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In Vitro Study on Cytotoxic and Apoptotic Property of *Chrysopogon zizaniodes* Root and *Acorus calamus* Rhizome Extracts Using UMR-106 Bone Cancer Cell Line.

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Abstract

Over the last few years, researchers have made a lot of efforts to explore anticancer agents from medicinal plants. In the present study, the cytotoxic and apoptotic properties of Chrysopogon zizaniodes root and Acorus calamus rhizome extracts were evaluated using the MTT assay with UMR-106 bone cancer cell lines. The IC_{50} value of *C. zizaniodes* and *A. calamus* was 265.5 µg / ml and 308.7 µg / ml, respectively, on the UMR-106 bone cancer cell line, and that of standard doxorubicin was 19.79 µg/ml. As the IC_{50} value of *C. zizaniodes* was comparatively less than that of *A. calamus*, the apoptotic experiment was continued with *C. zizaniodes*. Treatment of the UMR-106 cell line with sample *C. zizanioides* at concentrations of 160 µg/ml and 320 µg/ml was known to induce apoptosis.

Keywords: Cytotoxic, Apoptotic, *Chrysopogon zizaniodes*, *Acorus calamus*, UMR-106.

1. Introduction

In spite of immense progress in the medical field, the incidence of the multifactorial disease cancer is increasing worldwide [1]. Along with genomic alterations, risk factors like smoking, sedentary lifestyle, overweight, industrialization, urbanization, hormonal variations, etc, are other leading causes of cancer, which constitute a huge burden on society [2, 3, 4].

Chemotherapy is the most often used mode of treatment for treating many types of cancers. Though it is effective, depending on the type of drug and dosage used, this treatment is associated with the risk of adverse side effects and harm to other healthy cells [5, 6]. Many chemotherapeutic drugs are known to induce oxidative stress [7]. Besides high cost, lack of

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selectivity, drug resistance and high toxicity are the major limitations of chemotherapeutics used for cancer treatment [8, 9]. Therefore, today, as the development of new drugs is challenging, many researchers are striving hard to find safe, affordable and effective anticancer compounds [10].

To overcome the side effects induced by chemotherapeutics, the use of bioactive compounds from natural resources has increased significantly. A Number of phytochemicals, the foundation of traditional medicine, are known to possess high potential to fight against cancerous cells [11, 12, 13]. Phenolics and terpenoids, a class of naturally occurring organic compounds from various medicinal plants, have pharmacological properties including cytotoxic effects [14, 15]. So many products derived from plants are in clinical trials for anticancer properties.

Chrysopogon zizanioides, an evergreen perennial grass, belongs to the family Poaceae [16]. It is commonly called vetiver, grows densely in varied soil types and climatic conditions and is distributed worldwide [17]. Various parts of *C. zizaniodes* have been used since ancient times for multiple medicinal properties. In Ayurveda, *C. zizaniodes* root is utilized for blood purification, to solve the problems related to digestion and respiration (asthma, cough), snake bite, pain relief, to reduce urinary tract infection, as a stronger anticancer agent [17], reduce fatigue and also to enhance the immunity [18], etc. Hence, *C. zizaniodes* is known to possess many useful medicinal benefits.

Acorus calamus belongs to the family Acoraceae and is a perennial, semi-evergreen plant native to central Asia, cultivated throughout India [19]. It has a long history of ethnomedical applications since ancient times. It is well known for its uses in the treatment and management of migraine, body pain, gastrointestinal disorders, diabetes, depression, skin diseases, etc [20, 10]. Although it has been successfully used in various formulations [21], there is still scope for fully exploiting the potential of this plant as a medicinal agent.

Although the studies have clearly demonstrated the broad pharmacological properties of C. zizaniodes and A. calamus, many of their uses require scientific validation [22]. Hence, to explore the antiproliferative compounds, current research was undertaken to compare cytotoxic properties and also assess the apoptotic properties of *C. zizaniodes* root and *A. calamus* rhizome extracts using the bone cancer cell line UMR-106.

