

EVALUATION OF CYTOTOXICITY AND APOPTOTIC POTENTIAL OF *OPHIORRHIZA PECTINATA* ARN. – A POTENT ANTICANCER AGENT

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ABSTRACT

Objective: *Ophiorrhiza pectinata* Arn., a notable species belonging to the family Rubiaceae, is reported to be used in traditional and conventional medicines. The present study was aimed at the evaluation of the cytotoxic and apoptotic effects of the aqueous extract of *O. pectinata*.

Methods: Cytotoxic potential of the plant extract was analyzed using *Allium cepa* assay and the apoptotic effect of the plant extract was analyzed using Evan's blue staining technique.

Results: The extract was found to be cytotoxic at all tested concentrations, inducing several clastogenic and non-clastogenic aberrations at all the stages of mitosis. The clastogenic aberrations observed include chromosome coagulation, stickiness, chromosome bridges and fragments, nuclear lesions, and giant cells. Among non-clastogenic aberrations seen, the prominent ones were polyploidy, C-metaphase, stathmo-anaphase, ball metaphase, vagrants, tropokinesis, etc. The mitotic index was found to be decreasing with increase in concentration, whereas the abnormality percentage increased with an increase in the concentration of the plant extract. The *in situ* visualization of cell death by the Evan's blue staining method gave a positive relation between the concentration of the extract and its apoptotic activity.

Conclusion: As cytotoxicity and apoptosis are bioactivities related to anticancer potential, the study proves that the plant is of high therapeutic efficacy and requires more exploration toward its usefulness in drug discovery.

Keywords: *Ophiorrhiza*, Cytotoxicity, Apoptosis, Anticancer, Therapeutic.

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INTRODUCTION

Plants are the major source of natural medicines. Several products derived from plants have been used for thousands of years in traditional medicines to treat various human illnesses [1]. According to the reports of the World Health Organization, about 80% of the population in developing countries relies on traditional medicines for their health-care needs [2]. Although thousands of plants are well known for their ethnomedicinal properties, only 1–10% of these are scientifically studied for their potential medicinal use [3]. As the medicinal properties of all plants rely on their bioactive chemical constituents, large numbers of plants are constantly screened for the manufacture of new drugs with fewer side effects [4].

Recent years have witnessed the emergence of several dreadful diseases that wiped out thousands of lives. Cancer has become one such devastating disease, affecting millions of people worldwide. It accounts for more than 13% of all human deaths [5]. Although modern technology has developed synthetic drugs and therapies for the treatment, cancer still affects the quality of life. An alternative to this is the use of herbal-based drugs, and thus, medicinal plants become the principle source of anticancer agents [6].

Plants that are used in traditional and folklore medicines to treat and prevent diseases like cancer might possess compounds that could play a major role in the development of useful anticancer agents [7]. Agents with antiproliferative and apoptosis-inducing capacity are currently used in cancer treatment [8], so the assessment of cytotoxic and apoptotic efficacy of medicinal plants is important to validate their use as anticancer agents.

The present study was designed to assess the cytotoxic and apoptotic efficacy of the plant *Ophiorrhiza pectinata* Arn., belonging to the family

Rubiaceae. *Ophiorrhiza* L. is a notably species-rich genus consisting of 321 species, five varieties, and one subspecies [9] and found in wet tropical forests of South-East Asia, extending to Australia, New Guinea, and Pacific Islands. *Ophiorrhiza* species are small herbaceous or shrubby plants that possess many medicinal properties. They have been used in traditional and folk medicine as snakebite antitussive, and analgesic and for the treatment of ulcers, leprosy, gastropathy, and amenorrhea [10]. The presence of camptothecin (in some species), a cytotoxic alkaloid, which is the only natural occurring topoisomerase-I inhibitor [11,12] gives great importance to the genus in cancer research. The present study is the first attempt to analyze the cytotoxic and apoptotic efficacy of the plant, *O. pectinata* on *Allium cepa* root cells. The study tries to evaluate the potential of the plant to be used as an anticancer agent.

