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Research Article

APOPTOTIC AND CYTOTOXIC ACTIVITIES OF *STROBILANTHES VIRENDRAKUMARANA* VENU AND P. DANIEL IN *ALLIUM CEPA* AND HUMAN RED BLOOD CELLS

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ABSTRACT

Objective: The present study was aimed to explore the cytotoxicity of the aqueous shoot extract of *Strobilanthes virendrakumarana* Venu and P. Daniel in *Allium cepa* and human erythrocytes. It also focuses on the efficacy of the extract to induce apoptosis in the root primordia of *A. cepa.*

Methods: The aqueous extract of the shade-dried shoot was prepared. The cytotoxicity was evaluated both in *A. cepa* and human red blood cells (RBC) using standard protocols. The apoptotic and metabolic/mitochondrial activity evaluated with the help of Evans blue staining and 2,3,5-Triphenyl tetrazolium chloride (TTC) staining methods, respectively.

Results: Cytotoxic assay unveiled different types of aberrations which were dose-dependent. RBC assay revealed the capacity of phytocompounds in the extract to interact with the osmotic balance of RBC membrane and thereby modify it. Apoptosis induction was detected in *A. cepa* roots treated with various concentrations of the plant extract. The gradation in stain uptake indicates the apoptotic activity of the plant extract. The amount of generated formazon in TTC staining is an indirect evaluation of the metabolic/mitochondrial activity of the cell.

Conclusions: These results may be attributed to the presence of bioactive compounds in the plant extract. The staining techniques reveal the qualitative and quantitative evaluation of the potentiality of the extract. Thus, the present study opens a new avenue for further exploration of the plant for its bioactivities.

Keywords: Apoptosis, *Strobilanthes virendrakumarana*, Blebbing, Cytotoxicity, Cytostasis.

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INTRODUCTION

Humans trust in nature and low side effects makes plants invaluable in medicine since ancient times. The underexplored plants enhance the growing research field a fruitful and precious one in drug discoveries. The journey in search of medicine has the same age as that of mankind. The efficacy of plant-based drugs used in traditional medicine has been paying great attention because they are cheap with little side effects [1]. The free radicals generated in the body leads to cellular or oxidative stress, which eventually causes diseases such as diabetes, cirrhosis, and cancer [2]. Plants contain a rich source of free radical scavenging molecules such as phenols, flavonoids, vitamins, and terpenoids that hold antioxidant properties [3]. The first line and basic health services for people in remote areas and economically backward ones are herbal medicines [4].

Apoptosis induction is preceded by the distinct cellular threshold, and it directly regulates tumorigenesis [5]. It has been reported so earlier that the anticancer activity of various plants was by inhibiting the cell growth through apoptosis. The predominant change during apoptosis is the increase in endonuclease and proteolytic activity [6].

Nuclear condensation, membrane blebbing, and formation of apoptotic bodies are some of the most common features of apoptotic cells. The early process of apoptosis is cell shrinkage and pyknosis [7]. Apoptotic stimuli cannot produce caspase inhibition in erythrocytes, so it does not exhibit a functional death system. Even though they lack the essential mitochondrial apoptotic cascade (Apaf-1, cytochrome *c,* and caspase-9), they contain considerable amounts of caspase-3 and caspase-8 [8].

The secondary metabolites present in Acanthaceae point out its medicinal role and significance to researchers to study the various aspects of the family [9]. *Strobilanthes virendrakumarana* Venu and P. Daniel is a large shrub member in the genus *Strobilanthes* of Acanthaceae family. It is very common in semi-evergreen and moist deciduous forests at low elevations but endemic to Kerala. The vernacular name in Malayalam is Choru Kurinji as the shape and color of flower bud is similar to that of cooked rice. Morphologically, it can be identified easily by the yellow-red glands on the lower surface of leaves [10]. Flowering periodicity of the plant is about 10 years.

