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Cytotoxicity, apoptotic activity and phytochemical analysis of rhizome extract of *Amomum pterocarpum* Thwaites

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ABSTRACT

Cytotoxic and apoptotic effect of aqueous rhizome extract of *Amomum pterocarpum* was analysed using *Allium cepa* assay. *A. cepa* roots were treated with various concentrations (0.1%, 0.05%, 0.01% and 0.005%) of the extract for 24 h. Mitotic index and chromosomal aberrations were analysed. Several clastogenic and non clastogenic abnormalities were observed. Apoptotic effect was analysed using Evans blue test. In general a positive correlation has been observed between concentration of the extract and cytotoxic and apoptotic activities. Preliminary phytochemical analysis using standard methods showed the presence of bioactive constituents like alkaloids, steroids, flavonoids, terpenoids, phenols and tannins. The cytotoxic and apoptotic activities of *Amomum pterocarpum* rhizome extract thus reveals its therapeutic potential.

KEY WORDS: *Allium cepa, Amomum pterocarpum,* Apoptotic, Cytotoxic, Mitotic index.

1. INTRODUCTION

Amomum pterocarpum Thwaites is a rhizomatous herb which is included in the family Zingiberaceae. Members of Zingiberaceae have been attracted since long due to their culinary uses and biological and pharmaceutical activities (Pancharoen, 2000). Some species of *Amomum* are used as spices in traditional medicine for curing toothache, dysentery, diarrhoea, rheumatism, vomiting, lung diseases etc. *Amomum* also includes black cardamom which is used as a substitute for cardamom. Plants of this genus are remarkably pungent and aromatic. Even though numerous species of this genus have been used in traditional medicine in Asia for different purposes, little information is known on the biological activities of *A. pterocarpum.* This study thus tries to investigate the cytotoxic and apoptotic activities of the rhizome extract of *A. pterocarpum* on *Allium cepa* roots. The extract was also subjected to preliminary phytochemical screening to reveal its various phytoconstituents.

2. MATERIALS AND METHODS

Collection of plant material: Fresh plant material was collected from Vagamon, Idukki district, Kerala, India (geographical co-ordinates 9.6806°N, 76.8667°E; altitude 1100m above sea level).

Preparation of rhizome extract: Rhizome of the plant was washed thoroughly and chopped into small pieces. It was then shade dried and powdered. The extract was prepared with a mortar and pestle. 0.1% of the powdered material was dissolved in 100 ml distilled water to prepare the stock solution. Various concentrations of the extract (0.1%, 0.05%, 0.01% and 0.001%) were prepared in distilled water.

Cytotoxicity assay: The *Allium* test is a reliable screening procedure for chemicals and environmental agents which may represent environmental hazards. The adverse effects on chromosomes resulting in structural aberration provide an indication of likely toxicity (Akintonwa, 2009). Fresh and healthy onion bulbs were purchased. They were allowed to grow on sterilised soil. Onion bulbs with roots of length 1-2 cm were selected for assay. They were placed at the rim of the jar containing the extracts of different concentrations for 24 h in such a manner that the roots remain completely immersed. Distilled water was taken as negative control Methyl parathion as positive control. After the treatment, few healthy root tips were taken and washed thoroughly with distilled water. The roots were then fixed in modified Carnoy's fluid (1 acetic acid: 2 alcohol) for 1 h. The root tips were stored in 70% ethyl alcohol for further examination. Mitotic squash preparation was done using modified techniques (Sharma and Sharma, 1990). Root tips were treated with 1N HCl for 5 minutes for hydrolysis. The root tips were washed with distilled water and stained with 2% acetocarmine for 3 h. The roots were then de stained with 45% acetic acid, squashed and mounted on clean glass slides. Slides were screened for aberrations and data was taken from five fields which were selected randomly. The mitotic index (MI) was calculated as the number of dividing cells / the total number of cells in each field and shown as percentage. The percentage of chromosomal aberrations (CA) was calculated as the number of aberrant cells / the total number of cells counted. Slides were scanned under microscope (Olympus CX21 FSI, Japan) and photographs were taken using Amscope Mu1000 digital camera - FMA050 attached to the microscope.

Apoptosis assay: Apoptotic effect of the extract was analysed with Evans blue staining test. Onion bulbs with roots 4-5 cm were taken and washed thoroughly. They were placed at the rim of the jar containing the extracts of different concentrations for 24 h so that the roots remain completely immersed. Distilled water and Methyl parathion were taken as negative and positive control respectively. After the treatment, roots were washed thoroughly and were placed at the rim of the jar containing 0.25% (w/v) aqueous solution of Evans blue stain for 15 minutes (Baker and Mock, 1994). The roots were washed thoroughly with distilled water. Five stained roots with equal length were cut from each onion bulb and were transferred to test tubes with 3 ml N, N- dimethyl formamide for 1 h. The absorbance of Evans blue was read at 600 nm. All tests and analyses were done in triplicate and mean values were recorded.

