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# *In Vitro* Apoptosis Detection of MCF-7 and HCT116 Cells by Aqueous Plant Extract from *Stachytarpheta mutabilis using* PI Annexin V-FITC Flow Cytometry Assay

***Ramesh Londonkar[1*] and Maithilee Kesralikar[2]***

[1]*Professor, Department of Biotechnology,*
*Gulbarga University, Gulbarga (Karnataka), India.*
[2]*Ph.D. Research Scholar, Department of Biotechnology,*
*Gulbarga University, Gulbarga (Karnataka), India.*

*(Corresponding author: Ramesh Londonkar*)*

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**ABSTRACT: Cancer is one of the main global causes of mortality. Currently available treatment options include chemotherapy, radiation therapy, and surgery, all of which have undesirable side effects. In order to alleviate the negative effects of these therapies, combinational or alternative treatments derived from natural sources are required. Cancer chemopreventive potential of natural plant compounds with significant growth inhibition and apoptosis induction characteristics is being intensively researched. Extracts of plants are an abundant source of secondary metabolites. *Stachytarpheta mutabilis* is a member of the verbenaceae family and has been used as a folk remedy for ages. It has numerous pharmacological effects, but its anti-cancer potential is the most intriguing. This research addresses the induction of apoptosis in MCF-7 and HCT-116 cell lines after treatment with the aqueous fractions-isolated chemical. Apoptosis is a cell death process marked by distinct morphological and biochemical characteristics. A crucial step of apoptosis is the development of surface modifications by dying cells, which ultimately leads to their identification and ingestion by phagocytes. By coupling FITC (fluorescein isothiocyanate) to Annexin V, flow cytometry may be used to detect and quantify apoptotic cells on a single-cell basis. Our findings show that the anticancer effects of *S. mutabilis* on human MCF-7 and HCT-116 cell lines are promising. However, further study is required in light of its medicinal potential.**

**Keywords:** Phytochemical analysis. Apoptosis, flow cytometry, Chromatography, MTT assay, anticancer.

## INTRODUCTION

Cancer is uncontrolled cell division. The forecasts for new cancer cases, survival, and mortality are inconceivable. In 2021, there will be 2.3 million breast cancer cases worldwide, followed by 1.2 million instances of colorectal cancer (American cancer society, cancer facts). Although chemotherapeutic medicines are widely accessible, the problem of cancer treatment toxicity and tumour resistance has not yet been overcome (Somaida *et al.,* 2020). Since ancient times, plants have been utilised as folk medicine and herbal medicines to treat a variety of ailments. Plants have been used to treat and prevent many human and animal diseases since ancient times. Today, approximately 50% of the anticancer drugs used in chemotherapy are obtained from plants (Demir *et al.,* 2018). Secondary metabolites, which are abundant in plants, are crucial to the process of drug development. Numerous pure medicinal compounds derived from plants are used in chemotherapy medicines. Today, less toxic anticancer drugs originating from plants are gaining traction in therapeutic research (Chaudhry *et al*., 2019). *Stachytarpheta mutabilis* is a perennial plant of the verbenaceae family that grows in meadows and beside roadways. Additionally known as red porterweed. The plant's stem is comprised of a soft, woody stem, dark green leaves, and scarlet blooms. Antioxidants such as flavonoids, tannins, steroids, phenols, and alkaloids are abundant in this species. This species has shown anti-inflammatory, antioxidant, and antibacterial effects, according to studies. The herb has been utilised as a traditional remedy by several cultures around the globe. In this work, we employed human breast cancer and human colorectal carcinoma cell lines as in vitro experimental models to investigate the anti-cancer efficacy of the species *Stachytarpheta mutabilis.* These cell lines represent the two primary cancer forms with considerably high incidence globally.

Through an apoptotic test, anticancer activity against two cell lines, namely MCF-7 and HCT-116, were evaluated in the current research. Comparing these two tests has shown substantial findings. Apoptosis is a cell death process marked by distinct morphological and biochemical characteristics. Once initiated, apoptosis progresses with varying speeds depending on the cell

type, culminating in cell death and the creation of apoptotic bodies.