Traditional medicine in Malaysia and Indonesia uses filtrates of boiled *Strobilanthes* species leaves as antidiabetic, diuretic, antilytic, and laxative [11]. Moreover, its leaf extract has the ability to minimize the glucose level in blood and also reduces the risk of heart muscle/cardiovascular ailment [12]. The empirical evidence that supports the use of traditional herbs remains largely lacking [13]. *S. virendrakumarana* is a novel species; hence, the present study is the first report on the bioactivity of the plant. This study aims to project out the cytotoxic and apoptotic activities of aqueous shoot extracts of the plant.

MATERIALS AND METHODS

Collection of specimen

S. virendrakumarana was collected from Bhoothathankettu, Ernakulam district, Kerala. The voucher specimen was herbarized after proper taxonomic identification (CMPR 10953). Analytical grade chemicals were purchased (HiMedia® Chemical Laboratory, Mumbai, India). *Allium cepa* bulbs were procured from Agricultural University, Tamil Nadu. The plant shoots were shade dried and made into powder using a blender. An aqueous extract of the powder was prepared. All the photographs were taken with Leica DM2000 LED research microscope.

A. cepa assay

Rooted *A. cepa* bulbs were used for the evaluation of cytotoxicity. They are treated with different concentrations of shoot extract (0.1, 0.075,

and 0.05%) for 24 h. Hydrogen peroxide (0.1%) was taken as a positive control whereas distilled water was the negative control. Roots were excised from the bulbs after treatment. They were fixed in Carnoy's fluid (1 acetic acid:3 alcohol) for 1 h. It is followed by hydrolysis using one normal hydrochloric acid for a few minutes. Staining was done for 3 h in acetocarmine. Destained and mounted in 45% acetic acid. Mitotic squash preparations were done with standard protocol [14]. Images were acquired and cytotoxic parameters were calculated using the following formulas.

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$\text{Abnormality percentage} = \frac{\text{Number of aberrant cells}}{\text{Total number of cells}} \times 100$$

Evaluation of cytotoxicity using red blood cell (RBC) assay

For analyzing the cytotoxicity using RBC assay, stock solution (1%) of aqueous extract of *S. virendrakumarana* was prepared. Different dilutions were made from the stock (1%, 0.75%, and 0.5%). Normal saline solution (7%) was taken as the control. Blood samples were treated with different concentrations of extracts for 1 h. Morphological evaluation of RBC was done by preparing blood smears on microscopic glass slides. They are air dried and stained using May-Grunwald-Giemsa (MGG) method [15,16]. Slides were stained for 15 min with May-Grunwald and followed by Giemsa for 30 min without blotting. The slides were washed with phosphate buffer solution (pH = 7.2) for 20–30 s and allowed to air dry. It is followed by imaging the RBCs under an oil immersion microscope.

Evaluation of apoptotic activity

Evans blue is a reliable stain for determination of cell death. It was executed using the standard methodology with a slight modification [17]. Rooted onion bulbs were treated with different concentrations of shoot extract; namely, 0.1, 0.075, and 0.05% for 24 h. Distilled water and 0.1% hydrogen peroxide were taken as negative and positive controls, respectively. Both the treated and control roots were washed with distilled water and stained with 0.25% (w/v) aqueous solution of Evans blue for 15 min and washed twice. Roots were photographed for qualitative estimation. Five roots of equal lengths were excised from the bulb and drenched in 3 ml of N, N-dimethylformamide for 1 h to release the stain. Using a spectrophotometer, absorbance was measured at 600 nm against N,N-dimethyl formamide as blank.

Evaluation of metabolic/mitochondrial activity

2,3,5-Triphenyl tetrazolium chloride (TTC) staining is a novel approach to check the viability of cells. The roots of *A. cepa* along with the bulbs were treated with different concentrations of shoot extract, namely, 0.1, 0.075, and 0.05% for 24 h. The same set-up was followed for positive and negative control taken as 0.1% hydrogen peroxide and distilled water, respectively. All the roots were immersed in 0.5% (w/v) TTC stain for 5 h in dark [18]. Subsequently, the roots were washed in distilled water and excised from the bulbs. Photographs were taken, and five roots of equal length were immersed in 3 ml of 95% ethanol. Absorbance was measured at 490 nm using a spectrophotometer against 95% ethanol as blank.

