Assessment of *Punica granatum* L. extract on the mitotic arrest of plant bioassay system.

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Abstract: Analysis of mitotic process and description of chromosome aberrations are important aspects in plant genetics to assess the effect of medicinal plant extract. ***Ethnopharmacological relevance*:** *P. granatum* extracts have been reported to exert multiple health effects, *e.g.* anti-inflammatory, antibacterial, and antidiarrheal, etc. However, the antiproliferative effects were not as extensively studied, including those of protocatachuic acid, a major constituent of the extract. This *work aims at* analyzing the constituents of the acetone extract of the plant and testing its antiproliferative effects. ***Material and Methods*:** Fingerprint of *Punica granatum* L. polyphenol acetone extract was analyzed using HPLC, UV-detection. *Allium cepa* L. root meristem was used as bioassay system. The study groups included 3 concentrations of the extract (0.1, 0.5, 0.75/ 100 mL) with 3 exposure times for each (3, 6 and 12 hours) and control for each treatment. For every experiment, 5 samples with 1,000 cells were studied. Effects on cytological and ultrastructure assays were done. As well as biochemical and molecular analyses (estimation of total protein content, DNA ladder, detection of nuclease activity) were done. ***Results*:** HPLC authentication of the extract revealed the presence of 15 fractions, of which protocatechuic acid, O-H Benzoic, chlorogenic acid, phenol and quarecetin were quantified. The application of acetone extract resulted in changes in ultrastructure of all organelles, reverse the viability of meristem cells and induce programmed cell death. The inhibitory effect of acetone extract was found to be dose and exposure time dependent. The mitotic process was associated with metaphase arrest and the disappearance of telophase stage that abolish the continuity of mitotic activity. There was a significant increase in nuclease activity enzymes, which in turn enhanced the cleavage of DNA to induce a typical ladder form. ***Conclusion*:** The total polyphenol extract of *Punica granatum* L. reprogram *Allium cepa* L. meristematic cells resulting in programmed cell death.

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Key words: metaphase arrest, DNA ladder, HPLC fractions, disintegrated nuclear envelope.

1. Introduction

Mitosis is a mechanical event of the duplicated chromosomes in a mother cell that are precisely divided into two identical daughter cells. This process follows five stages and is terminated by cytokinesis. The cells should pass through two checkpoints before entering into mitosis. First is the G2 checkpoint in response to DNA damage, (**Harper and Elledge 2007**), followed by the antephase checkpoint that is activated to prevent mitotic defects in response to a number of stresses, changes in chromatin structure and spindle damage before the cells reach the point of 'no-return' **(Chin and Yeong 2010).** The antephase checkpoint which was first introduced by **Bullough and Johnson (1951)** exists between the G2 and early prophase **(Matsusaka and Pines, 2004).** The cell cycle control system is arrested when cells detect DNA damage. Meanwhile, the premature onset of mitosis leads to another type of cell death that is mitotic catastrophe (**Bucher and Bitten 2008**). Mitotic catastrophe occurs either during or after mitosis **(Vitale *et al*., 2011).** A new type of cell death that takes place at metaphase is defined as metaphase fragmentation where condensed chromosomes lose viability and are progressively degraded **(Heng *et al*.,2004** and **Touati *et al.*, 2013).** Chromosome fragmentation does not exhibit the typical oligosomal DNA degradation of apoptosis **(Stevens *et al*., 2010).** Cell death is the consequence of inappropriate cell development with different paths.

Medicinal plants with multimethods of extraction have been of a great interest in research in cell biology. *Punica granatum* L. (pomegranate) has a widespread ethno medical uses, and is extensively used in traditional medicine for its rich and multifarious active constituents which may possess various pharmacological and toxicological activities. Pomegranate extract was used in multipurpose for its antioxidant properties (**Garcea *et al*., 2005** and **Bekir *et al.*,2013);** anti-inflammatory effect **(Ismail *et al.*, 2012** and **Bekir *et al*.,2013);** antibacterial effect **(Vasconcelos *et al*., 2006** and **Quattrucci *et al.*, 2013**); in carcinogenesis **(Hong MY. *et al*.,2008** and **Joseph *et al.*,2013);** and for its potential to arrest the cell cycle progression **(Nair.*et al*., 2011** and **Aqil *et al.*,2012**).

In the present study we investigate the potency of *Punica guanatum* L. polyphenol extract to perturb the cell cycle progression in the plant bioassay *Allium cepa* L. leading to a programmed death of the cells. The morphological characterization of chromosome abnormalities and the description of different hallmark involved in the process of plant cell death indicate a positive correlation between depression in the mitotic index, metaphase arrest, the disappearance of cytokinesis, alteration in ultrastructure of cell organelles and formation of DNA laddering. Depression in mitotic activity in consequence of certain biochemical defeats and specific cellular alteration and their cytological analysis are discussed in the present investigation.

2.Materials and Methods

Preparation of total polyphenol extracts (Makris and Kefalas, 2004)

A weight of 100 g of air dried pericarps of *Punica granatum* L. were repeatedly extracted in 300 ml of 70% acetone at 40°C. The collected extract were filtered and concentrated under reduced pressure (using Rotavap inustrument).The tested concentrations were obtained by dissolving 0.1, 0.5 and 0.75 g of each extract in 100 ml distilled water giving concentrations of 0.1% (1000 µg/ml), 0.5% (5000 µg/ml) and 0.75% (7500 µg/ml).

Fingerprint of phenolic compounds using HPLC

A weight of 0.1 g dried extract of *Punica granatum* L. total polyphenol extract was extracted in 3 ml of spectral grade methanol and filtered through a 0.2 µm membrane filter prior to HPLC analysis according to **Tuzen (2003)** and the modification according to the unpublished PhD thesis of ***Soliman, (2002)***. HPLC analysis was performed on a Hewlett-Packard HPLC (Model 1100) using a hypersil C18 reversed-phase column (250 x 4.6 mm) with 5 µm particle size. A constant flow rate of 1 ml/ min was used with two mobilephases: (A) 0.5% acetic acid in distilled water at pH 2.6, (B) 0.50% acetic acid in 99.5% acetonitrile. The elution gradient was linear starting with (A) and ending with (B) over 35 min, using UV detector set at wavelength 254 nm. Phenolic compounds of each sample were identified by comparing their relative retention times with those of the standards mixture chromatogram. The concentration of each compound was calculated on the basis of peak area measurements.

Cytological studies.

