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Screening of cytotoxicty, metabolic inhibition and possible apoptotic cell death induced by *Gomphostemma heyneanum* Wall. ex Benth. var. *heyneanum* using *Allium cepa* root tips

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ABSTRACT

Present study was carried out to examine the cytotoxic potential of aqueous extract of Gomphostemma heyneanum var. heyneanum and its ability to affect mitochondrial activity and membrane integrity of the cells using meristematic root tips of Allium cepa. Cytotoxic screening revealed the mitodepressive ability and caused many clastogenic and aneugenic aberrations like nuclear lesion, nuclear budding, micronucleus formation, cytoplasmic shrinkage, giant cell formation, C-metaphase, chromosome bridge at anaphase etc. were noticed many of which were signs of apoptotic induction caused by treatment with plant extract. Mitochondrial activity screened using TTC staining showed the adverse effect of extract on mitochondrial respiratory enzymes and the reduction in metabolic activity in treated roots. Evans blue test evaluated the cell viability and cell death caused by the treatment in Allim cepa root tips, revealing the membrane damage potential of aqueous extract of G. heyneanum var. heyneanum. Cytogenetic aberrations with apoptotic signs together with reduction in metabolic activity due to mitochondrial disfunction and cell death caused by the membrane damage are all indications of possible apoptotic potential of G. heyneanum var. heyneanum. These effects can be effectively confirmed by further in vitro and in vivo studies and can be targeted as a candidate in cancer treatment.

KEY WORDS

Gomphostemma heyneanum var. heyneanum, Allium cepa assay, Apoptosis, Chromosome abnormalities, Cytotoxicity.

INRODUCTION

Plants have always been a source for treating many ailments and are likely to be a major source for the construction of novel compounds with enhanced biological properties. Plant derived compounds have an additional advantage that they have less side effects as compared to synthetic drugs. Secondary metabolites are attributed for the wide array of biological and pharmacological properties exhibited by the plants [1]. A large number of plant derived natural compounds are used for treating many pathological conditions including cancer and many more are in clinical trials. Cancer is regarded as the leading cause of morbidity and mortality worldwide. Secondary plant compounds have great potential for cancer therapy and can play an important role in chemo prevention [2].

The Allium cepa test is a simple and reliable method to record chromosomal aberrations in mitotic cells [3]. It is as an excellent assay for assessing chromosomal damage and is a sensitive test that has been used for the determination of anti-mitotic, cytotoxic, and, or genotoxic effects of various chemical substances and herbal extracts [4,5]. Naturally occurring cytotoxic

compounds have been considered to be a valuable tool of lead compounds in the development of potential anticancer drugs [6].

Remarkable advances in cancer biology and cancer genetics have led to the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. Studies have shown that some oncogenic mutations can disrupt apoptosis, leading to tumor initiation, progression or metastasis. Rational strategies that can manipulate cell suicide programs will help to produce new therapies that are less toxic and mutagenic than current treatment regimens [7]. Studies have shown that plant derived compounds can induce apoptotic pathways that are blocked in cancer cells and induction of apoptosis is considered as an important marker of cytotoxic antitumor agents [8, 9].

Lamiaceae, the mint family has a rich tradition of being used in various ethano-medicinal preparations because of its curative and preventive properties. *Gomphostemma heyneanum* var. *heyneanum* belonging to Lamiaceae is a less exploited plant used by the tribes of Wayanad, Kerala for treating rheumatism, dysentery and diarrhoea. It is a sub shrub with four angled stems and densely covered with stellate hairs. Flowers are in racemes, yellow coloured and thickly packed.

Present study was designed to evaluate the clastogenic and aneugenic effects induced by aqueous extract of *Gomphostemma heyneanum* var. *heyneanum* using Allium cepa assay. Mitochondrial activity which reflects the cell viability and metabolic inhibition was examined using the 2, 3, 5-triphenyl-tetrazolium-chloride (TTC) assay. Loss of cell viability due to compromises made to cell membrane integrity was studied using the Evans blue staining method.

MATERIALS AND METHODS

Plant material:

Aerial parts of *Gomphostemma heyneanum Wall. ex Benth. var. heyneanum* were collected from Nelliyampathy Forest Area (coordinates 10° 33' 19.9'' N 76° 42' 51.8'' E). The specimen was authenticated and voucher specimen (CALI 123772) was deposited in the Herbarium of Department of Botany, University of Calicut.

