Isolation and characterization of anti-cancer compounds from ethanol extract of *Vetiveria zizanioides* (Nash) roots and their cytotoxic effects

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Abstract
This study investigates the anti-cancer potential of compounds isolated from the ethanol extract of Vetiveria zizanioides (Nash) roots. The roots were subjected to sequential solvent extraction using ethanol, chloroform and petroleum ether, followed by Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC) to isolate active fractions. The ethanol extract exhibited superior cytotoxicity against MCF-7 human breast cancer cell lines, with an IC50 value of 44.6 µg/ml, compared to chloroform (IC50 56.54 µg/ml) and petroleum ether (IC50 99.63 µg/ml) extracts. Among the fractions obtained from the ethanol extract, the second fraction demonstrated the highest cytotoxic activity with an IC50 value of 29.1 µg/ml. Spectral analyses (UV, IR, NMR and LC-MS) identified flavonoids as the major bioactive constituents.

The second fraction induced apoptosis in MCF-7 cells, as evidenced by DNA fragmentation, lactate dehydrogenase (LDH) release and caspase-3 activation. Furthermore, the p53 gene was upregulated by 1.20 and 1.21-fold at concentrations of 200 µg/ml and 300 µg/ml respectively. These findings highlight the potential of V. zizanioides ethanolic extract as a promising source of anti-cancer agents. Further studies are warranted to elucidate the molecular mechanisms and to evaluate therapeutic efficacy in in vivo models.

Keywords: MCF-7 cell line, Flavonoids**,** Apoptosis**,** Caspase-3, p53**,** DNA fragmentation Cytotoxicity assay**,** Traditional medicine, Breast cancer.

Introduction

Plants have long been utilized for treating various diseases, especially in economically disadvantaged regions. The scientific assessment of traditional medicine derived from tropical plants has been a focal point of research. Natural compounds are foundational in the discovery of bioactive molecules and serve as lead compounds in developing drugs for human diseases[3]. Cancer, a critical global health issue, has shown a marked increase, particularly in developing nations such as India. Among cancers affecting women, breast cancer ranks as the second most prevalent[24], with approximately 80,000 new diagnoses and 40,000 deaths annually in India alone[22]. Globally, breast cancer remains the most common form of cancer in women, with significant mortality rates due to metastases. The balance between cell proliferation, differentiation and apoptosis in the mammary gland is crucial for normal breast development. Imbalances in these processes can trigger mutations leading to cancer[1,15,17,18].

Conventional anti-cancer drugs are effective in only a fraction of cases, approximately one-third of breast cancer patients[10]. Moreover, these treatments are associated with severe side effects including stroke, blood clots and other complications. As a result, there is a pressing need to explore alternative agents for the prevention and treatment of breast cancer. Studies highlight that dietary substances like plant extracts, fruits and vegetables can reduce the risk of breast cancer[6]. Medicinal plants have historically been significant sources of novel pharmaceuticals and are increasingly recognized for their potential in oncology[11,12]. Therefore, plant-based remedies are being evaluated for their therapeutic efficacy.

Therefore, in this study we aim to evaluate the extracts of *Vetiveria zizanioides* for their therapeutic efficacy in cancer. The objective of this research is to isolate and characterize bioactive anti-cancer compounds from the ethanol extract of *Vetiveria zizanioides* roots, to assess the cytotoxic activity of the extract against human breast cancer cells (MCF-7) and investigate the underlying mechanisms of its ability to induce cell death in these cells.

Material and Methods

Collection of Plant Sample: The roots of *Vetiveria zizanioides* (Nash) were collected from the foothills of Maruthamalai in the Western Ghats region, Coimbatore. The plant was identified and confirmed as *V. zizanioides* by the Botanical Survey of India, Coimbatore, Tamil Nadu, India (Ref. No: BSI/SRC/5/23/2011-12/Tech-1673) (Fig. 1).

Chemicals: The chemicals used in this study included (3-(4,5-dimethyl thiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate-

buffered saline (PBS), DMEM and antibiotics, which were obtained from Sigma Aldrich and Himedia, Mumbai.

Fig. 1: *Vetiveria zizanioides*
A photograph showcasing the morphology of the *Vetiveria zizanioides* (Vetiver Grass), including its tall, narrow leaves and extensive root system commonly used for soil conservation, aromatic oil extraction and phytoremediation.

Cell Culture: The human breast cancer cell line (MCF-7) was obtained from the National Centre for Cell Science (NCCS), Pune and cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C in an environment with 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week.

Preparation of Extract: Powdered roots were subjected to hot extraction using solvents of varying polarities: low polar (petroleum ether), moderate polar (chloroform) and high polar (ethanol) in a Soxhlet extractor for three days. The extraction process was repeated three times. The extracts obtained were filtered and evaporated using a rotary evaporator and freeze dryer to yield crude dried extracts which were stored at room temperature until use.

TLC Analysis: Dried extract of *V. zizanioides* (100 mg) was weighed using an electronic balance and dissolved in 10 ml of ethanol. The solution was centrifuged at 3,000 rpm for 5 minutes at room temperature and the supernatant was used as the test extract. Various concentrations (2µl, 4µl, 6µl, 8µl, 10µl) of the test extract were loaded onto a 10 × 10 cm silica gel TLC plate using a Hamilton syringe and the Camag Linomat 5 instrument. Organic solvents such as toluene-ethyl acetate (9.3:0.7), benzene-ethyl acetate (1:1) and chloroform-toluene-methanol (6:2.5:1.5) were used as the mobile phase. The twin trough developing chamber was saturated with mobile phase vapors for 10 minutes before the TLC plates were resolved in the presence of mobile phase up to 8 cm. The plates were then dried to evaporate solvents, visualized under UV light (254 nm and 366 nm) in a photo documentation chamber (Camag Reprostar 3) and images were captured.

***In vitro* Cytotoxic MTT Assay:** Monolayer cells were detached with trypsin-EDTA to produce single-cell suspensions. Viable cells were counted using the trypan blue exclusion assay with a hemocytometer. The cell suspension was diluted with medium containing 5% FBS to achieve a final density of 1×10^5 cells/ml. Each well of a 96-well plate was seeded with 100 µl of the cell suspension (10,000 cells/well) and incubated at 37°C in 5% CO_2, 95% air and 100% relative humidity for 24 hours to allow cell attachment. After 24 hours, the cells were treated with serial concentrations of test samples dissolved in DMSO. Four additional 2-fold serial dilutions were prepared and 100 µl of each dilution was added to the appropriate wells.

The plates were incubated for 48 hours followed by the addition of 15 µl of MTT (5 mg/ml) in PBS and further incubated for 4 hours. Formazan crystals were dissolved in 100 µl of DMSO and absorbance was measured at 570 nm using a microplate reader. The percentage of cell inhibition was calculated using the formula:

% Cell Inhibition = 100 − (Absorbance of sample / Absorbance of control) × 100

Spectral Analysis: The second-fraction of the ethanolic extract was analysed within the UV-visible range of 200-800 nm using a UV-visible spectrophotometer (Shimadzu Pharma Spec UV-1700). A concentration of 1 mg/mL of the ethanolic extract (second fraction) was prepared for analysis which was employed to study organic compounds.

