



Research Article

Investigating antioxidant and anticancer properties of *Achyranthes aspera* leaf extracts against B16F10 melanoma cells: *In-vitro* and *in-silico* study

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Abstract

Background: Melanoma is a highly aggressive skin cancer with considerable metastatic potential, highlighting the need for novel therapeutic strategies. Natural products, particularly plant-derived extracts, represent promising sources for anticancer drug discovery owing to their diverse phytochemical constituents. This study evaluated the antioxidant and anticancer potential of *Achyranthes aspera* leaf extracts against B16F10 melanoma cells. **Methods:** Aqueous and ethanolic extracts of *A. aspera* leaves were subjected to phytochemical screening. Total phenolic content (TPC) and total flavonoid content (TFC) were determined. Antioxidant activity was assessed using DPPH, nitric oxide scavenging, and FRAP assays. Cytotoxicity against B16F10 cells was evaluated using the MTT assay, while apoptosis was confirmed by DAPI staining. Molecular docking was performed to investigate the binding affinity of major phytoconstituents toward tyrosinase-related protein 1 (TYRP1), a potential melanoma-associated target. **Results:** Phytochemical screening revealed flavonoids, tannins, terpenoids, saponins, and phenolic compounds. The aqueous extract exhibited higher TPC (102.56 mg GAE/g) and TFC (140.87 mg QE/g) and consequently showed superior antioxidant activity across all assays. It also demonstrated significant, concentration-dependent cytotoxicity against B16F10 cells, with a lower IC₅₀ value (40.65 µg/mL) than the ethanolic extract (75.21 µg/mL). DAPI staining showed nuclear fragmentation and chromatin condensation, confirming apoptosis. Molecular docking identified spathulenol and squalene as prominent compounds, exhibiting binding affinities of -7.9 and -7.6 kcal/mol, respectively, toward TYRP1. **Conclusions:** *A. aspera* leaf extracts, particularly the aqueous extract, exhibit promising antioxidant and anticancer activity, potentially mediated through apoptosis. Spathulenol and squalene may contribute to TYRP1 inhibition. Further *in-vivo* studies are warranted.

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Introduction

Cancer, characterized by uncontrolled cell growth and invasion, remains a formidable global health challenge, with melanoma representing a particularly aggressive form of skin cancer. Melanoma is responsible for a considerable share of skin cancer-related mortality because of its high spread potential and resistance to standard therapy in advanced stages (1). Current

treatment options, such as surgery, chemotherapy, and radiation, often face limitations including systemic toxicity and acquired resistance, underscoring the urgent need for novel therapeutic strategies. According to global estimates, new cancer cases increased from 19.3 million in 2020 to 19.97 million in 2022. Similarly, cancer-related deaths remained alarmingly high, with approximately 10 million fatalities in 2020, declining slightly to 9.7 million in 2022, underscoring its position as a predominant cause of global mortality (2–4).

Natural products, particularly plant-derived extracts, have long been recognized for their potential in anticancer drug discovery due to their rich chemical diversity and bioactive properties (5). This is especially crucial in developing countries facing significant economic burdens related to cancer (6). Consequently,

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research teams in these regions are actively exploring medicinal plants to uncover their cytotoxic properties. A key focus of research in these regions involves identifying natural compounds from plant that exhibit specific cytotoxicity that targets cancer cells while protecting healthy cells, offering a promising avenue for designing targeted therapies to elicit fewer adverse effects than conventional treatments (7). Such strategies are increasingly relevant in the era of precision oncology, where the emphasis lies on personalized therapeutic approaches tailored to individual patient profiles.

The genus *Achyranthes* L., part of the Amaranthaceae family, comprises approximately 21 species of perennial or annual herbs. These species are predominantly found in tropical and subtropical regions (8). One well-known species, *Achyranthes aspera* L., is commonly referred to as Prickly Chaff Flower or Aghara. In both Ayurvedic and Unani systems, different parts of plant are employed for managing diverse health conditions such as leprosy, asthma, arthritis, skin ailments, wounds, insect and snake bites, gastrointestinal issues, respiratory infections, parasitic infestations, gum problems, and sexually transmitted diseases (9).

To date, researchers have isolated and identified 58 significant compounds from several parts of plant namely flavonoids, tannins, terpenoids, saponins, phytosterols, and phenolics (10–14), each known for their therapeutic properties like antioxidant (15,16), anti-inflammatory (17), antimicrobial (18), antihyperlipidemic (19), anti-diabetic (20), hepatoprotective (21), diuretic (22) and other essential medicinal benefits (23).

Several studies have reported the anticancer potential of *A. aspera* leaf extracts against pancreas epithelial cancer cell line, including MiaPaCa-2 and Panc 10.05 (24). However, its efficacy may differ across various cancer types due to variations in genetic profiles and signaling pathways. The B16F10 murine melanoma cell line is a widely used in-vitro model for investigating melanoma progression and therapeutic responses (25). Emerging research indicates that natural antioxidants play crucial role in reducing oxidative stress a major contributor to melanoma proliferation and metastasis while selectively inducing cytotoxicity in malignant cells (26). Despite these findings, the potential anticancer properties of *A. aspera* leaf extracts against B16F10 cells remain insufficiently studied. Furthermore, comparative analyses of extracts derived from different plant parts and their effects on various cancer types could help elucidate bioactive compounds and their underlying mechanisms, thereby advancing targeted therapy development.

Tyrosinase-related proteins (TYRPs) are members of the Cu⁺⁺/Zn⁺⁺ metalloenzyme family that involve a set of sequence homologies that play key regulator of melanogenesis. The initial discovery of TYRP1/gp75 emerged from the characterization of an autoantibody found in a metastatic melanoma patient. Both TYRP1 and tyrosinase contain an M-box domain capable of interacting with the microphthalmia-associated transcription factor (MITF), a regulator essential for pigmentation, proliferation, and differentiation (27). Tyrosinase, functioning as the rate-limiting enzyme in melanin biosynthesis, facilitates hydroxylation of tyrosine followed by its oxidation during the pigment production process. Consequently, developing pharmaceutical agents that inhibit tyrosinase, a key enzyme in the melanin pathway, has become a significant area of focus. Structures of target protein were choosen from protein data bank (PDB) and target Human tyrosinase enzyme's PDB code was 5M8M (28). Additionally, melanoma risk is greatly increased by the TYR and TYRP1 variants. Melanoma is an aggressive malignant tumor of the

melanocyte that is accountable for skin cancer-related deaths. Phase 1 clinical trial using a recombinant human monoclonal antibody against TYRP1 show that melanogenesis inhibition may be an effective treatment approach.