Five bulbs of *Allium cepa* L. bulbs (var. Giga 6 Mohassan) were used for each concentration. *Allium* bulbs were germinated in tap water at room temperature. When the roots reached 3-4 cm, they were treated with the tested total polyphenol extracts of *Punica granatum* L. for 3, 6 and 12 h for each concentration, control roots were simultaneously soaked in tap water then the roots were detached, fixed in Carnoy's fixative (ethyl alcohol: glacial acetic acid 3:1 (v/v)) for 24 hours, repeatedly washed in water, then Feulgen's squash technique was carried out (***Colman 1938***). Six temporarily slides were prepared for each treatment and control. At least 1000 cells per slide were examined under 40x magnification for mitotic analysis. The number of total cells in the mitotic division was scored and the percentage of cell division was calculated (MI), percentage of mitotic phases, percentage and type of abnormalities in each mitotic phases. The most common abnormalities were pictured by microphotography. The significance between the mean results and control was determined by (ANOVA) test in the statistical analytical system (SPSS) software.

Ultrastructure Studies

Tissue specimens of treated and untreated *Allium cepa* L. root tip (about 1mm) were fixed in 2% glutaraldehyde in 0.05 mol/L sodium cacodylate buffer (pH 6.9) for 2 h at 4°C. The tissue was rinsed in sodium cacodylate buffer then postfixed in 4% osmium tetraoxide for 1 h at 4°C. The dehydration process was carried out through a graded of ethanol water series, then a graded of propylene oxide/ethanol series. The root tips were gradually infiltrated with resin by placing them for 24 h in each of series of resin/propylene oxide mixtures, followed by three changes in 100% Epoxy resin. Finally, materials were embedded in freshly prepared resin mixture and polymerized in oven at 60°C for 48 h. ultrathin sections (0.1 µm) were cut using ultra microtome, mounted on copper grids and stained with 0.5% uranyl acetate for 30 min and lead citrate for 30 min as described by **Reynolds, (1963)**. Observations were carried out using Philips Electron Microscope, Electron Microscope center, Faculty of Medicine, Ain Shams University.

Biochemical and Molecular Analysis

Estimation of total protein content in treated and untreated *Allium cep* L. roots was carried out using 0.8 g of fresh tissue. The tissues were powdered in liquid N_2 to a fine powder. Total soluble protein were extracted by 1 ml 80% ethyl alcohol, precipitated for 15 min at 4000 rpm at -5°C and dissolved in 1 ml phosphate buffer (pH 7.0). The protein extract were stained using Coommassie Brilliant Blue (G-250 Sigma) and the absorbency was recorded at 595 nm according to **Bradford, (1976)** method.

DNA ladder formation assay

To examine DNA fragmentation as a marker of programmed cell death, genomic DNA was isolated by CTAB reagent from *Allium cepa* L. roots treated with *Punica granatum* L. total polyphenol extract and

untreated cells using the method of **Isaac, (1994)**. *Allium cep* L. roots (0.3 g) were powdered in liquid nitrogen. Each sample was incubated for 60 min at 65°C in 9 ml prewarmed CTAB extraction buffer, then mixed with an equal volume of chloroform–isoamyl alcohol (24:1). After gentle shaking for 5 min, the mixture was centrifuged for 15 min at 10000 rpm. The chloroform–isoamyl alcohol extraction was repeated when necessary. DNA was recovered by centrifugation for 10 min at 10000 rpm, followed by washing with 70% ethanol, and dissolved in 1ml TE buffer. To detect DNA fragmentation, samples were run on a 1% (wt/vol) agarose gel and stained with 0.5 µg of ethidium bromide per ml **(Tada *et al.*, 2001)**. The bands were visualized under UV transilluminator then were photographed by Polaroid camera for further documentation using Gel Pro Analyzer version 3.1 for windows95-NT (Media Cybernetics 1993-1997).

Detection of nuclease activity

Nuclease activity was assayed by measuring the release of acid-soluble material from denatured calf thymus DNA following the method of **Blank and Mckeon, (1989)**. Assay mixtures containing DNA calf thymus, NaCl and $ZnCl_2$ and root samples were incubated for both 10 min at 37°C. After addition of 2 ml of 15% perchloric acid, suspensions were held on ice for 10 min, then centrifuged for 15 min at 2000 rpm. The absorbency was recorded at 260 nm.

$$\text{Units /ml} = \frac{A_{260} \text{ of sample} - A_{260} \text{ of blank X dilution X } 1242}{10}$$

Determination of cell viability

Cell death was assessed by membrane permeability of dead cells with propidium iodide and indicated by the intense red fluorescence which was determined by fluorescence microscope according to **Harris and Oparka, (1994).** Briefly, treated and control root tip sections were simultaneously stained with 0.5 mg/mL propidium iodide (PI) in water for 5 min at 37°C and immediately observed by epifluorescence microscopy using a fluorescence microscope (Olympus BX50 Flourescent Microscope,). The microscope was fitted with a green filter set for PI fluorescence, while viable cells of control were stained in a drop of propidium iodide and were observed using phase contrast microscope.

Aim of the work

The present work is conducted to particularize the initiation of programmed cell death and make a procedural study to define the eligible hallmark accompanied with decline in number of cells entering mitosis assessed by the potential of *Punica granatum* extract.

3. Results

Table1. Mitotic and phase index and percentage of mitotic abnormalities of *Allium cepa* L. root tip cells treated with *Punica granatum* L. total polyphenol extract

Treatment	Total No. of cells	Mitotic index ± SD	% of Mitotic Abnormalities	Prophase	Metaphase	Anaphase (+Telophase)
Control						
3 hrs.	5032	3.80 ±0.53	8.30	17.04	42.75	40.21
6 hrs.	5076	4.26 ±0.70	8.80	28.45	37.25	34.30
12 hrs.	5051	4.42 ±0.23	9.23	32.10	35.66	32.24
0.1g/100ml						
3 hrs.	5037	2.83* ±0.13	13.60*	24.00	45.60	30.40
6 hrs.	5012	1.68** ±0.25	44.00**	20.90	50.30	28.80
12 hrs.	5025	1.15** ±0.34	29.55**	23.20	51.10	25.70
0.5 g/100ml						
3 hrs.	5005	1.52** ±0.19	45.41**	32.70	50.00	17.30
6 hrs.	5012	1.07** ±0.08	36.12**	27.30	56.30	16.40
12 hrs.	5047	0.93** ±0.12	42.25**	18.20	62.60	19.20
0.75 g/100ml						
3 hrs.	5068	0.96** ±0.35	28.80**	10.42	63.48	26.10
6 hrs.	5015	0.48** ±0.43	36.44**	14.45	70.00	15.55
12 hrs.	5000	0.00** ±0.00	00.00	00.00	00.00	00.00