Statistical data

The recorded result values of the experiment were statistically analyzed using SPSS 20 (SPSS Inc., Chicago, IL, USA) to determine the mean separation and significance of treatments. All data were expressed as mean ± SE and differences between corresponding controls and treatments were considered statistically significant at $p<0.05$. Analysis was performed in SPSS software.

RESULTS

The aqueous shoot extract of *S. virendrakumarana* brought out divergent aberrations in *A. cepa* assay which include both clastogenic and non-clastogenic (Fig. 1). A positive correlation between extract concentration and abnormality percentage was noticed. The mitotic index declined to rise in the concentration of the extract. It revealed a hike in the negative control (78.47%) and was low with 30.51% in positive control. The abnormality percentage ranges between 72.83% and 10.62% (Table 1). The major clastogenic aberrations were nuclear lesions, pulverized ball metaphase, coagulated anaphase, nuclear budding, etc. Sticky stathmo-anaphase, pole to pole metaphase, scattered metaphase, tropokinesis at metaphase, vagrants at anaphase, and cytostasis and chained metaphase were some of the non-clastogenic aberrations obtained.

The RBC suspension treated with normal saline solution which was taken as the control retained its shape after 1 h. They were round or spherical in nature. However, the blood cells treated with aqueous shoot extract resulted in blebbing of cell membranes (Fig. 2). The 1 h extract treatment ultimately altered the shape of RBCs by a crenation appearance.

The Evans blue staining resulted in dose-dependent gradation in roots treated with different concentrations of *S. virendrakumarana* extract. The positive control made maximum cell death, which is indicated by highly stained root tips (Fig. 3). Root tips treated with distilled water (negative control) were colorless after staining. The absorbance value increases with a rise in concentration. Minimum absorbance was observed in the negative control (Fig. 4).

The TTC staining results were in reverse of Evans blue staining. The maximum absorbance was recorded with roots treated with negative control. The absorbance value decreases with increasing concentration of the plant extract (Fig. 4). The chromogenic effects on roots reduced with increment in concentration of the extract. Hence, the lowest concentration treated roots were highly colored.

DISCUSSION

Cytotoxicity assay using *A. cepa* is a simple, inexpensive, and reliable method to evaluate the toxic potentiality of an extract. The resulted chromosomal aberrations revealed the mitoclastic potential of the extract of *S. virendrakumarana*. It includes both clastogenic and non-clastogenic abnormalities. Clastogenic aberrations are those which directly affect the chromosomes, whereas non-clastogenic ones will affect the spindle fibers.

The adhesion of proteinaceous material of chromatin is called stickiness [19]. It was suggested that stickiness is the depolymerization of nucleic acids of chromatin. It may lead to incomplete separation of

Table 1: Mitotic index and abnormality percentage of *Allium cepa* cells treated with different concentrations of aqueous extract of *Strobilanthes virendrakumarana*

Concentration (%)	Mitotic index (%)	Abnormality (%)
Negative control (distilled water)	78.47±2.53	10.62±0.41
0.05	66.6±3.63	18.3±1.11
0.075	51.9±3.58	31.57±5.5
0.1	30.51±2.73	62.38±3.15
Positive control (hydrogen peroxide)	21.57±2.14	72.83±3.36