Test system and chemicals:

Healthy Onion bulbs (*Allium cepa* L., 2n=16) free from pesticides, procured from TNAU were used for the present study. Chemicals used were Evans blue (Himedia), TTC (Himedia) and N, N-dimethylformamide (Himedia).

Preparation of test solution and control for the studies:

Fresh aqueous extract of *Gomphostemma heyneanum* var. *heyneanum* was prepared using mortar and pestle. Stock solution of one percentage was prepared and various concentrations (0.005, 0.01, 0.05 and 0.1%) were prepared by subsequent dilution using distilled water. Distilled water and Methyl parathion (0.01%) served as negative and positive control respectively.

Allium cepa assay:

Healthy and uniform sized onion bulbs were selected for the study. Outer scales were removed to facilitate root formation and were placed in sterilized sandy soil for germination. Germinated onion bulbs with healthy roots of about 1-2 cm were selected and washed in distilled water. Rooted onion bulbs were then placed in the rim of vials containing various concentrations of the extract (0.005, 0.01, 0.05 and 0.1%) and controls in such a way that only the root tips remained immersed in the solution. Different time intervals (1/2, 1, 2 and 3 hours) were assigned for the treatment and after which root tips were collected and fixed in modified Carnoy's fluid for 1 hour. Squash preparations were made according to standard protocol [10]. Preparations were viewed under Leica DM2000 microscope and photomicrographs were taken using the top mount camera Leica DMC2900. Mitotic index and percentage of abnormal cells induced were the parameters considered for the cytotoxic studies and were calculated as follows:

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

$$\text{Chromosome aberration \%} = \frac{\text{Number of aberrant cells}}{\text{Total number of cells}} \times 100$$

TTC Assay:

Germinated onion bulbs were exposed to treatment and control as mentioned earlier for 24 h. Ten root tips each were excised from treatments and controls, then they were immersed separately in 0.5% 2, 3, 5- triphenyl tetrazolium chloride (TTC) at 35±1°C for 15min in dark. The root tips were subsequently rinsed with distilled water and coloured triphenyl formazan complex formed were extracted from roots using 95% ethanol and absorbance was measured at 490nm.

Evans blue staining:

Evans blue staining method is an effective method to study the loss of cell viability. In the present study, germinated onion bulbs were subjected to different treatments and control as mentioned previously for a period of 24 hrs. Control and treated roots were subsequently stained using 0.25% (w/v) of aqueous Evans blue for 15min, followed by washing in distilled water for 30 min. Ten root tips of almost equal size were excised and soaked in 3ml. of N,N-dimethylformamide for 1hour at room temperature to release Evans blue. The absorbance of of released Evans blue was then measured at 600nm.

Statistical analyses:

Statistical analyses were performed using SPSS 20 (SPSS Inc., Chicago, IL, USA). The data obtained from the experiments were subjected to one-way ANOVA and in order to validate the results, Duncan's multiple range test was performed. The results are expressed as mean ± SE and $P < 0.05$ was considered statistically significant.

RESULT

Cytotoxic assay using *Allium cepa*

Present study evaluated the cytotoxic potential of *G. heyneanum* var. *heyneanum* using *Allium cepa* root tips. Mitotic activity in terms of mitotic index was one of the parameters taken for cytotoxic evaluation.

Table 1. Effect of different concentrations of aqueous extracts of *G. heyneanum* var. *heyneanum on Allium cepa* root meristem at different time periods.NC: Negative control; PC: positive control, SE: Standard error. Means with the same superscript letter within the column do not differ statistically at 0.05 level (P < 0.05; DMRT test).