2. Materials and Methods

2.1. Extraction of plant samples

Chrysopogon zizanioides (Vetiver) root and *Acorus calamus* (Vasambu) rhizome are the two samples used in the present research work. The plant samples were collected from Tamil Nadu, India, and identification was confirmed

by a botanical specialist. Using methanol as a solvent, the extracts of *C. zizanioides* root and *A. calamus* plant rhizome were prepared.

2.2. MTT assay to study the cytotoxic effects of plant extracts on UMR-106 bone cancer cell lines.

MTT assay measures the metabolic activity of viable cells via mitochondrial dehydrogenases. The vital ingredient of the principle is [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] or MTT. It is a water-soluble tetrazolium salt, which yields a yellowish solution when prepared in salt solutions without phenol red. Mitochondrial dehydrogenase enzymes of the living cells convert the dissolved MTT into an insoluble purple formazan [(2E, 4Z) – (4, 5-Dimethylthiazol-2-yl)-3, 5-diphenyl formazan] by cleavage of the tetrazolium ring. This formazan can be solubilised using Dimethyl Sulfoxide, acidified isopropanol or other solvents like pure propanol and spectrophotometrically, the resulting purple solution is measured. Any deviation in cell number results in a corresponding variation in the quantity of formazan formed, denoting the degree of effects induced by the test material.

2.2.1. Materials required

a. MTT

b. Dimethyl Sulfoxide

c. Carbon Dioxide incubator, Tecan plate reader

2.2.2. Test solution preparation

Standard solution was prepared using 10 mg Doxorubicin stock, and then serial two- fold dilutions were prepared from 100 µg to 3.125 µg by utilizing Dulbecco's Modified Eagle Medium (DMEM) plain media.

Preparation of the test sample

32 mg/ml stock for cytotoxicity studies was prepared using Dimethyl Sulfoxide. The two-fold serial dilutions were prepared from 3.2 mg/ml to 10 µg /ml using Dulbecco Modified Eagle Medium plain media for treatment.

2.2.3. Cell lines and culture medium

The cell lines required for the experiment were purchased from the American Type Culture Collection. The stock cells were cultured in Dulbecco Modified Eagle Medium supplemented with 10% inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO2 at 37 °C. The Viability of the cells was checked and centrifuged. Further 50,000 cells/well were seeded in a 96-well plate and incubated in 5 % CO_2 incubator at 37 °C for 42 hours.

Procedure

The cell culture suspension was centrifuged, and the cell count was adjusted to 5 x 10^5 cells/mL using the media containing 10% FBS. Added 100 µl dilute cell suspension (50000 cells/well) to all 96 wells of the microtiter plate. To the cell suspension in the microtiter plates, 100 µl of different concentrations of test drugs was added after 24 hours. Then the plates were incubated in 5% CO_2 atmosphere for 24 hours at 37°C. After incubation, 10 µl of MTT (5 mg/ml in PBS) was added to all wells, and then the plates were incubated in a 5% CO_2 atmosphere for 4 hours at 37°C. Now, collected the supernatant, centrifuged and added 100 µl DMSO to the pellet. Then the solution was transferred to the respective wells, and the plates were shaken gently to stabilize the formazan formed.

Using a microplate reader, absorbance was measured at 590 nm, and the percentage of growth inhibition was calculated using the following formula. From the dose-response curve, the amount of test drug required to inhibit cell growth by 50% (IC50 value) is determined.

2.2.4. Calculation of inhibition:

$$\% \text{ Inhibition} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \text{x } 100$$

2.2.5. Statistical Evaluation

Half maximal inhibitory concentration (IC_{50})

IC_{50} or half maximal inhibitory concentration is the measure of efficacy of the compound in inhibiting biochemical or biological function. This quantitative measure designates the quantity of inhibitor, drug or any other material required for the specific biological process by half.

By constructing the dose response curve and analyzing the consequences of various concentrations of antagonist on reversing agonist activity, the half maximal inhibitory concentration (IC_{50}) can be determined.

From the curve fit nonlinear regression analysis, based on a sigmoid dose variable response curve, computed using Graph Pad Prism 6 (San Diego, Graph Pad, CA, USA), the half maximal inhibitory values for cytotoxicity tests were derived.

