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Evaluation of antioxidant, anti-inflammatory and anticancer activities of root stock of *Monochoria vaginalis* – An *in vitro* study

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Abstract
Monochoria vaginalis is a plant belonging to the Pontederiaceae family known to have several medicinal properties. It is a weed found in rice (Oryza sativa) fields and is used as a vegetable. Even though the plant root stock and leaves have cooling, bittersweet, aromatic and diuretic properties, some of the phytochemical and pharmacological properties in this plant still remain unexplored. The objective of the present study was to explore the antioxidant, antimicrobial and anticancer characteristics of the methanolic extract of root stock of *Monochoria vaginalis* through *in vitro* assays. Phytochemical studies were carried out using three different solvents such as petroleum ether, chloroform and methanol. DPPH radical scavenging assay, trypsin inhibitory assay, protein denaturation assay, MTT assay and apoptosis detection by acridine orange and ethidium bromide double staining method were carried out. Phytochemical analysis revealed the presence of phenols, flavonoids, alkaloids, glycosides, terpenoids and steroids. Methanolic extract exhibited significant antioxidant and anti-inflammatory activities. Cytotoxicity studies showed significant mortality against HeLa cell lines. Extracts possess significant anti-inflammatory and anticancer activities, validating its traditional uses. This study provides evidence for the plant's potential as a natural antioxidant, anti-inflammatory and anticancer agent.

Keywords: *Monochoria vaginalis*, HeLa cell line, antioxidant, anti-inflammatory, apoptosis

Introduction

Medicinal plants have been utilized for thousands of years, and the World Health Organization (WHO) recognizes them as a vital source of diverse drugs. Approximately 80% of individuals in developed countries rely on traditional medicines derived from plants. Plants contain numerous bioactive compounds which have various therapeutic properties that are essential for human health [1]. In most developing and developed countries, there is an enormous surge in the use of herbal medicine for disease prevention and treatment. Herbal based drugs or medicinal plants are believed to promote healthier living than synthetic drugs [2].

In spite of the remarkable growth of traditional medicine in each society, herbal medicines have played a key role in treating disease [3]. Medicinal plants are known to play vital roles as sources of active anti-inflammatory agents [4]. *In vitro* cytotoxicity assays are implemented to evaluate the toxic potential of chemical and natural materials in cell culture models to help detect the ability of plant extracts to affect cell viability, cellular growth, and cell damage [5].

Monochoria vaginalis (Carpet Weed) from the Pontederiaceae family has been traditionally used for its medicinal properties. Studies have demonstrated its antioxidant and anti-inflammatory activities [6]. The plant's root stock and leaves possess cooling, bittersweet, aromatic, and diuretic properties [7]. Previous investigations have reported the presence of glycosides, flavonoids, and tannins in *Monochoria vaginalis* extracts [8]. Other studies have evaluated the antioxidant and cardio protective potential of *Monochoria vaginalis* [6] its protective nature against acetaminophen induced necrotic damage [9] and its phytochemical properties [10].

This study aims to evaluate the phytochemical properties, anti-inflammatory and anticancer activity of methanolic extract of *Monochoria vaginalis* root stock. Understanding its potential will contribute to the development of plant based anti-inflammatory and chemotherapeutic drugs.The investigation of *Monochoria vaginalis'* bioactive compounds and their therapeutic effects will provide valuable insights into its traditional medicinal uses and potential applications in modern medicine.

Materials and methods

1. **Plant Material**
   Monochoria vaginalis collected, identified and authenticated.

2. **Cell Line**: HeLa (Human cervical cancer) cell line from National Centre for Cell Sciences (NCCS), Pune, India. Chemicals and reagents used were of analytical grade.

3. **Preparation of plant Extract**
   A 100gm dried powder of the root stock of *Monochoria vaginalis* was subjected to successive soxhlet extraction using a series of solvents of increasing polarity starting from petroleum ether, choloroform and methanol respectively for 72 hrs at a temperature not exceeding the boiling point of the solvent. The step was repeated with a new set of dried powder and solvent until the required quantity was achieved. The extracts were filtered using Whatman filter paper (No.1), while hot and concentrated in a rotary evaporator. The yield of methanolic extract of *Monochoria vaginalis* (MEMV) was 5.06 %. The concentrate was suspended in DMSO for *in vitro* studies.