FT-IR Spectral Analysis: Infra-red spectroscopy was employed for chemical identification, based on selective absorption in the infra-red region. The ethanolic extract (second fraction) of *V. zizanioides* was scanned on an FT-IR spectrometer (Spectrum 100, Perkin Elmer, USA) in the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}, averaging 25 scans. This analysis was performed at Sastra University, Thanjavur, Tamil Nadu, India.

^{1}H, ^{13}C NMR Spectral Analysis: ^{1}H and ^{13}C NMR spectroscopy were utilized for structural determination. The NMR spectra of the ethanolic extract (second fraction) were scanned on a Bruker 300 Ultrashield NMR spectrophotometer at 300 MHz. The analysis was conducted at Sastra University, Thanjavur, Tamil Nadu, India.

LC-MS Spectral Analysis: Molecular screening of compounds present in the ethanolic extract (second fraction) of *V. zizanioides* was carried out using LC-HR-ESI-MS/MS analysis. The LC-MS conditions included a discontinuous gradient elution with a flow rate of 0.2 mL/min using acetonitrile and acidified water as mobile phases. Mass spectrometry data were collected in both negative and positive ionization modes.

DNA Fragmentation Analysis: MCF-7 cells (3 x 10^6/ml) were seeded into 6-well plates and were incubated at 37°C

in a 5% CO_2 atmosphere for 24 hours. The cells were washed with medium, treated with extract and standard drug and incubated again at 37°C in 5% CO_2 for another 24 hours. At the end of the incubation period, the chromosomal DNA of the cancer cells was prepared using the Roche apoptotic DNA ladder kit. The cells were harvested and lysed with lysis buffer for 10 minutes. The samples were then mixed with isopropanol, passed through a filter and washed. The DNA was eluted from the filter, treated with RNAse at 37°C for 30 minutes, loaded onto 2% agarose gel and electrophoresed at 50 V/cm for 3 hours. The gel was visualized under a UV transilluminator and photographed.

Determination of LDH activity in extract-treated cell supernatants: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS. To each well of the 96-well microtiter plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was removed and the monolayer was washed once with medium. Subsequently, 100 µl of different extract concentrations was added to the cells in the microtiter plates. The plates were incubated at 37°C for 3 days in a 5% CO_2 atmosphere. Microscopic examination was performed and observations were recorded every 24 hours.

After 72 hours, the supernatant was collected from individual wells, centrifuged at 2000 rpm for 10 minutes and transferred into clean vials. From each sample, 100 µl was transferred to a fresh, clean 96-well plate. Then, 100 µl of the reaction mixture from the kit was added and the plate was incubated at room temperature for 30 minutes in the dark. The absorbance was measured using a microplate reader at a wavelength of 490 nm. The percentage activity was calculated relative to the untreated control samples.

Determination of the effect of extract on Caspase-3: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS. To each well of a 6-well plate, 2 ml of the diluted cell suspension was added. After 24 hours, when a partial monolayer was formed, the supernatant was removed and the monolayer was washed once with medium. Different extract concentrations prepared in DMEM were added to the cells. The plates were incubated at 37°C for 3 days in a 5% CO_2 atmosphere. Microscopic examination was conducted and observations were recorded every 24 hours. After 72 hours, the cells were scraped and centrifuged at 2000 rpm for 10 minutes to separate the pellet. The pellet was re-suspended in 50 µl of chilled cell lysis buffer and incubated on ice for 10 minutes.

The mixture was centrifuged at 15,000 rpm for 1 minute and the supernatant was transferred to a fresh tube. The vial was maintained on ice and the protein concentration of the sample was measured using the Bradford assay. The samples were diluted to a concentration of 4 mg/ml. A volume of 50 µl of the sample was mixed with 50 µl of 10 mM DTT followed by 5 µl of 4 mM DEVD-p-nitroanilide and incubated at 37°C for 120 minutes. The absorbance was measured using a microplate reader at a wavelength of 405 nm.

Reverse Transcriptase PCR Procedure: The mRNA expression levels of p53 were analysed using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the MCF-7 cells were cultured in 60 mm Petri dishes and maintained in DMEM medium for 48 hours. The DMEM medium was supplemented with FBS and amphotericin. The required concentrations of the test sample (200 µg/ml and 300 µg/ml) were added and the cells were incubated for 48 hours. Total cellular RNA was isolated from the untreated (control) and treated cells using the Trizol reagent, following the manufacturer's protocol.

cDNA was synthesized from the total isolated RNA using a reverse transcriptase kit, following the manufacturer's instructions (Thermo Scientific). A reaction mixture of 20 µl was subjected to PCR for amplification of p53 cDNA using specifically designed primers procured from Eurofins India. As an internal control, the housekeeping gene GAPDH was co-amplified with each reaction.

The amplification conditions for the p53 gene involve an initial denaturation at 95°C for 5 minutes. This is followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 1 minute. A final extension is performed at 72°C for 10 minutes. An oligo dT primer was used for first-strand synthesis. Forward primer 5' CTGAGGTTGGCTC TGACTGTACCACCATCC 3' and reverse primer 5' CTCATTCAGCTCTCGGAACATCTCGAAGCG 3' were used for second-strand synthesis. The product size was 370 base pairs.

Results

***In vitro* cytotoxicity assay of ethanol, chloroform and petroleum ether extracts of *V. zizanioides in* MCF-7cell lines:** Table 1 presents the percentage of cell inhibition achieved by plant extracts obtained from roots of *V. zizanioides* using different solvents: ethanol, chloroform and petroleum ether, at varying concentrations (Figures 2 to 4). The plant extract obtained using ethanol demonstrated the highest potency, achieving complete cell inhibition (100%) at 150 µg/ml, with an IC50 value of 44.6 µg/ml, indicating its strong bioactivity. The chloroform-based extract showed moderate effectiveness, reaching 100% inhibition at 300 µg/ml with an IC50 value of 56.54 µg/ml, suggesting that it requires a slightly higher concentration to achieve similar effects as the ethanol extract.

On the other hand, the petroleum ether extract was the least effective, achieving 84.99% inhibition at the maximum tested concentration (300 µg/ml) and an IC50 value of 99.63 µg/ml, significantly higher than the other two extracts. These

findings indicate that the plant extract obtained with ethanol is the most effective in inducing cell inhibition followed by the chloroform extract, while the petroleum ether extract demonstrates comparatively weaker bioactivity.