2.3. Apoptosis studies by flow cytometry using the UMR-106 cell line treated with *C. zizanioides*

Using DMEM cell culture medium, for a 6-well plate, 1 x 10^6 cells per well were plated a day before induction of apoptosis. The dead floating cells in the well were separated after 18 hours by pipette, and new culture medium was added to restore the original volume. To induce apoptosis, the cells

were treated with 160 µg/ml and 320 µg/ml *C. zizanioides* and incubated at 37° C, 5% CO_2 for 24 hours. Later, the cell culture medium was collected in 15 ml tubes. The cells were separated from the well plates using a cell scraper, 1 ml of medium was added to each well, and the contents were transferred into ria vials. Centrifuged at 45000 rpm for 5 minutes at 4° C, and the supernatant was discarded. Twice, the cells were washed with cold PBS and resuspended at a concentration of 1 x 10^6 cells per ml in 1 ml 1X binding buffer. Aliquoted 500 µl cell suspension, 10 µl propidium iodide and 5 µl Annexin V were added. At room temperature, the suspension was incubated in the dark. After the incubation, as soon as possible, within one hour, the cells were analyzed using a flow cytometer.

3. Results and Discussion

3.1. MTT assay of UMR 106 bone cancer cell line to study the cytotoxic effects of medicinal plant extracts.

Samples *C. zizanioides* and *A. calamus* have shown 265.5 µg/ml and 308.7 µg/ml IC_{50} inhibition values, respectively, in the UMR 106 cancer cell line. IC_{50} value of the standard Doxorubicin was 19.79 µg in UMR 106 cells. Details of the MTT assay of doxorubicin, plant extracts *C. zizanioides* and *A. calamus* are given in Table 1, Figure 1. and Figure 2.

Table 1: Details of MTT assay of doxorubicin, plant extracts C. *zizanioides* and *A. calamus*

Compound name	Conc. µg	OD at 590 nm	% Inhibition	IC_{50}
Control	0	0.556	0	-
Doxorubicin	3.125	0.457	17.81	19.79
	6.25	0.41	26.26	
	12.5	0.321	42.27	
	25	0.256	53.96	
	50	0.167	69.96	
	100	0.108	80.58	
C. zizanioides	10	0.51	8.29	265.5
	20	0.444	20.22	
	40	0.409	26.51	
	80	0.362	34.98	
	160	0.325	41.55	
	320	0.19	65.89	
A. calamus	10	0.523	5.94	308.7
	20	0.51	8.27	
	40	0.475	14.57	
	80	0.431	22.48	
	160	0.351	36.87	
	320	0.261	53.06	

