Research article

Phytochemical analysis, *in-vitro* antioxidant and cytotoxicity activities of *Flaveria trinervia* ethanolic extract

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ABSTRACT

Introduction and Aim: Crude herbal drugs and commercial extracts obtained from plant sources continue to play a significant role in healthcare, particularly in developing countries where traditional medicine practices are deeply rooted. The aim of the study was to estimate the antioxidant (DPPH) and cytotoxic effects of the ethanolic extract of *Flaveria trinervia* on raw 246.7 cells.

Materials and Methods: In the Indian town of Thoothukudi's Kovilpatti village, *F. trinervia* was isolated. The entire plant was air-dried before being crushed, and 1 kg of the powdered ethanolic extract was employed in the Soxhlet apparatus for continuous extraction. By utilizing GC-MS analysis, *F. trinervia* ethanolic extract phytochemical assessment was studied. The antioxidant potential of *F. trinervia* was examined using the DPPH test. Raw 246.7 cell lines were used to test the ethanolic extract of *F. trinervia* for cytotoxic activity.

Results: *F. trinervia* extracts were found to contain a variety of biologically active phytoconstituents, including ergosterol, octadecanoic acid, propanoic acid, and isopropyl palmitate, according to phytochemical analysis. Compared to the negative control, the ethanolic extract of *F. trinervia* considerably showed antioxidant activity and cytotoxicity. Ascorbic acid antioxidant activity with DPPH was 98.12%, whereas *F.trinervia* ethanolic extract's antioxidant activity was 78.91%. The anticancer efficacy of *F. trinervia* ethanolic extract against uncultured 246.7 cells was evaluated using the MTT assay. By charting cell viability vs extract concentration, the IC_{50} value was determined. The MTT assay identified raw cells IC_{50} at 24 hrs as 200 µg/mL of ethanolic *F. trinervia* extract.

Conclusion: Active phytoconstituents are present in the *F. trinervia* whole plant ethanolic extract. Because of the presence of phytoconstituents, the extracts have excellent antioxidant activity and good cytotoxicity activity against raw 246.7 cell lines. The study recommended using whole plant extract from *F. trinervia* to treat several metabolic diseases.

Keywords: *F. trinervia;* ethanolic extract; phytochemical analysis; antioxidant activity; cytotoxicity.

INTRODUCTION

F. trinervia, is a plant belonging to the Asteraceae family, commonly known as yellow tops. This plant is grown in various countries of the world (1). It has also been introduced to other regions and is sometimes considered a noxious weed. In India, the plant is widely distributed in Kerala, Tamil Nadu, and Andhra Pradesh. *F. trinervia* grows up to 75 cm tall and has lanceolate or elliptic leaves. The leaves are yellowish-green, 1-7.5 cm long, opposite, and may have petioles or be upper sessile. The plant produces inflorescences called capitula, which are compact heads with numerous yellow flowers (2, 3). The flowers have very short ray florets and are arranged in congested axillary heads. The margin of the leaves is toothed, and the leaves have three veins originating from the base.

The researchers conducted a phytochemical analysis of the ethanolic extract of *F. trinervia*. Phytochemical analysis involves identifying and quantifying the chemical constituents present in plant extracts (4, 5). This analysis helps in understanding the potential medicinal properties of the plant. Additionally, the researchers evaluated the antioxidant activity of the extract. Furthermore, the cytotoxicity of the *F. trinervia* ethanolic extract was assessed (6, 7). Cytotoxicity studies involve examining the effects of a substance on living cells, specifically whether it can induce cell death or inhibit cell growth. These studies are important for understanding the potential toxicity or anti-cancer properties of plant extracts.

MATERIALS AND METHODS

Chemical and reagents

Ethanol, ascorbic acid, DMEM, PBS, and distilled water chemicals were purchased from Sigma Aldrich Mumbai, India.

Plant collection and authentication

F. trinervia was collected in June 2021 from Kovilpatti village in Thoothukudi district, Tamil Nadu, India. Identification and authentication were carried out at St. Xavier College Department of Botany in Palayamkotaai, Tamil Nadu, India. Plant materials were collected, washed, dried in the shade, and powdered. These pulverized materials were then analysed further.