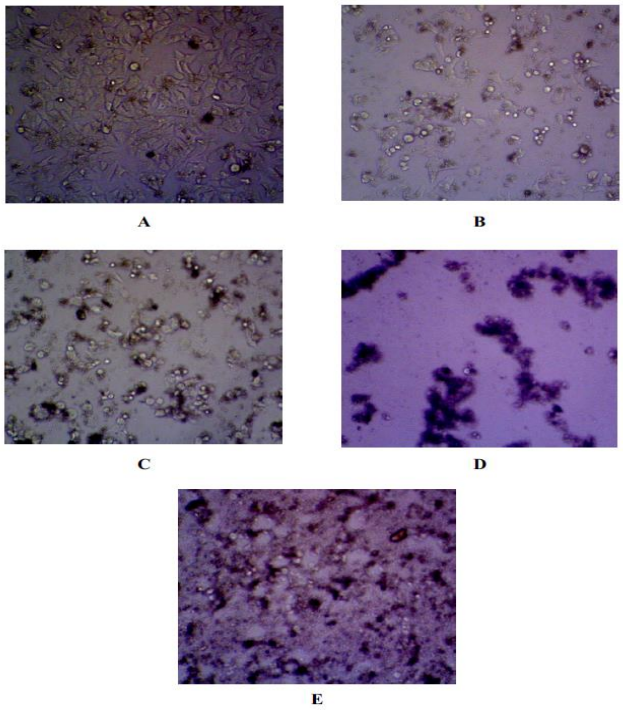

Fig. 2: Photographic documentation of the cytotoxic effects of the crude ethanol extract of *Vetiveria zizanioides* root on the MCF-7 human breast cancer cell line. The images correspond to different concentrations of the extract: A - 18.75 µg/ml, B - 37.5 µg/ml, C - 75 µg/ml, D - 150 µg/ml, E - 300 µg/ml.

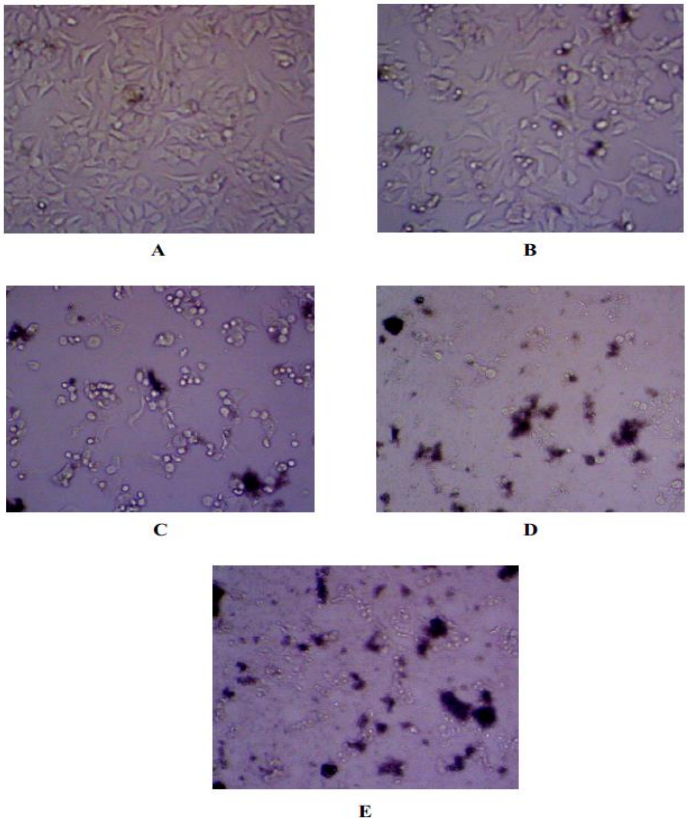

Fig. 3: Photographic documentation of the cytotoxic effects of the crude chloroform extract of *Vetiveria zizanioides* root on the MCF-7 human breast cancer cell line. The images correspond to different concentrations of the extract: A - 18.75 µg/ml, B - 37.5 µg/ml, C - 75 µg/ml, D - 150 µg/ml, E - 300 µg/ml.

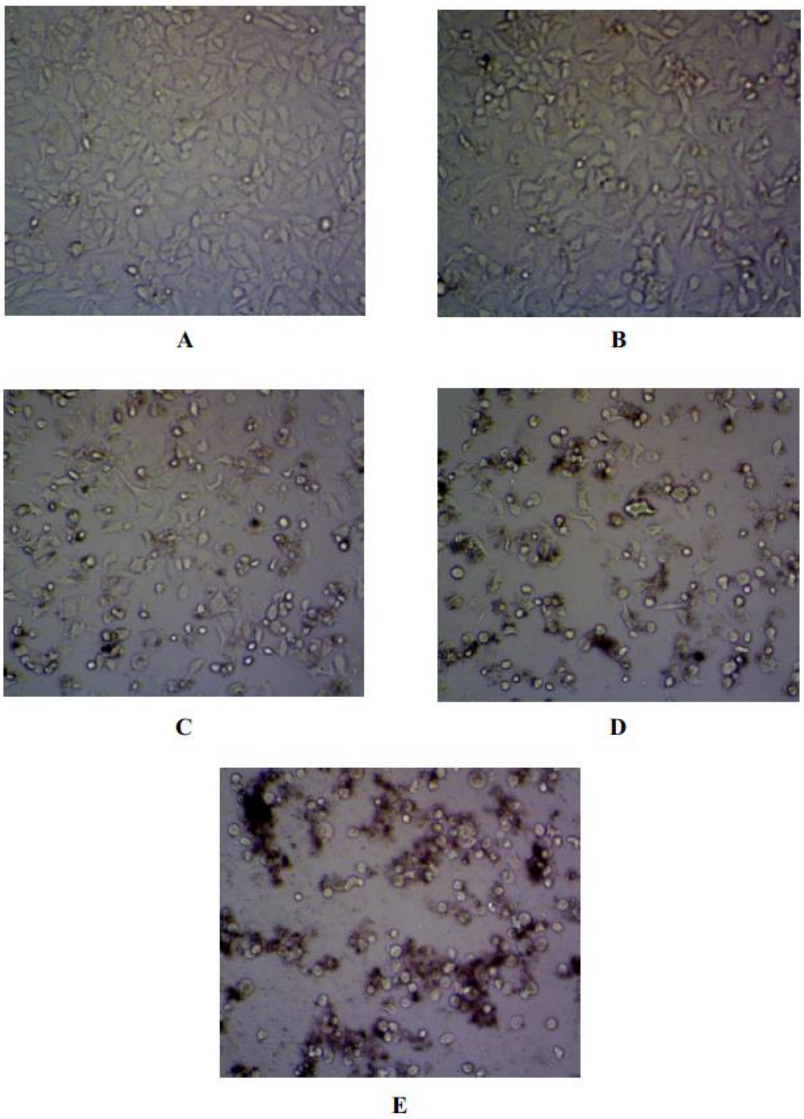

Fig. 4: Photographic documentation of the cytotoxic effects of the crude petroleum ether extract of *Vetiveria zizanioides* root on the MCF-7 human breast cancer cell line. The images correspond to different concentrations of the extract: A - 18.75 µg/ml, B - 37.5 µg/ml, C - 75 µg/ml, D - 150 µg/ml, E - 300 µg/ml

Thin Layer Chromatography analysis for ethanol extract: TLC separation of the ethanol extract was conducted using silica gel 60 F254 pre-coated TLC plates. The chromatogram for the ethanol extract was developed with three different mobile phases: chloroform-toluene-methanol (6:2.5:1.5), toluene-ethyl acetate (9.3:0.7) and benzene-ethyl acetate (1:1). The ethanol extract was applied in five different concentrations (2, 4, 6, 8 and 10 µL) for each mobile phase. Spots were visualized using a UV chamber and images were captured under UV light at 254 nm and 366 nm (Fig. 5). Among the mobile phases, the highest resolution was achieved with benzene-ethyl acetate (1:1), yielding Rf values of 0.27, 0.46, 0.67, 0.81 and 0.97 (Fig. 5).