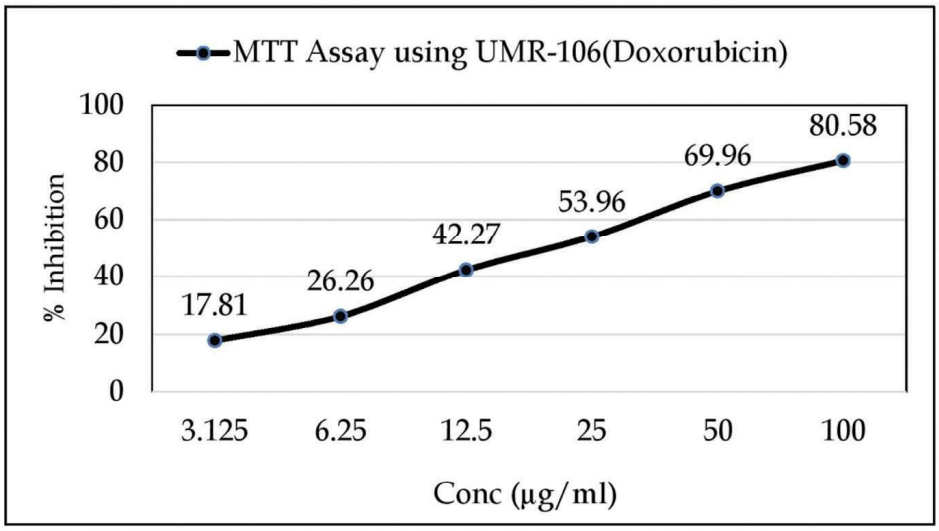


Figure 1: MTT assay of Doxorubicin using UMR-106 cells

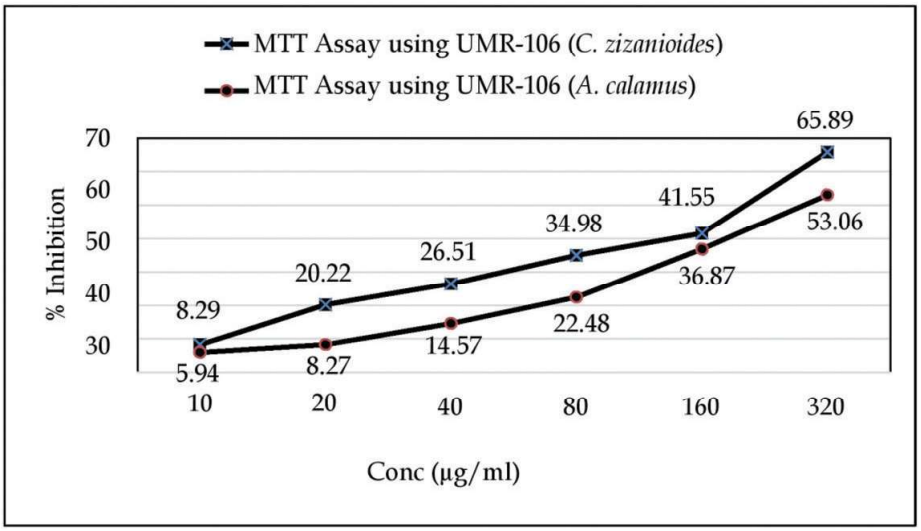


Figure 2: MTT assay of plant extracts C. zizanioides and A. calamus using UMR 106 cells

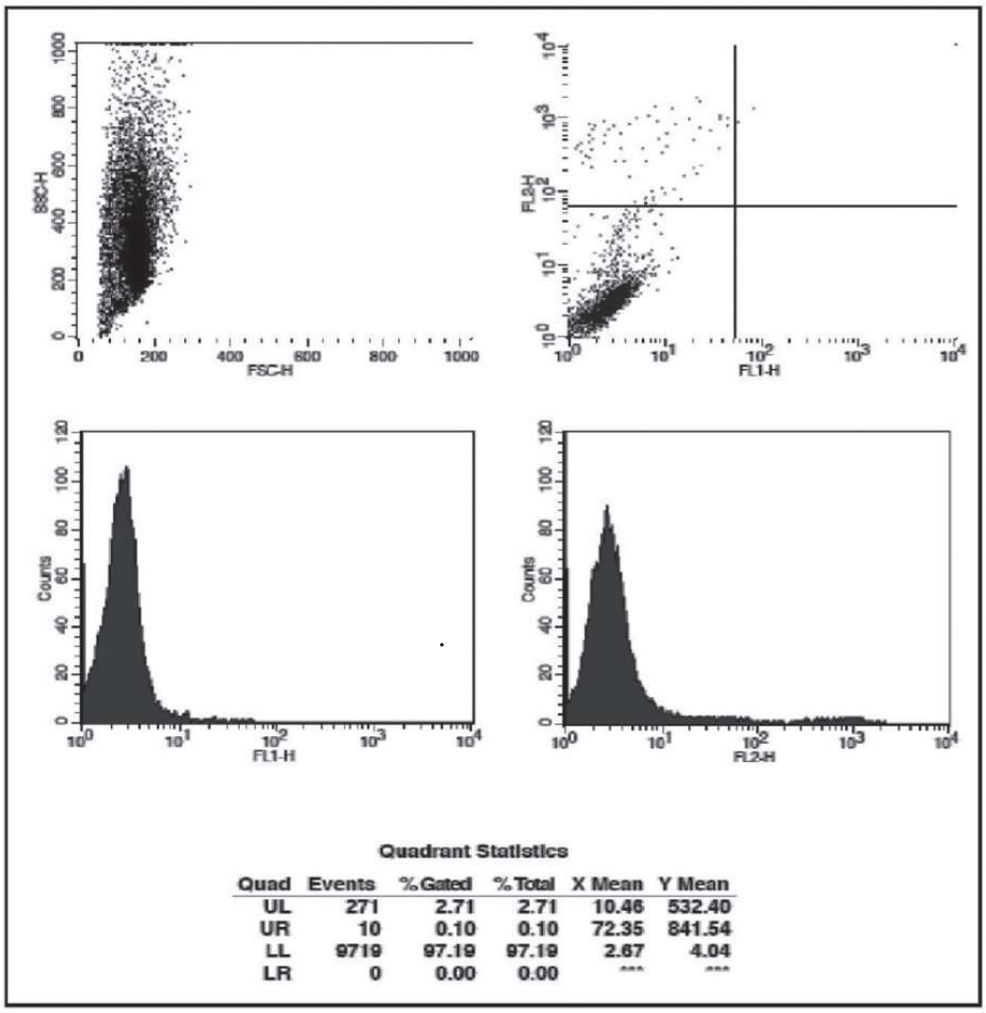
The results show that the cytotoxic value of *C. zizanioides* is lower than that of *A. calamus*. Hence, the apoptotic property of *C. zizanioides* was considered for further study.