Statistical Analysis: The data of MI and CA are represented in percentage mean ± SE. For statistical analysis, one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMR) (Duncan, 1955) were used. All statistical analyses were done by using the computer software SPSS 20.0 for Windows. Results with $p < 0.05$ were considered to be statistically significant.

Preliminary Phytochemical Analysis: Qualitative phytochemical tests were done for detecting the presence of alkaloids, phenols, tannins, flavonoids, terpenoids and cardiac glycosides by the following standard methods suggested by Trease and Evans (1983); and Harbourne (1983).

Test for Alkaloids: A small portion of the crude extract was dissolved in 5 ml 1% HCl on a steam bath. Dragendorff's reagent and Mayers reagent were then added separately. Formation of turbidity or precipitation was taken as the presence of alkaloids.

Test for Phenols and Tannins: Plant extract was treated with 2 ml of 2% $FeCl_3$ reagent. A blue-green or black colouration shows the presence of phenols and tannins.

Test for Flavonoids: A chip of magnesium metal strip was added to the crude extract and a few drops of concentrated HCl was added. The occurrence of a red or orange colouration indicates the presence of flavonoids.

Test for Terpenoids: Crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. After adding 2 ml of concentrated H_2SO_4 it was heated for about 2 minutes. Presence of greyish colour shows the presence of terpenoids.

Test for Steroids: The extract was dissolved in 3 ml $CHCl_3$ and then filtered. Concentrated H_2SO_4 was added to the filtrate to form a lower layer. A reddish brown colour indicates steroids.

Test for Cardiac Glycosides: The extract was dissolved in 2 ml glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was under laid with concentrated H_2SO_4. A brown ring in between the layers indicates the presence of a deoxy sugar, characteristic of cardiac glycosides. A violet ring may form just above this ring and gradually spreads through this layer.

Test for Saponins: Crude extract was added to 5ml of distilled water in a test tube and shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

3. RESULTS AND DISCUSSION

The rhizome extract considerably affected mitotic index and there is a positive correlation between the concentration of the extract and percentage of chromosomal aberration (Table.1). The highest concentration reduced the mitotic index to 18.20 % and the chromosomal aberration increased up to 24.92 %.

A broad spectrum of clastogenic and non clastogenic aberrations were observed. Nuclear lesions (Fig.1I) were very common in all the treatments. Lesions develop by the inhibitory action of phytochemicals on DNA biosynthesis which leads to the clastogenic effect of the extract (Akaneme and Amaefule, 2012). The lesions can also develop from the dissolution of protein components of the chromatin, induced by the extract (Udo, 2014). Pulverised chromatin was another common abnormality observed (Fig.1B). Pulverisation may be due to the premature condensation of chromosome as a result of the action of active phytochemicals present in the extract (Knuutila, 1981). C-mitosis, (Fig.1F) another abnormality, occurs by the inactivation of spindle formation which results in delay in the division of centromere. Micronuclei (Fig.1H) developed from lagging chromosomes. Nuclear erosion (Fig.1D) is another prominent aberration which occurs from the partial dissolution of nucleoproteins which become visible in the nucleus as erosion zones (Caetano-Pereira, 1999). Spindle disturbances which resulted in displacement and disturbances of chromosome at various mitotic stages were also visible. Diagonal metaphase is one such aberration (Fig.1G). Stickiness is another common abnormality (Fig.1C) noted which may be due to several causes like DNA condensation (Osterberg, 1984), depolymerization of DNA (Abraham and Koshy, 1979) and physical adhesion of chromosomal proteins (Patil and Bhat, 1992). It is interpreted that stickiness reflects highly toxic and usually irreversible effect that probably results in cell death (Liu, 1992; El-Ghameryet, 2000; Tipirdamaz, 2003). Evandri (2000) suggest that chromosome bridges and fragments (Fig.1E) are signs of clastogenic effects resulting from chromosome and chromatid breaks. Binucleated cell formation (Fig.1A) may be because of the inhibition of cytokinesis following telophase (Sutan, 2014). These anomalies can be considered as biomarkers of genotoxic events and chromosomal instability.

Apoptosis was detected by Evans blue staining method on the basis of its penetration to nonviable cells (Panda, 2011). Evans blue stain taken up by *A. cepa* roots indicates cell death. In higher concentration of plant extract (Fig.2) severe cell death was observed. Dose dependent intensity of the Evans blue stain of the *Allium* root tips indicates cell death induction due to cytotoxicity of the extract (Table.2). The concentration of the extract and the intensity of the dye absorbed is positively correlated (Fig.2A-F). Apoptosis was high at higher concentrations of the extract. Evans blue uptake as an apoptotic parameter is an indicator of cytotoxicity.

Phytochemical analysis of the rhizome extract showed the presence of alkaloids, phenols, tannins, flavonoids, terpenoids and cardiac glycosides (Table 3). The presence of various bioactive compounds in the plant reveals its therapeutic potential. The presence of compounds like alkaloids accounts for its cytotoxic and apoptotic activity. Their potential uses in reducing human cancer cell lines (Staerk, 2000) have been widely studied. The

present study thus reveals the cytotoxic potential of *A. pterocarpum* rhizome extract which can be used as a source for potential drugs.