All the values are expressed in mean±SE

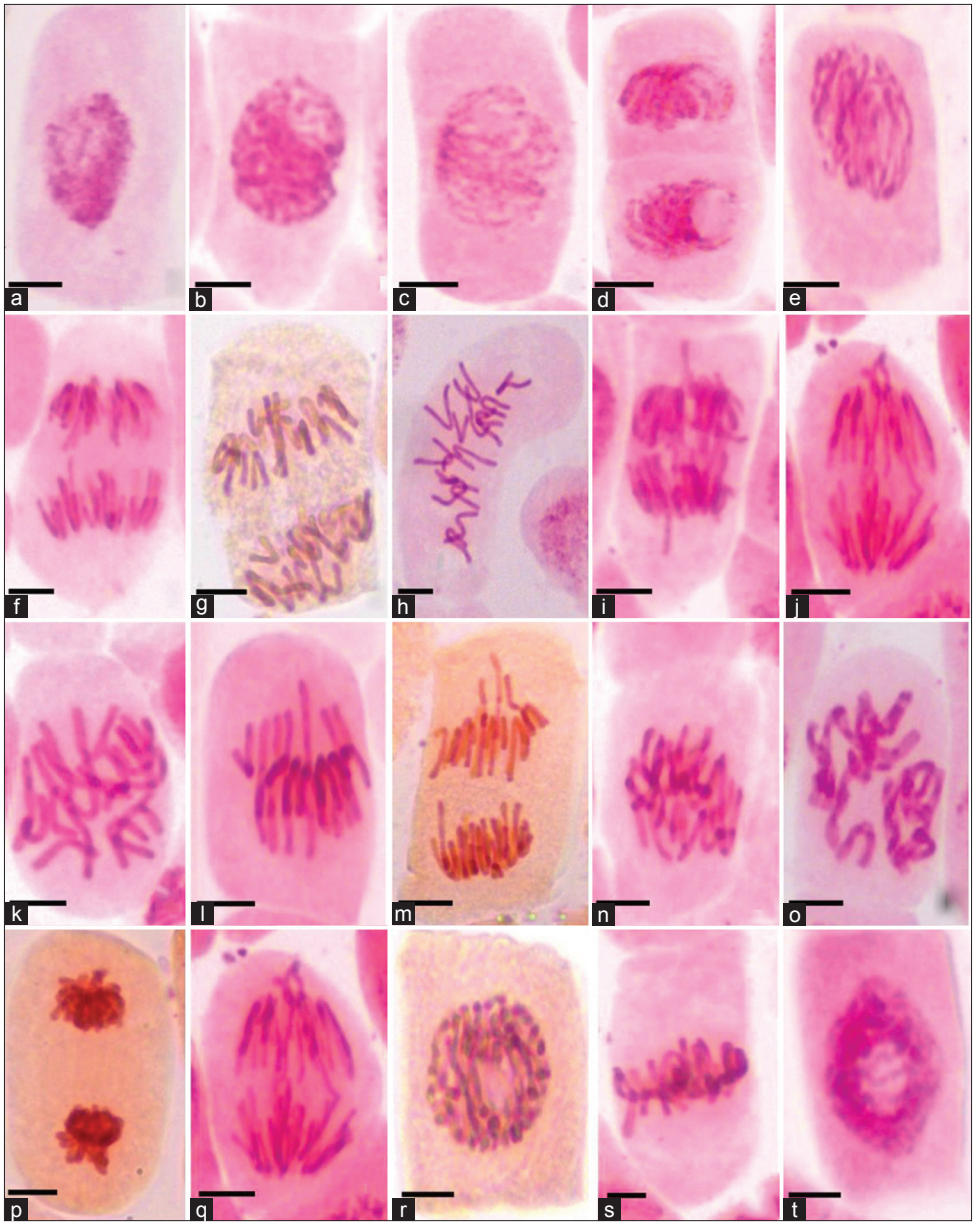

Fig. 1: Chromosomal aberrations induced by shoot extract of *Strobilanthes virendrakumarana* during *Allium cepa* assay (a) nuclear erosion, (b) lesion at prophase, (c) pulverized chromatin at prophase, (d) nuclear lesion, (e) chromatin erosion in ball metaphase, (f) sticky anaphase, (g) coagulated anaphase, (h) Pole to pole metaphase, (i) sticky stathmo-anaphase, (j) sticky anaphase showing multiple bridges and vagrants, (k) scattered metaphase, (l) cytostasis, (m) vagrants at anaphase, (n) tropokinesis at metaphase, (o) chained metaphase, (p) coagulated anaphase, (q) sticky anaphase showing multiple bridges and vagrances, (r) ball metaphase showing lesion, (s) chromosome dumping in a hypoploid cell, (t) single nuclear lesion at early prophase (bar – 10 µm)

daughter chromosomes [20]. The formation of multiple bridges and vagrants may be attributed to the breaks of chromosomes and reunion of broken ends [21]. It leads to the loss of genetic content.