This research investigates the potential of *Achyranthes aspera* (AA) leaf extracts as an anticancer agent. We prepared ethanol and aqueous extracts, quantified their phenolic and flavonoid content, and evaluated their cytotoxicity against B16F10 melanoma cells for the first time. The MTT assay measured cell viability, and DAPI staining observed nuclear morphology. Additionally, molecular docking simulations explored the interaction between AA's bioactive compounds and the Human TYRP1 protein, a known factor in melanoma. This study aims to fill a significant knowledge gap regarding AA's anti-melanoma properties.

Materials and Methods

Material

Ethanol and aluminium chloride were procured from Loba Chemie Pvt. Ltd., Mumbai, India. Gallic acid and quercetin were procured from Sigma-Aldrich, India. DPPH, Griess reagent, Folin–Ciocalteu reagent, ascorbic acid, and dimethyl sulfoxide (DMSO) were procured from Thermo Fisher Scientific, India.

Collection and authentication

A. aspera specimens collected from Sangli district in Maharashtra state, India were botanically authenticated by Botanical Survey of India, Pune, (BSI/WRC/Iden.Cer./2022/1804220006437). Harvested leaves were thoroughly cleaned and subsequently shade dried for a period of 3–4 weeks. Once dried, leaves were powdered mechanically and resulting fine powder was preserved in a sealed container for next use.

Preparation of Extract

Dried *Achyranthes aspera* leaves were finely powdered and subjected to extraction using two different solvents. For the ethanolic extract, 1 kg of powdered leaf material was extracted in a Soxhlet apparatus using 99.9% ethanol (29). Extraction was continued until exhaustive extraction was achieved. The solvent was subsequently recovered and removed using a rotary evaporator under controlled conditions. The concentrated extract was further dried until complete removal of the solvent, yielding the ethanolic extract. For the aqueous extract, 500 g of finely powdered leaf material was subjected to maceration with 10% chloroform water IP for 8–10 days at room temperature (30). The mixture was filtered through filter paper, and the filtrate was concentrated by evaporation at 45–50°C until a dry extract was obtained. Aqueous and ethanolic extracts were stored under refrigerated conditions until further analysis and biological evaluation.

Cell culture and maintenance

B16F10 murine melanoma cells were procured from the National Centre for Cell Science (NCCS), Pune, India, and cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin–streptomycin (100 U/mL and 100 µg/mL, respectively) at 37°C in a humidified atmosphere of 5% CO₂. Cells were monitored regularly for morphology and confluency. At approximately 70–80% confluency, the cells were washed with sterile PBS, detached using accutase, resuspended in fresh complete medium, and subcultured into new tissue culture flasks. Cells in the logarithmic growth phase were used for subsequent experiments.

Phytochemical analysis

Presence of various phytoconstituents in crude aqueous and ethanolic extracts of *Achyranthes aspera* leaves was determined through qualitative analysis subsequent standard protocols.

Total phenolic content (TPC)

By spectrophotometry and Folin-Ciocalteu reagent, TPC of aqueous and ethanolic extracts was derived (31). Standard Gallic acid (10-100 µg/ml) was utilised to create a calibration curve. Standards and samples were aqueous and ethanolic extracts at 1 mg/mL, and a 1 mL aliquot was mixed using 5 mL of tenfold diluted Folin-Ciocalteu reagent and 4 mL of 7.5% w/v sodium carbonate solution. After 30 minutes of incubation at 45°C, the mixture was tested three times for absorbance at 765 nm. Measured in mg of gallic acid equivalent per gram of extract, TPC was calculated by interpolating the absorbance of the extracts against the standard curve.

Total flavonoid content (TFC)

TFC of extracts was measured via an AlCl₃ colorimetric assay (31) by Quercetin (CAS no. [117-39-5](#)) as the standard. To establish a calibration curve, 1 ml of Quercetin (10-100 µg/ml) were mixed with (4 ml). After incubating for 5 minutes, 0.3 ml of 10% AlCl₃ was added to every tube, following addition of 0.3 ml of 5% NaNO₂ solution. An orange-yellow tint was produced when 2 ml of a 1M NaOH solution was introduced at 6th minute and volume was brought to 10 ml using distilled water. A spectrophotometer was used in duplicate to measure absorbance of these solutions at 510 nm.

Aqueous and ethanolic extracts of *Achyranthes aspera* (1 mg/mL) were subjected to the identical protocol. TFC was determined by interpolating extracts absorbance Vs standard curve and is stated as mg of Quercetin equivalent (QE) per gram of extract.

Antioxidant activities

DPPH assay

Free radical scavenging potential of aqueous and ethanolic extracts was evaluated by a DPPH assay by technique of Nithianantham et al. (1998) by modifications (32). In a volumetric flask, 19.7 mg of DPPH was liquefied in methanol and then diluted to a final volume of 100 mL to prepare a 0.5 mM DPPH solution.

To assess radical scavenging activity, 1 mg/mL stock solutions of aqueous and ethanolic extracts were prepared in methanol. These were serially diluted to concentrations of 10–100 µg/mL. One milliliter of each dilution was mixed with three milliliters of DPPH solution for the test. At room temperature, samples were left to incubate in the dark for half an hour. A methanol blank (1 mL methanol + 3 mL DPPH) served as the negative control. At 517 nm, a spectrophotometer was used to measure liquids' absorbance. Three replicates were performed for each measurement. The standard employed in the experiment was ascorbic acid. The extract's 50% inhibitory concentrations (IC₅₀ values) were determined using graph of concentration versus % inhibition.

To conclude the degree of inhibition, the following equation was used:

$$\% \text{ of inhibition (I\%)} = [(A_c - A_s) / A_c] \times 100 \quad (1)$$

Where A_c is absorbance value of control (solution consisting of methanol and DPPH) and A_s is absorbance value of reaction mixture (consisting of DPPH and sample).

Nitric Oxide (NO) Radical Scavenging Assay

Griess-Ilosvay reaction by sodium nitroprusside was used to evaluate ability of ethanolic and aqueous extracts to scavenge nitric oxide radicals (33). 1 mL of various concentrations of each extract individually (200, 400, 600, 800, and 1000 g/ml) was mixed with 0.5 ml (10 mM) Sodium nitroprusside dissolved in pH 7.4 phosphate buffer saline. Combination was incubated at a temperature of 25°C for a duration of 3 hours. Subsequently, a volume of 1 ml of Griess reagent was then added to the reaction mixture following incubation period. Mixture's absorbance at 546 nm was measured next another 30 minutes of incubation at room temperature. Nitric oxide is being scavenged actively, as evidenced by the declining absorbance.