Phytochemical analysis whole plant ethanolic extract of *F. trinervia* by conventional method and GC-MS

Traditional analysis was employed by Vijayalakshmi et al. and Lokapur *et al.,* (8, 9) to screen preliminary phytochemical studies of the entire F. trinervia plant ethanol extract, whereas Jayakar *et al.* (10) used GC-MS analysis for phytochemical screening investigations.

Antioxidant activity of *F. trinervia* ethanolic extract

The DPPH was used to analyse the free reactive oxygen species in the ethanolic extract of *F. trinervia*. 24 mg of DPPH were dissolved in 100 mL of methanol to create the solution. The DPPH stock solution was filtered through methanol to produce a usable mixture that had an absorbance of about 0.973 at 517 nm. In a test tube, 100 mL of the ethanolic extract from *F. trinervia* and 3 mL of the DPPH working solutions were combined. As a standard, three mL of DPPH solution in 100 mL of methanol is usually offered. After that, the tubes were placed in complete darkness for 30 minutes. As a result, the absorbance was determined to be 517 nm.

% of antioxidant activity= [(A control−A test) ÷ A control] × 100

Cytotoxicity of *F. trinervia* ethanolic extract

Cell culture maintenance

In the study, the raw cell line was used, which was obtained from the National Centre for Cell Sciences cell repository located in Pune, India. To keep the Raw 264.7 cell line alive and support its growth, DMEM culture media was used. 10% FBS and DMEM culture media, which provides essential nutrients and growth factors necessary for the cells' survival and proliferation. To the media, 100 µg/mL of streptomycin and penicillin was added. Penicillin and streptomycin are antibiotics that inhibit the growth of bacteria, ensuring a sterile environment for cell culture. The cells were maintained at 37°C, which helps to mimic the physiological conditions necessary for cell growth and function. Additionally, the cells were cultured in an environment with 5% CO_2, which helps to regulate the pH balance and provides the necessary carbon dioxide for cell metabolism. This standardized culture condition of temperature, humidity, and CO_2 concentration provides an optimal environment for the growth and maintenance of the Raw 264.7 cell line, allowing researchers to perform experiments and evaluate the effects of the *F. trinervia* ethanolic extract on these cells (11).

MTT assay

MTT assay of the *F. trinervia* ethanolic extract was studied by following the method developed by Jayakar *et al*. (12). Raw cells were placed in cell culture plates (96-well) plates and treated with different concentrations of *F. trinervia* ethanolic extract. The concentrations ranged from 0 to 500 µg/mL. Additionally, a control group without the extract was included.

The treated cells were incubated for 24 hours at 37°C in a humidified environment with 95% air and 5% CO_2. The *F. trinervia* cells that had ethanolic extract were then rinsed with fresh sterile culture medium after the incubation period. MTT 5 mg/mL treated the cells. A multi-well plate reader was used to measure the absorbance of the formazan product produced by live cells at 540 nm following the MTT dye's 4-hour incubation. The enhanced absorbance values are linked to enhanced cell viability as measured by the absorbance readings. In comparison to the control group, the results were presented as a percentage of viable cells. The IC_{50} values were determined from the dose-response curve generated by plotting the percentage of viable cells against the concentrations of *F. trinervia* ethanolic extract. The optimal doses of the extract were also investigated over time.

Inhibition (%) = (Control-Test) x 100

IC_{50} was calculated by using data obtained from the MTT assay. The dose-response curve plots the percentage of viable cells against the concentrations of *F. trinervia* ethanolic extract.

Apoptosis was studied by using the AO/EB staining

The study followed the method by Shilpa *et al.,* (13). To 100 µL of AO, 100 µL of EB in PBS was added, resulting in a total volume of 200 µL. *F. trinervia* extract treated RAW 264.7 cells stained with AO and EB (100 µg/mL). Stained cells observed under the fluorescent microscope with a magnification of 40X were used to examine the stained cells. The researchers observed the cells under the microscope and identified the presence of apoptosis-like characteristics.

RESULTS

Phytochemical analysis of *F. trinervia* ethanolic extract

Bioactive phytochemicals have been identified using the GC-MS investigation of the ethanolic extract. Table 1 lists the various phytoconstituents found in the ethanolic extract of *F. trinervia*.