*Significant compared to its control at 0.05 level.
**Significant compared to its control at 0.01 level

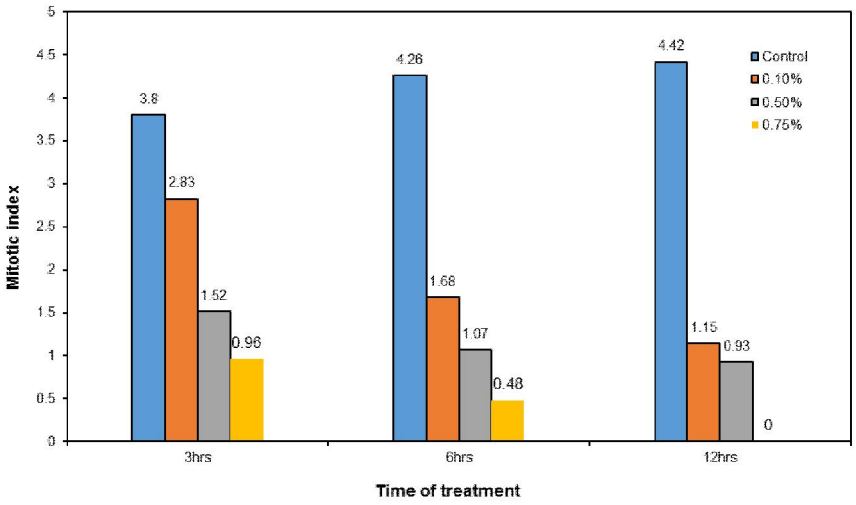


Figure 1. Mitotic index of *Allium cepa* L. root tip cells treated with *Punica granatum* L.total poyphenol extract

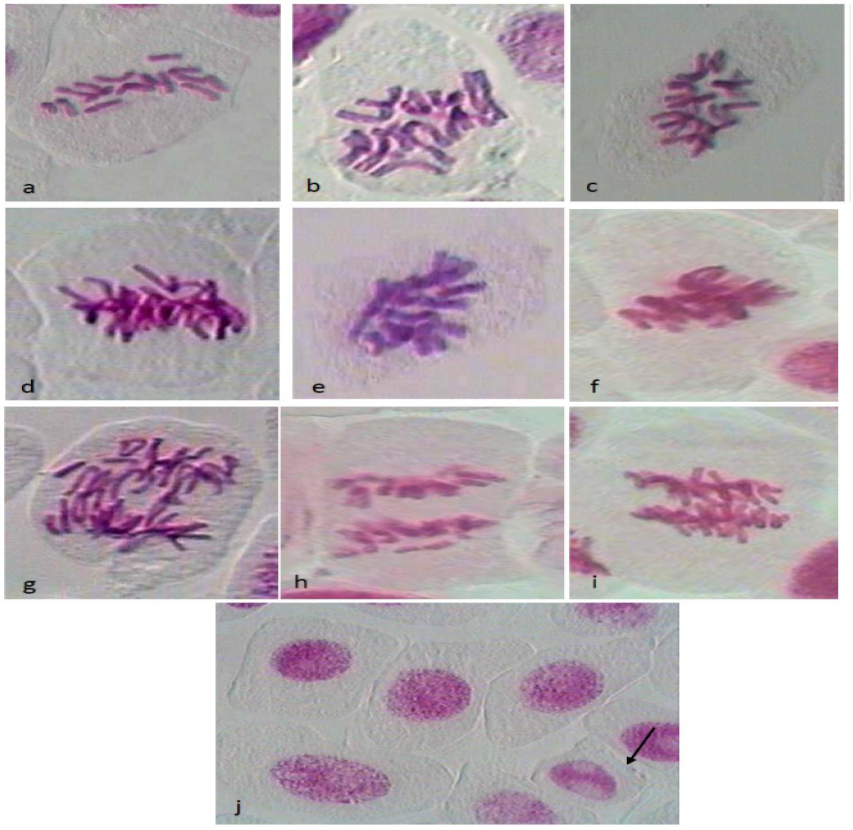


Figure 2. Types of mitotic abnormalities in *Allium cepa* L. root tip cells treated with *Punica granatum* L. total polyphenol extract were: a) C-metaphase with condensed chromosomes after treatment with 0.5%. **b)** C-metaphase after 0.1%. **c)** C-metaphase after 0.75%. **d)** Light sticky metaphase with laggard chromosomes after 0.5%. **e)** Light sticky C-metaphase with condensation in some of the chromosomes after treatment with 5%. **f)** Sticky and disturbed metaphase after 0.75%. **g)** Disturbed anaphase with failure of assembly of spindle apparatus after treatment with 0.5%**. h)** Failure of segregation into two chromatids each pole receive only one set of chromosomes after treatment with 0.5%. **i)** Sticky anaphase with 'male' segregation after treatment with **5%. j)** Appearance of giant cells after treatment with 0.1% (arrow point to the normal size of interphase cell, 20x).

Cytological Analysis

A gradual decrease in mitotic indices of *Allium cepa* L. root cells was clearly found after the application of *Punica granatum* L. total polyphenol extract as compared to their comparative control. The constant decrease was dose and time dependent (Table 1). The mitotic process was completely halted after 12h treatment with 0.75%, with a normal morphological feature of root tips. The maximum frequency of mitotic index was 2.83±0.13 which was achieved after 3 h exposure to the least concentration (0.1%) of the total polyphenol extract that was *significant at 0.05 level. On the other hand, 0.48± 0.43 was the minimum mitotic index frequency induced in root tip cells treated with 0.75% for 6 h that was highly **significant at 0.01 level. This was followed by discontinuity of cell proliferation progress. A complete absence of telophase stage was remarkable after all treatments compared to its normal presence during the proliferation of untreated cells (Table 1). Moreover, the limited numbers of cells entered into M-phase were arrested at metaphase stage. The highest ratio of metaphase accumulation reaching 70% after treatment with 0.75% extract for 6 h. While 45.60% was the least metaphase accumulation ratio that was induced after 3 h exposure to 0.1% *Punica granatum* total polyphenol extract. The accumulation in metaphase stage was on the response of both prophase and anaphase after all treatment with the extract. C-Metaphase, induction of spindle disturbance and stickiness were the common type of mitotic abnormalities. Giant cells were pictured and a complete absence of micronucleus was notable (Fig. 1).