Subsequent equation was used to calculate % of NO inhibition:

$$[(A_0 - A_1) / A_0] \times 100 \quad (2)$$

Where A₀ - absorbance of control and A₁ - absorbance of treated sample.

Ferric reducing antioxidant power

By improved version of approach proposed by Dudonne et al. (2009), the antioxidant capacity to reduce ferric ions was assessed in both water and ethanolic extracts (34,35). A 2 mM solution of FeCl₃ was added to every well, with 80 µl total volume. A 1 mg/ml stock solution of every extract was ready by combining 10 mg of extract with 10 ml of ethanol. Test tubes were used to prepare serial dilutions of a stock solution at 1000, 800, 600, 400, and 200 µg/ml. To matching wells, 80 µl of each dilution and 80 µl of FeCl₃ were added. Subsequent to the 5-minute incubation time, 40 µl of O-Phenanthroline monohydrate was introduced to each well, and the absorbance was assessed three times using a 593 nm wavelength. A standard known as ascorbic acid was utilized in this investigation.

Cytotoxicity study

Cell viability was evaluated by MTT assay (36,37). Using a hemocytometer, the cells were counted after they were separated with accutase and suspended in medium. Every well of a 96-well plate was seeded with around 50,000 cells, and after 24 hours of incubation in a 5% CO₂ incubator, cells were removed. Cells were exposed to ethanolic and aqueous extracts in triplicate after incubation, with dilutions of 6.25, 12.5, 25, 50, 100, and 200 µg/ml applied, respectively. Control samples were prepared using DMSO. Following the initial incubation period of 24 hours, 20 µL (5mg/ml in PBS) of MTT reagent was added to every well and allowed to incubate for an additional three hours. Once the media had been delicately detached, 100 µL of DMSO was added and gently shaken for 15 minutes to dissolve the formazan crystals. In order to determine the IC₅₀ of each extract and cell viability, the microplate reader was utilised to quantify formazan absorbance at 570 nm. Equations were then derived from this data.

$$\% \text{ Cell viability} = [\{\text{Test absorbance/Control absorbance}\} \times 100] \quad (3)$$

$$\% \text{ Inhibition} = 100 - \% \text{ Cell viability} \quad (4)$$

Morphological investigation by DAPI staining

Protocol of DAPI nuclear staining described by Frozza *et al.* in 2017 was followed (38). After 24 hours of culture at 37°C, in a

CO₂ incubator, cells were transferred to a 24-well flat-bottom microplate that included coverslips. After that, cells were subjected to the corresponding extracts. Following procedure, cells were fixed with 4% paraformaldehyde for half an hour and subsequently rinsed with PBS. Afterwards, five minutes of incubation at room temperature in dark was used to stain the nuclei with a 20 µL DAPI (1µg/mL) solution. Morphological changes indicative of apoptosis were observed using a fluorescence microscope. Randomly selected fields were examined to quantify the number of apoptotic cells.

Molecular docking studies

The earlier testified 3D structure of human tyrosinase related protein 1 (TYRP1) by PDB: 5M8M having 2.65 Å resolutions was retrieved from RCSB-PDB (<https://www.rcsb.org/>) (28,39,40). Downloaded 3D Crystal structure of the target protein was ready by removing water molecules along with earlier bound ligand groups. Further, structure of prepared target protein was protonated by adding polar hydrogen atoms. There after the stereochemical adaptability and overall quality of prepared protein structure were evaluated with help of PROCHECK and ProSA-web server (41). The binding pocket present in target protein was forecast by CASTp 3.0 server (42). The optimal ligand binding site in each target protein was predicted using the identified binding sites. The structures of phytochemicals found in *Achyranthes aspera* were retrieved from PubChem in SDF file set-up and chosen based on previously published literature. (2E) Hydroquinone, 2-furaldehyde, p-benzoquinone, 5-(hydroxymethyl), eugenol, 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, phytol, spathulenol, and 3,7,11,15-tetramethyl-2-hexadecen-1-ol. In order to facilitate molecular docking with the target protein, squalene were used. Prepared protein and downloaded ligand structures were further used for in silico molecular docking study.

In the current study, binding potential of phytochemicals with target protein was determined using molecular docking technique. Molecular docking studies are useful for learning about ligand-target protein interactions and binding energies. Molecular docking was carried out using Auto Dockvina through PyRx0.8 (43–45). Imported into PyRx 0.8 and run through an energy minimization procedure were PDB files containing all of the phytochemicals and proteins. In order to include residues from binding site, grid box was used in Vina workspace. An exhaustiveness level of 8 was used by default. According to the selected target protein (PDB: 5M8M), 9 different conformations were predicted for every phytochemical. Minimum binding affinity of each molecule was also considered while deciding where to place it. The use of BIOVIA Discovery Studio 2020 allowed for the display, analysis, and docking interaction of stored conformations.

Statistical analysis

All three trials were conducted independently, and outcomes were stated as mean ± SD. The statistical analysis included comparing different extracts using t-tests and ANOVA. At P < 0.05, statistical significance was established.

Results

Exploration of phytochemicals

Initial phytochemical investigation of aqueous and ethanolic extracts from *Achyranthes aspera* leaves revealed the presence of diverse phytochemical compounds (Table 1).

Table 1: Phytochemical analysis of *Achyranthes aspera* leaves extracts

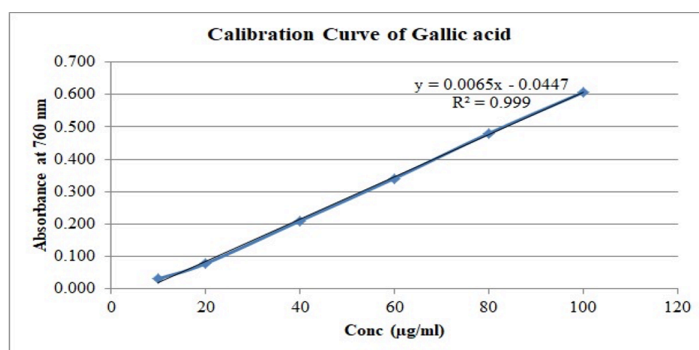
Chemical constituents	Observation	Ethanolic extract of AA (EA)	Aqueous extract of AA (AA)
Alkaloids (Mayer's Test)	Formation of Yellow ppt	-	+
Alkaloids (Dragendroff's Test)	Formation of Orange red ppt	-	+
Flavonoids (Shinoda Test)	Formation of reddish pink colour	+	+
Flavonoids (Lead Acetate Test)	Formation of Yellow ppt	+	+
Terpenoids (Salkowski Test)	Formation of red colour	-	+
Tannins and phenols (FeCl₃ Test)	Formation of blue colour	+	+
Anthraquinones (Borntrager's Test)	Ammonical layer converted to reddish-pink	+	-
Saponins (Foam Test)	Formation of Foam	-	+
Proteins (Million's Test)	White ppt, changed to red	+	-

+ = Present, - = Absent

Total phenolic content

Results were presented as GAE per gram of extract for both ethanolic and aqueous extracts of *Achyranthes aspera* leaves, as evaluated by Folin-Ciocalteu reagent method. Figure 1 shows the linear regression equation used for quantification, which was obtained from the Gallic acid standard curve. Ethanolic extract showed a TPC of 94.64 mg GAE/g, while aqueous extract contained 102.56 mg GAE/g.