4. **Cell Line and Culture**
   HeLa cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and

antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

5. Preliminary phytochemical screening

Preliminary phytochemical screening was carried out for petroleum ether, chloroform and methanol extracts for the detection of phytoconstituents as per the standard conventional protocols [11-14].

6. *In vitro* antioxidant studies

The plant extract was subjected to free radical scavenging by DPPH assay. The plant extract was subjected to quantification of phytoconstituents such as total flavonoids, phenolics and antioxidant capacity.

6.1 DPPH radical scavenging activity

The determination of the free radical scavenging activity of MEMV was carried out using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay [15]. The antioxidant activity of various extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The reduction capability of DPPH radicals was determined by the decrease in the absorbance at 517 nm. Ascorbic acid was used as standard.

6.2 Determination of total phenolic content

The total phenolic content in the water extract was determined by folin- ciocalteu method [16]. For the preparation of calibration curve 1 ml aliquots of 50, 100, 150 and 200 µg/ml gallic acid solutions were mixed with 5 ml folin-ciocalteu reagent (diluted ten-fold) and 4 ml (75 g/l) sodium carbonate. The final volumes were made up to 10ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve of gallic acid was prepared and the results were expressed as mg GAE (Gallic acid equivalents)/g dry extract.

6.3 Determination of total flavonoids

Total flavonoid content was measured with the aluminum chloride colorimetric assay [17]. Methanolic extracts at a concentration of 400µg/ml and different dilution of standard solution of quercetin (10- 100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO2 was added. After 5 minutes, 0.3ml of 10% AlCl3 was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Total flavonoid content of the extract was expressed as percentage of quercetin equivalent per g dry weight of sample.

6.4 Determination of total antioxidant activity

The antioxidant activity of the extract was evaluated by the phospho molybdenum method [18]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6M sulphuric acid, 0.28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in place of extract was used as the blank. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

7. Anti-inflammatory studies

7.1. Trypsin (proteinase) inhibitory assay [19]

It is demonstrated that proteinase implicate the tissue damage during the inflammatory reactions. Proteinases abundantly exist in lysosomal granules of neutrophils. Therefore proteinase inhibitors provide the significant level of production. The reaction mixture (2 ml) contains {0.06 mg proteinase or trypsin, 1 ml 20 Mm Tris HCl buffer (pH 7.4) and 1 ml test sample/ standard drug, Diclofenac sodium, of different concentration 50,100 and 200 µg/ml. The mixture is incubated at 37°C for 5 min and then I ml of 0.8% (w/v) casein or 4% (w/v) bovine serum albumin is added. The mixture is incubated for an additional 20 min. 2 ml of 70% perchloric acid or 5% trichloroacetic acid (TCA) is added to terminate the reaction. Cloudy suspension is centrifuged at 3000 rpm for 10 minutes or 2500 rpm for 5 minutes and the absorbance of the supernatant is read at 210 nm or 217 nm against buffer as blank. The experiment is performed in triplicate. The percentage inhibition of proteinase inhibitory activity is calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{adsorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \text{x100}$$

7.2. Inhibition of protein denaturation [20]

To evaluate the anti-inflammatory effects of the extracts, the protocol described by Padmanabhan, 2012 was used with small modifications. A volume of 1ml of extracts (petroleum ether, chloroform and methanol) or of diclofenac sodium at different concentrations (100, 200, 500µg/ml) was homogenised with 1ml aqueous solution of bovine serum albumin (5%) and incubated at 27ºC for 15 minutes. BSA and distilled water constituted the control tube. Denaturation of protein was made by placing the mixture in a water bath for 10 minutes at 70^0C. The mixture was kept at room temperature and the activity of each mixture was measured at 660nm. Each test was done three times. The following formula was used to calculate inhibition percentage

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \text{ x 100}$$

8. *In vitro* anticancer studies

The viability of cells was evaluated by MTT assay and direct observation of cells by inverted phase contrast microscope.

8.1 Cytotoxicity assay by direct microscopic observation and by MTT assay [21, 22]

Entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope and microscopic observation was recorded as images. Any detectable changes in the morphology of the cells,

such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

HeLa cells were seeded in 96-well plates and treated with different concentrations of MEMV root extracts (10-100 µg/mL). Cell viability was assessed using MTT reagent after 48 hours.

8.2 Determination of apoptosis by Acridine Orange (AO) and Ethidium Bromide (EtBr) double staining [23]

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EtBr is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. HeLa cells were treated with of root stock of MEMV at different concentrations for 48 hours. Cells were stained with AO and EtBr and observed under a fluorescence microscope.