Studies have shown that apoptotic cells contain thrombospondin binding sites, lose sialic acid residues, and expose a phospholipid like phosphatidylserine (PS) on their surface (Badmus *et al*., 2015). In the plasma membrane, phosphatidylcholine and sphingomyelin are found on the outside leaflet of the lipid bilayer, whereas phosphatidylserine is more often found on the inner surface facing the cytosol (Pumiputavon *et al*., 2017). There is evidence that both active platelets and senescent erythrocytes display PS on the surface of the cell membrane. New evidence suggests that PS is translocated to the plasma membrane's outer layer during the apoptotic process, when the membrane's phospholipid asymmetry is destabilised. Membrane integrity is maintained throughout this stage of apoptotic cell death (Kowalczyk *et al.,* 2019).

This PS exposure may serve as a universal and early indicator of cell death. Because it binds to negatively charged phospholipids like PS in the presence of $Ca^{2+}$ and shows minimal binding to phosphatidylcholine and sphingomyeline, annexin V, a member of a recently discovered family of anticoagulant proteins, the annexins, has proven to be a useful tool for detecting apoptotic cells (Wassim *et al*., 2020). Prior to the morphological changes associated with apoptosis and the breakdown of membrane integrity, changes in PS asymmetry, as evaluated by Annexin V binding to the cell membrane, were discovered. It is possible to discriminate between intact, early apoptotic, late apoptotic, and necrotic cells by staining them simultaneously with FITC-Annexin V (green fluorescence) and propidium iodide (red fluorescence) (Faye *et al*., 2021; Ogbiko *et al.,* 2019). Previous studies (Badmus *et al*., 2015) have revealed many alterations on the surface of apoptotic cells, including the expression of thrombospondin binding sites, the removal of sialic acid residues, and the exposure of a phospholipid like phosphatidylserine (PS). Phospholipids in the plasma membrane are unevenly distributed between the inner and outer leaflets, with phosphatidylcholine and sphingomyelin being more exposed on the outer leaflet of the lipid bilayer and phosphatidylserine being more prominently observed on the inner surface facing the cytosol (Pumiputavon *et al*., 2017). PS has been observed to be exposed on the plasma membrane of active platelets and senescent erythrocytes. It was recently discovered that PS is translocated to the plasma membrane's outer layer during apoptosis, when cells break down their phospholipid asymmetry. At this point in the process, the cell membrane is still functional (Kowalczyk *et al*., 2019).

This exposure to PS may be a signature (early and extensive) for identifying cells in the process of dying. Annexin V, a member of the recently discovered family of anticoagulant proteins known as the annexins, has been shown to be a useful tool in the detection of apoptotic cells (Wassim *et al.*, 2020). This is because annexin V preferentially binds to negatively charged phospholipids like PS in the presence of $Ca^{2+}$, while showing minimal binding to phosphatidylcholine and sphingomyeline. PS asymmetry alterations were found before to morphological abnormalities associated with apoptosis and before membrane integrity was compromised by evaluating Annexin V binding to the cell membrane. It is possible to distinguish between intact cells, early apoptotic cells, and late apoptotic or necrotic cells by staining them concurrently with FITC-Annexin V (green fluorescence) and the propidium iodide (red fluorescence).

## MATERIAL AND METHODS

**Phytochemical analysis.** Leaves of *Stachytarpheta mutabilis* were taken from the More Nursery in Pune, Maharashtra, as experimental plant material in January 2020, and was characterised in the Department of Botany at Gulbarga University in Kalaburagi. After being cleaned with water and allowed to dry at room temperature, the leaves were ground up using a grinder into a coarse powder in order to remove any residues. A soxhlet extraction of the crushed powder was carried out using methanol at a ratio of 1:10 ratio. Secondary metabolic products such as phenolic, flavonoids, steroid and saponin, and alkaloid were found to be present in the plant after it was subjected to a phytochemical analysis. Steroid-like compounds were isolated from a crude aqueous extract by the use of TLC (thin layer chromatography), HPLC (high performance liquid chromatography), and NMR (nuclear magnetic resonance) techniques.

**Cell lines and culture medium.** HCT116 and MCF-7 cell lines were obtained from ATCC, and stock cells were grown until confluent in DMEM supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 IU/ml), and streptomycin (100g/ml) in a humidified environment containing 5% carbon dioxide at 37 degrees Celsius. Using a cell dissociating solution (0.2% trypsin, 0.02% EDTA, and 0.05% glucose in PBS), the cell was dissociated. The cells are tested for viability and centrifuged. Additionally, a 96-well plate was seeded with 50,000 cells per well and cultured for 24 hours at 37°C, 5% $CO_2$ incubator.