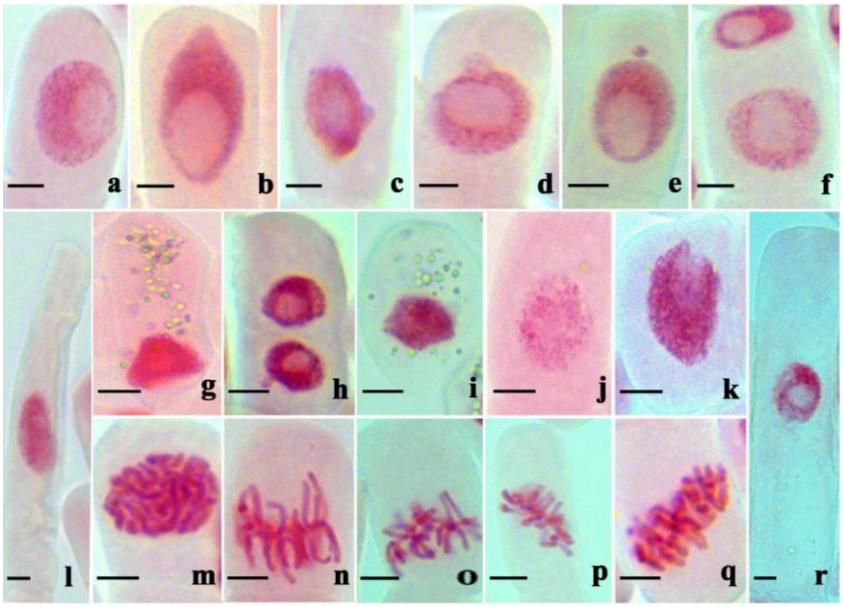
Treatment duration (hrs.)	Concentration (%)	Mitotic Index %±SE	Abberation %±SE
1/2	NC	92.53±3.31^{a}	0^{a}
	PC	13.00±1.36^{b}	98.20±1.53
	0.005	87.86±1.17^{b}	25.66±0.66^{a}
	0.01	85.96± 1.24$^{a, b}$	27.70±0.81^{a}
	0.05	84.16±1.24$^{a, b}$	27.23±0.52^{a}
	0.1	82.40±1.45^{a}	30.30±1.00^{b}
1	NC	92.93± 4.62^{a}	0^{a}
	PC	8.86±1.03^{a}	98.36 ±1.30^{b}
	0.005	83.63±0.81^{c}	27.0333±0.69^{a}
	0.01	81.36±1.10^{c}	31.1667±0.54$^{b, c}$
	0.05	79.16±0.89$^{a, b}$	29.6000±0.40^{b}
	0.1	78.10±0.87^{a}	32.9000±0.72^{c}
2	NC	92.30±3.31^{a}	0^{a}
	PC	8.73±1.12a	98.69±0.80
	0.005	81.80±0.72^{d}	31.36±1.01^{a}
	0.01	76.53±0.90^{c}	33.16±0.98^{a}
	0.05	72.06±0.31^{b}	36.83±1.14^{b}
	0.1	69.66±0.55^{a}	40.53±0.69^{c}
3	NC	90.10±5.52^{a}	0^{a}
	PC	7.70±1.02^{a}	99.16±0.64^{a}
	0.005	68.40±0.55^{c}	35.50±0.98^{a}
	0.01	67.03±0.82^{b}	39.56±0.71^{b}
	0.05	63.40±0.49^{a}	42.93±1.39^{c}
	0.1	62.06±0.54^{a}	47.66±0.64^{d}

Table 1 summarizes the cytological effect of plant extract on mitotic index and chromosome abberations. From the results it can be interpreted that the plant extract has significant cytotoxic potential as the mitotic index reduced and chromosomal aberrations increased in a dose and duration dependent manner. Among the

different concentrations of plant extract tested, maximum cell division (87.86±1.17) was with lowest concentration (0.005%) and mitotic index was minimum (62.06±0.54) at highest concentration (0.1%) for 3hrs treatment. Reduction in mitotic index can be taken as an indication of mitodepressive and cytotoxic potential of the plant extract.

Mitotic aberration percentage was the second parameter considered for evaluating the cytotoxic potential. In the case of treatments with different concentrations of plant extract, high percentage of mitotic aberration (47.66±0.64) was with highest concentration (0.1%) and at longest duration (3hrs) whereas minimum (25.66±0.66) was noticed with lowest concentration (0.005%) for shortest duration. Treatments with distilled water which served as negative control showed maximum mitotic index with no aberrations, but positive controls exhibited a high percentage of aberration with minimum mitotic activity. An array of mitotic aberrations was noticed in the present study which could be classified into clastogenic and aneugenic aberrations as shown in Fig. 1 and Fig. 2. Major clastogenic aberrations noted were nuclear lesions, nuclear budding, nuclear peak, giant cells, pulverized chromatin, coagulated chromatin and chromosome bridges. Aneugenic aberrations which mostly result from spindle disturbances were reflected in the form of lagging chromosomes, anaphase bridges, shift in MTOC, formation of micro and macro cells, diagonal anaphase, stathmo-anaphase and micronucleus formation.