Table 1: Preliminary phytochemical analysis of *F. trinervia* ethanolic extract

S. No	Chemical groups	Ethanolic extracts of *F. trinervia*
1	Alkaloids	-
2	Glycosides	-
3	Carbohydrates	+
4	Steroids	+
5	Triterpenoids	+
6	Flavanoids	+
7	Tannins	-
8	Phenolics	+
9	Saponins	-
10	Fixed oils and fats	-
11	Proteins & free amino acid	-
12	Volatile oil	-
13	Mucilage and gums	-

+ (Present); - (absent)

Table 1: GC-MS analysis of *F. trinervia* ethanolic extract and its biological importance

S. No.	Compound name	m/z	Molecular formula	Area %	Biological activity
1	1,3-Cyclopentanedione	55.10	$C_5H_6O_2$	80.13	flavouring agent
2	1-Propyne	58.20	C_3H_4	37.91	-
3	Catecholborane	65.20	$C_6H_4BO_2$	19.05	Antimicrobial
4	2-Methoxy-4-vinyl phenol	135.20	$C_9H_{10}O_2$	79.69	antibacterial activity
5	3-Cyclohexen-1-ol, 3-methyl	113.10	$C_7H_{12}O$	76.41	inhibit of enzymes
6	Creatinine	55.10	$C_4H_7N_3O$	58.44	Indicator
7	Pentafluoropropionic acid, tridecyl ester	69.20	$C_3HF_5O_2$	39.30	Cytotoxic activity
8	3-Deoxy-d-mannoic lactone	102.10	$C_6H_{10}O_5$	68.34	Anticancer and Antibacterial
9	Propanoic acid	54.20	$C_3H_6O_2$	50.36	Antimicrobial activity
10	2-fluoro-5-hydroxy-1-ribofuranosyl	73.10	$C_8H_{11}FN_2O_5$	74.93	Cytotoxic activity
11	isopropyl palmitate	55.20	$C_{19}H_{38}O_2$	50.52	Antimicrobial, emollient, and antistatic agent.
12	Tetradecanoic acid	57.20	$C_{14}H_{28}O_2$	80.72	Antioxidant and anti-inflammatory
13	Cyclotriacontane	69.20	$C_{30}H_{60}$	71.94	Enzyme inhibition activity
14	n-Hexadecanoic acid	129.20	$C_{16}H_{32}O_2$	79.26	Antioxidant (14)
15	Tetracosyl pentafluoropropionate	55.20	$C_{27}H_{49}F_5O_2$	79.23	-
16	Octacosyl heptafluorobutyrate	69.20	$C_{32}H_{57}F_7O_2$	63.74	Antioxidant, antitumor, Antiulcer and Antibacterial
17	cis-Vaccenic acid	95.20	$C_{18}H_{34}O_2$	66.33	Hypolipidemic effect
18	Octadecanoic acid	69.20	$C_{18}H_{36}O_2$	78.19	Anticancer activity
19	Ergosterol	55.20	$C_{28}H_{44}O$	72.14	Antitumor
20	Tetratriacontyl trifluoroacetate	71.20	$C_{36}H_{69}F_3O_2$	75.67	Antioxidant

Antioxidant activity of *F. trinervia* ethanolic extract

The maximum absorption of DPPH occurs at 517 nm, which corresponds to a purple colour. When DPPH reacts with a free-radical scavenger antioxidant and forms DPPHH, the decrease in absorbance is likely associated with changes in the electronic structure of the molecule. The gathering of additional electrons in the radical form can lead to a shift in the absorption spectrum and a decrease in the intensity of the purple colour, resulting in decolourization (Fig. 1 and Table 2).

Table 2: *F. trinervia* ethanolic extract showed an antioxidant activity of 78.91% (200 µg), IC_{50}=25 µg/ml, and ascorbic acid showed an antioxidant activity of 98.12% (200 µg), IC_{50}= 3.125 µg/ml with DPPH

Concentration (µg)	*F. trinervia* ethanolic extract (% of Inhibition)	Ascorbic acid (% of Inhibition)
200	78.91	98.12
100	68.65	91.64
50	64.54	86.29