Ultrastructure Analysis

Ultrastructural changes in the meristematic cells of *Allium cepa* L. treated with *Punica granatum* L. total polyphenol extract was followed up by electron microscope. Meristematic cells of untreated root tips possessed a large rounded nucleus, dense cytoplasm, and well organized organelles (Fig. 3a). Treatment with 0.1% for 3 h revealed the appearance of electron dense cytoplasm and normal organelles structure and the nucleus appeared in normal structure with an entire nuclear envelope (Fig. 3b). The sub-cellular organelles showed gradual changes following the application of median dose of the extract (0.5% for 6h). Figure 3c. illustrates the formation of several elongated organelles that lacking internal structure while, Figure 3d exemplifies the aggregation of many spherical vacuoles aside around the nucleus and as a result of this aggregation the nuclear shape has been changed while the chromatin material as well as the nuclear membrane remained unchanged. Following treatment with high dose (0.75% for 12h) of extract the vacuolar system increased in frequency and size (Fig. 3e). Additionally, the conspicuous electron microscopic observation of treated *Allium cepa* meristematic cells is the accumulation of endoplasmic reticulum and its lumen appears dilated as compared with their control (Fig. 3e). Moreover, Figure 3f shows the presence of irregular nucleus with disintegrated envelope while, some nuclear materials were fragmented from the nucleus into the cytoplasm. The other striking feature is the recession of the plasma membrane from the cell wall in early stage of treatment (Fig. 3g). Finally, treatment with *Punica granatum* L. total polyphenol extract caused the movement of the debris of the cytoplasm to the periphery of the cell because the vacuolar system occupied most cell volume (Fig. 3h).

Biochemical and Molecular Analysis

The depressive action of *punica granatum* L. total polyphenol extract on protein synthesis was obvious in the present study. A progressive decrease in soluble protein was clearly observed after all treatments as compared with their control, this depression was time and concentration dependent (Fig. 4). This reduction effect of the extract was vigorous as all protein content in treated *Allium cepa* L. root were less than their counterpart control. Application of 0.1% of the extract for 6h caused the formation of the maximum amount of soluble protein reaching 0.24±0.11g/g fresh weight, in contrast 0.1±0.28g/g fresh weight was the minimum amount of soluble protein occurred after the treatment with 0.75% *punica granatum* L. total polyphenol extract for 12h.

DNA of untreated *Allium cepa* L. roots as well as roots treated with 0.5% and 0.75% for 6 and 12h respectively of *Punica granatum* L. total polyphenol extract was analyzed by agarose gel electrophoresis. The extract successfully cleaved the genomic DNA of *Allium cepa* L. root cells and gave a genome-specific fingerprint of DNA fragments (Fig. 5). The total number of fragments generated by extract was recorded for the detection of the ladder pattern of degraded DNA. The total number of cleaved fragments was dose dependent. Total polyphenol extract was able to produce 4 fragments in *Allium cepa* L. root cells treated with 0.5% for 6h, while the total number of fragments induced after treatment with 0.75% of total polyphenol extract for 12h was 6 fragments. These fragments ranged from 1358 to180 base pairs. This DNA pattern shows integer multiples of approximately 180 base pairs of DNA.