METHODS

Collection of plant material

Fresh plant materials (whole plant) of *O. pectinata* were collected from Kulamavu, Idukki district, Kerala, India. The geographical coordinates of the place correspond to 9° 41' 53.21" N and 76° 55' 38.74" E at an altitude of 800 m above sea level. The taxonomic authentication of the collected plant materials was done by Dr. M. Sabu, Professor, Angiosperm Taxonomy Division, Department of Botany, University of Calicut, Kerala, India. The voucher specimen (CALI no: 143972) was deposited in the Calicut University Herbarium.

Test material and chemicals used

A. cepa (Onion) bulbs, 2n=16, free from pesticides and other forms of growth inhibitors were procured from agricultural vendors and used as the test material. Evan's blue stain and N, N-dimethylformamide were purchased from HiMedia Chemical Laboratory (Mumbai, India).

Preparation of plant extract

The collected plants were thoroughly washed to remove all the dirt and dust and were shade-dried. After drying, they were powdered in a blender and stored in moisture free sealed container. For the preparation of the plant extract, 1 g of the powder is dissolved in 100 ml of distilled water using chilled mortar and pestle. This solution of 1% concentration served as the stock solution. From the stock solution, extracts of various concentrations (0.1%, 0.05%, 0.01%, and 0.005%) were prepared.

Evaluation of cytotoxicity

A. cepa root tip assay was done for analyzing the cytotoxic potential of the plant extract. For this, fresh and healthy, uniformly sized onion bulbs were selected and the outer dry scales were removed without destroying the root primordia and grown on sterilized, moist sand. On germination, onion bulbs with roots of length

1–2 cm were collected at the time of peak mitotic activity (9–10 am), standardized by repeated trial and error, and washed in distilled water. After thorough washing, the bulbs were suspended in different concentrations of the plant extract for 24 h treatment. Care was taken that only roots of the bulbs get immersed in extracts. Distilled water and hydrogen peroxide (0.1%) were used as the negative control (NC) and positive control (PC), respectively. After treatment, roots were cut from the bulbs and washed in distilled water, followed by fixation in modified Carnoy's fluid (1 acetic acid:2 ethanol) for 1 h. Mitotic squash preparation was done following the methodology of Sharma and Sharma (1990) with slight modifications [13]. The fixed root tips were washed with distilled water and hydrolyzed in 1 N hydrochloric acid for 5 min. The roots were then washed and stained with 2% acetocarmine for 3 h. After destaining using 45% acetic acid, the roots were squashed and mounted in 45% acetic acid on clean glass slides. The mounted slides were observed under