3.2. Apoptosis study by flow cytometry using UMR-106 cell lines treated with *C. zizanioides*

From the studies carried out, it was seen that the treatment of sample *C. zizanioides* with 160 µg/ml and 320 µg/ml concentration was known to induce apoptosis after 24 hours of incubation in the UMR-106 cell line in a concentration-dependent manner. But in the untreated control, the amount

of early, late apoptosis and the necrosis reported was 0.0 %, 0.1 % and 2.71 % respectively (Figure. 3).

Concentration of 160 µg/ml showed an early and late apoptosis of 6.06% and 5.54 % respectively, in UMR-106 (Figure. 4). Concentration of 320 µg/ml showed an early and late apoptosis of 13.39% and 11.99% respectively, in UMR-106 (Figure. 5). The necrosis was found to be 7.60% and 6.81% at concentrations of 160µg/ml and 320µg/ml, respectively. The Standard Doxorubicin at 25 µM has shown 22.02% early, 16.09% late apoptosis and 6.82% necrosis in UMR-106 cells (Figure. 6). Histograms of the untreated UMR-106 cell line, cell line treated with 160 µg/ml, 320 µg/ml and standard Doxorubicin 25 µg/ml are given in Figure 3 to Figure 6, respectively. Details of flow cytometry analysis of apoptosis on UMR 106 cells are summarised in Table 2 and Figure 7.



Quadrant Statistics

Quad	Events	%Gated	%Total	X Mean	Y Mean
UL	271	2.71	2.71	10.46	532.40
UR	10	0.10	0.10	72.35	841.54
LL	9719	97.19	97.19	2.67	4.04
LR	0	0.00	0.00	***	***