Table.1. Mitotic index and chromosomal aberrations in *A. cepa* root tip cells induced by rhizome extract of *A. pterocarpum*

Treatment duration (h)	Concentration (%)	Mitotic index (Mean±S.E.)	Abnormalities (Mean±S.E.)
24 h	C--	71.49±1.60^{d}	0.000 ^{a}
	C++	12.08±1.75^{a}	62.18 ± 8.55^{d}
	0.005	31.79±6.29 ^{c}	14.81 ± 3.25^{b}
	0.01	27.52±2.76 ^{b,c}	18.00 ± 0.55 ^{c,b}
	0.05	24.70±1.90 ^{b,c}	22.28 ± 2.23 ^{c,b}
	0.10	18.20±4.67 ^{a,b}	24.90 ± 9.01 ^{c}

C-- = Negative control; C++ = Positive control

Each value (mean ± S.E.) represents mean of five replicates. Means in a column followed by the same superscript letters are not significantly different (p< 0.05, one way ANOVA, DMR test).

Table.2. Cell death in *A. cepa* roots visualised by Evans blue Staining

Concentration %	A 600	Concentration %	A 600	Concentration %	A 600
C--	0.077±0.01 ^{a}	0.005	0.097±0.01 ^{a,b}	0.05	0.143±0.03 ^{b}
C++	0.903±0.01 ^{c}	0.01	0.120±0.01 ^{a,b}	0.10	0.153±0.02 ^{b}

C-- = Negative control; C++ = Positive control

Each value (mean ± S.E.) represents mean of three replicates. Means in a column followed by the same superscript letters are not significantly different (p< 0.05, one way ANOVA, DMR test).

Table.3. Phytochemical screening of rhizome extract of *A. pterocarpum*

Chemical Test	Result	Chemical Test	Result	Chemical Test	Result
Alkaloids	+++	Saponins	++	Phenols	++
Flavonoids	++	Steroids	+++	Terpenoids	++
Cardiac glycosides	+++	Tannins	++		

Key: ++ = Moderate, +++ = Abundant

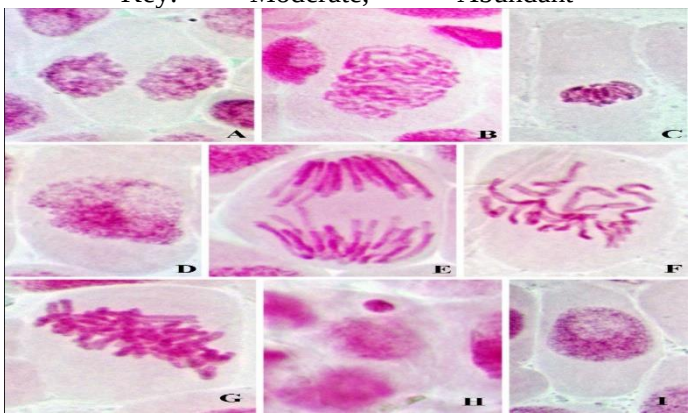

Fig.1. Chromosomal aberrations induced by *A. pterocarpum* extract in root tip cells of *A. cepa*.

A) Binucleate cell, B) Pulverised chromatin at prophase, C) Sticky metaphase, D) Nuclear erosion at interphase, E) Chromosomes bridge at anaphase, F) C metaphase, G) Diagonal metaphase, H) Micronucleus at interphase, I) Nuclear lesion at interphase.

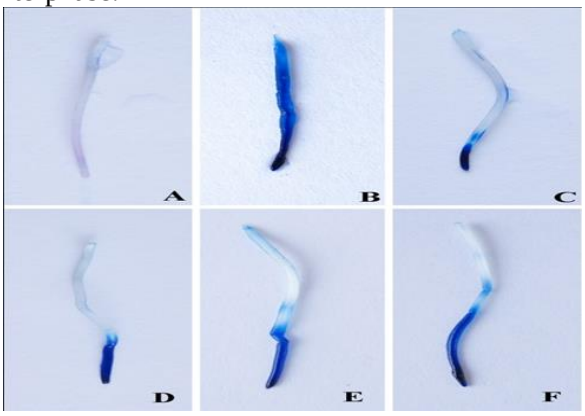

Fig.3. A–F Apoptosis induced in root tissues of *A. cepa* treated with different concentrations of plant extract

A) Negative control B) Positive control C) 0.005% D) 0.01% E) 0.05% F) 0.1%

4. CONCLUSION

The results of the present study proved that the extract have cytotoxic and apoptotic potential. Phytochemical analysis revealed the presence of bioactive compounds which may be responsible for this activity. Further study and isolation of compounds are necessary for determining the specific bioactive compounds accountable for the cytotoxic potential. In addition, further studies in animal model may reveal the efficacy of *A. pterocarpum* in cancer treatments.

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