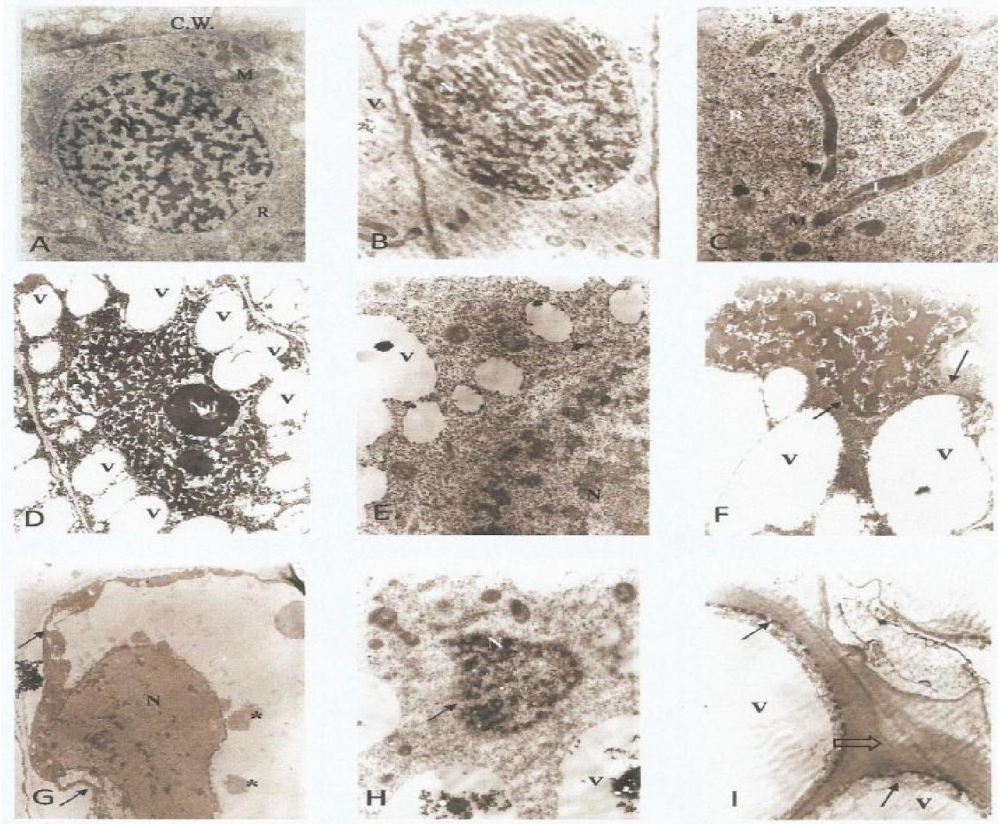


Figure 3. Example of alterations in ultrastructure of *Allium cepa* root tips after all treatments with polyphenol extract: A) organelles in untreated root tip cells: the cytoplasm with large nucleus (N) with entire envelope, endoplasmic reticulum (ER) ramifies through the cytoplasm, ribosomes (R) gives a beaded appearance, mitochondrion (M) with double membrane (x= 5.200). B) The appearance of dense cytoplasm with organelles having normal membrane, vacuole (V) containing some cytoplasmic contents after 1% (x= 5.200). C) The appearance of long undifferentiated organelles after 1% (x=14.000), D) The aggregation of many spherical vacuoles around the nucleus lead to its irregular shape, the appearance of nucleolus (Nu) after 0.5% (x= 7.000). E) Disintegrated nuclear envelope after 0.75% (x=14.000). F) Large aggregated vacuoles (V) around the irregular nucleus, dilated endoplasmic reticulum (arrows) after 0.75% (x=9.800). G) The irregular nucleus (N) and disintegrated nuclear envelop enable many nuclear material to get out in the cytoplasm (astriks) after 0.75% (x=7.000). H) Vacuoles (V) containing granular material and cytoplasmic debris, disintegrated nuclear envelope (arrow) after 0.75% (x=11.500). I) Large vacuole, the debris of cytoplasm moved to the margin of the cell (arrows) and thick fibrillar cell wall (large arrow) after 0.75% (x=5.500).

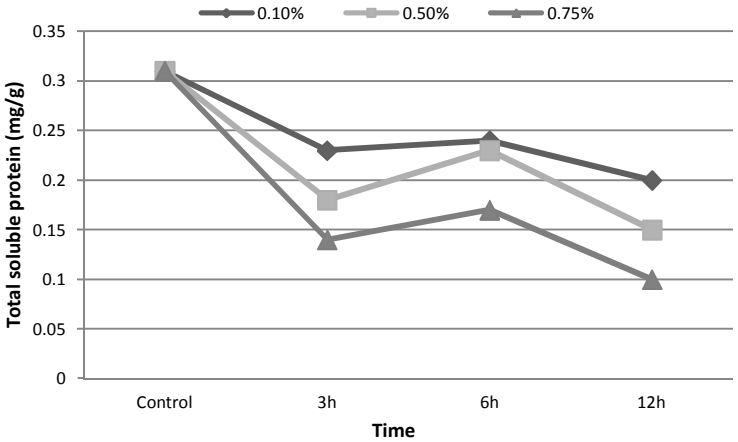


Figure 4. Effect of *Punica granatum* L. total polyphenol extract on total soluble protein of *Allium cepa* L. roots

Nuclease activity assay was carried out using nuclease extracts from *Allium cepa* L. root cells treated with 0.5% and 0.75% of *Punica granatum* L. total polyphenol extract for 6 and 12h respectively. A universal increase in nuclease activity was found. Figure 6 demonstrates that the nuclease activity increment was highly significant as compared with their corresponding control and their results were treatment dependent. The total polyphenol extract increase the nuclease activity to 3.48±0.88units/g after 6h exposure to 0.5% of *Punica granatum* total polyphenol extract and increased sharply after treatment with 0.75% of *Punica granatum* total polyphenol extract for 12h reaching 9.08±0.091units/g fresh weight.

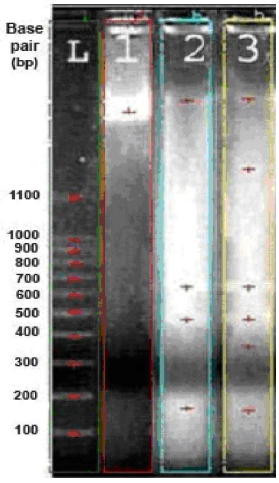


Figure 5. Agarose gel showing induction of DNA laddering in *Allium cepa* L. roots by total polyphenol extract of *Punica granatum* L. Numbers to the left of the figures indicate DNA size in bp. L, marker corresponds to a 100-bp ladder; Lane 1, untreated control; Lane 2, 0.5% of *Punica granatum* L. total polyphenol extract for 6h; Lane 3, 0.75% of *Punica granatum* L. total polyphenol extract for 12h.

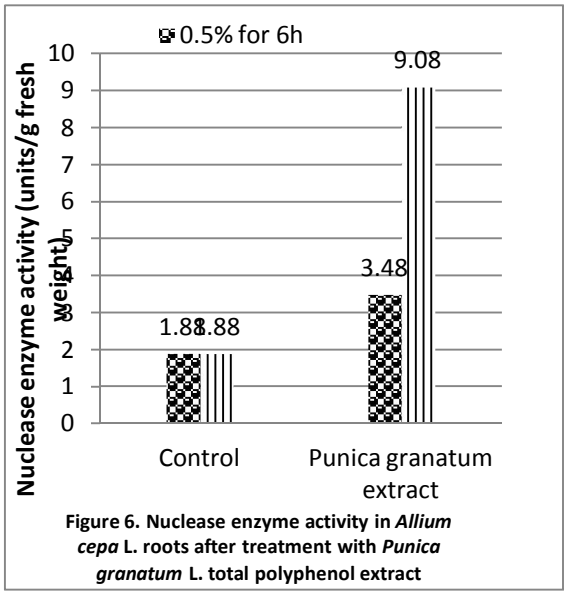
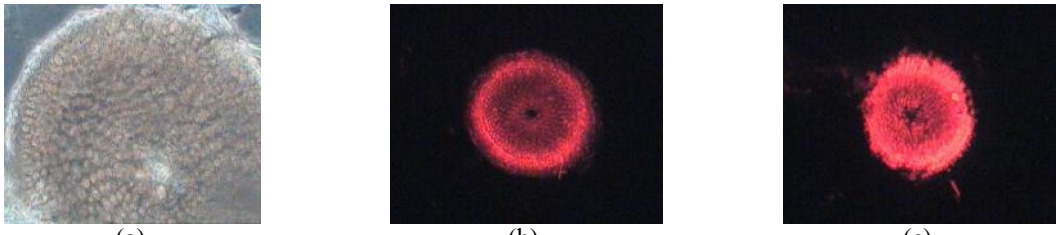


Figure 6. Nuclease enzyme activity in *Allium cepa* L. roots after treatment with *Punica granatum* L. total polyphenol extract

Treated *Allium cepa* L. roots undergoing programmed cell death showed a dramatic loss of viability, as indicated by the intense red fluorescence contributed by propidium iodide which was assessed by fluorescence microscope. The increase in color intensity was treatment dependent, whereas the untreated controls showed no red fluorescent. Treatment with 0.5% of *punica granatum* L. total polyphenol extract for 6h pictured a striking result, this treatment has exerted a selected phase of cell viability; selective cells were reprogrammed to die while the rest of cells were only captured in the program (some cells were appeared completely stained with fluorescent red color, while the other cells appeared unstained). Additionally, the application of 0.75% for 12h of *Punica granatum* L. total polyphenol extract to *Allium cepa* L. roots was able to reprogram all cells to die thus there was a dense red fluorescent color formed and covered all cells as can be seen in Figure 7.