Figure 3: UMR-106 untreated cells

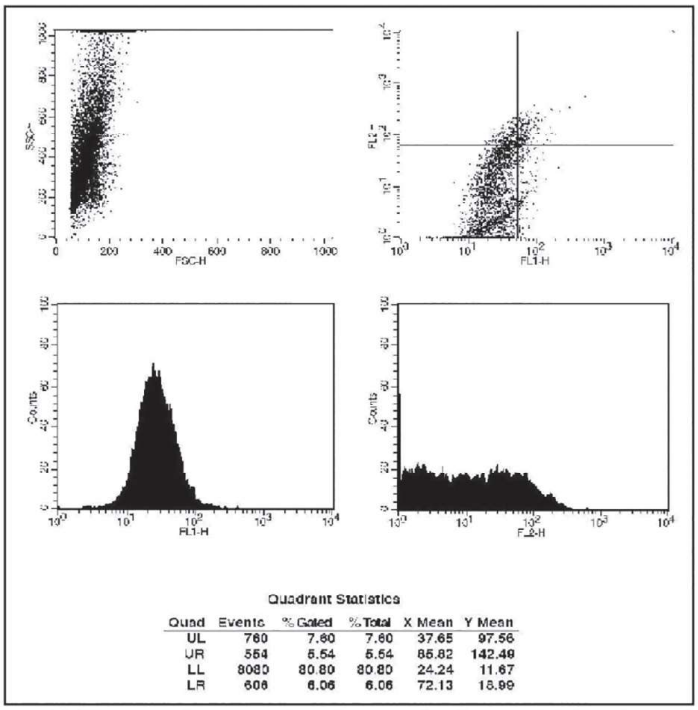


Figure 4: UMR 106 cells treated with sample C. *zizanioides* 160 µg/ml

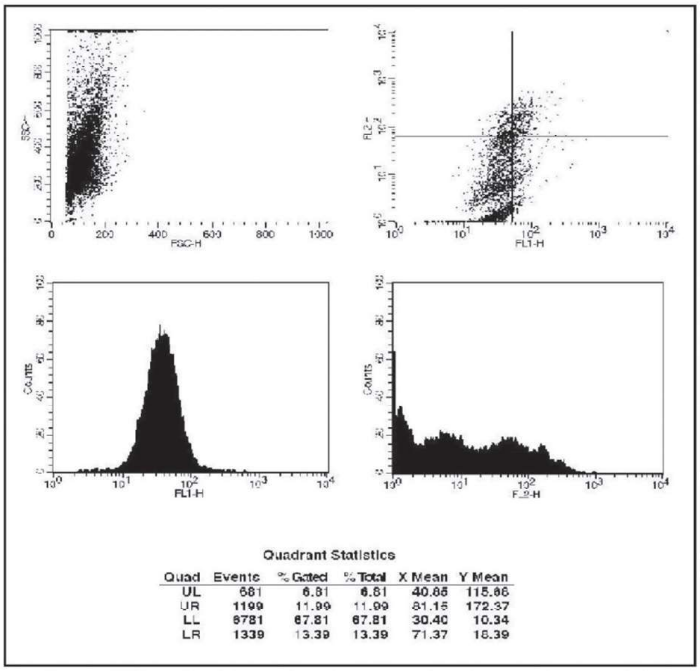


Figure 5: UMR 106 cells treated with Sample *C. zizanioides* 320 µg/ml

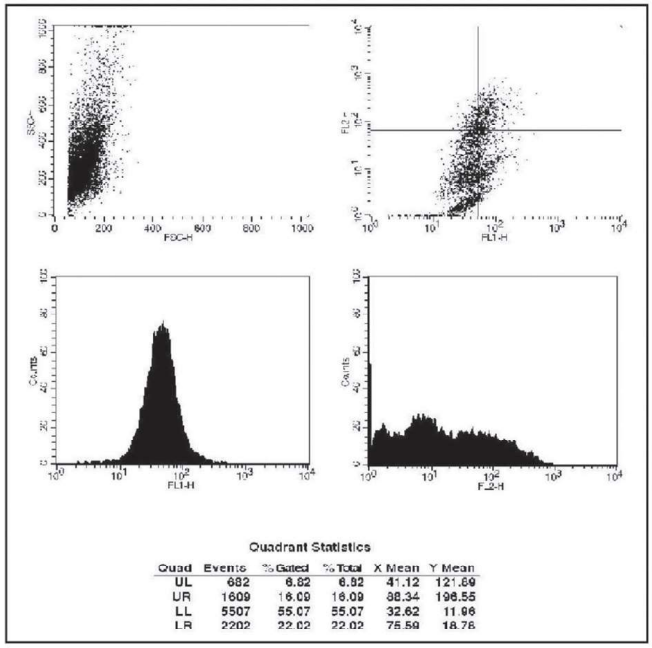


Figure 6: UMR 106 cells treated with standard Doxorubicin 25 µg/ml

Table 2: Flow cytometry analysis of apoptosis in UMR 106 cells

Sample in µg/ml	Viable cells %	Early apoptotic %	Late Apoptotic %	Necrotic cells %
Control	97.19	0.00	0.10	2.71
C. zizanioides 160	80.80	6.06	5.54	7.60
C. zizanioides 320	67.81	13.39	11.99	6.81
Doxorubicin 25	55.07	22.02	16.09	6.82