Preparative Thin Layer Chromatography analysis for ethanol extract: PTLC plates were used to isolate compounds from the ethanol extract to collect the five fractions (0.27, 0.46, 0.67, 0.81 and 0.97) by using the same mobile benzene-ethyl acetate (1:1). Sample (6 µl) was used to loaded in the PTLC plate with the help of using Hamilton Syringe and Camag Linomat 5 instrument.

***In vitro* cytotoxicity studies for Ethanol fractions:** The cytotoxicity study was carried out for five fractions. The five fractions were screened for its cytotoxicity against MCF-7 cell line to determine the IC50 (Inhibition of concentration) by MTT assay. It was found that the inhibition of concentration showed in second fraction (29.1µg/ml) as in fig. 6. The results are tabulated in table 2.

Table 2 outlines the results of a cytotoxicity assay performed on MCF-7 cell lines for five different fractions obtained in ethanol extract at varying concentrations (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL and 100 µg/mL). The table shows the percentage of cell inhibition for each fraction achieved at these different concentrations.

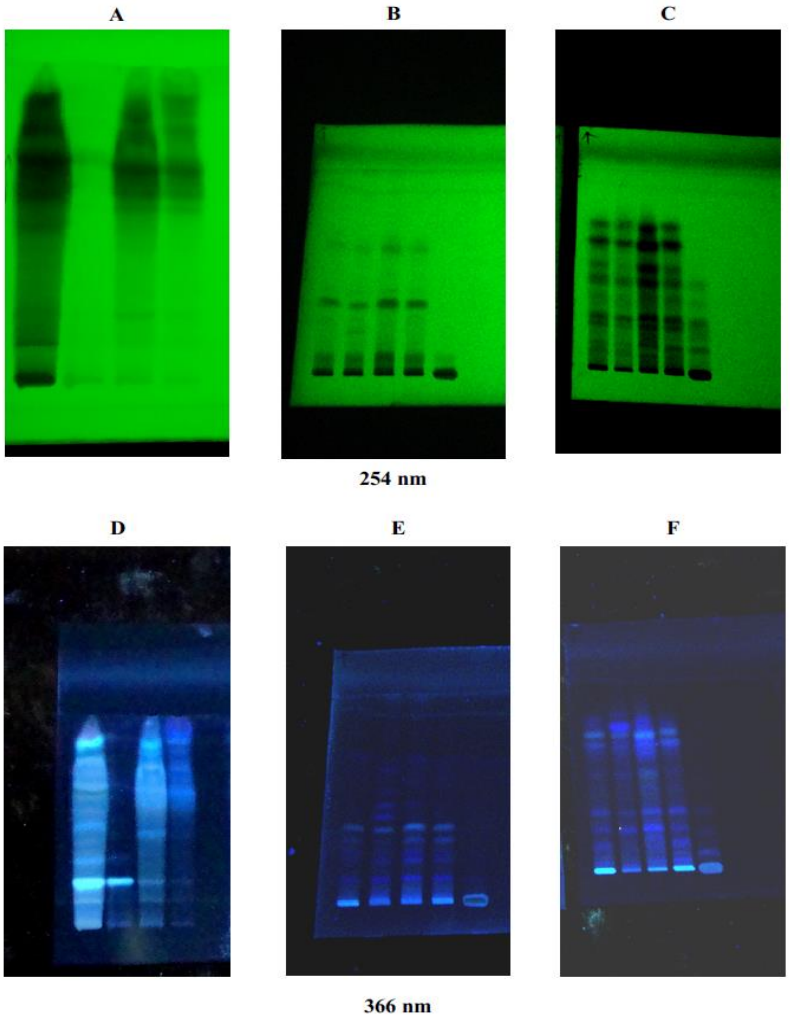

Fig. 5: TLC Chromatogram of Crude Ethanol Extract of *Vetiveria zizanioides* root
The TLC chromatogram of the crude ethanol extract of *Vetiveria zizanioides* root is displayed in six panels. Panels A and D illustrate the chromatogram using a mobile phase composition of chloroform:toluene:methanol in the ratio 6:2.5:1.5. Panels B and E represent the chromatogram with a mobile phase of toluene:ethyl acetate in the ratio 9.3:0.7. Panels C and F depict the chromatogram with a mobile phase comprising benzene:ethyl acetate in a 1:1 ratio. The ethanol extract was applied at varying concentrations on the tracks as follows: Track 1 - 2 µl, Track 2 - 4 µl, Track 3 - 6 µl, Track 4 - 8 µl and Track 5 - 10 µl.

Table 1
Cytotoxicity assay of ethanol, chloroform and petroleum ether extracts of *V. zizanioides in* MCF-7cell lines

Concentration (µg/ml)	Ethanol (% Cell Inhibition)	Chloroform (% Cell Inhibition)	Petroleum Ether (% Cell Inhibition)
18.75	15.22	0.38	1.60
37.5	41.85	31.13	9.18
75	70.11	61.97	39.58
150	100	98.56	69.98
300	100	100	84.99
IC50 (µg/ml)	44.6	56.54	99.63

The key findings revealed distinct inhibitory effects for the five fractions analyzed. The first fraction showed a progressive increase in cell inhibition with increasing concentration, achieving nearly complete inhibition (99.63%) at 100 µg/mL and an IC_{50} value of 36.08 µg/mL. The second fraction demonstrated a gradual rise in inhibition, with 83.01% inhibition at 50 µg/mL and 100% inhibition at 100 µg/mL, yielding the lowest IC_{50} value of 29.14 µg/mL. In contrast, the third fraction exhibited lower inhibition compared to the first two, peaking at 70.41% inhibition at 100 µg/mL and an IC_{50} value of 72.29 µg/mL. The fourth fraction displayed moderate inhibition, with a peak of 93.02% at the highest concentration of 100 µg/mL and an IC_{50} value of 63.14 µg/mL.