(a) (b) (c)

Figure 7. Changes of *Allium cepa* L. roots cell viability treated with *Punica granatum* total polyphenol extract, in hand cut sections: a) untreated root (control) and pictured with phase contrast (cannot be visualized under fluorescent emission). b) after 6h. exposure to 0.5% polyphenol extract. c) complete loss of viability after 12h.exposure to 0.75% extract.

Identification of the punica granutum L. polyphenol extract

Table (2). The polyphenol profile of the pomegranate pericarp representing the constituent, relative retention time and their percentage as revealed by UV detector of HPLC.

Peak n⁰	*Compound*	*Retention Time*	*Area*	*Area %*	*Height*	*percentage*
1		3.283	2798353	1.09	285553	1.71
2		3.39	2283989	0.89	230156	1.38
3		4.333	4502590	1.76	26072	0.16
4		20.473	17752510	6.94	1254047	7.51
5	Procatechines	20.78	41268007	16.13	2660662	15.94
6		21.03	23840619	9.32	2658959	15.93
7		21.2	40330068	15.76	3019491	18.09
8		21.483	71709702	28.03	3992991	23.92
9	O-H Benzoic	22.023	11099565	4.34	796923	4.77
10		22.317	13495175	5.27	965515	5.78
11	Chlorogenic	22.707	8494313	3.32	321580	1.93
12	Phenol	23.587	1760080	0.69	86099	0.52
13		24.093	797	0	0	0
14	Quarecetin	31.11	15915479	6.22	378986	2.27
15		33.807	608793	0.24	16968	0.1
		Total	255860040	100	16694002	100.01

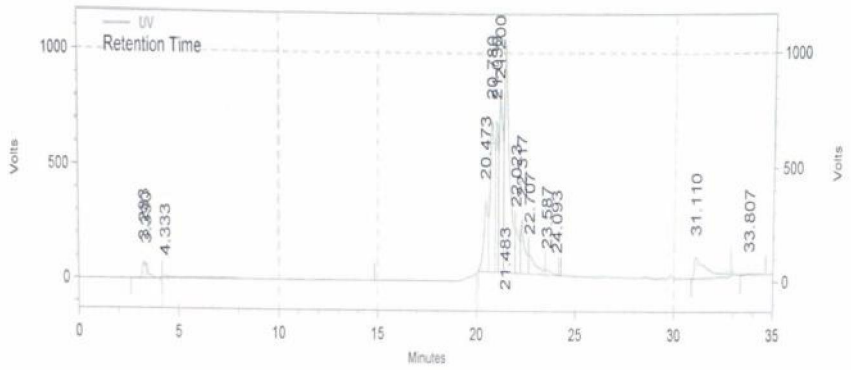


Figure 8. HPLC-UV (wavelength 254 nm) chromatograms of thepolyphenol extracted from promegranate pericarp.

HPLC profile of *punica granatum* L. polyphenol extract was conducted to represent the major constituent of the extract assessed by our biosystem. The extract contains mixture of phenolic compounds. Protocatechuic as simple tannins is observed in peak number 5 with retention time (RT) 20.78 and some phenolic acids (hydroxyl benzoic acid and chlorogenic acid) can be seen in peaks 9 and 11 with R.T 22.02 and 22.70 respectively. The phenol compound occurred in peak 12 with R.T. 23.58, while flavonoid compound (quarecetin) exists in peak 14 with R.T 31.11. The concentration of each phenolic compound is dictated in Table 2. The highest (identified) percentage contribution is shown by protocatechuic acid (15.94%), whereas the lowest one (0.53 %) is the phenol compound. There are some unidentified compounds present together with the pervious phenolic mixture (Fig 8).

4.Discussion

Cytological Analysis

Depression in mitotic index was previously reported after the use of several plant extracts as: *Artimisia herba alba* **(Shehata *et al.*, 1999)**, *Cyperus rotundus*, the aqueous extracts of seven plants used as antihypertensive agents in Argentine folk medicine **(Anibal *et al.*, 2002),** *Rosmarinus officinalis* **(Tawab *et al.*, 2004)** and *Withania obtusifolia* **(Tawab, 2004).** However, the decrease in mitotic index inherent with morphological symptoms as hardness and darkness of root tips has been defined as toxicity as previously reported by **Mohamed and El-Shimy, (1995)** and **Shehab *et al.*, (1995).** Checkpoints help to enforce the correct sequence of events after environmental insults such as DNA damage or spindle damage. This mechanisms arrest cell cycle progression in response to damage, allowing the cells time to repair DNA or

complete spindle assembly before cell cycle progression resumes **(Murray, 1995** and **Bucher and Britten 2008**). The decrease in the mitotic indices is due to that most of cells might adapted to G_2 checkpoint *(DNA Damage Checkpoint)* as the cell-cycle control system prevents or delays entry into mitosis in response to the incomplete DNA replication or DNA damage which sent a negative signals to a series of protein kinases which arrest the cycle at *DNA damage checkpoints.* The change in kinases activities in the form of phosphorelation and dephosphorelation lead to the uncompromising activity of cyclin dependent kinases thereby blocking entry into mitosis. This interpretation is consistent with that proved by arsenic trioxide which induced G_2 phase delay by **Park *et al.,* (2001)** and **McCollum *et al.,* (2005).**

