1.

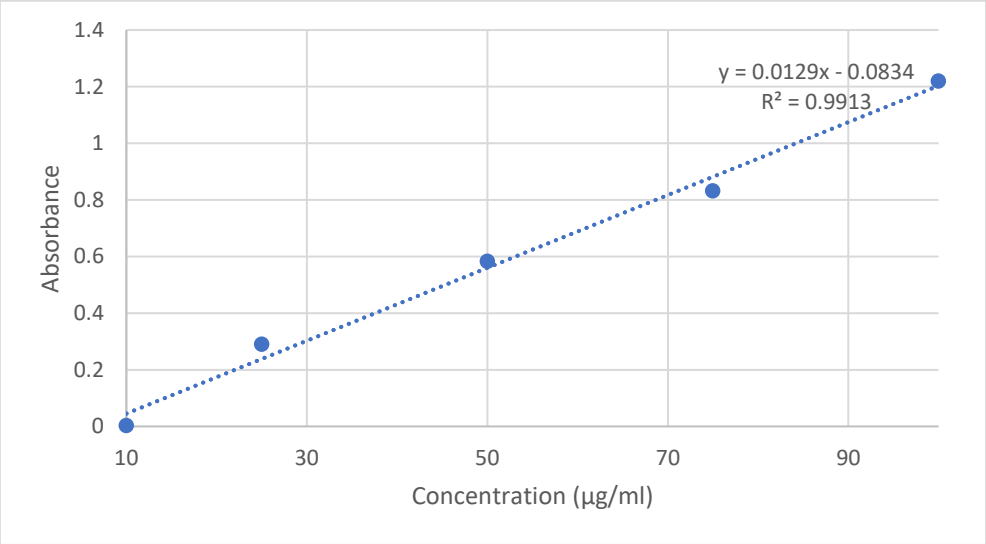


Figure 1: Standard Curve of Gallic acid

Table 3: Total Phenolic content of ethanolic and aqueous extracts

SI.No	Extract	Concentration(µg/ml)	Total phenolic content (mg/GAE/g)
1	Ethanolic extract	500	2.93
		1000	5.26
2	Aqueous extract	500	4.63
		1000	2.87

Total flavanoid content: The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method. A standard calibration curve was prepared using rutin, and the results were expressed as rutin equivalents (RE) per gram of dry extract weight. The flavonoid content of Ethanolic extract and aqueous extract are tabulated in Table 4 and fig 2.

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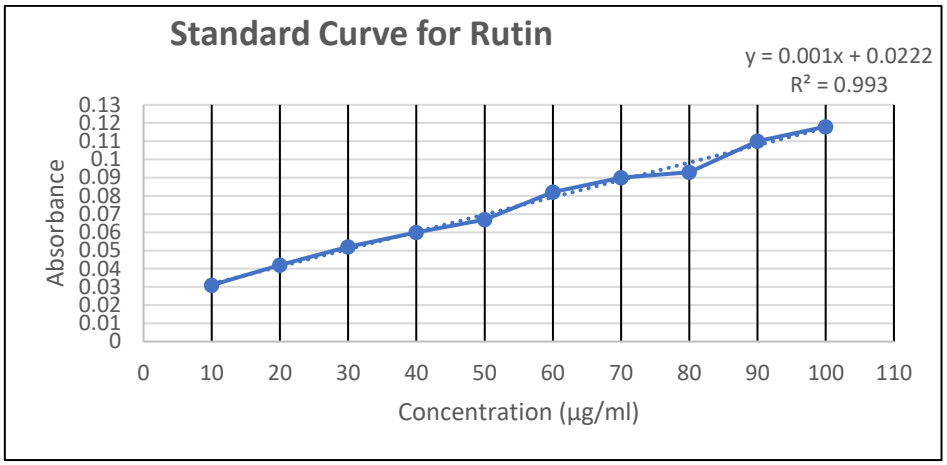