Finally, the fifth fraction demonstrated a strong inhibitory effect, achieving 96.65% inhibition at 100 µg/mL and the highest IC_{50} value among the fractions at 96.55 µg/mL. This assay reveals that the 1st and 2nd fractions are the most potent against MCF-7 cell lines, with low IC_{50} values indicating high cytotoxic activity. The 5th fraction, while achieving significant inhibition at higher concentrations, has the least potent IC_{50} among the tested fractions. The data indicates varying levels of cytotoxicity among the fractions, reflecting their potential for further investigation in anti-cancer research.

Table 2
Cytotoxicity assay for different ethanol fractions in MCF-7 cell lines at different concentrations

Concentration (µg/ml)	% Cell Inhibition (1st Fraction)	% Cell Inhibition (2nd Fraction)	% Cell Inhibition (3rd Fraction)	% Cell Inhibition (4th Fraction)	% Cell Inhibition (5th Fraction)
6.25	0.64	3.21	0.27	1.01	2.73
12.5	8.41	10.46	1.56	3.67	10.93
25	33.60	37.83	6.97	11.11	32.91
50	61.34	83.01	25.61	20.20	75.62
100	99.63	100	70.43	93.02	96.46
IC50 (µg/ml)	**36.08 µg/ml**	**29.1 µg/ml**	**72.99 µg/ml**	**63.14 µg/ml**	**96.55 µg/ml**

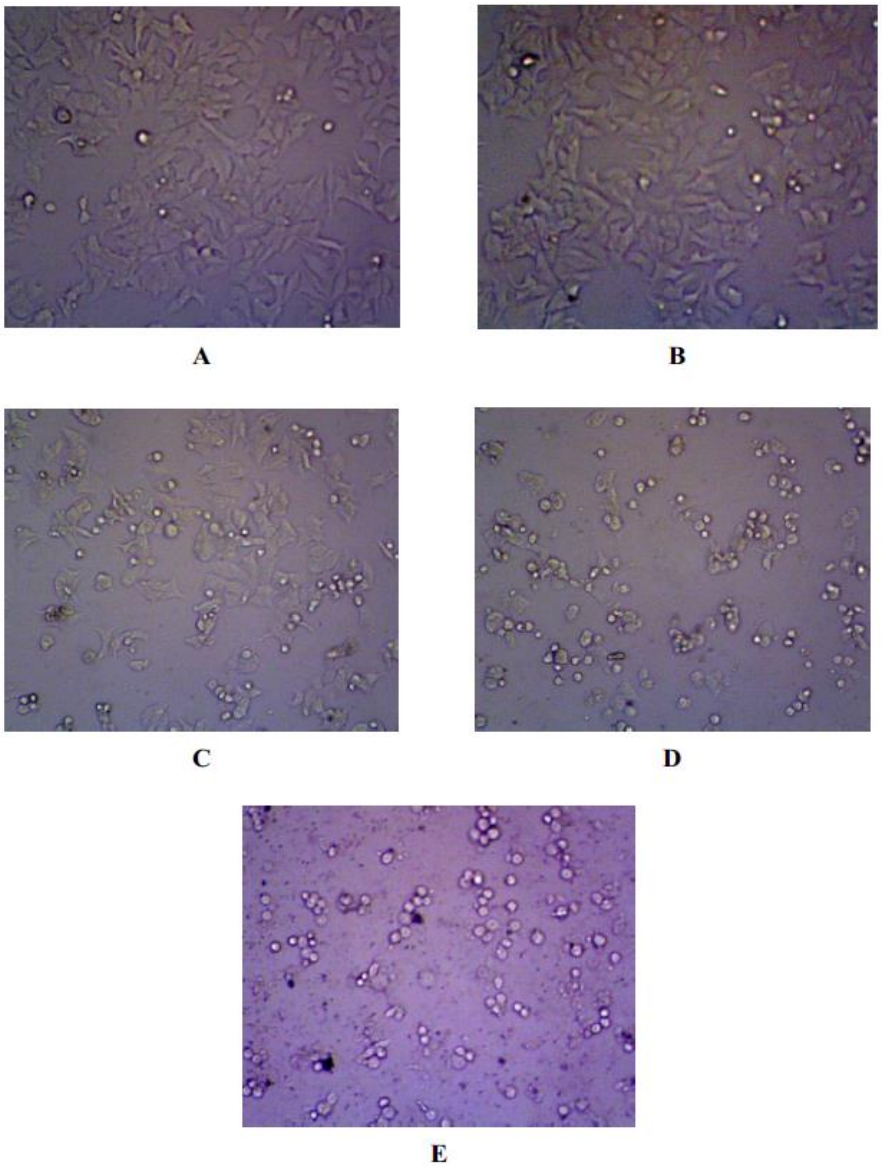

Fig. 6: Photographic Documentation of Cytotoxic Effects of the Ethanolic Second Fraction of *Vetiveria zizanioides* on MCF-7 Human Breast Cancer Cell Line. The images correspond to different concentrations of the extract: A - 6.25 µg/ml, B - 12.5 µg/ml, C - 25 µg/ml, D - 50 µg/ml, E - 100 µg/ml.

Spectral Analysis: The second fraction was subjected to spectral analysis techniques including UV, IR, NMR and LC-MS, to identify and confirm the unknown compounds present in the fraction.

UV-Visible Spectral Analysis: The UV-Visible spectrum is presented in fig. 7. The maximum absorbance was observed within the range of 200–400 nm.

IR Spectral Analysis: The IR spectra (in cm^{-1}) of the basic nucleus structure revealed the presence of an OH stretch (hydrogen bonded) at 3428.50 cm^{-1}, CH stretch (aliphatic ring) and a ketone group at 1665 cm^{-1}. Aromatic C=C was observed at 1459 cm^{-1}, CO stretch at 1267 cm^{-1} and 1036 cm^{-1}, with an aromatic ring signal at 604 cm^{-1}. Additional signals included a C–O stretch at 1126 cm^{-1} and a CH_3 bond at 1383 cm^{-1} (Fig. 8) for the second fraction.