9. Statistical analysis

Results were expressed as mean ± S.D and all statistical comparisons were made by means of one way ANOVA test

Results and Discussion

A screening study was conducted to identify different types of phytochemicals present in various extracts of *Monochoria vaginalis*. The *in vitro* antioxidant potential and cytotoxic studies of plant extracts were also tested to get an insight into the active components responsible for their medicinal properties.

1. Phytochemical Screening

Preliminary phytochemical analysis of *Monochoria vaginalis* revealed the presence of different types of secondary metabolites in petroleum ether, chloroform and methanol fractions as shown in Table 1.

Table 1: Phytochemical screening of *Monochoria vaginalis*

Constituents	Petroleum ether	Chloroform	Methanol
Alkaloids	-	-	+
Flavonoids	-	+	+
Phenolics	-	+	+
Tannins	-	+	+
Glycosides	-	-	+
Steroids	+	+	-
Saponins	-	-	+
Terpenoids	+	+	-
Carbohydrates	-	+	+
Proteins & amino acids	-	-	+

+ sign indicates the presence of constituents - sign indicates the absence of constituents

2. *In vitro* antioxidant studies

The plant extracts were subjected to free radical scavenging activities such as scavenging of superoxide, hydroxyl free radical and DPPH assay. The plant extracts were subjected to quantification of phytoconstituents such as total phenolics, flavonoids and antioxidant capacity.

2.1. DPPH radical scavenging activity

The petroleum ether, chloroform and methanol extracts of *M.vaginalis* exhibited a significant increase in DPPH activity especially at a concentration of 500µg/ml. A concentration dependent assay was carried out with these extracts and the results were summarised in Figure1. Among five different concentrations used in the study (5 to 500µg/ml), methanol extract showed maximum scavenging activity. Percent (%) scavenging activity was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis.

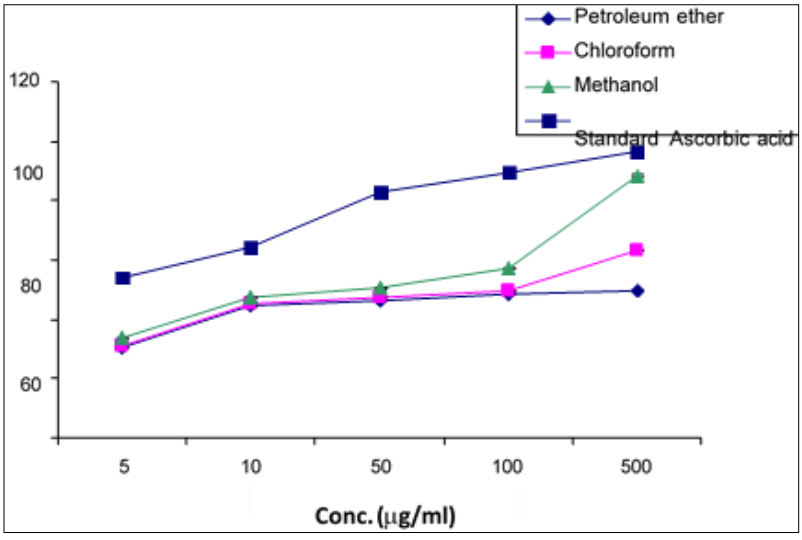


Fig 1: DPPH radical scavenging activity of different extracts of *M.vaginalis*

2.2. Determination of total phenolics, flavonoids and antioxidant activity of *Monochoria vaginalis*

The methanolic extract of *Monochoria vaginalis* showed high radical scavenging ability when compared to other extracts. Also the methanolic extract contain almost all phytochemicals. Hence MEMV was used for the evaluation of phytoconstituents.

MEMV showed high amount of phenolics, flavonoids and total antioxidant activity.The results are summarized in table 2.