The metaphase accumulation may be due to inability to perform metaphase checkpoint *(The Mitotic Checkpoint)*, by stabilizing activity of mitotic cyclin dependent kinases (M-CdK)and preventing cells to exit from mitosis **(Murray, 1995).** Thus, the data in the present study confirm that both entry into and exit from mitosis is blocked in treated cells suggesting that *Punica granatum* total polyphenol extract may interfere with the balance between cyclin condensation required for participation into mitosis and its ubiquitinous destruction by anaphase promoting complex (APC) requisite to exit from mitosis **(Morgan, 1997** and **Tawab *et al*., 2004).** The ability of *Punica* polyphenol extract to abolish and block metaphase to anaphase transition inferred the use of tubulin subunits as main target. This explanation coincides with the earlier establishment of **Sorger *et al.,* (1997)** in their study where most microtubule-damaging agents that inhibit normal spindle formation, either by increasing microtubule stability or by depolymerization, conduced cells to arrest at metaphase-to-anaphase transition by the action of *The Spindle-Attachment Checkpoints.* Since that, the disassembly of mitotic spindle induces a strong signal that greatly prolongs the metaphase stage and blocks sister-chromatid separation **(Shah** and **Cleveland, 2000)** and may results to segregation of the whole chromosomes towards the extreme poles (Fig.2h)**.** The deficiency in tubulin equilibrium by means of polymerization and depolimerization of microtubules as there expansion are essential for the formation phragmoplasts by new microtubules results in failure of the cytokinesis process **(Murata *et al*., 2013).** The mitotic arrest at metaphase stage as a result of change in mitotic spindle was previously observed by several authors (**Anibal *et al.,* 2002; Sasaki *et al.,* 2002; Tawab *et al*., 2004** and **Weikang, 2005).**

However, *Punica* polyphenol extract mimics the model of taxol. The taxol has been previously reported by **Parekh and Simpkins (1997),** where they find that taxol binds tightly to the microtubules, stabilizing them, and then arrest at metaphase for the dividing cells. This process indicates that microtubules must not be only polymerized but also depolymerized during mitosis. It is obvious to note that the *Punica* polyphenol extract and *Rosemarinus offisinales* water extract (**Tawab *et al*, 2004**) delay the progress of cell cycle. Firstly, both extracts obstruct most of all the cells at G_2-check point; secondly they block the sneaking cells at metaphase check point. These cases conflict with the effect of the vinblastine as cells are: firstly allowed to enter M-phase, then are blocked at metaphase (**Jordon *et al*.1992**)

Therefore, the presence of either a limited number of divided cells or abnormal cells sneaking into M-phase whether these cells are delayed and/or blocked – will not be able to exit from the cell cycle. The explanation of the failure of chromosomes to segregate properly at the metaphase-to-anaphase transition may be a consequence of defeats in its regulatory mechanisms which results to abnormalities **(Cahill *et al.,* 1998)**. The cells unable to perform checkpoint adaptation may instead induce a program of cell death or may simply fail to proliferate, remaining quiescent in mitosis depending on the stress intensity, a function of the type and amount of cell damage **(Pelayo *et al.,* 2003)**. The anti-tubulin colcemid was sufficient to block Hela S3 cells at *the Mitotic Checkpoint* and led to the appearance of apoptic cells after their initial accumulation in mitosis **(Steven *et al*., 1994)**. In addition, **Blgosklonny *et al*., (1997)** confirmed that disrupting the dynamics of tubulin polymerization or depolymerization and the functions of microtubules with treatment induces mitotic arrest and programmed cell death in dividing cancer cells.

Concerning the cytological results observed in the present study, the cell division was arrested in order to initiate programmed cell death. This commencement of that process is ascertained by the failure of the cytokinesis process which abolished the presence of telophase stage as compared to the untreated cells (Table 1) and the presence of giant cells.

Ultrastructure Analysis

Total polyphenol extract of the tested medicinal plant (*Punica granatum* L.) was able to change the ultrastructure of *Allium cepa* L. meristematic cells and increase the number and size of the vacuolar system**. Betra *et al.,* (1983)** and **Fuzinatto *et al.,* (2007)** suggested that the increase in number and size of vacuolar system, many of which appeared to have

cellular debris in various stages of degeneration, indicate the presence of autophagic and autolysis processes. While, the disappearance of plasmodesmata and the formation of dilated endoplasmic reticulum could be a direct effect of changes, ultimately leads to the programmed cell death. **Lawen, (2003)** elucidated that cells lose contact with their neighbors as a result of formation of dilated endoplasmic reticulum. This aspect was previously explained by **Madeo *et al.*, (1997)** who confirmed that the formation of dilated endoplasmic reticulum leads to programmed cell death in yeast. The same finding was proved by **Bakeeva *et al.*, (2005).** The changes in nuclear morphology has been described in some forms of plant programmed cell death, including aerenchyma formation in response to hypoxic stress **(Gunawardena *et al.*, 2001a)** and in BY-2 tobacco cells in response to oxidative stress **(Houot *et al.*, 2001). Madeo *et al.*, (1997)** proved that the induction of disintegrated nuclear envelope and the occurrence of several randomly distributed nuclear fragments in the cytoplasm of yeast cells are resemble to the late stage of animal programmed cell death. The detachment of the plasma membrane from the cell wall of any cell is considered as a hallmark of programmed cell death in plant cells as mentioned by **Beers and McDowell, (2001)**. Moreover, **Schussler and David, (2002)** proved that the change in the position or structure of plasma membrane occurs early in plant programmed cell death while in animal cells is one of the last events to occur in apoptosis. Additionally, this alteration in plasma membrane was progressively observed in soybean cells treated with 1,2-bis (2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid as the cell death program advances **(Zuppini *et al.*, 2004)**. The systematic changes in nuclear structure, condensation of cytoplasm as well as the maintenance of organelle integrity in the periphery of the cell till the late stage of death process was firstly introduced and pictured by **Kerr *et al.*, (1972)** considering them as a genetically controlled cell death.

Biochemical and Molecular Analysis

The decrease in total soluble protein was previously reported after the use of *Rosmarinus officinalis* extract (**Tawab *et al.* 2004).** This conspicuous decline in gene expression reflects a highly inhibition effect of the polyphenol extract on protein synthesis process of *Allium cepa* root cells by interfering with the translation process from RNA to protein either by blocking the binding of aminoacyl-tRNA molecule to ribosomal A-site, blocking the peptidyl transferase reaction on ribosome or by blocking the translocation reaction on ribosome (**Jiménez, 1988 and Lord *et al.*, 1991).** However, changes in protein synthesis in mice liver through the induction of programmed cell death by treatment with cadmium and found that it was able to suppress functional activity of tRNA, this effect may account for the decreased activity of the whole translation process by causing a significant inhibition of amino acid incorporation, decreasing the protein synthesis **(Ivanov *et al.*, 2002)**. Changes in protein synthesis and protein degradation before or during senescence, a well-known process of plant cell death, have been reported in many plant species **(Camp *et al.*, 1984; Hosseini and Mulligan 2002; Wang *et al.*, 2013; Budic *et al.*, 2013; Karmous *et al.*, 2014 and Chondrogian *et al.*, 2014)**.