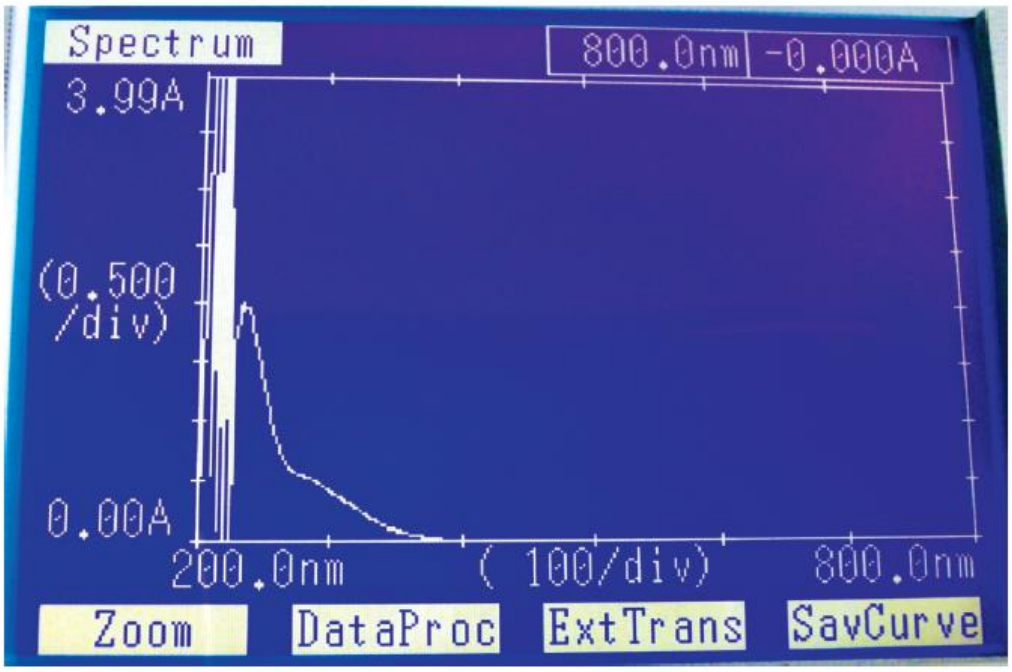

Fig. 7: UV-Visible spectral analysis of the ethanolic second fraction derived from the roots of *Vetiveria zizanioides*.

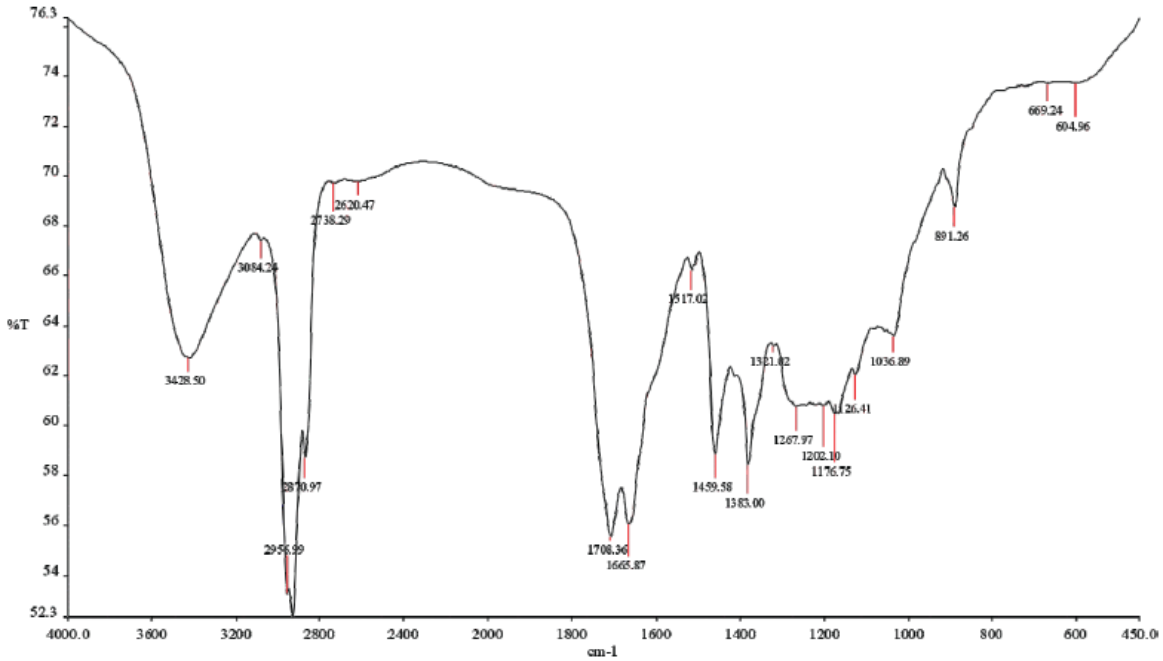

Fig. 8: FTIR analysis of the ethanolic second fraction derived from the roots of *Vetiveria zizanioides*, conducted using a Spectrum 100 spectrometer (Perkin Elmer, USA) with a scanning range of 4000–400 cm^{-1}, a resolution of 4 cm^{-1} and an average of 25 scans.

NMR Spectral Analysis

^{1}H NMR ($CDCl_3$): The ^{1}H NMR spectrum showed an aromatic CH signal at δ 7.4 and signals at δ 4.52 and 4.8 indicated the R-C=CH of the aromatic ring. Methoxy group presence was confirmed by signals at δ 3.9, 4.5–4.9 and 2.32–2.7. Signals at δ 1.2–1.8 represented the presence of a long-chain aliphatic ring. A hydroxyl (O-H) signal was observed at δ 9.8 (Fig. 9) for the second fraction.

^{13}C NMR ($CDCl_3$): Aromatic carbon signals appeared at δ 119, 121, 122, 153 and 154, while aliphatic carbons showed signals in the range of δ 10–35. A C=C bond was observed at δ 103 (Fig. 10) for the second fraction.

LC-MS Spectral Analysis: The LC-MS analysis identified a group of compounds in the second fraction of *V. zizanioides* with flavonoid nuclei including Hydroquinidine, 3',4',7-Trimethylquercetin, Luteolin 3'-sulfate, Kaempferol, Acacetin, Delphinidin-3-O-beta-glucopyranoside, 2',6-Dihydroxy flavanone, Quercetin 3-O-Methyl Ether Peracetate, 4,6-Dimethyl-3-(4'-hydroxyphenyl) coumarin, 3-Hydroxy-3'-methoxyflavone, Peoridin-3-O-beta-D-glucoside, Kaempferol-3-O-rutinoside, Iridin, Myricetin-3-Galactoside and Luteolin 7-(6'''-acetylallosyl-(1→2)-glucoside).

DNA Fragmentation Analysis: A genomic DNA fragmentation assay was conducted on *V. zizanioides* second fraction-treated MCF cells (500 and 750 µg/ml) to analyse apoptosis. DNA electrophoresis revealed that the tested extract induced DNA fragmentation in a concentration dependant manner (Fig. 11).