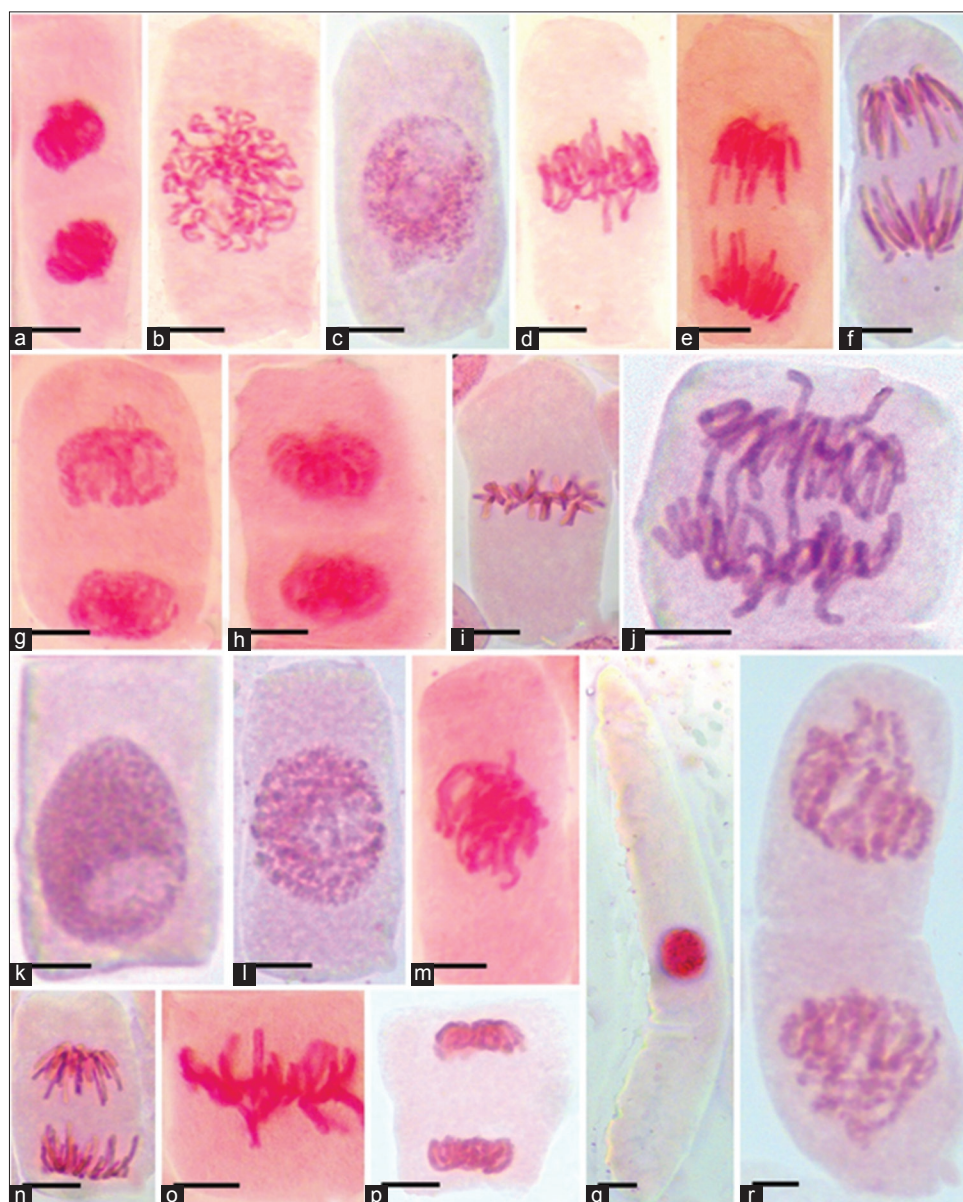


Fig. 1: Clastogenic aberrations induced by aqueous extract of *Ophiorrhiza pectinata* on *Allium cepa* root tip cells. (a) Coagulated anaphase, (b) ring chromosomes in early ball metaphase, (c) pulverized early prophase, (d) sticky metaphase, (e) sticky anaphase, (f) fragments at anaphase, (g) sticky late telophase, (h) pulverized chromatin at cytokinesis, (i) hypercondensed chromosomes, (j) chromosome bridges at C-anaphase in a hyperploidy cell, (k) single nuclear lesion at early prophase, (l) pulverized prophase, (m) coagulated metaphase, (n) chromosome erosion at anaphase, (o) sticky hypercondensed chromosomes at metaphase, (p) telophase showing chromosome erosion, (q) giant cell showing cell shrinkage, (r) chromatin gaps and erosion at late cytokinesis. Bar = 10 μ m

40× objective of light microscope (Leica DM 2000 LED, Germany) and photomicrographs were taken. Six different fields were randomly selected to calculate the number of mitotic cells, aberrant cells, and total number of cells. Mitotic index (%) and abnormality percentage (%) were calculated using the formulae,

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

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

Evaluation of apoptosis

In situ visualization of cell death using Evan's blue staining method [14] with slight modifications was followed for assessing the apoptotic effect of the plant extract. Onion bulbs with roots of length 4–5 cm were selected and washed with distilled water. The bulbs were then treated with different concentrations of the plant extract for 24 h. The bulbs are so placed that only roots get immersed in the extract.