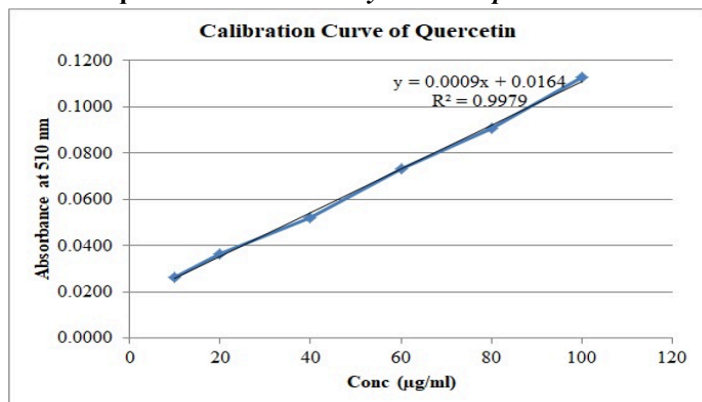
Figure 1: Calibration curve of Gallic acid used for determination of total phenolic content in ethanolic and aqueous extracts of *Achyranthes aspera* leaves



Total flavonoid content

TFC in both extracts was purposive by a linear regression equation derived from a standard curve of Quercetin (Figure 2). Ethanolic extract had a TFC of 96.33 mg QE/g, while aqueous extract contained 140.87 mg QE/g.

Figure 2 Calibration curve of Quercetin used for determination of total flavonoid content in ethanolic and aqueous extracts of *Achyranthes aspera* leaves



Determination of Antioxidant actions

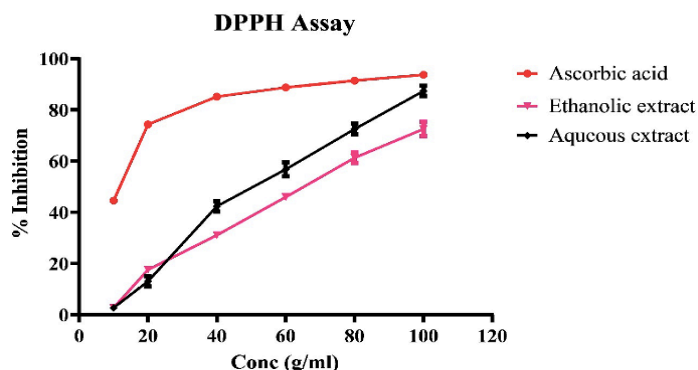
DPPH radical scavenging assay

Free radical DPPH is extremely stable. DPPH has an advantage over other free radicals generated in vitro, such the hydroxyl radical and superoxide anion, as it is not influenced by firm side reactions like enzyme inhibition and metal ion chelation. At 517 nm, the freshly prepared DPPH solution absorbs the most light, turning it a deep purple hue. The purple hue of DPPH free radicals is reduced when antioxidant molecules convert them into a colorless or bleached form, such as DPPH or a substituted, comparable hydrazine. DPPH free radicals are neutralized by hydrogen atoms. This is likely accomplished by reducing the DPPH's absorbance in the 517 nm band, which is caused by a free-radical attack on the DPPH.

Reducing power of *Achyranthes aspera* leaves ethanolic and aqueous extracts was assessed in the current study using a DPPH radical scavenging assay, and concentrations that provided 50% inhibition (IC₅₀) values or, in other words, the amount needed to scavenge 50% DPPH free radicals were used to determine these extracts reducing power. % DPPH free-radical scavenging action for both extracts at various concentrations is displayed in Figure 3.

Both extracts had varying levels of antioxidant capabilities when associated to standard, ascorbic acid. Ascorbic acid was determined to have an IC₅₀ value of 11.59 µg/ml. The ethanolic extract exhibited moderate antioxidant action by an IC₅₀ value of 64.81 µg/ml, whereas the aqueous extract had a much greater free radical scavenging activity of 48.68 µg/ml.

Figure 3: Inhibition of DPPH by ethanolic and aqueous extracts of *Achyranthes aspera*

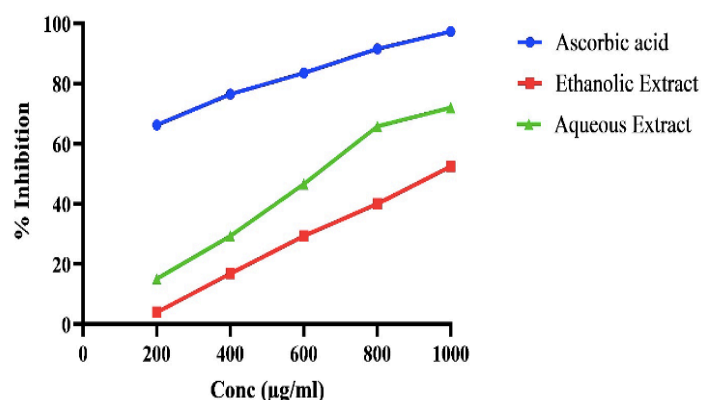


NO Radical Scavenging Assay

NO has high reactivity and instability when exposed to aerobic environments. It interrelates by O₂ to produce stable nitrates & nitrite via intermediates NO₂, N₂O₄, and N₃O₄. Using Griess reagent, it is estimated. Ethanolic extract exhibited a minimum nitric oxide scavenging activity of 4% at 200 µg/ml, while extreme action observed was 52.44% at 1000 µg/ml. Aqueous extract had a minimum nitric oxide scavenging activity of 15.11% at 200 µg/ml. Conversely, the maximum activity observed was 72.00% at 1000 µg/ml. These results were compared to those of standard Ascorbic acid, which exhibited a minimum 66.22% at 200 µg/ml and a maximum activity of 97.33% at 1000 µg/ml. The percentage of inhibition exhibited a positive correlation with the concentration of both extracts. However, aqueous extract exhibited a greater level of nitric oxide radical scavenging action associated to ethanolic extract, as depicted in Figure 4.