25	59.12	79.74
12.5	47.31	71.83
6.25	32.39	65.58
3.125	26.75	57.21

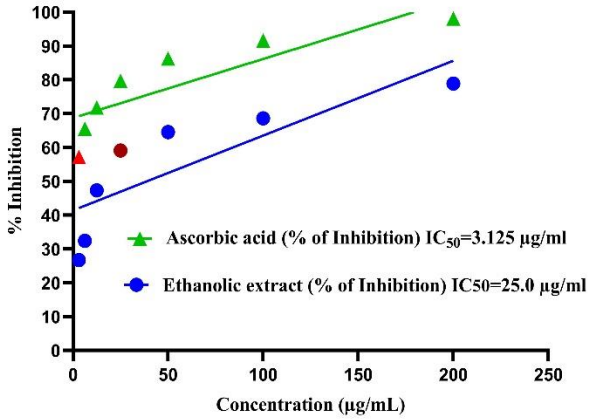

Fig. 1: *F. trinervia* ethanolic extract showed an (IC_{50}=25 µg/ml) antioxidant activity of 78.91%, and ascorbic acid (IC_{50}= 3.125 µg/ml) showed an antioxidant activity of 98.12% with DPPH.

MTT assay *F. trinervia* ethanolic extract

In the current study, the MTT assay was used to evaluate the influence of ethanolic *F. trinervia* extract treatment on the cell viability of RAW 264.7 cells. It is one of the most used in vitro model systems to evaluate the cytotoxic effects of many toxic substances and plant extracts against cancer cell lines. The viability of RAW 264.7 cells decrease with an increase in the concentration of the ethanolic extracts. Only viable cells have the ability to reduce MTT tetrazolium into a coloured formazan product. The cytotoxic activity was expressed as a percentage of cell viability in RAW 264.7 cell lines when compared with the control and the plant extracts revealed more cytotoxicity towards cancer cell line RAW 264.7. Concentrations in the range of 0-400 µg/ml for ethanolic extracts of *F. trinervia* respectively were used for the study. *F. trinervia* ethanolic showed a cytotoxic effect towards RAW 264.7 cell lines. In this Data is expressed as mean SEM (n=3). Statistical significance was determined using one-way ANOVA (* $p < 0.05$, **$p < 0.01$) as compared to standard and control groups. The results exposed morphological changes and cellular shrinkage resulting in cell death caused by the extracts in the renal cancer cell lines (Figure 3). In Figure 3: Cellular shrinkage, cell reduction and membrane damage were observed (Magnification for RAW 264.7 cells under were 45X). The survivability of cells to the *F. trinervia* leaf ethanolic extract (Figure 2). In-vitro, growth inhibition effects were observed in the RAW 264.7 cell line. All the extracts were evaluated in triplicates concerning concentration (0, 25, 50, 100, 200, 300 and 400 µg/ml) by serial dilution. Concentrations, 200 µg/ml of ethanolic extracts were the most effective inhibit the growth of the 50% RAW 264.7 cell lines.

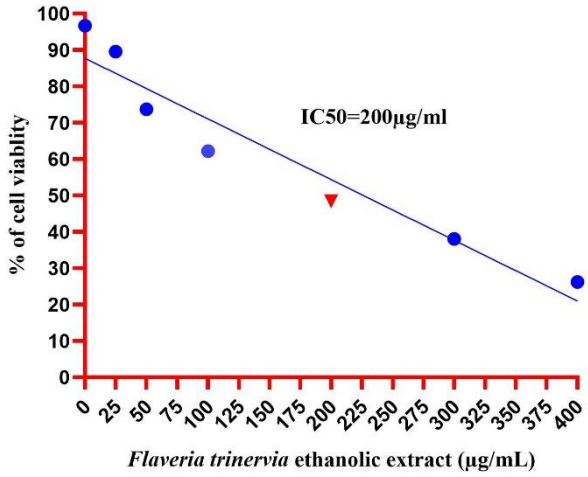

Fig. 2: *F. trinervia* ethanolic extract cytotoxicity against raw 246.7 cells (IC_{50}=200 µg/ml)