Pulverization occurs in the DNA synthesis phase (S phase) due to the lysis of the nuclear membrane [22]. When comparing with the normal chromosomes, the pulverized ones replicate later. This asynchrony results from the fusion of cells containing asynchronous nuclei [23]. The positive correlation of abnormality percentage and extract concentration indicates the genotoxic phytocompounds present in the aqueous extracts of *S. virendrakumarana*. The fall in the mitotic index is due to the mitodepressive property of the extract.

RBC assay using MGG method is used for the morphological evaluation of natural products on RBCs. It is simple, low cost, and commercially available one, which makes the MGG stain as an advantage over several others [15]. The crenation appearance of blood cell projects out the capacity of compounds present in aqueous extract of

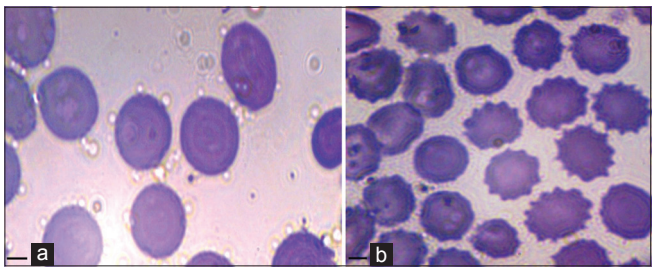

Fig. 2: (a) Control showing normal circular erythrocytes, (b) effect of aqueous shoot extract of *Strobilanthes virendrakumarana* on human red blood cells showing crenation appearance (bar – 2 µm)

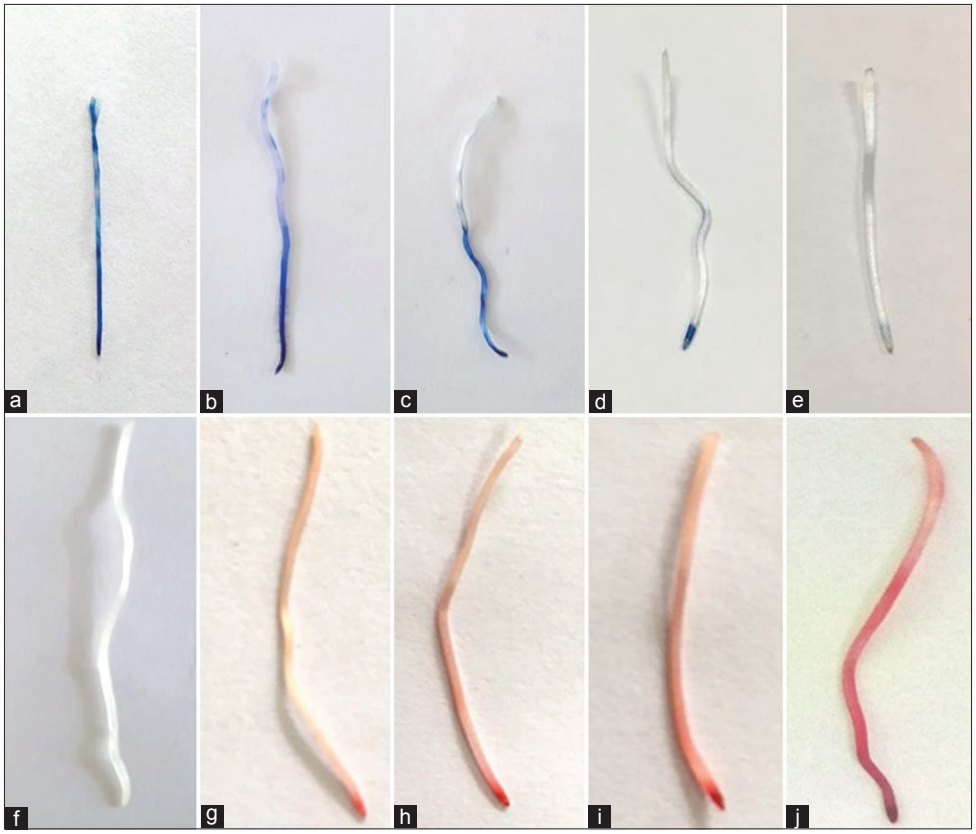

Fig. 3: Effect of different concentrations of *Strobilanthes virendrakumarana* aqueous shoot extract in *Allium cepa* roots (a) positive control, (b) 0.1%, (c) 0.075%, (d) 0.05%, (e) negative control, (f) positive control, (g) 0.1 %, (h) 0.075%, (i) 0.05%, (j) negative control, (a-e: Evans blue stain and f-j: Triphenyl tetrazolium chloride stain)