Fig. 1. (a). Single nuclear lesion (b). Nuclear peak formation with lesion (c). Nuclear peak (d). Nuclear budding and lesion (e). Micronucleus (f). Macro and micro cells with lesions (g). Coagulated chromatin (h). Binucleate cell with nuclear lesion and erosion (i). Bizarre nucleus (j). Pulverized chromatin showing apoptosis (k). Nuclear erosion (l). Cytoplasmic shrinkage in giant cell (m). Ball metaphase (n). Sticky metaphase in hypoploid cell (o). Partial C-metaphase (p). Diagonal C-metaphase (q). Sticky metaphase with chromosome gaps (r). Giant cell with nuclear lesion. *Bar* = 10 µm



The overall result thus suggests that the plant extract has both turbagenic and mitodepressive potential. The reduction in mitotic index and wide spectrum of mitotic aberrations observed throughout the cytotoxic studies are indicative of cell death with apoptotic symptoms.

TTC staining

TTC staining was employed in the present study as an indicator to evaluate the effect of plant extract on mitochondrial metabolism. In TTC staining 2, 3, 5-triphenyl tetrazolium chloride is reduced to red formazan by mitochondrial enzymes [11]. The result showed a dose dependent decrease in mitochondrial activity as visualised by decrease in staining and absorbance (Fig. 3). Positive control remained unstained with minimum absorbance indicating least mitochondrial activity and negative control showed maximum activity. In the case of treatments, root tips treated with highest concentration (0.1% for 24 hrs)

exhibited low staining and least absorbance indicating mitochondrial dysfunction and adverse effect of plant extract on mitochondrial function.

Fig. 2. (a). Chromosome bridge at anaphase (b). Chromosome bridges at coagulated anaphase (c). Chromosome bridge at sticky statmoanaphase (d). Diagonal anaphase (e). Diagonal anaphase with multiple bridges (f). Equitorial separation showing chromosome bridge at anaphase (g). Shift in MTOC at telophase (h). Sticky telophase (i). Chromatin erosion at cytokinesis (j). Coagulated chromatin at cytokinesis (k). Coagulated chromatin with nuclear lesion at late cytokinesis (l). Giant cell with chromosome bridge at anaphase (m). Nuclear lesion at cytokinesis (n). Nuclear peak formation at early cytokinesis (o). Nuclear erosion at cytokinesis (p) Sticky chromatin with persistent bridge at cytokinesis (q). Sticky chromatin at early cytokinesis. *Bar* = 10 µm

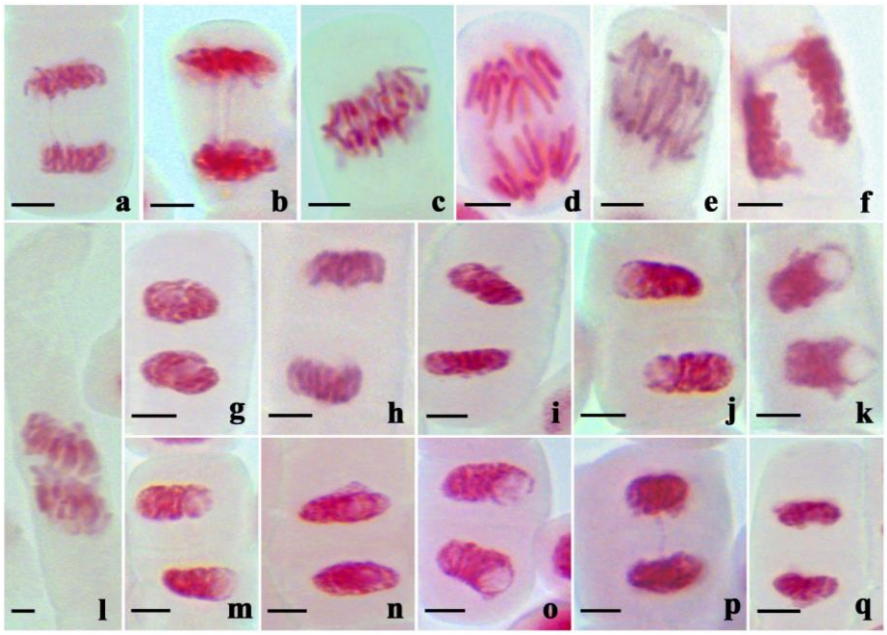
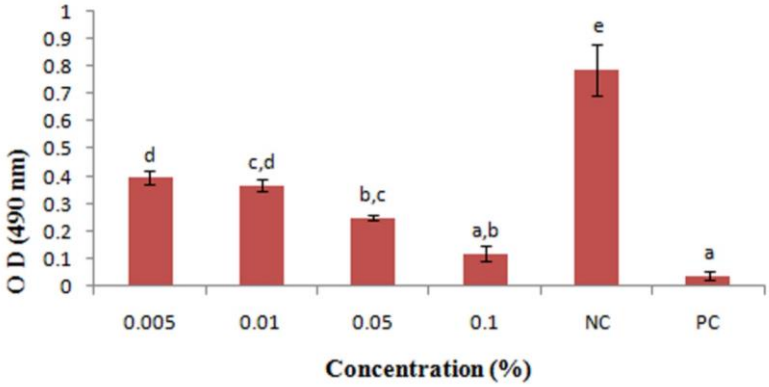