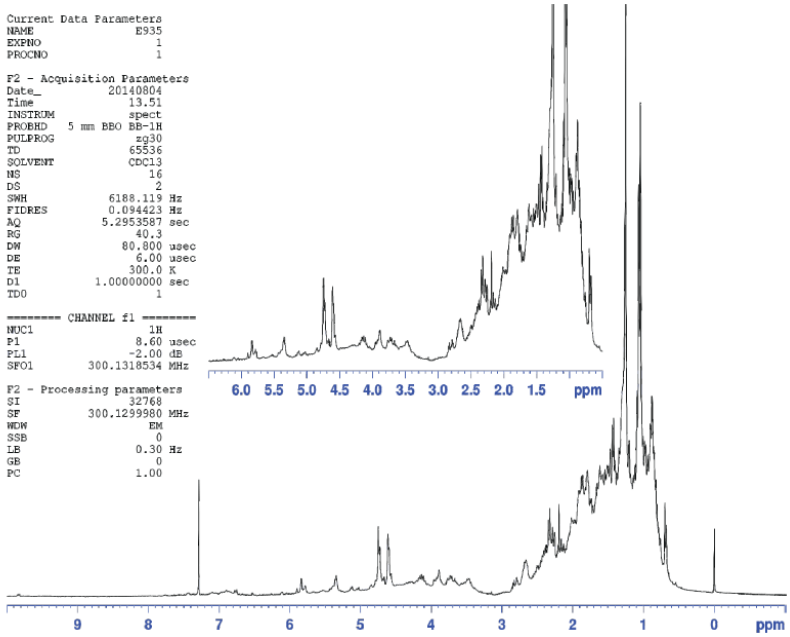

Fig. 9: ^{1}H NMR analysis of the ethanolic second fraction derived from the roots of *Vetiveria zizanioides*.

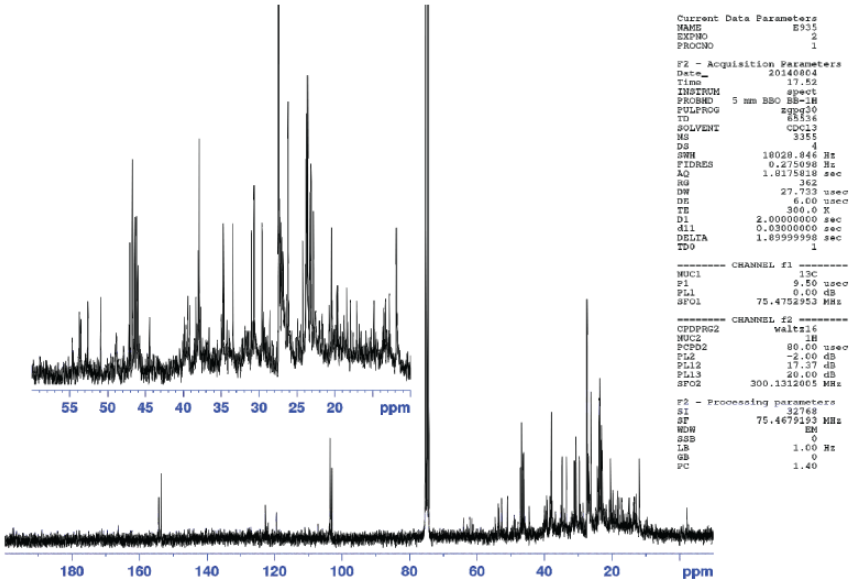

Fig. 10: ^{13}C NMR analysis of the ethanolic second fraction derived from the roots of *Vetiveria zizanioides*.

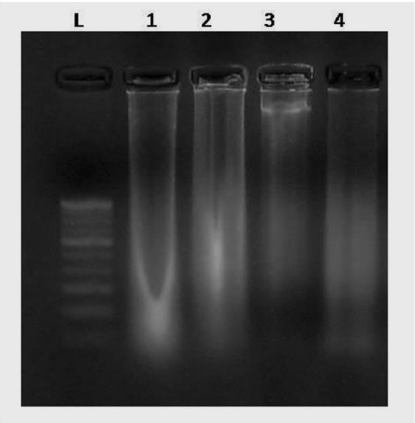

Fig. 11: DNA fragmentation analysis of MCF-7 cells treated with the ethanolic second fraction of *Vetiveria zizanioides*. The DNA ladder (L) serves as a molecular size marker, while lane 1 corresponds to cells treated with 750 µg/ml of the ethanolic second fraction and lane 2 represents cells treated with 500 µg/ml of the same fraction. Lane 3 shows the DNA profile of the untreated control cells and lane 4 represents the standard treatment with 5 µg/ml. This analysis highlights the effects of the ethanolic fraction on DNA integrity in MCF-7 cells.

Table 3
Effect of extract in MCF-7 cell line by LDH (Lactate dehydrogenase assay)

Ethanol fraction	Concentration (µg/ml)	LDH Level (IU/ml)
Fraction 2	1000	92.97
	500	64.50
	250	40.52
	125	21.65
	62.5	7.29
	Control	12.50

Table 4
Effect of extract on Capsase-3 in MCF-7 cell line

S.N.	Test Drug	Test Concentration (µg/ml)	Caspase-3 Activity
1	Fraction 2	1000	1.30
2		500	0.95
3	Camptothecin	5	3.15
4	Control	-	0.68

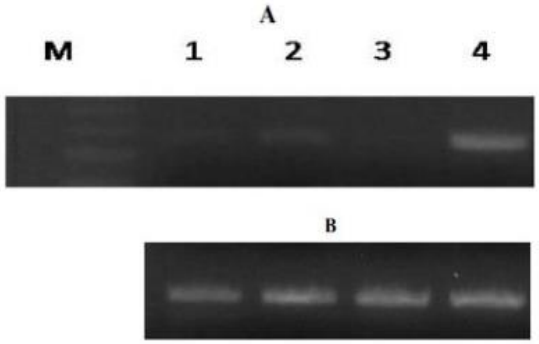

Fig. 12: Reverse Transcriptase PCR analysis of the p53 gene in MCF-7 cells treated with the ethanolic second fraction of *Vetiveria zizanioides*.
In panel A, lane M represents the marker, lane 1 shows the cells treated with the ethanolic second fraction at a concentration of 200 µg/ml, lane 2 represents cells treated with 300 µg/ml of the same fraction, lane 3 corresponds to the untreated control cells and lane 4 depicts cells treated with doxorubicin at a concentration of 1.5 µg/ml. Panel B presents a similar arrangement: lane 1 shows cells treated with the ethanolic second fraction at 200 µg/ml, lane 2 at 300 µg/ml, lane 3 represents untreated control cells and lane 4 represents cells treated with 1.5 µg/ml of doxorubicin.

LDH activity: Table 3 shows the effect of the ethanol extract (Fraction 2) on MCF-7 cells using the lactate dehydrogenase (LDH) assay. The table demonstrates the cytotoxic effect of ethanolic fraction 2 on MCF-7 cells, as indicated by LDH release, which is a marker of cell membrane damage and cytotoxicity. The LDH levels increase with the concentration of fraction 2, suggesting a dose-dependent increase in cytotoxicity. At the highest concentration (1000 µg/ml), the LDH level is 92.97 IU/ml, indicating significant cell damage.

Lower concentrations show reduced LDH levels with the lowest concentration (62.5 µg/ml) yielding only 7.29 IU/ml of LDH release. The control group shows an LDH level of 12.50 IU/ml, indicating minimal natural cell damage. These results suggest that fraction 2 exhibits a potent cytotoxic effect on MCF-7 cells, particularly at higher concentrations.