Fluorescent staining for apoptosis

Acridine orange/ethidium bromide (AO/EB) staining is a commonly used fluorescent staining method to assess the morphological changes and apoptotic features of cells (Fig. 3). The staining was performed on raw 246.7 cell lines to investigate the effects of *F. trinervia* ethanolic extract on these cells. The staining method involves the use of two fluorescent dyes, acridine orange and ethidium bromide. Acridine orange stains both live and dead cells, producing a green fluorescence in live cells. Ethidium bromide, on the other hand, only enters cells with compromised membrane integrity, such as dead or apoptotic cells, and produces an orange-red fluorescence. By using AO/EB staining, researchers can distinguish between live cells (green fluorescence) and dead or apoptotic cells (orange-red fluorescence). Live cells that have intact membranes are permeable to acridine orange and appear green under fluorescence microscopy. Dead or apoptotic cells, with compromised

membranes, are permeable to ethidium bromide and display an orange-red fluorescence. The AO/EB staining fluorescence pattern provides insights into cell viability and membrane integrity. It allows researchers to visually observe and analyse the morphological changes in the raw 246.7 cells induced by the *F. trinervia* ethanolic extract. By comparing the number and distribution of live and dead/apoptotic cells, researchers can assess the extract's effects on cell survival and apoptotic processes.

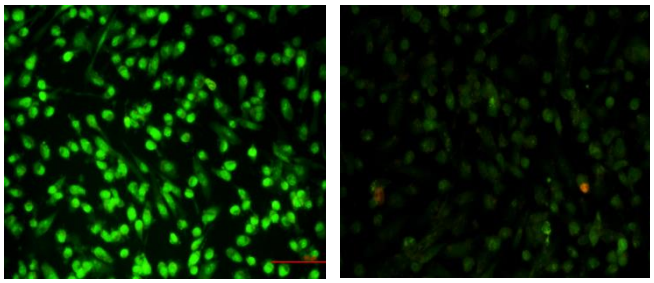

Fig. 3: A: Raw 246.7 control cells, B: *F. trinervia* ethanolic extract treated raw 246.7 cells (24 hr).

DISCUSSION

Since ancient times, plants and their preparations have been used as medicines. The Indian ancient Vedic literature known as Ayurveda is the science of wellness and good health. It is a collection of conventional and cultural medical philosophies. Ayurveda-based modern drug research programmes have found widespread support in the current healthcare system. Natural chemicals originating from plants are less hazardous to healthy cells and more tolerated; thus, recent medication discovery is interesting to them (15). Numerous phytochemicals have been identified as the active components in these plant species, which have been found to inhibit the growth and progression of free radicals and cytotoxicity in cancer patients. Numerous pathways exist via which phytochemicals have anticancer effects (16). The present study investigated the antioxidant and cytotoxic activities of the ethanolic extract of *F. trinervia*. The study found that the extract exhibited significant scavenging abilities on DPPH, a free radical, indicating its potential as an antioxidant. This is consistent with a previous study by Hoskeri et al. in 2011 (17), which also reported antioxidant activity of the ethanolic extract of *F. trinervia*. The reducing power of the extract was found to be significant even at lower doses, suggesting its ability to protect tissues against oxidative damage, including cancer. The DPPH free radical method is a sensitive way to determine the antioxidant activity of plant extracts, and the results presented in Figure 1 and Table 2 showed the amount of the ethanolic extract required to inhibit 50% of DPPH activity (IC_{50}=25 µg/ml). Furthermore, the study evaluated the cytotoxic activity of the ethanolic extract against RAW 264.7 cells, and Figures 2 and 3 displayed the amount of extract needed to inhibit 50% of cell growth (IC_{50}=200 µg/ml. The extract exhibited potent cytotoxic activity against RAW 264.7 cells at low concentrations. Overall, the study combined indigenous knowledge with laboratory assessments to demonstrate the in-vitro antioxidant and cytotoxic activities of the ethanolic extract of *F. trinervia*. The findings suggest that this plant extract has effective antioxidant and potential natural anticancer properties.

CONCLUSION

The biological activity of phytoconstituents is the most important aspect. However, to gain a complete picture of a plant's phytochemical content and biological activities, selecting the most promising component is critical by examining several parts of that plant. The antioxidant activity (DPPH) and cytotoxicity (raw 246.7 cells) of *F. trinervia* ethanolic extract were investigated in this study. The ethanolic extract of *F. trinervia* contains active phytoconstituents. The presence of phytoconstituents in the section results in good antioxidant activity and cytotoxicity against raw 246.7. This study examined the antioxidant activity (DPPH) and cytotoxicity (raw 246.7 cells) of *F. trinervia* ethanolic extract. *F. trinervia* ethanolic extract contains active phytoconstituents. The extract's presence of phytoconstituents results in good antioxidant activity and cytotoxicity against raw 246.7 cells.

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CONFLICT OF INTEREST

Nil.

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