Roots treated in distilled water were taken as the NC and those treated in hydrogen peroxide (0.1%) were taken as the PC. The treated roots were washed and kept in 0.25% (w/v) of Evan's blue aqueous solution for 15 min. After thorough washing in distilled water, the

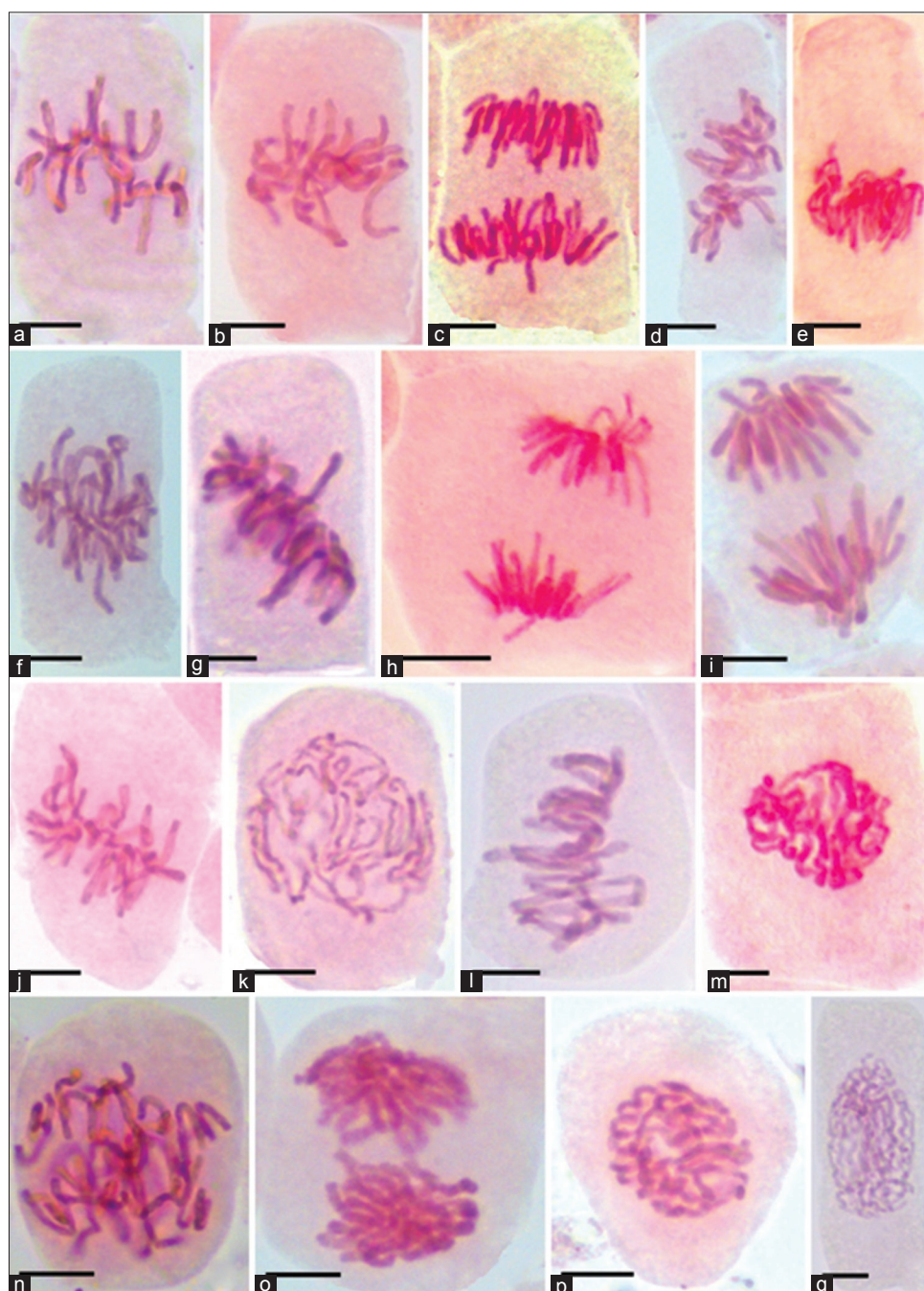


Fig. 2: Non-clastogenic aberrations induced by aqueous extract of *Ophiorrhiza pectinata* on *Allium cepa* root tip cells. (a) Tropokinesis, (b) vagrant metaphase in a hypoploid cell, (c) vagrants at anaphase, (d) diagonal partial C-metaphase, (e) chromosome clumping at metaphase, (f) disturbed metaphase, (g) diagonal metaphase showing vagrants, (h) shift in MTOC at anaphase, (i) diagonal partial C-anaphase, (j) partial C-metaphase, (k) scattered ball metaphase, (l) pole-to-pole metaphase, (m) chained ball metaphase, (n) stathmo-anaphase, (o) stellate anaphase, (p) ball metaphase, (q) early ball metaphase. Bar = 10 μm

roots with dead cells, that have stained blue, were photographed. For quantitative estimation of the cell death, five stained roots of equal length were excised from each onion bulb and immersed in 3 ml of N, N-dimethylformamide for 1 h. The absorbance of the released Evan's blue stain was spectrophotometrically measured with the help of UV-visible spectrophotometer (Elico, India) at a wavelength of 600 nm and the values were recorded. All the tests were done in triplicates.

Statistical analysis

All the statistical analyses were done using the SPSS Version 20 software program. The data obtained were subjected to one-way analysis of variance and Duncan's multiple range tests to validate the results. The results were expressed as mean \pm standard error and those with * $p < 0.05$ were treated as statistically significant.

RESULTS

Evaluation of cytotoxicity