**Purification of compound from the crude methanolic extract.** The compound was purified from crude methanolic extract using TLC (Thin Layer Chromatography), Column column fractionation and HPLC (High Performance Liquid Chromatography).

**MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).** The monolayer cell culture was trypsinized and the cell count was adjusted to 5.0 × 105 cells/ml using respective media containing10% FBS. To each well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates (Penichepavia *et al.,* 2018). The plates were then incubated at 37°C for 24hrs in a 5% $CO_2$ atmosphere. After incubation, the test solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in

PBS) was added to each well. The plates were incubated for 4 h at 37°C in a 5% $CO_2$ atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and the concentration of test drug needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line.

**Calculation of Inhibition.** % Inhibition = ((OD of Control – OD of sample)/OD of Control) × 100.

**Statistical evaluation: IC50 Value.** The half-maximal inhibitory concentration quantifies a compound's capacity to inhibit a biochemical or physiological activity (IC50). The half-inhibitory concentration (IC50) is a quantitative measure of how much of a drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, such as an enzyme, cell, cell receptor, or microbe) by fifty percent (Ahmed *et al*., 2020). The IC50 of a drug may be determined by constructing a dose-response curve and analysing the effect of different dosages of antagonist on agonist activity (Fouzat *et al.*, 2022). The half-maximal inhibitory concentration (IC50) of an antagonist may be calculated by evaluating the percentage of the agonist's biological response that can be inhibited at a given concentration. The IC50 values for the cytotoxicity tests were determined using nonlinear regression analysis (curve fit) using a sigmoid dose-response curve (variable) and Graph Pad Prism 6 for the computations (Graph pad, San Diego, CA, USA).

**Assessment of cell populations by fluorescence activated cell sorting (FACS).** Numerous cancer treatments attempt to promote apoptosis as a method of eliminating cancer cells (Naghavi *et al.*, 2021). The day before to apoptosis induction, 1 × 106 cells were seeded into each well of a 6-well plate using DMEM cell culture medium. After about 18 hours of monitoring for floating (dead) cells, the wells are emptied using a pipette. Two separate concentrations of the chemical, 80 g/ml and 160 g/ml, were utilised to induce apoptosis in cells. Aliquots comprising 500 mL of cell suspension are treated with 10 mL of Propidium Iodide (PI) and 5 mL of Annexin V. After being blended, the suspension is left to incubate in the dark at RT for 15 minutes. As soon as possible after the conclusion of the incubation period, the cells were analysed using a flow cytometer (within 1 hour). Annexin/PI staining reveals an increase in the percentage of cells experiencing early and late apoptosis (Zahra *et al*., 2022).