Figure 4: Inhibition of Nitric oxide radical scavenging activity by ethanolic and aqueous extracts of *Achyranthes aspera*

Nitric oxide radical scavenging activity

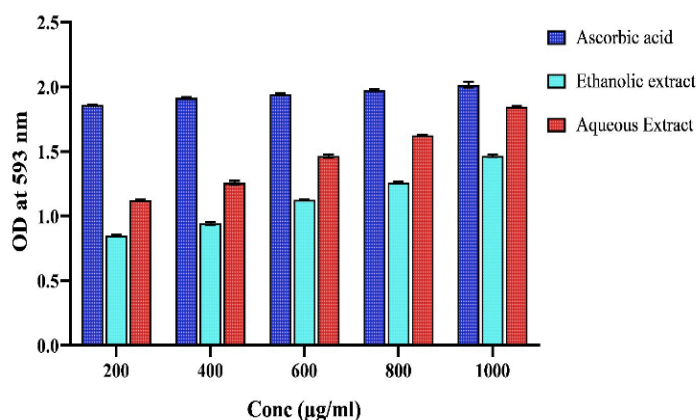


Ferric reducing antioxidant power

Figure 5 demonstrates reductive capabilities of ethanolic and aqueous extracts derived from *Achyranthes aspera*. Overall, aqueous extract exhibited higher FRAP activity than ethanolic extract. Moreover, aqueous extract exhibited similar reductive potential to that of Ascorbic acid at higher concentrations.

Figure 5 Ferric reducing antioxidant potential of ethanolic and aqueous extracts of *Achyranthes aspera*

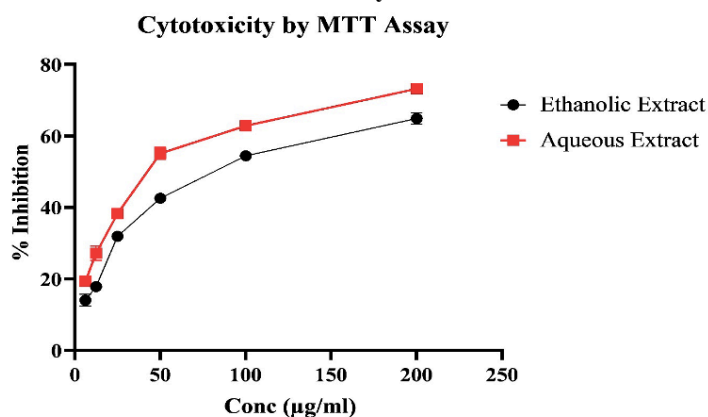
FRAP Assay



Cytotoxicity study

Mitochondrial succinate dehydrogenase activity is measured in MTT assay by measuring decrease of the yellow-colored MTT compound. This technique was used to create an insoluble, dark purple-colored formazan in this work using extracts from the leaves of *Achyranthes aspera*. This formazan formation, indicative of cellular vitality, correlates with metabolic activity in cells. Individually extracts demonstrated concentration-dependent growth inhibition (**Figure 6**). Aqueous extract notably inhibited cell growth by more than 50% at lesser concentrations, whereas ethanolic extract showed slightly weaker activity associated to ethanolic extract.

Figure 6: Cytotoxic effects of ethanolic and aqueous extracts of *Achyranthes aspera* leaves on B16F10 cell line by MTT assay



IC₅₀ value for aqueous extract of *Achyranthes aspera* was determined to be 40.65 ± 2.71 µg/ml, that was significantly lower associated to IC₅₀ value of ethanolic extract (75.21 ± 5.98 µg/ml).

Morphological investigation by DAPI nuclear staining

Distinction among apoptosis plus necrosis relies on characteristic nuclear changes. DAPI, nuclear stain that fluoresces blue under a fluorescence microscope, was utilized in this study. Employing DAPI nuclear labeling enabled the detection of apoptosis-associated modifications in B16F10 cells treated with ethanolic and aqueous extracts from *Achyranthes aspera*.

The cells unstained by treatment (Control) (**Figure 7A**) showed entire nucleus by a weak, even blue staining, as shown by microscopic investigation. On other hand, cells exposed with IC₅₀ dose of both extracts showed occurrence of apoptotic bodies and small nuclei by significant chromatin condensation, blebbing, and nuclear fragmentation (**Figure 7B and 7C**). Results of quantitative study show that the aqueous extract treatment at the IC₅₀ level had more noticeable effects, compared to the aqueous extract, which showed slightly lesser impact (**Figure 8**).

Figure 7 (A-C): DAPI Nuclear staining of B16F10 cell line in control and presence of plant extracts

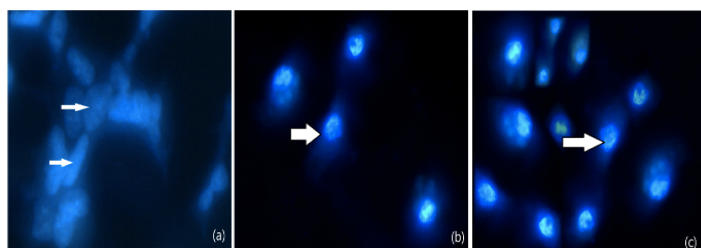
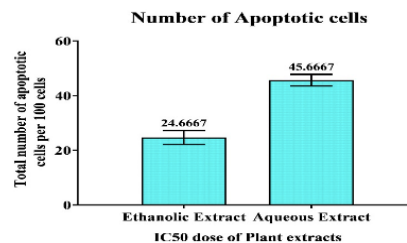


Figure 8: Quantitative results for the number of apoptotic cells per 100 cells



Molecular docking studies

Using the PROCHECK and ProSA-web servers, the quality evaluation and binding site analysis of the generated protein structure were conducted. As shown in **Figure 9a**, the Ramachandran plot showed that, following protein processing, 85.8% of the 5M8M residues were located in the preferred regions. With a z-score of -8.57 for PDB: 5M8M, the overall quality of the models for both of the produced protein structures was determined to be satisfactory. z-scores of every determined protein chain in both PDB: 5M8M are plotted in **Figure 9b**. Binding pocket analysis indicated that only one pocket is present in the targeted macromolecule (PDB ID: 5M8M) with an area of 13110.387 and a volume of 19898.415 (**Figure 10**).