The plant extract of *O. pectinata* was found to be cytotoxic at all the tested concentrations, inducing several clastogenic and non-clastogenic aberrations in *A. cepa* root tip cells in all the mitotic stages. The major clastogenic aberrations observed were coagulated anaphase (Fig. 1a), sticky metaphase (Fig. 1d), sticky anaphase (Fig. 1e), hypercondensed chromosomes (Fig. 1i), chromosome bridges at C-anaphase in a hyperploid cell (Fig. 1j), pulverized prophase (Fig. 1l), chromosome erosion at anaphase (Fig. 1n), giant cell showing cell shrinkage (Fig. 1q), etc. The major non-clastogenic aberrations induced were tropokinesis (Fig. 2a), vagrants at anaphase (Fig. 2c), chromosome clumping at metaphase (Fig. 2e), disturbed metaphase (Fig. 2f), shift in microtubule organizing center (MTOC) at anaphase (Fig. 2h), partial C-metaphase (Fig. 2j), scattered ball metaphase (Fig. 2k), pole-to-pole metaphase (Fig. 2l), chained ball metaphase (Fig. 2m), stathmo-anaphase (Fig. 2n), stellate anaphase (Fig. 2o), ball metaphase (Fig. 2p), etc.

Mitotic index was found to be decreasing with an increase in the concentration of the plant extract when compared with control. A gradual reduction of mitotic index was observed from lower to higher concentrations of the plant extract. The highest concentration of the plant extract (0.1%) exhibited a mitotic index percentage of 28.56 ± 2.93 and the lowest concentration (0.005%) showed a higher mitotic index percent of 73.68 ± 6.85 . The abnormality percentage increased from 29.95 ± 4.44 at 0.005% to 85.86 ± 3.55 at 0.1% of the aqueous extract. Mitotic index thus obtained indicated a dose-dependent reduction in the number of normal dividing cells and a dose-dependent increase was found in the number of aberrant cells, both signifying the cytotoxic potential of the plant. The mitotic index and abnormality percentage induced by various concentrations of the extract are given in Table 1.