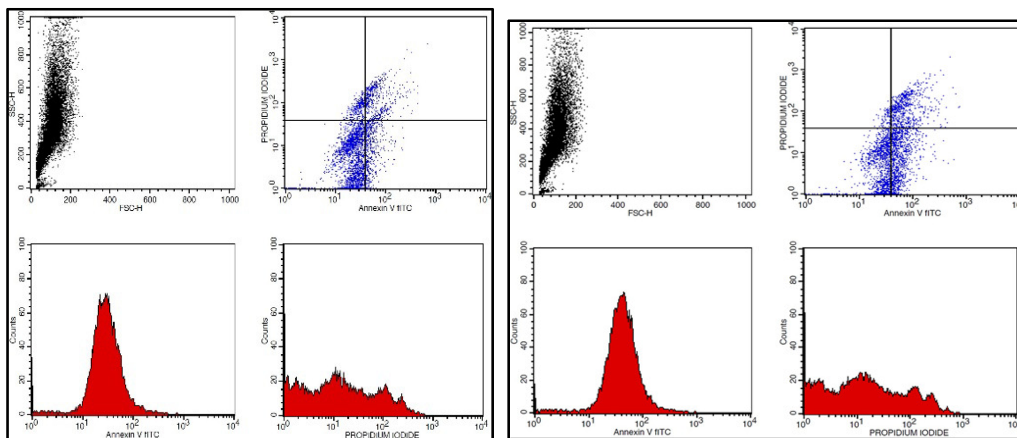


**Fig. 1.** MCF-7 cells treated with Sample 80µg/ml (left) and 160µg/ml (right).

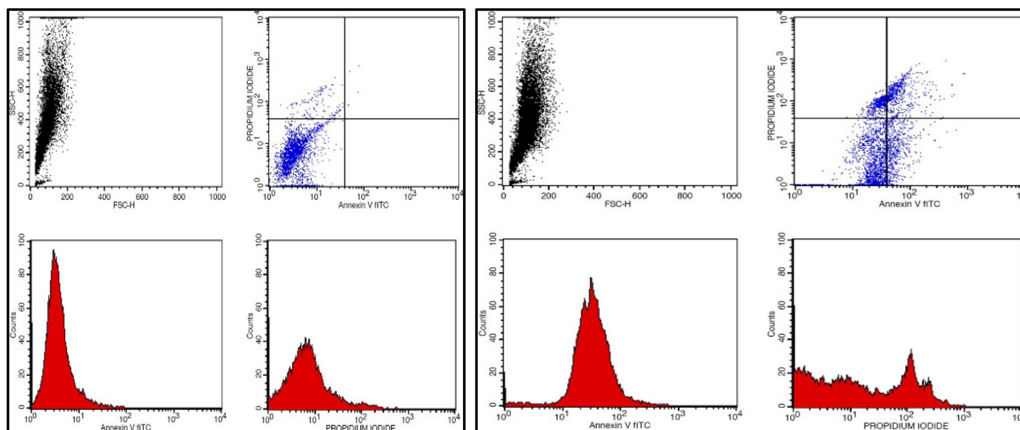


**Fig. 2.** MCF-7 cells treated with control (left) and standard vincristine 25µM (right)

Selected cell lines exhibited antiproliferative activity in response to S. mutabilis extracts. Therefore, it was determined whether the cytotoxic effects of *S. mutabilis* extracts were associated with their capacity to trigger apoptosis in cells. Apoptosis induction of medicinal plant extract is an important characteristic required in cancer drug screening (Dixon *et al.*, 1997). It is a more favorable mode of cell death than necrosis since cell death via necrosis can cause inflammation during treatment.. Apoptosis is characterized by chromatin condensation, cell membrane shrinkage, and DNA fragmentation. In this study, the apoptotic cell population from *S. mutabilis* extract-treated cells was firstly analyzed by flow cytometry (Kiaris and Schally 1999). The flow cytometry Annexin V FITC and PI analysis demonstrated that apoptosis was induced after 24 hours. Fig. 1 shows MCF-7 cells treated with pure compound in two different concentrations 80 µg/ml and 160 µg/ml. For the untreated or control cells, the percentages of viable, early apoptotic, late apoptotic, and necrotic cells were 95.54, 0.01, 0.44, and 4.01 respectively. In same manner standard vincristine the percentage for viable, early apoptotic, late apoptotic and necrotic cells were 42.84, 32.14, 21.49, and 3.53. Cells treated with 80µg/ml concentration showed the percentage of viable, early apoptotic, late apoptotic and necrotic cells 62.35, 13.96, 14.88 and 8.81 respectively.

Similarly 160µg/ml concentration treated cells showed 50.64, 17.42, 19.00 and 12.94 percentage of viable, early apoptotic, late apoptotic and necrotic cells respectively. The flow cytometry analysis is summarised in Table 1.

**Table 1: Flow cytometry analysis of Apoptosis detection of MCF-7 and HCT-116 cells.**

| FACS Analysis of MCF-7 and HCT-116 cell lines | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Sample Group** | **Viable cells (%)** | | **Early Apoptotic (%)** | | **Late Apoptotic (%)** | | **Necrotic Cells (%)** | |
| | **MCF-7** | **HCT-116** | **MCF-7** | **HCT-116** | **MCF-7** | **HCT-116** | **MCF-7** | **HCT-116** |
| Control | 95.54 | 97.85 | 0.01 | 1.21 | 0.44 | 0.15 | 4.01 | 0.79 |
| 80µg/ml | 62.35 | 77.61 | 13.96 | 17.71 | 14.88 | 4.59 | 8.81 | 0.09 |
| 160µg/ml | 50.64 | 56.84 | 17.42 | 24.63 | 19.00 | 14.51 | 12.94 | 4.02 |
| Vincristine 25µM | 42.84 | 44.74 | 32.14 | 41.85 | 21.49 | 11.97 | 3.53 | 1.44 |