Figure 9 a) Ramachandran plot showing 85.8% residues in the favoured region and b) Z-Score plot for experimentally determined protein chains.

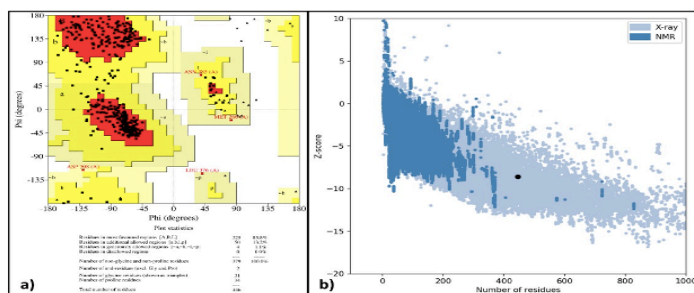
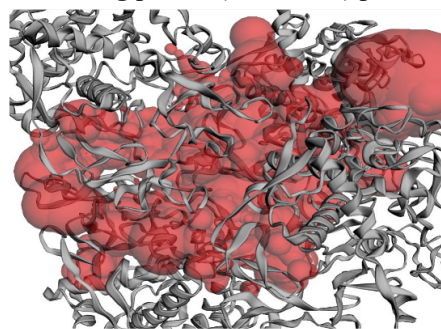


Figure 10: Binding pocket (red colour) present in 5M8M



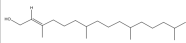
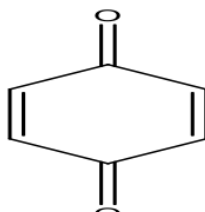
Molecular docking was done to determine binding potential of selected phytochemicals with TYRP1. PDB: 5M8M was selected to perform the molecular docking. It is one of widely applied virtual screening methods. In the present work, PyRx 0.8 program was used to carry out molecular docking study (45,46). Phytochemical from the *Achyranthes aspera* were downloaded from PubChem database and docked with 5M8M using PyRx program. The obtained binding affinity is denoted in Table 2, respectively.

Table 2: The binding affinity of phytochemicals against the human tyrosinase related protein 1 (PDB: 5M8M) along with interacting residue and type of interaction

Sr.No.	Name of Molecule	Binding Energy (kcal/mol)	Interactions		
			Conventional H bond	Pi Alkyl	Vdw
1	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol	-6.9	CYS113	TYR226, TYR462, ILE128, VAL126, ARG118, LYS233,	GLY116, THR112, ARG230, PRO445, SER106, GLY107, ARG130, PRO242, GLY461, GLN236, LEU460
2	2-Furaldehyde,5-(Hydroxymethyl)	-5.4	ARG131,SER245, SER243,	LEU136, PRO247	VAL129, LEU246, ATG130, PHE244, PHE144, GLU140, HIS143
3	3,5- Dihydroxy-6-Methyl-2,3-Dihydro-4H-Pyran-4-one	-5.7	GLU232, TRP117, GLY116	LEU229, LYS233	ARG130, GLN236, ILE128, GLY461, TYR462, LEU460, VAL126, ARG118, ARG114, PRO115
4	Eugenol	-6.4	GLY116, GLN236	TYR226, LEU229, LYS233, PRO115, ARG118, VAL126	ARG230, ARG114, TRP117, GLU232, ILE128, GLY461, TYR462
5	Hydroquinone	-5.1	-	ILE1287, VAL126	TYR462, GLN236, ASER243, GLY461, LEU460, GLY116, PRO115, TRP117, ARG114
6	p-benzoquinone	-5	SER245	PRO247, LEU136	PHE244, PHE144, LEU246, ARG131, GLU140
7	Phytol	-7.1	ARG114, GLY116, TRP117	ARG230, LEU229, PRO115, LYS233, ILE128, VAL126, ARG118, VAL447	PRO445, SER106, MET452, GLU451, CYC113, PRO242, GLY461, GLN236, TYR462, LRU460, GLU232
8	Spathulenol	-7.9	TRP117	LEU229, PRO115, LYS233, ILE128, VAL126	GLU232, LEU460, TYR462, SER243, PRO242, LEU127, GLY461, GLN236, GLY116, ARG118, CYC113, ARG114, THR112
9	Squalene	-7.6	-	MET452, VAL447, PRO115, TYR226, VAL126, PRO445, LYS233, LEU229, ILE128, ARG118	PRO446, TRP444, ARG114, CLU232, CYC113, GLY461, GLY116, LEU127, ARP117, ARG114, TYR462, GLN236, GLU451, GLY107, SER106, ARG230, LEU460, SER243, PRO242, GLN236,
10	5-FU (Reference)	-5.6	GLY116, TRP117, PRO242		ILE128, GLN236, GLY461, PRO115, ARG114

Spathulenol was exhibited good binding affinity of -7.9 kcal/mol as compared to other phytochemicals from *Achyranthes aspera*. The interaction was visualized via software (BIOVIA Discovery Studio) and interacting residues with type of interaction formed between phytochemicals and protein structure is represented in **Table 3**.