Evaluation of apoptosis

Evan's blue staining method revealed the apoptotic effect of the plant, *O. pectinata*. Fig. 3 shows the gradual increase in the intensity of the stain uptake by *A. cepa* roots treated with lower to higher concentrations of the plant extract. As the intensity of the stain absorbed by root cells is related to cell death, this indicated the increasing apoptotic effect of the plant extract.

Quantitative estimation of the cell death by spectrophotometric method indicated a dose-dependent increase in apoptosis from lower to higher concentrations of the plant extract. The absorbance of Evan's blue stain for various concentrations of the plant extract on *A. cepa* root tip cells is summarized in Table 2.

DISCUSSION

Cytotoxicity is an important bioactivity of medicinal plants that can be related to their anticancer potential [15]. Various secondary metabolites present in the plant produce cytotoxic effect by disturbing the normal cell cycle [16] and inducing different forms of chromosomal aberrations. The extract of *O. pectinata* induced aberrations at all mitotic stages and this could be the synchronized effect of the chemical

Table 1: Mitotic index and abnormality percentage induced by various concentrations of the aqueous extract of *Ophiorrhiza pectinata* on *Allium cepa* root tip cells treated for 24 h

Concentration of the plant extract (%)	Mitotic index $\% \pm SE$	Abnormality percentage $\pm SE$
NC	83.26 ± 2.25^c	7.41 ± 3.20^a
PC	27.50 ± 1.12^a	86.71 ± 2.01^d
0.005%	73.68 ± 6.85^c	29.95 ± 4.44^b
0.01%	49.80 ± 6.85^b	67.01 ± 3.62^c
0.05%	33.85 ± 4.59^a	83.33 ± 3.50^d
0.1%	28.56 ± 2.93^a	85.86 ± 3.55^d

Means in a column with similar superscripts are not significantly different at * $p < 0.05$ as determined by Duncan's multiple range tests. NC: Negative control, PC: Positive control, SE: Standard error

Table 2: Absorbance of Evan's blue stain at various concentrations of the aqueous extract of *Ophiorrhiza pectinata* on *Allium cepa* root tip cells treated for 24 h

Concentration of the plant extract (%)	Absorbance at 600 nm
NC	0.02 ± 0.003^a
PC	0.44 ± 0.032^d
0.005%	$0.06 \pm 0.005^{a,b}$
0.01%	0.08 ± 0.001^b
0.05%	0.14 ± 0.0009^c
0.1%	0.16 ± 0.005^c

Means in a column with similar superscripts are not significantly different at * $p < 0.05$ as determined by Duncan's multiple range tests. NC: Negative control, PC: Positive control

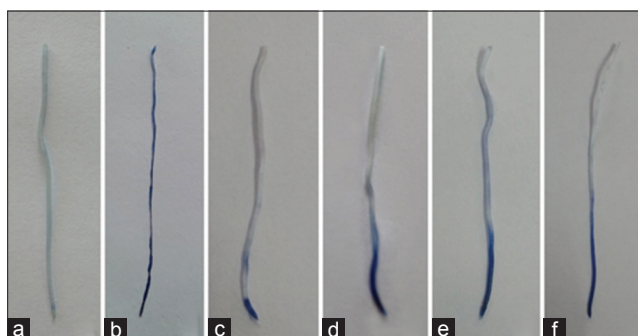


Fig. 3: Apoptosis induced by different concentrations of plant extract of *Ophiorrhiza pectinata* on *Allium cepa* root tip cells. (a) Negative control, (b) positive control, (c) 0.005%, (d) 0.01%, (e) 0.05%, (f) 0.1%

constituents in the extract that blocks DNA synthesis or inhibits spindle formation.

Nuclear lesions observed could be the inhibitory effect of the extract on DNA biosynthesis [17]. Pulverization was another abnormality observed. This might be due to the premature condensation of the chromosomes [18]. Stickiness might occur from improper folding of the chromosome fibers [19] and chromosomal bridges and fragments arise due to the breaks in chromosomes and chromatids [20]. Alteration in the viscosity due to the depolymerization of the DNA might be the reason for chromosome coagulation. Hypercondensed chromosomes were also one of the prominent abnormalities seen. Sosnikhina *et al.* (2003) described the aberration as irregular compaction and stickiness of chromosomes [21]. Formation of giant cell could be attributed to the disruption in "S" phase and thereby cell expansion leading to enlarged cell size.