Fig. 3.TTC staining - Graph showing the effect of *G. heyneanum* var. *heyneanum* aqueous extract on mitochondrial activity. (NC) Negative control - Distilled water; (PC) Positive control - 0.1% Methyl parathion. Mean value with in graph followed by the same letters are not significantly different at P < 0.05 as determined by Duncan's multiple range tests.



Evans blue staining

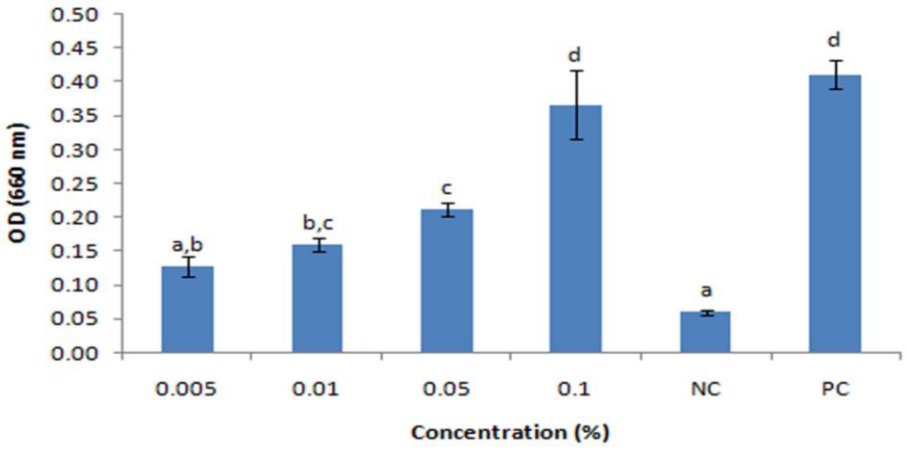
Evans blue staining is a dependable stain to determine cell viability and an indicator of plasma membrane integrity. Dead cells which have lost their membrane integrity take up more dye and become intensely coloured. N, N-dimethyl formamide serve as an

excellent solvent to release dye from the cell and the absorbance of Evans blue containing solvent with spectrophotometer helps to quantify the amount of dye absorbed.

Root tips treated with positive control became intensely stained whereas those with negative control did not take up any stain. A gradation in the intensity of dye absorbed by the treated root meristems which in turn is a measure of cell death is shown in Fig. 4. Spectrophotometric determination of cell death showed that cell death is more with root tips treated with higher concentration (0.1%), suggesting a dose dependent increase in absorbance value.

It can thus be inferred that the plant extract has an antagonistic effect on both cell viability and membrane integrity. It is interesting to note that loss of membrane integrity is a hall mark of apoptotic cell death and the study clearly depicts the ability of plant extract to induce cell death evoking apoptotic symptoms.

Fig. 4. Evan blue staining - Graph showing the effect of *G. heyneanum* var. *heyneanum* aqueous extract on cell viability. (NC) Negative control - Distilled water; (PC) Positive control - 0.1% Methyl parathion. Mean value with in graph followed by the same letters are not significantly different at P < 0.05 as determined by Duncan's multiple range tests.



DISCUSSION

Present study displayed the mitodepressive action of *Gomphostemma heyneanum* var. *heyneanum* and its ability to induce spindle disturbances. The lowering of mitotic index may be attributed to inhibition of DNA synthesis at S-phase [12].

Many serious mitotic aberrations were noticed during present investigation. Among these, nuclear lesions were common in almost all treatments using different concentrations of plant extract. The nuclear lesions are indication of nuclear poison [13]. Nuclear lesions offer cytological evidence for inhibitory action of DNA biosynthesis [14]. Binucleate formation may be due to the inhibition of phragmoplast formation during cytokinesis [15]. The phytochemicals in the plant extract may be interfering with cytokinesis process and is responsible for the inhibition of phargmoplast formation resulting in binucleate cells.