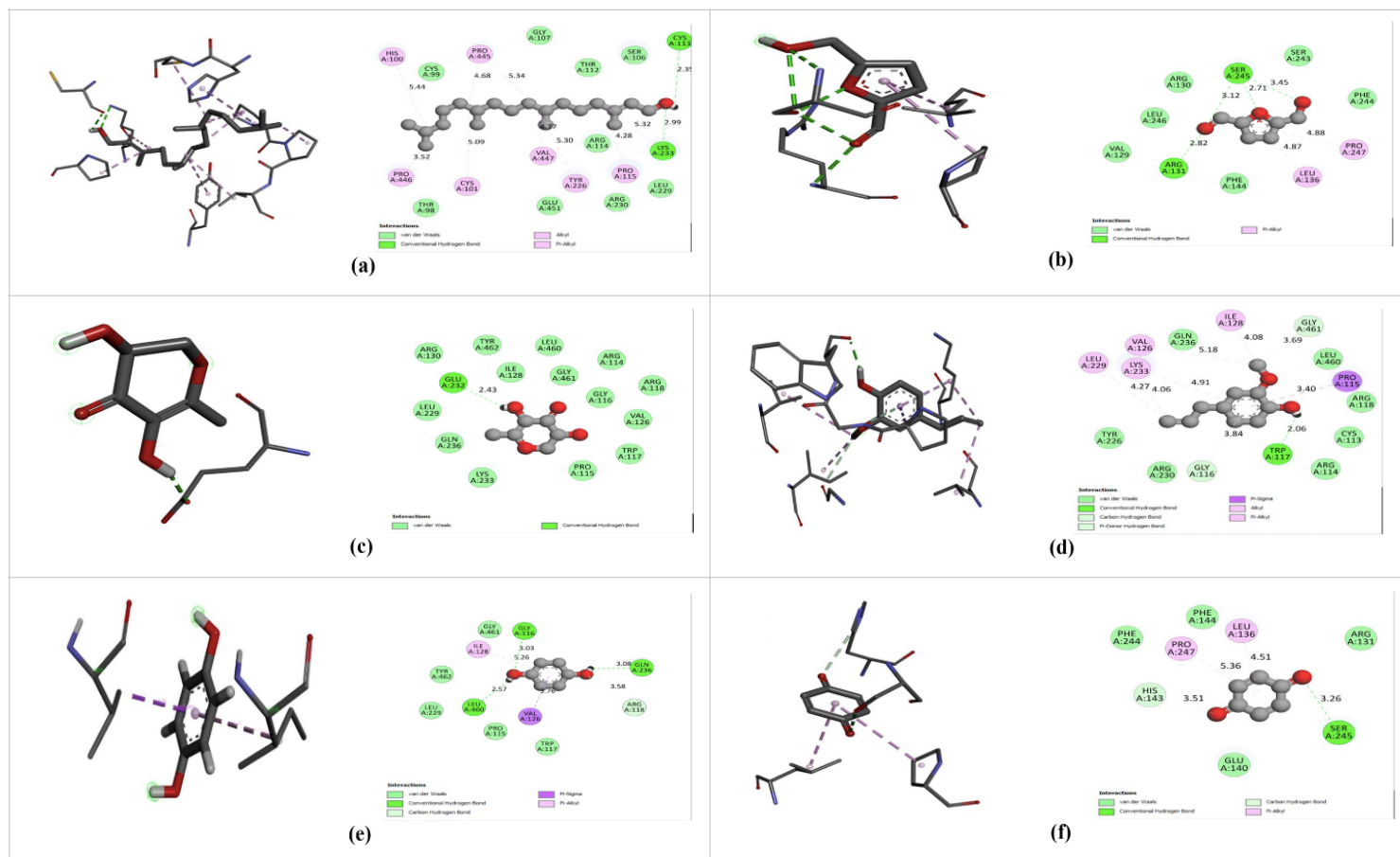
Table 3: Structure of chemical constituents found in *Achyranthes aspera* leaves

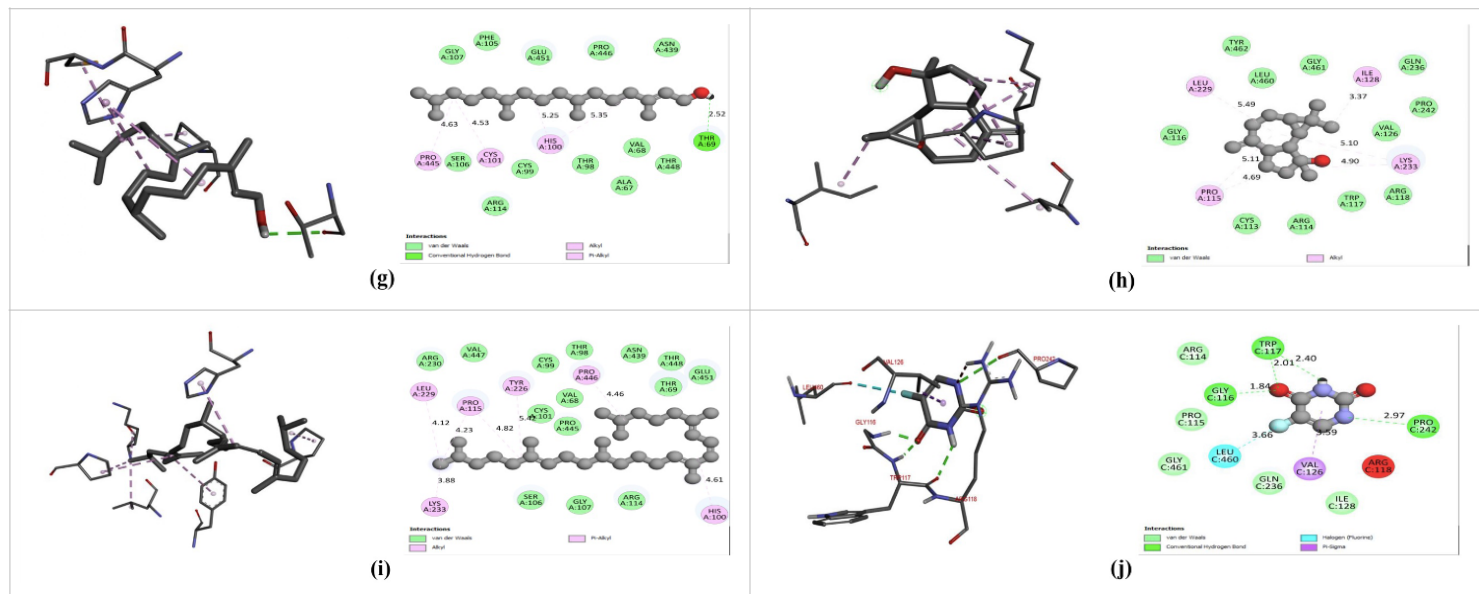
Sr. No.	Name of Constituent	Structure	Sr. No.	Name of Constituent	Structure
1	(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol		6	p-benzoquinone	

2	2-Furaldehyde,5-(Hydroxymethyl)		7	Phytol	
3	3,5- Dihydroxy-6-Methyl-2,3-Dihydro-4H-Pyran-4-one		8	Spathulenol	
4	Eugenol		9	Squalene	
5	Hydroquinone		10	5-FU (Reference)	

Spathulenol showed interactions with TRP117, LEU229, PRO115, LYS233, ILE128, VAL126, GLU232, LEU460, TYR462, SER243, PRO242, LEU127, GLY461, GLN236, GLY116, ARG118, CYC113, ARG114, THR112 amino acid residues. Extending to that Squalene also showed good binding affinity of -7.6 showing interactions with MET452, VAL447, PRO115, TYR226, VAL126, PRO445, LYS233, LEU229, ILE128, ARG118, PRO446, TRP444, ARG114, CLU232, CYC113, GLY461, GLY116, LEU127, ARP117, ARG114, TYR462, GLN236, GLU451, GLY107, SER106, ARG230, LEU460, SER243, PRO242, GLN236. The 3D visualization of interaction between protein and subjected phytochemicals were represented in **Figure 11A-11J**, respectively.