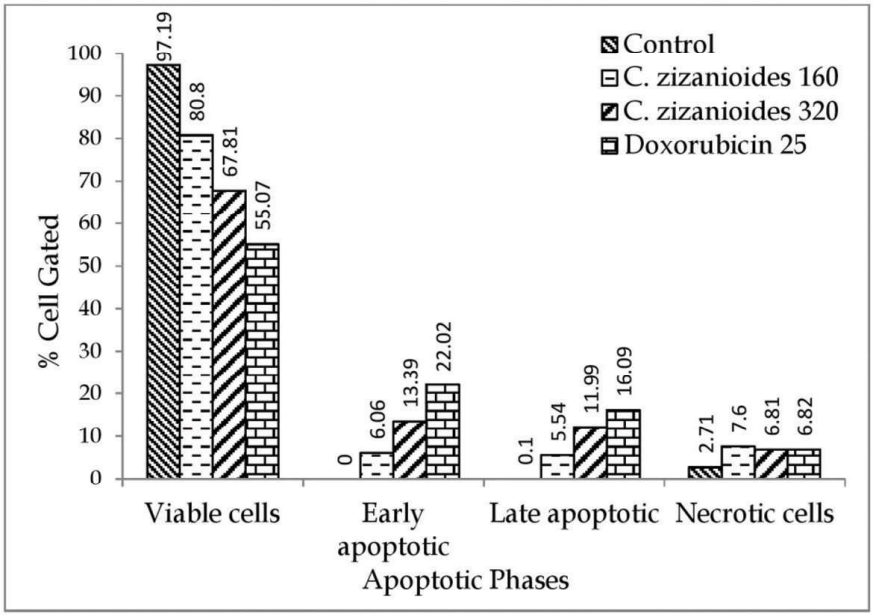


Figure 7: Flow cytometry analysis of apoptosis in UMR 106 cells

From the findings of the present study, it is clear that both *A. calamus* rhizome and *C. zizanioides* root extracts induce cytotoxic effects on UMR 106 bone cancer cell lines. However, since the cytotoxic value of C. zizanioides was lower than that of A. calamus, C. zizanioides was selected for the apoptotic study. Results of the apoptotic study using *C. zizanioides* root on UMR 106 bone cancer cell lines after 24 hours of incubation revealed apoptotic properties.

A long history of usage of these two plants for treating various problems clearly provides the broad pharmacological activities, including antiproliferative property [23]. But the amount of chemical composition varies depending on the part of the plant [24]. Hence, based on the previous reports, the rhizome of A. calamus and the root of C. zizanioides were selected for the study.

Rhizome extract of *A. calamus* showed promising anticancer activity [25] and significantly inhibited the multiplication of J774 cancer cell lines. [26, 27]. *C. zizanioides* is one of the promising natural remedies for treating various ailments, including the reduction of proliferation of cancer cells [17]. Evaluation of the ethanolic root extract of *C. zizanioides* by Madhuri et al [17] revealed the significant growth inhibition in both oral and prostate cancer cell lines.

Thus, the results of the current research study are also in line with earlier reports, and hence clearly justify the cytotoxic effect of both samples and the apoptotic property of *C. zizanioides.* Further studies are required to shed more light on the particular bioactive molecule responsible for these properties.

Conclusion

The identification of new medicines from natural resources for treating cancer is a trend in recent times. *A. calamus* and *C. zizanioides,* being important traditional medicinal plants, have shown cytotoxic effects on UMR 106 bone cancer cell lines. The cytotoxic value of *C. zizanioides* is lower than that of *A. calamus*. An apoptotic study conducted using C. zizanioides at concentrations of 160 µg/ml and 320 µg/ml induced apoptosis in the UMR-106 cell lines. Hence, *C. zizanioides* can be a potential source of a drug for the development of medicine to treat cancer.

Conflict of interest

The author declares that there is no conflict of interest.

Author contributions

The author conceived the study, performed the experiment and wrote the article.

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