Apoptosis has been described as a form of cellular suicide because death appears to result from induction of active processes within the cell itself. The dying cell then undergoes rapid changes, which are reflected in both its structure and biochemistry. One of the morphological characters manifested by apoptotic cell is cytoplasmic shrinkage [16] and it was prominent in many treated cells. Giant cells are formed when the cell division becomes affected especially in the S phase followed by mitotic arrest and induction of cell expansion which results in larger cell size [17]. Giant cells are subjected to various kinds of stress and normally cannot cope with the enormous size of the protoplasm thus they are liable for injury [18]. Nuclear budding observed may be assumed to be derived from anaphase laggards that independently form a nuclear envelope in telophase before integrated into the nucleus or from remnants of broken anaphase bridges [19].

Ball metaphase is formed by the fusion of chromosomes into a compact group in the middle of the cell which may be due to the toxicity of the compounds in plant extract [20]. Sticky chromosomes observed are presumably due to intermixing of chromatin fibres resulting in

subchromatid connections between chromosomes [21]. Chromosome stickiness is irreversible and may cause cell death which may be attributed as one of the reason for decline in mitotic index [22].

Micronuclei, one among the serious aneugenic aberrations noticed are the results of acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of the mitotic cells. Prominent anaphase bridges were observed as a major aberration throughout the study. Bridge formation could be due to chromosomal breaks and reunion of broken ends of chromosomes [23]. Chromosome bridges may lead to a number of different outcomes, including chromosome breakage, polyploidy, aneuploidy, and possibly cell cycle arrest [24]. This shows that the plant extract is a clastogen that can induce chromosome breaks and it can also act as an aneugen that induces lagging chromosomes [25].

The inability of cells and microorganisms to reduce the colourless electron acceptor triphenyltetrazolium chloride (TTC) to a red formazan precipitate is commonly used as a means of screening for cells that have a dysfunctional respiratory chain [26]. In the present study, decrease in TTC staining and corresponding decrease in absorbance measured, manifests the toxic effect of *G. heyneanum* var. *heyneanum* on mitochondrial activity. Mitochondria have several functions in the cell, including energy production, calcium buffering, and regulation of cellular apoptosis [27]. The mitochondria have many proteins and activation of these sets into motion programmed cell death pathways that result in the demise of the cell. In many of these pathways, permeabilization of mitochondrial membranes is a critical event that results in release of various molecules from the mitochondrial intermembrane space that are crucial for apoptosis [28]. Thus, a variety of key events in apoptosis focus on mitochondria, including the release of caspase activators, changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation-reduction, and participation of pro- and antiapoptotic Bcl-2 family proteins, all of which later leads to mitochondrial dysfunction [29].

Evans blue staining was employed to detect the loss of membrane integrity. Following apoptotic trigger, a chain of events leads to cell shrinkage, membrane blebbing, nuclear condensation, loss of membrane integrity and enzymatic DNA degradation [30]. It could be presumed that nuclear changes occurring as a part of apoptosis followed by loss of membrane integrity is the reason for the uptake of Evans blue stain by root cells indicating the loss of viability of the cells.

Cytotoxic effect of *Gomphostemma heyneanum* var. *heyneanum* extract manifested by its mitodepressive, clastogenic and aneugenic activity together with loss of plasma membrane integrity as portrayed by Evans blue staining and mitochondrial dysfunction depicted by TTC staining, points towards the possible apoptotic potential of the plant extract. Earlier studies have reported the presence of various phytochemicals like phenols, terpenoids, saponins *etc*. in *G. heyneanum* var. *heyneanum* [31, 32]. These phytochemicals may be responsible for the cytotoxic and apoptotic symptoms induced by the plant extract in *Allium cepa* root meristem.

CONCLUSION

Cytotoxicity assays and assessment of membrane integrity are basics in screening anti-cancer activity, Present study revealed the cytotoxic potential of *Gomphostemma heyneanum* var. *heyneanum* as evident from the chromosomal abnormalities and mitotic inhibition induced by different concentrations of plant extract. Loss of membrane integrity and metabolic inhibition together with cytotoxicity and mitotic inhibition caused by plant extract can be taken as symptoms of apoptotic cell death induced by treatment. However more in-depth and detailed studies are needed to clarify the apoptotic potential and possible anti-cancer activity of *G. heyneanum* var. *heyneanum*, which can then be exploited effectively as anti-proliferative agent in cancer treatment.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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