Figure 11: 3D interaction between selected phytochemicals and PDB: 5M8M (A-J)





Discussion

In current years, there has been increasing attentiveness in exploring herbal medicines for treating cancer, driven by their diverse phytochemical conformations and several biological properties (47). Present study investigated ethanol & aqueous extracts derived from *Achyranthes aspera* leaves, revealing significant potential in preventing cancer, particularly against the B16F10 melanoma cell line. Preliminary phytochemical analysis of these extracts detected flavonoids, terpenoids, tannins, saponins, phytosterols and phenolic compounds phenolic compounds.

Polyphenols are prominent plant chemicals known for their antioxidant effects. Among them, phenolic compounds act as radical scavengers, contributing significantly to antioxidant activity (48,49). Plants generally show a sturdy correlation between their TPC and TFC, which parallels their capability to scavenge free radicals. This connection underscores their potential to exhibit anticancer properties (50,51). The ethanolic extract and aqueous extract of *Achyranthes aspera* leaves shown TPC values of 94.64 mg GAE/gm and 102.56 mg GAE/gm, respectively. These findings advise that *Achyranthes aspera* leaves are a major source of phenolic compounds. It is observing that ethanolic extract and aqueous extract of *Achyranthes aspera* leaves showed TFC of 96.33 mg QE/g and 140.87 mg QE/g, correspondingly.

Reactive free radicals have a important impact on physiological processes of living cells, particularly in initiation of pathological states during prolonged stress. The toxicity of free radicals in biological systems necessitates the evaluation of plant extract's capability to scavenge these radicals. Antioxidant capability of plant extracts was assessed in this study using the FRAP test, the NO radical scavenging activity assay, and DPPH radical scavenging assay. One trusted way to measure an antioxidant's free radical scavenging capabilities is DPPH assay.

DPPH assay was utilized in this study as a reliable predictor of possible antioxidant action, as demonstrated by percentage inhibition of DPPH (Figure 3). The plotted graph depicting the relationship between concentration and % inhibition of DPPH exhibited a positive correlation, indicating that antioxidant prospective of extracts increased as their concentration increased. At higher concentrations, radical scavenging activity of t aqueous

extract was equivalent to that of ordinary Ascorbic acid, and it was much higher than that of the ethanolic extract.

Significant involvement of nitric oxide in many inflammatory processes is widely recognized. The continuous production of this radical has a direct toxic effect on tissues and plays a role in the vascular collapse observed in cases of septic shock. Additionally, the long-term presence of nitric oxide radicals is linked to the development of different carcinomas and inflammatory conditions (52,53). Toxicity of nitric oxide is significantly enhanced upon its reaction by superoxide radical, resultant in formation of extremely reactive peroxyxynitrite anion (54). Nitric oxide produced as a result of the chemical reaction involving sodium nitroprusside undergoes a reaction by oxygen, resulting in formation of nitrite. Both extracts demonstrate the ability to hinder the generation of nitrite by engaging in direct competition by oxygen during reaction involving NO. The current study showed that aqueous extract of exhibited a greater level of nitric oxide radical scavenging action compared to ethanolic extract.

Subsequently, reducing capacity of both extracts was assessed using the reduction reaction involving the conversion of Fe^{3+} to Fe^{2+} . The reduction of Fe^{3+} is frequently employed as a means to assess electron donating activity, a crucial mechanism in the action of phenolic antioxidants. This activity has been found to exhibit a strong correlation with several other antioxidant activities (35). The capacity of an agent to undergo reduction can be a valuable predictor of its potential antioxidant action. A compound with a higher absorbance value is indicative of a greater ferric reducing power. The reductive potentials of both extracts exhibited a positive correlation, indicating that the reductive potential of the extracts increased as their concentration increased. But the aqueous extract at higher concentration showed significant free radical scavenging action than ethanolic extract.

To estimate cytotoxic effects, we exposed the B16F10 cell line to ethanolic and aqueous extracts. Both extracts exhibited concentration-dependent growth inhibition, illustrated in Figure 6. Aqueous extract notably suppressed cell growth by over 50% even at lower concentrations. Furthermore, compared to ethanolic extract, aqueous extract demonstrated slightly enhanced activity. To investigate how *Achyranthes aspera* possesses anticancer properties relate to apoptosis, B16F10 cells were exposed to both extracts. After treatment, cells underwent DAPI staining. The

staining outcomes indicated notable changes in nuclear morphology among the treated cells, marked by formation of apoptotic bodies.

Phytochemicals of *Achyranthes aspera* underwent virtual screening in contradiction of human tyrosinase related protein 1 to assess their binding affinity and identify optimal conformations. Molecular docking analysis revealed robust binding affinity of these phytochemicals to protein structure signified through PDB code 5M8M. Notably, Spathulenol showed least binding affinity among compounds, followed by Squalene and Phytol. Despite these differences, the aqueous extract of *Achyranthes aspera* leaves demonstrated significant antioxidant and cytotoxic action against cancer cells, findings corroborated through docking analysis.

Conclusion

Both leaf extracts of *Achyranthes aspera* had high concentrations of phenolic and flavonoid chemicals, which are known to have potent cytotoxic and antioxidant effects. These bioactive components have the ability to inhibit cancer and antioxidants, and molecular docking studies of the TYRP1 protein have shown that they bind to each other.

Though useful, in-vitro assays for cytotoxicity and molecular binding may not be able to capture all of the nuances of biological systems in living organisms, which were the mainstays of the present study. To validate these findings and better understand the safety, efficacy, and pharmacokinetic profile of *Achyranthes aspera* extracts, further investigations using *in-vivo* models are essential. Such studies would help confirm detected cytotoxic properties and elucidate mechanistic pathways in a physiological environment.

Current study highpoints favourable cytotoxic and molecular interaction properties of *Achyranthes aspera* leaf extracts. However, advancing this research through *in-vivo* experiments and deeper mechanistic exploration will be critical for harnessing its full therapeutic prospective in anticancer applications.

Statements and Declarations

Ethical statement: This article does not include any animal experiments.

Availability of data and material: Datasets utilized and/or analyzed in this study will be made public by corresponding author upon sensible request.

Funding: Non-funded.

Competing interests: No competing interests

Authors' contributions

Authors have contributed to conceptualization and strategy of study. Initial draft of manuscript was authored by Prakash Nargatti, with subsequent inputs and revision provided by all authors. All authors have carefully reviewed manuscript and permitted final manuscript.

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