Table 2: Determination of total phenolic, flavonoid and antioxidant activity of MEMV

M.vaginalis extract	Total antioxidant activity (mg ascorbic acid/g dry extract)	Phenolic contents (mg GAE/g dry extract)	Flavonoids (mg QE/g dry extract)
Methanol	195.0 ± 8.06	26.85 ± 1.56	1.32 ± 0.15

Values are mean ± SD (n=3)

3. Anti-inflammatory studies

3.1. Trypsin (proteinase) inhibitory assay

Protein denaturation is a well-documented cause of inflammation. Anti-inflammatory agents are substances that can inhibit protein denaturation. In this investigation, *in vitro* anti-inflammatory effects of different extracts (Petroleum ether, chloroform and methanol) of *Monochoria vaginalis* were evaluated against the denaturation of bovine serum albumin. While investigating the anti-proteinase potentials of various solvent extracts of *Monochoria vaginalis*, a significant dose dependent reduction in proteinase activity was observed in methanol extract treatment. It showed highest inhibition at 200µg/ml. The result obtained at different concentrations was given in Figure 2

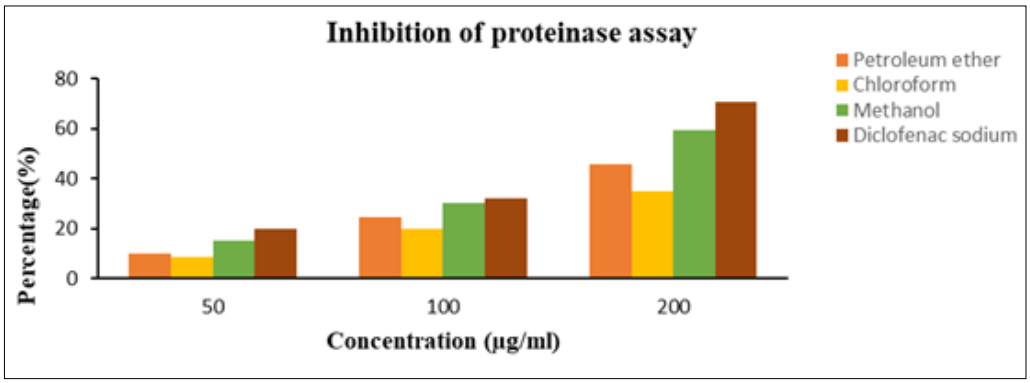


Fig 2 : Inhibition of proteinase activity

3.2. Inhibition of protein denaturation

The anti-inflammatory activity of petroleum ether, chloroform and methanol extract of *Monochoria vaginalis* was evaluated against denaturation of egg albumin method. Methanol extract at 500 µg/ml showed maximum inhibition. The denaturation of tissue protein is well documented cause of inflammation. In this study, we found dose dependent inhibition of protein denaturation due to administration of *M.vaginalis* extracts, which indicates anti-inflammatory potentials on it.

Further during the execution of the assay, we used gradual heating to increase the temperature to 70°C from 37°C (instead of heating rapid) [24] to prevent formation of irregular clumps resulting from protein coagulation resulting from evaporation of water molecules from egg white and thermal denaturation of egg protein [25]. The exact mechanism through which the extract mediated its anti-denaturation effect is unknown at present. But it may be due to interaction of its flavonoids, tannins and alkaloids with aliphatic region around lysine residue on the albumin protein [26]. The results shown in Figure 3. The effect of plant extract was studied by comparing with the standard diclofenac sodium.

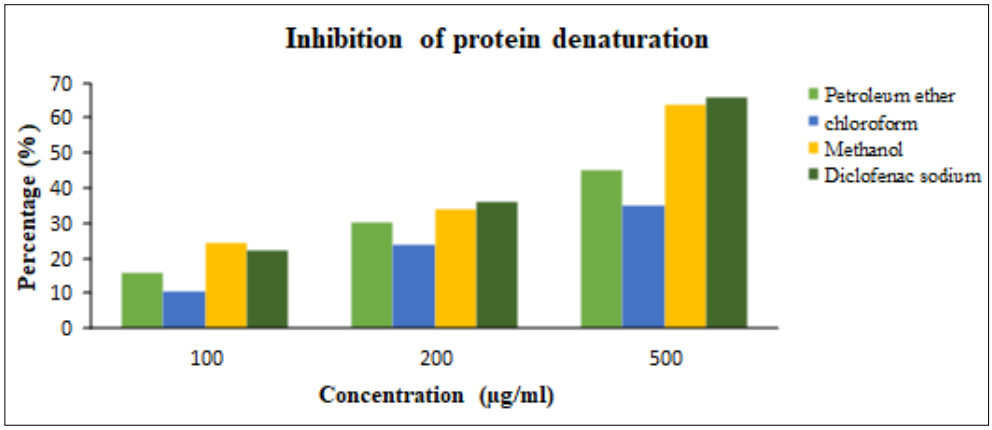


Fig 3: Inhibition of protein denaturation activity of different extracts of *M. vaginalis*

4. *In vitro* anticancer studies

HeLa cell line was used for determining anticancer effects of methanol extracts of root stock of *M. vaginalis*. The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope followed by MTT assay.