Effect of ethanolic fraction 2 on Caspsase-3 activity: Table 4 presents the effect of ethanolic fraction 2 and a standard drug, camptothecin, on Caspase-3 activity in MCF-7 cells. Caspase-3 is a key enzyme involved in apoptosis (programmed cell death) and its activity indicates the induction of apoptosis.

Fraction 2 shows a concentration-dependent effect, with Caspase-3 activity increasing from 0.95 at 500 µg/ml to 1.30 at 1000 µg/ml. This suggests that fraction 2 induces apoptosis in MCF-7 cells, though less effectively than camptothecin. Camptothecin, used as a standard apoptosis-inducing agent, exhibits a significantly higher Caspase-3 activity (3.15) at a much lower concentration (5 µg/ml), indicating its potent apoptotic effect. The control group has the lowest Caspase-3 activity (0.68), representing the baseline activity in untreated cells. Overall, the results indicate that fraction 2 induces apoptosis in MCF-7 cells, its efficacy, at the given concentration, is lower compared to camptothecin.

Reverse Transcriptase PCR analysis: The results of this study indicate a upregulation of the p53 gene by fraction 2 (Fig. 12). Both the test sample (treated) and the standard (Doxorubicin) were compared to the control. Compared to the control (lane 3, fig. 12), the test samples exhibited a 1.20- and 1.21-fold upregulation at 200 and 300 µg/ml respectively. Meanwhile, the standard showed a 2.58-fold upregulation relative to the control (lane 4, fig. 12).

In summary, this study demonstrates that fraction 2, derived from the ethanolic extract of *Vetiveria zizanioides*, effectively inhibited cancer cell proliferation. The cytotoxic activity of fraction 2 is likely attributable to its iron-chelating properties, which may induce apoptosis through p53 and caspase-dependent pathways, ultimately leading to genomic DNA fragmentation.

Based on these findings, compounds isolated from fraction 2 of the ethanolic extract of *V. zizanioides* hold promise as potential agents for cancer chemoprevention or chemotherapy. Nevertheless, further research is needed to unravel the exact molecular mechanisms underlying the anticancer effects of these bioactive compounds.

Discussion

For centuries, plants have played a significant role in traditional medicine for treating various diseases. In India, breast cancer ranks as the second most prevalent cancer after cervical cancer, with approximately 115,251 new cases diagnosed and 53,592 deaths recorded in 2008[9]. Projections by the Indian Council of Medical Research indicated a rise in breast cancer cases to 106,124 by 2015 and 123,634 by 2020. Medicinal plants have gained considerable scientific interest as complementary and alternative treatments[16]. Plant-derived chemotherapeutic agents like vinblastine, taxol, camptothecin and podophyllotoxin are integral to cancer management[20]. Modern analytical techniques have enabled the isolation of novel natural compounds from plants through fractionation and purification.

These efforts primarily aim to identify bioactive agents for direct use as drugs or as lead compounds for developing semisynthetic drugs. However, systematic screening has only been conducted for 5-15% of the approximately 250,000 higher plant species[6,7]. To explore novel therapeutic strategies, cell lines are widely used to evaluate the effects of new compounds on cancer cells. The current study aims to investigate the anticancer potential of ethanolic fractions of *V. zizanioides* roots in human breast cancer (MCF-7) cell lines.

The preliminary *in vitro* screening of *V. zizanioides* root extracts in ethanol, chloroform and petroleum ether against MCF-7 cells revealed IC50 values that highlight the ethanolic extract's superior activity. Similar findings were observed in previous studies where a dose-dependent activity was reported for crude *Morinda lucida* extracts against MCF-7 cells. While the crude form demonstrated activity, a clear dose-dependent response was noted. This observation aligns with results from other studies showing increased activity with higher concentrations[2,13,19]. Among the tested extracts, the ethanolic extract exhibited greater cytotoxicity than the chloroform and petroleum ether extracts, warranting further investigation into its potential.

The present study confirms that the crude ethanolic extract of *V. zizanioides* demonstrated superior cytotoxicity (44.6 µg/ml) compared to chloroform (56.54 µg/ml) and petroleum ether extracts (99.63 µg/ml) in MCF-7 cell lines. Cytotoxicity assays serve as critical preliminary tools for identifying plant extracts with potential anticancer properties[5]. If these effects are replicated *in vivo*, it supports the traditional use of these plants in cancer treatments.

Baskar and coworkers[4] have conducted similar work, utilizing thin layer chromatography (TLC) to analyze and standardize plant extracts. TLC, as a globally accepted

method, enables efficient screening and quality evaluation of herbal products.

In this study, the crude ethanol extract was separated using TLC with benzene and ethyl acetate (1:1) as the mobile phase. Under UV light at 254 and 365 nm, over five distinct spots were identified. Subsequent cytotoxicity testing using MTT assays on these fractions revealed that the second fraction exhibited the highest activity. This fraction underwent further purification via PTLC and spectral analysis (UV, IR, NMR, LC/MS) to identify its compounds.

Spectral analyses revealed that the second fraction contained flavonoids, with UV absorption maxima at 200 nm and IR spectra confirming functional groups like OH stretching (3428.50 cm^{-1}). The NMR data supported the presence of aromatic and aliphatic rings, along with functional groups such as methoxy and hydroxyl groups. LC/MS identified 15 compounds, including Hydroquinidine, Trimethylquercetin, Luteolin-3'-sulfate, Kaempferol and Myricetin-3-Galactoside, among others. These results confirmed flavonoids as the primary bioactive components. Similar observations were made for the presence of the flavonoids by Wenying and co workers[25].

The study also assessed LDH release as an indicator of cytotoxicity and apoptosis, suggesting that the second fraction induced cell death through membrane damage, likely due to its lipophilic compounds. It has been demonstrated that volatile plant-derived compounds are well-known inhibitors of cancer cell growth[14].

Furthermore, the study investigated caspase-3, a key enzyme in apoptosis. Results indicated that the second fraction upregulated caspase-3 levels by 1.20 and 1.21-fold at concentrations of 200 µg/ml and 300 µg/ml after 48 hours, respectively, consistent with findings by other researchers[21,26]. The ethanolic root extract of *V. zizanioides* inhibits MCF-7 cell growth through apoptosis induction. Further research is needed to develop therapeutic formulations based on these findings.

Conclusion

The findings of this study underscore the potential of the ethanolic root extract of *Vetiveria zizanioides* as a source of bioactive compounds with anti-cancer properties. The ethanol extract demonstrated significant cytotoxicity against MCF-7 breast cancer cells, with its second fraction exhibiting the most potent activity. The presence of flavonoids, as identified through spectral analyses, appears to contribute to the observed effects, which include apoptosis induction via caspase-3 activation and p53 upregulation.

This research provides a strong foundation for developing plant-based chemotherapeutic agents, highlighting the need for further *in vivo* studies and clinical trials to validate the therapeutic potential of *V. zizanioides*. These results also reinforce the importance of exploring traditional medicinal plants as valuable resources for modern oncology.

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