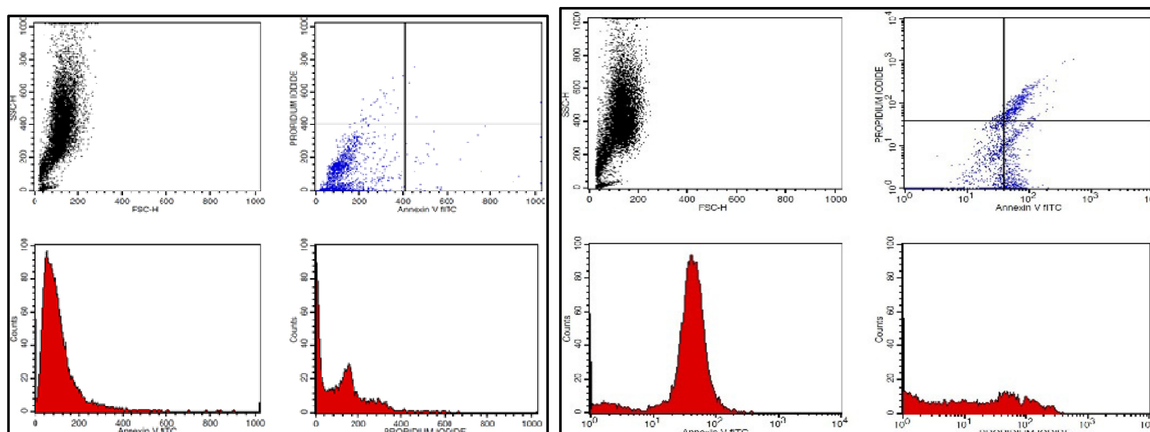


**Fig. 3.** HCT-116 cells treated with control (left) and standard vincristine (25µM) (right).

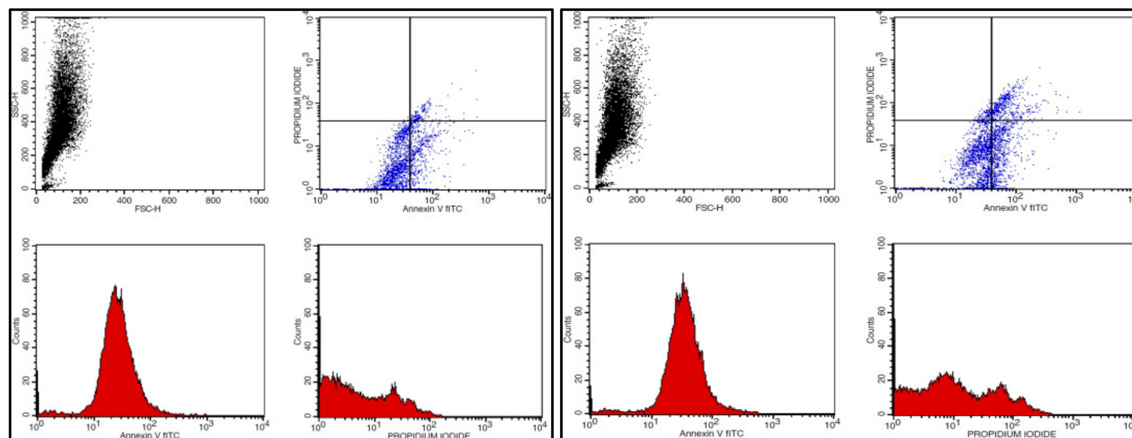


**Fig. 4.** HCT-116 cells treated with Sample 80µg/ml (left) and 160µg/ml (right).

Annexin-V positive, propidium iodide negative, cells were defined as apoptotic (Sadeghi *et al*., 2020). Necrotic cells were confirmed to be positive for both Annexin-V and propidium iodide. Similarly, cells were considered to be alive when they were negative for both Annexin-V and propidium iodide. The viability of untreated HCT-116 cell lines was found to be 97.85%, with 1.21 % showing signs of early apoptosis, 0.15 % showing signs of late apoptosis, and 0.79 % showing signs of necrosis. There was a decrease in viability from 44.74% to 11.97% in late apoptosis, 1.44% in necrosis, and 41.85% in early apoptosis when vincristine was used. Cells exposed to 80 ng/ml showed percentages of viability of 76.61%, apoptosis at 17.71%, apoptosis at 4.59%, and necrosis at 0.09%, respectively. Viable cell percentage, early apoptotic cell percentage, late apoptotic cell percentage, and necrotic cell percentage were respectively 56.84%, 24.63%14.51%, and 4.02% in cells treated at 160 g/ml. At the beginning of the apoptotic process, phosphatidyl-serine (PS) on the plasma membrane serves as a recognition site for phagocytes (Kumar *et al*., 2022). The calcium-dependent protein annexin V may attach to free phosphatidyl-serine (PS) chains on the membrane's outermost layer, as reported by Reda *et al*. (2020). Apoptosis was shown to be one of the main ways of cell death produced by the plant extracts at two doses in this research, as the proportion of cells undergoing late apoptosis was considerably higher in MCF-7 cells compared to the HCT-116 cell line at 80 g/ml and 160 g/ml.

## RESULTS AND DISCUSSION

Traditional medicine has made great strides in recent years in developing unique natural medications that are poisonous to malignant but not normal cells. Plant extracts have been utilised to treat a wide variety of medical conditions because they are a source of very inexpensive phytochemicals with significant therapeutic potential. Phytochemical analysis was used to determine the presence of phenolics, flavonoids, steroids, saponins, and alkaloids in the leaf extracts (El Huneidi *et al*., 2020). A steroid-like molecule was identified and purified using TLC, HPLC, and NMR from the aqueous fraction. The purified fraction is a stearic acid-like molecule, as shown by FTIR, NMR, and XRD analyses, which helped to understand its structure. The MTT test was used to measure the cytotoxic activity of compound fractions in HCT116 and MCF7 cell lines subjected to 80 and 160 micrograms per