4.1. Anticancer assay by direct microscopic observation

The anticancer effects of MEMV at different concentrations were observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope. Observable morphological changes of cells such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells as indicators of cytotoxicity were noted in all plates. More viability was noted in concentration control, 6.25 and 12.5 µg/ml seeded plates. All observations were recorded as images as shown in the Figure 4. In order to confirm the maximum percentage viability of cells, MTT assay was carried out.

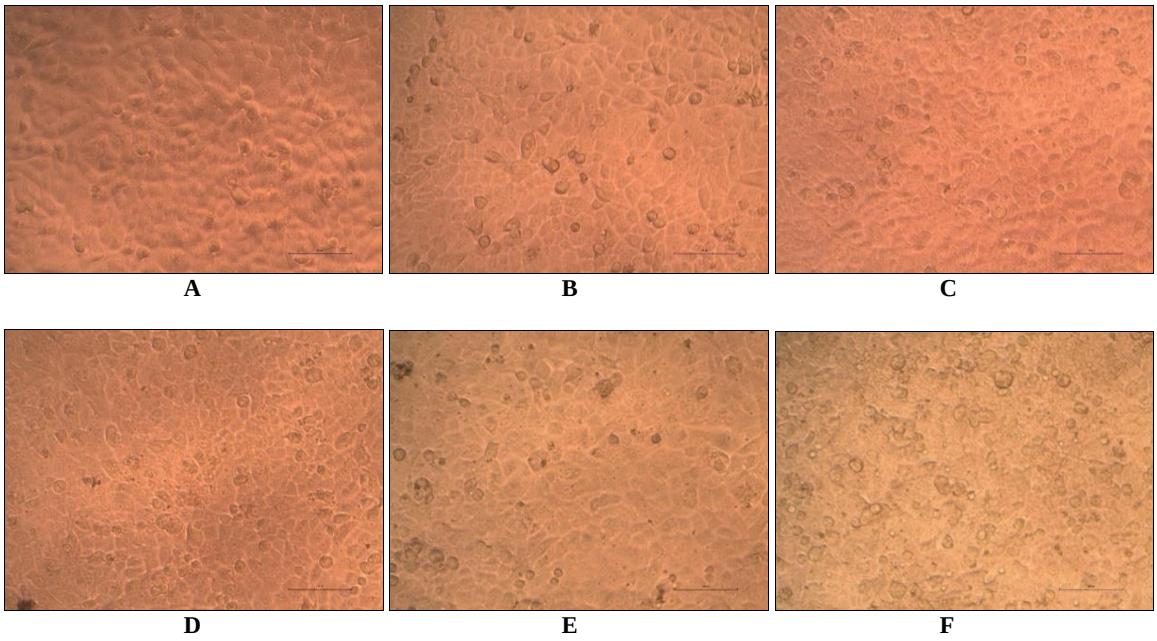


Fig 4: Cell images. Untreated control **(A)** and cells administered with various concentrations of sample **(B)** 6.25 µg/ml **(C)** 12.5 µg/ml **(D)** 25 µg/ml (E) 50 µg/ml and (F) 100 µg/ml

From the above images, increasing concentration of plant extract has significant role in decreasing cell viability. A significant change in cell morphology can also be observed.

4.2. Cell growth profile in MTT assay

The cytotoxic activity of MEMV at different concentrations (6.25, 12.5, 25, 50 and 100µg/ml) against HeLa cells was assessed *in vitro* by MTT proliferation assay. It showed a significant decrease in cell viability. As the concentration of sample increases, it induced cell toxicity and showed a decrease in cell viability and finally at a dose of 100µl of MEMV only 62.39 68% cells were viable. These results indicated that MEMV showed significant potentiality against the viability and proliferation of cervical cancer cell (HeLa) cell lines. The photographs of cytotoxicity were provided under Figure 5.

4.3. Determination of Apoptosis by Acridine Orange (Ao) and Ethidium Bromide (EtBr) Double Staining

To evaluate whether the extract from the plant *M.vaunalis* induced apoptosis in the HeLa cells, morphological analysis by microscopic examination of acridine orange/ethidium bromide stained target cells was performed. Microscopic examination revealed that the extract induced apoptosis in target HeLa cells after 24hr treatment. Photographs of acridine orange/ethidium bromide-stained control HeLa cells were given in Figure 6.