Among the non-clastogenic aberrations observed, tropokinesis might have occurred due to abnormal orientation of spindle fibers. The

same defect of the spindle fibers would be the reason for diagonal or disorientation of chromosomes and shift in MTOC [22]. Hyperploid cells would have emerged due to multipolar mitosis [23]. Pole-to-pole orientation of chromosomes at metaphase was also observed in cells, which would have resulted due to irregular pathways of spindle assembly and altered spindle activity [24]. Ball-shaped arrangement of chromosomes due to the localized action of the spindle apparatus at the center that makes the chromosomal centromeres to be at the equator and arms to radiate at different directions was also observed in different mitotic stages [25]. Stathmo-anaphase, where the chromosomes remain connected by partial overlapping of the arms, was also observed in cells. Stellate arrangement of chromosomes might have occurred as a result of the spindle disturbances or due to the clumping of daughter chromosomes to a star-like form [26].

Mitotic index is a reliable parameter to measure cytotoxicity within living organisms [27]. Reduced rate of mitotic index is an index of cytotoxic potential of the plant extract. A dose-dependent decrease of mitotic index with increasing concentration of the extract was observed. Similarly, a dose-dependent increase of abnormality percentage was also observed. According to Anjana and Thoppil (2013), reduced mitotic index indicates the presence of cytotoxic substances in the extract that inhibits mitotic activities and produces aberrant cells by its genotoxic effects on the treated root tip cells [7]. Sudhakar *et al.* (2001) explained that the reduced mitotic activities might be due to the blockage in DNA synthesis or G2 phase in cell cycle that inhibits the cells to enter mitotic phase [28].

A. cepa root tip cells treated with the plant extract might suffer from genotoxic stress and such cells either complete nuclear division or undergo apoptosis [29]. Apoptosis is a biological response of the cells with DNA damaged that forms an excellent marker to screen compounds for developing potent anticancer agents [30]. The main aim of using anticancer agents is the induction of apoptosis or cell death-related signals that disturb cancer cell proliferation [4]. Cell death that marks cytotoxicity could be revealed by Evan's blue staining method [31]. The stain deeply penetrates non-viable cells. Thus, the intensity of stain uptake by treated and control roots of *A. cepa* provides indirect evidence of cell death as dead cells are deeply stained than viable cells [32]. The dose-dependent increase in the uptake of the stain by the root tip cells indicated higher rate of cell death at higher concentrations of the extract signifying the apoptotic potential of the plant. Spectrophotometric determination of apoptosis also signified the same.

CONCLUSION

Evaluation of the cytotoxicity of *O. pectinata* mediated by the assessment of apoptotic efficacy revealed that the plant may be formulated as a potent therapeutic agent. The various chromosomal aberrations induced by the plant extract on the root tip cells of *A. cepa* indicate its antimitotic activity, thus preventing cell division. A dose-dependent reduction of mitotic index values and a dose-dependent increase of abnormality percentage marked the cytotoxic potential of the plant. *In situ* visualization of cell death by Evan's blue staining suggested an indirect evidence of apoptosis or programmed cell death, which is an important property for anticancer agents.

A. cepa assays are extensively used to evaluate the cytotoxic, antimitotic, genotoxic, and apoptotic potential of medicinal plants and further works are required to confirm the specificity of the plant for therapeutic use. However, the results suggest that the plant is an effective source for anticancer studies.

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AUTHORS' CONTRIBUTIONS

The first author designed the work, performed the laboratory experiments, collected the data, and prepared the manuscript.

The second author reviewed the manuscript and made proper corrections to improve its quality.

CONFLICTS OF INTEREST

Both the authors declare that there are no conflicts of interest with respect to the publication of this research article.

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