Figure 2: Standard Curve of Rutin

Table 4: Total flavonoid content of ethanolic and aqueous extracts

SI.No	Extract	Concentration(µg/ml)	Total phenolic content (mg/GAE/g)
1	Ethanolic extract	500	3.93
		1000	9.96
2	Aqueous extract	500	2.63
		1000	7.56

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were assessed at 500 and 1000 µg/mL, at 500 µg/mL, the aqueous extract showed a higher TPC (4.63 mg GAE/g) than the ethanolic extract (2.93 mg GAE/g), while at 1000 µg/mL, the ethanolic extract surpassed the aqueous extract (5.26 vs. 2.87 mg GAE/g), demonstrating concentration-dependent solubility. The ethanolic extract showed a higher total flavonoid content (9.96 mg Rutin/g) compared to the aqueous extract (8.56 mg Rutin/g), indicating better extraction efficiency with ethanol. This suggests that ethanol may be more effective in extracting flavonoid-rich constituents from the plant material [15].

***In-vitro* antioxidant activity:**

The ethanol and aqueous crude extracts were investigated for anti-oxidant activity by DPPH radical scavenging method. Results are shown in Figure 3. DPPH assay results confirmed the antioxidant potential of both extracts. The ethanolic extract demonstrated stronger activity, with a maximum inhibition of 66.89% at 500 µg/mL and compared to the aqueous extract's 59.75% inhibition. Though both extracts showed lower activity than the standard ascorbic acid the results affirm the antioxidant capacity, likely due to the presence of phenolics and flavonoids identified through phytochemical tests Phenolics and flavonoids exhibit strong antioxidant activity by scavenging free radicals and chelating metal ions. They also contribute to cytotoxic effects by inducing apoptosis and disrupting cancer cell signaling pathways.

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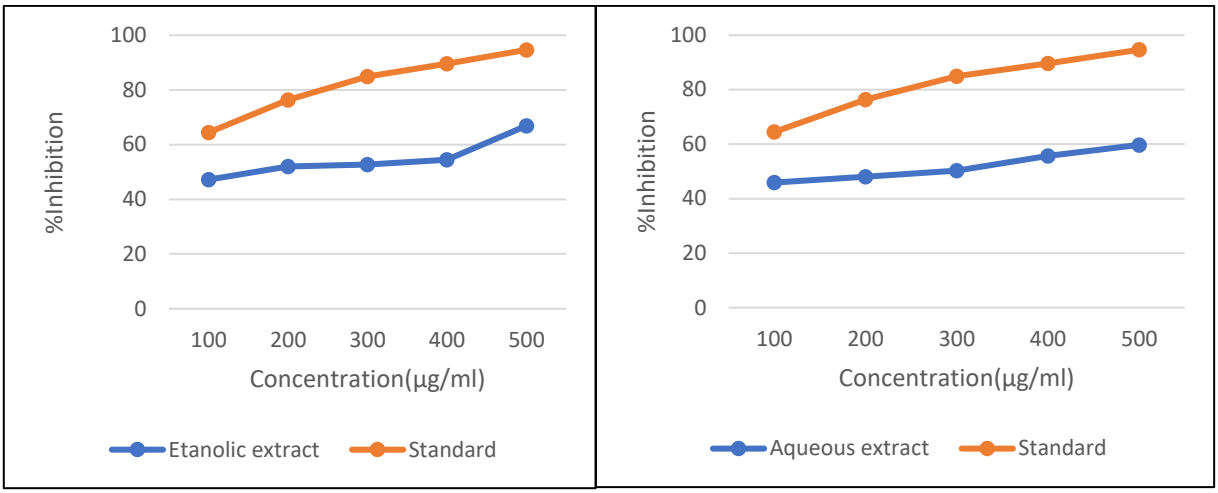


Figure 3: Antioxidant potential of ethanolic and aqueous extracts

GC-MS analysis:

The extract was analyzed for its phytochemicals using GC-MS, which identified 38 compounds. Analysis of GC-MS data for retention time, molecular formula and molecular weight with reference to NIST database library helped to identify the presence of important secondary metabolites. Most of the compounds were eluted between 25 -35 min analysis time. GC- MS chromatogram for extract and spectrum for different identified compounds were shown in the fig-4 and tabulated in Table 5

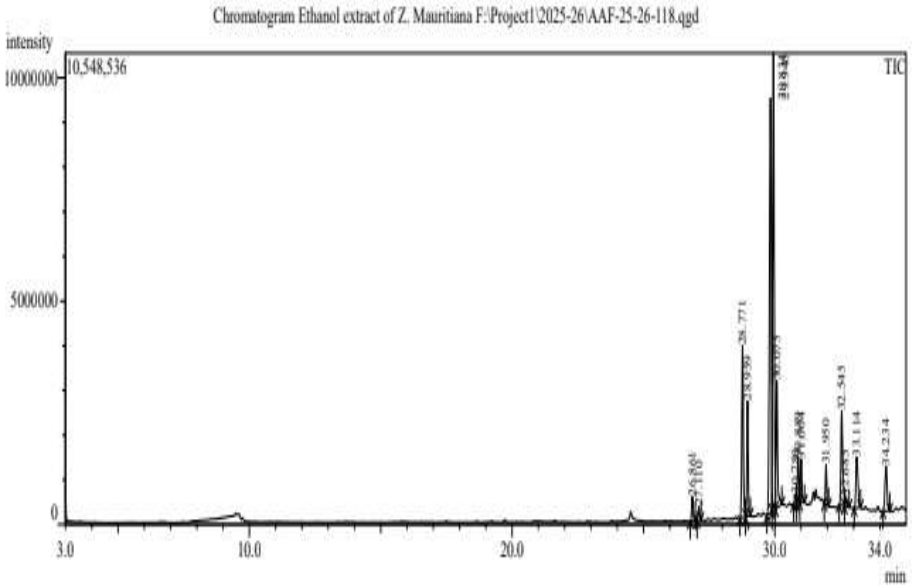


Figure 4: GC-MS Profile of Ethanolic extract of *Z. mauritiana* Lam. Seeds

Table 5: Phytochemicals identified in ethanol extract by GC-MS

SI. No	Name of the compound	Retention time	Molecular formula	Molecular weight	Category
1	(E)-4-(3-Hydroxyprop-1-en-1-yl)-2-methoxyphenol	26.861	C10H2O3	180	Phenyl propanoid
2	Tetradecanoic acid	27.110	C14H28O2	228	Saturated fatty acid
3	n-Hexadecanoic acid	28.771	C14H28O2	256	Saturated fatty acid
4	Hexadecanoic acid, ethyl ester	28.959	C18H36O2	284	Fatty acid ester
5	cis-9-Hexadecenal	29.834	C16H30O	238	monounsaturated fatty aldehyde
6	(E)-9-Octadecenoic acid ethyl ester	29.946	C20H38O2	310	fatty acid ester
7	Octadecanoic acid, ethyl ester	30.075	C18H36O2	312	fatty acid ethyl ester
8	Oleic Acid	30.780	C18H34O2	282	Fatty acid
9	9-Eicosenoic acid, (Z)-	30.889	C20H38O2	310	Fatty acid
10	Eicosanoic acid, ethyl ester	31.004	C22H44O2	340	Saturated fatty acid
11	Docosanoic acid, ethyl ester	31.950	CH248O2	368	Fatty acid ester
12	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	32.545	C57H104O6	884	Triglyceride
13	Octadecanoic acid, 2,3-dihydroxypropyl ester	32.685	C21H42O4	358	Lipids

***In - Vitro* Cytotoxic Evaluation** The extract was evaluated at concentrations of 20, 40, 60, 80, and 100 µg/mL, with 10 µM Rotenone serving as the standard. The corresponding % inhibition values observed at these concentrations were 0.55, 3.87, 6.08, 9.39 and 11.05 respectively, while Rotenone exhibited 7.73% inhibition. The ethanolic extract was further evaluated for its cytotoxic potential against the N2a (neuroblastoma) cell line.