millilitre of extract, respectively. Following established protocols, we observed that the IC50 values for the HCT-116 cell line and the MCF-7 cell line, respectively, were 91.6 and 98.8 g/ml. The Annexin-V/PI apoptosis detection test was employed to verify apoptosis induction, since it is often utilised to discriminate between early and late phases of apoptosis. Together, PI and annexin V are utilised to identify apoptotic cells and distinguish them from necrotic ones. PI may penetrate late apoptotic or necrotic cells and stain DNA since the membrane has lost its integrity. The use of Annexin V and PI allows for the differentiation of cells at various phases of the apoptotic process. The entrance of PI into the nucleus, where it binds to DNA, distinguishes late-stage apoptotic cells and necrotic cells from those that bind solely to Annexin V. In a study (Kar *et al.,* 2022). In the early stages of apoptosis, phosphatidyl-serine (PS) on the outer layer of the plasma membrane functions as a recognition site of phagocytes. Calcium-dependent annexin V (annexin V) binds to phosphatidyl-serine (PS) on the membrane's outermost layer. Apoptosis was shown to be one of the main ways of cell death produced by the plant extracts at two doses in this research, as the proportion of cells undergoing late apoptosis was considerably higher in MCF-7 cells compared to the HCT-116 cell line at 80 g/ml and 160 g/ml. Treatment with the *S.mutabilis* extract shifted the cell population of the MCF-7 and HCT-116 cells toward apoptosis, as shown by flow cytometry. These findings show that *S.mutabilis* extract may trigger cell death, especially in the MCF-7 cell line (Tamboli *et al*., 2022).

## CONCLUSION

The findings of this investigation show that the aqueous extract of *S. mutabilis* might be a promising anticancer drug. Apoptosis is thus valuable as a marker for measuring the efficiency of potential cancer prevention therapy. Following therapeutic treatments, assessing viable and apoptotic cells is facilitated by cell-based assays (Vikas and Anil 2019). By assessing the efficiency of chemicals in vitro, it is possible to find prospective drugs for the treatment of diseases. Using cell-based tests, the therapeutic effectiveness of newly discovered drugs and biologics for the treatment of cancer may be evaluated. The findings of this investigation demonstrate conclusively that the extract was able to suppress cancer cell proliferation by inducing apoptosis. The intriguing finding indicates that the extract may include bioactive chemicals with anticancer properties that need isolation and further characterisation.

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**Conflict of Interest.** None.

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