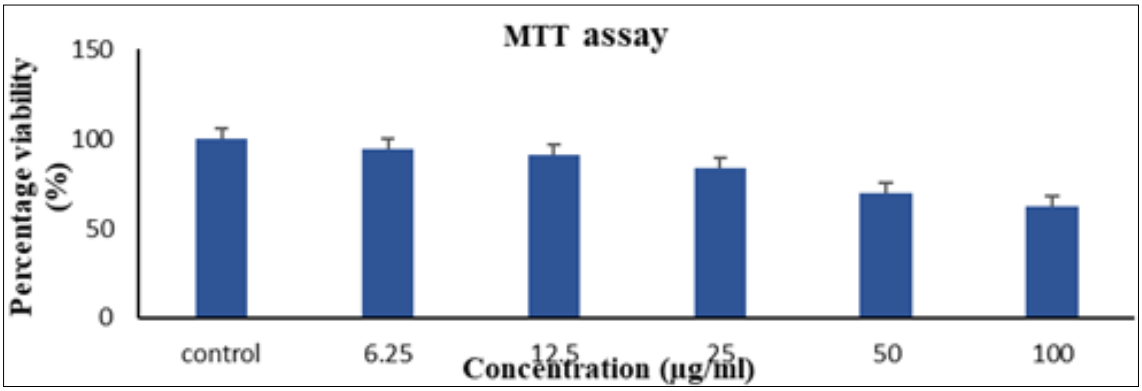
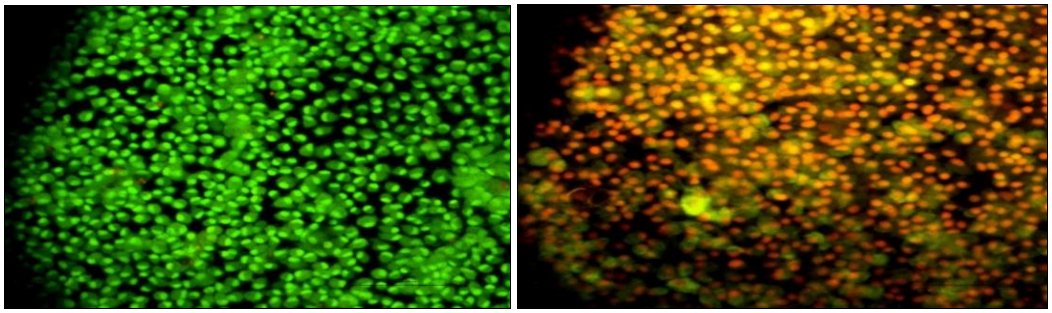


Fig 5: Anticancer effect of plant extracts on HeLa cell line using MTT assay



A B

Fig 6: Phase contrast microscopic image showing Acridine orange/ Ethidium bromide stained Hela cells. (A) Control (B) treated with methanic extract of root stock of *M. vaginalis*.

From the pictures it could be observed that treatment of cells with 100µg/ml of extracts produced significant changes in nuclear integrity. Control cells with intact green nuclei exhibited red fluorescence following compound treatment. The presence of ethidium bromide stained red nuclei indicated compromised membrane integrity which is a hall mark of apoptosis.

Conclusion

Monochoria vaginalis, a common aquatic herbaceous plant, exhibits diverse pharmacological activities. Traditionally, its roots are used for medicinal purposes, while the rest of the plant is consumed as a vegetable. Phytochemical analysis reveals the presence of alkaloids, flavonoids, phenolics, tannins, glycosides, terpenoids, and steroids in its roots. This study demonstrates the antioxidant activity of *M. vaginalis* root stock in various extracts, with the methanol extract exhibiting the highest free radical scavenging activity. The methanolic extract shows significant anti-inflammatory potential and anticancer activities.

These findings suggest that *M. vaginalis* roots possess antioxidant, anti-inflammatory, and anticancer properties, attributed to the synergistic effects of phytochemicals such as alkaloids, phenolic compounds, and flavonoids. In conclusion, *Monochoria vaginalis* has potential therapeutic applications, particularly as an anticancer agent, due to its cytotoxic properties. Further research and development of novel cancer treatments are warranted.

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