S. virendrakumarana to react with the cell membrane. It may modify the osmotic transport balance or erythrocyte membrane ion transport. It could decrease the membrane integrity and thereby alter the shape of the membrane through blebbing.

Proteins and cellular molecules participate in energy using a process called apoptosis [24]. It is induced by genotoxic stimuli which on prolonged exposure results in cell death. The roots treated with the highest concentration of the extract were highly stained since the genotoxic compounds within the extract may react with the cells. The negative control (distilled water) retained the color of the roots. The accrual of color intensity in staining confirms the effect of the extract on cell death.

The TTC is a colorless stain generally used as an indicator to check the viability of seeds. It is regarded as the indirect, partial measure of the functionality of mitochondrial electron transport chains [25]. Electrons for the reduction of colorless TTC to colored formazan were produced from the mitochondrial electron transport system. The insoluble formazan production (red color) in TTC staining was inversely proportional to the concentration of the extract. The highest concentration treated roots have a lesser capacity to produce the color. These may be due to the lack of electrons which indirectly indicates the inhibition of mitochondrial activity by phytocompounds in the aqueous extract.

Mitochondria play a major role in apoptosis. Disruption of the membrane potential of mitochondria release cytochrome *c.* This event is crucial in triggering caspase activation [26]. The results from Evans blue and TTC staining can be correlated. The mitochondrial activity was less in roots treated with highest extract concentration. In the same way, apoptosis was hiked in higher concentration. It can be assumed that enhanced apoptosis may be due to the release of cytochrome *c* through which mitochondrial activities are lowered.

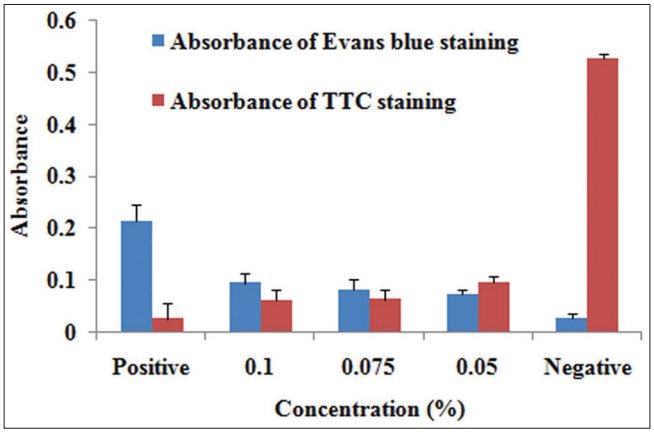

Fig. 4: Graph showing the effect of *Strobilanthes virendrakumarana* aqueous shoot extract on cell death and metabolic/mitochondrial activities

CONCLUSIONS

The alarming spread of cancer created awareness among people about the importance of therapeutic drugs from plants. The drug discovery from natural products initiated from ancient time itself due to its low side effects and availability. The current work declared the presence of phytocompounds in *S. virendrakumarana* which are capable of apoptosis. This property can be used for the treatment of cancer by enhancing cancer cell death. It is evident that the extract is a source of cytotoxic potent compounds. As a novel species, the bioactivities of the plant are anonymous to science. Through this work, the cytotoxicity and apoptotic activity of the plant are unveiled. Thus, *S. virendrakumarana* must be further explored for its bioactivities on *in vitro* systems and

isolation of compounds for better understanding of the mechanism behind it.

AUTHORS' CONTRIBUTIONS

RC collected plant material, performed experimental analysis and was involved in writing a draft paper and is the corresponding first author. JET designed the study project, contributed to experiment guidance, manuscript editing, finalization and is the second author. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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