The cytotoxic evaluation of the seed extract revealed a concentration-dependent decrease in cell viability. At 100 µg/mL, the % cell viability was 88.95%, compared to 99.45% at 20 µg/mL, indicating increasing cytotoxic potential with higher concentrations. The IC_{50} value was determined to be 137.46 µg/mL, suggesting moderate cytotoxicity. The standard Rotenone (10 µM) exhibited 92.27% cell viability, validating the assay and allowing comparison with the extract's activity. The results are tabulated in Table 6 and fig 5.

Table 6: Cytotoxic Effect of *Z. mauritiana* Seed Extract on N2a Cells

Si. No	Concentration(µg/mL)	Absorbance at 540nm	Percentage Inhibition	IC $_{50}$
1	Control	1.81	0	390.41 µg/mL
2	20	1.8	0.55	
3	40	1.74	3.87	
4	60	1.7	6.08	
5	80	1.64	9.39	
6	100	1.61	11.05	
7	Rotenone (Standard)	1.67	7.73	

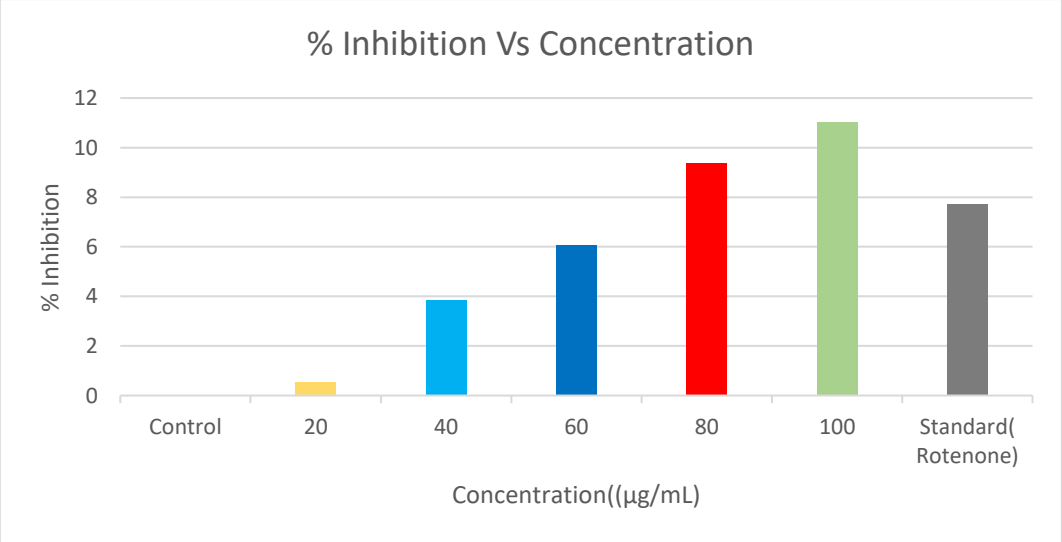


Figure 5: Concentration vs % Cell viability of N2a cell line

CONCLUSION

The comprehensive phytochemical, and biological evaluation of *Ziziphus mauritiana* seed extract highlights its potential as a natural source of therapeutic compounds. The seed demonstrated rich antioxidant content, including flavonoids, phenolics. The ethanolic extract exhibited only mild cytotoxicity against N2a neuroblastoma cells, its safety profile and bioactive richness suggest potential for use in supportive or adjunct neuroprotective therapies. These findings lay a strong foundation for further exploration of *Z. mauritiana* in the development of plant-based therapeutic agents.

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