The decrease in cellular protein content in our work may be in part explained by increasing in proteolytic activities**.** It is suggested that the decrease in intracellular protein content of rice culture during senescence process could be due to a high rate of protein turnover in cells entered stationary phase by changing in the environmental conditions or due to leakage of proteins from senescing cells (**Hosseini and Mulligan 2002)**. Additionally, large increase in normal proteolytic activity during the senescence of ephemeral flower, daylily have been documented (**Valpuesta *et al.*, 1995; Guerrero *et al.*, 1998; Karmous *et al.*, 2014).** However, increase in proteolytic activity was observed during oxidative stress and hypersensitive response of plant cell (**Delpozo and Lam, 1998; Lakimova *et al.*, 2013 and Aoki *et al.*, 2014).** The induction of cell death by chemical treatment during tomato cell culture indicated the critical role of proteolysis enzymes activity **(Avila and Devarenne 2013)**. The process of programmed cell death is not usually expressed by synthesis of *de novo* protein **(Budic *et al.*, 2013)**; instead protein synthesis inhibitors can induce programmed cell death **(White, 1996; Chinnaiyan and Dixit, 1996; Wang *et al* 2013).** Therefore, increase in proteolytic activity in the present work may be a reason by which the cellular protein content in *Allium cepa* L. root cells was depressed during chemically induced programmed cell death **(Farr and Cohen-Fix, 1999** and **Yi et al. 2012; Xie *et al.*, 2014)**.

Detection of DNA fragmentation is currently one of the most frequently used techniques in the study of cell death **(Thomas *et al.*, 2006; Hanna, et al. 2013; Zou et al. 2014).** DNA laddering was detected throughout the death process by routine agarose gel electrophoresis in our study revealing the active degradation of genomic DNA into multimers of 180 bp. It was suggested that this pattern of DNA fragmentation may be a universal marker of nuclear change during plant programmed cell death **(Ning *et al.*, 2002)**. The degradation of DNA has been obtained by several stimuli during programmed cell

death **(Peters and Chin, 2007** and **Lombardi *et al.*, 2007)**. Although plant programmed cell death occurring without laddering has also been observed by **Xiong *et al.*, (2006).** This systematic DNA fragmentation was associated with significant increase in nucleases activity. The nucleic acid catabolism seams to be catalysed by endonuclease enzymes capable of digesting both single-stranded DNA (ssDNA) and double stranded DNA (dsDNA) **(Langston *et al.*, (2005)**.The increase in nuclease activity signifies the representing of some specific endonucleases with strong activity accumulated in the treated cells **(Sodmergen *et al.*, 1991 and Yupsanis *et al.*, 2004)).** The fragmentation of nuclear DNA into specific segments by nuclease enzymes, proved by ladder formation, confirms a point of no return to the cell programmed to die and hastens cell to its final destination. This result was considered the final hallmark of cell death especially after the visualization of cell death obtained by the cell viability test.

The cell viability technique has shown its outstanding feasibility for evaluating the state of *Allium cepa* L. root cells. This type of analysis constitutes a valuable complement of other biochemical or analytical studies. Propidium iodide is an impermeable membrane dye and generally cannot cross the membrane of viable cells as pictured in Fig.6 a **(Sasaki *et al.*, 1987)**. In contrast, dead cells may exhibit nonspecific uptake of dye during fluorescence labeling through increased membrane permeability. Plasma membrane in normal cells is intact and the phosphatidylserine (PS) is sequestered along the inner surface of the cytoplasmic membrane. However, the plasma membrane remodeling, including the early transverse redistribution of phosphatidylserine, is a general feature occurring in cells that has been programmed to die **(Fadok *et al.*, 1992).** This redistribution may be due to the inactivation of phosphatidylserine flippase enzyme **Aupeix *et al.*, (1997).** Therefore, the appearance of few cells in the hand cut section of *Allium cepa* L. root in the course of cells stained with propidium iodide is an additional explanation for the exaggerated low percentage of dividing cells. Accordingly, propidium iodide readily enters into nonviable cells, binds to damage DNA and intercalates between the bases with little or no sequence preference for purine or pyrimidine base pairs and with a stoichiometry of one dye per 4-5 base pairs of DNA **(Waring, 1965).**These findings might explain the observed evidence that has been occurred during programmed cell death induced in *Allium cepa* L. root cells by the acetone extract. The membrane of control cells was intact and able to exclude the stain. On the other hand, the usage of median dose has changed the integrity of the plasma membrane and infiltrated the stain among the membrane of some cells and bound to fragmented DNA. Moreover, the continuous exposure of root cells to high dose of treatment caused the exposure of phosphatidylserine on the outer surface of the plasma membrane and significantly decreased the membrane potential thus all cells became stained (**Obara *et al.*, 2001; Madeo *et al.*, 2002** and **Andrew *et al.*, 2003; Marcus *et al.*, 2012; Sadik 2013; Liu *et al.*, 2014).** Our results proved that cell viability of examined root cells, showed a decreased manner which was concomitant with the decrease in protein content as previously reported by **Hosseini and Mulligan, (2002).** Moreover, the viability method provides the real view of the general physiological state of the cells. It has been claimed that nucleoid degradation is an early event in the death process of hyphae of *Streptomyces antibioticus* and this degradation precedes the rupture of the plasma membrane **(Miguélez *et al.*, 1999).** This is only based on the ultrastructural aspect of the nucleoid within specific hyphae (i.e. a disorganization of the electron-dense nucleoid and a continuous well-stained membrane structure). However in the present study, viability method elucidated that the nuclear degradation was started in the late stage of cell death which was accompanied with the high decrease in cell viability.

It could be concluded that the total polyphenol extract of the tested medicinal plant (*Punica granatum* L.) has the ability to reprogram *Allium cepa* L. meristematic cells to induce plant programmed cell death and clarify the morphological changes induced during the process. The severe decline in mitotic index, the metaphase arrest, the failure in separation into two daughter cells, the formation of giant cells, the disintegrate nuclear envelope and finally the formation of DNA ladder fragments are correlative hallmarks for plant programmed cell death ascertained by loss of cell viability. Since the cell death of *Allium cepa* L. meristematic cells lacks several features of animal apoptosis (especially the formation of numerous apoptotic bodies), programmed cell death in *Allium cepa* L. meristematic cells model should be classified as nonapoptotic bodies former although, there is no current system to classify nonapoptotic programmed cell death in plants as such in animals**.**

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