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Research Article

Evaluation of Cytotoxicity and Genotoxicity of *Inula viscosa* Leaf Extracts with Allium Test

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I. viscosa has been used for years in folk medicine for its anti-inflammatory, antipyretic, antiseptic, and paper antiphlogistic activities. In this study, cytotoxic and genotoxic effects of *I. viscosa* leaf extracts on the root meristem cells of *Allium cepa* have been examined. Onion bulbs were exposed to 2.5 mg/ml, 5 mg/ml, and 10 mg/ml concentrations of the extracts for macroscopic and microscopic analysis. Tap water has been used as a negative control and Ethyl methanesulfonate (EMS) ($2 \cdot 10^{-2}$ M) has been used as a positive control. The test concentrations have been determined according to doses which are recommended for use in alternative medicine. There has been statistically significant ($P < .05$) inhibition of root growth depending on concentration by the extracts when compared with the control groups. All the tested extracts have been observed to have cytotoxic effects on cell division in *A. cepa*. *I. viscosa* leaf extract induces the total number of chromosomal aberrations and micronuclei (MNC) formations in *A. cepa* root tip cells significantly when compared with control groups. Also, this paper shows for the first time the induction of cell death, ghost cells, cells with membrane damage, and binucleated cells by extract treatment. These results suggest the cytotoxic and genotoxic effects of the *I. viscosa* leaf extracts on *A. cepa*.

1. Introduction

Medicinal herbs have been used in folk medicine for millennia. Simply, in recent times, scientific study of their effects has flourished. Nevertheless, some of them can cause adverse effects or have the potential to interact with other medications [1]; moreover, there is little information on the potential risk to health of such herbs [2]. Based on their long-term use by humans, one might expect herbs used in traditional medicine to have low toxicity. It is known that green plants in general are a primary source of antimutagens as well as natural toxic agents [3], and many plants contain cytotoxic and genotoxic substances. Recent investigations have revealed that many plants used as food or in traditional medicine have mutagenic effects and cytotoxic and genotoxic effects in vitro and in vivo assays [4–7]. This raises concern about the potential mutagenic or genotoxic hazards resulting from the long-term use of such plants. Many plants contain mutagenic and/or carcinogenic substances [8, 9] and their use has been correlated with high rate of tumor formation in some human populations [8, 10–13].

Inula viscosa (L.) Aiton (syn. *Cupularia viscosa* G. et G., *Dittrichia viscosa* Greuter) (Compositae); common name, "sticky fleabane" is a perennial weed that is found in most of the Mediterranean basin [14–16]. *I. viscosa* has been used for years in folk medicine for its antiinflammatory [17], antipyretic, antiseptic, and antiphlogistic activities [18, 19], and in the treatment of diabetes [20]. Aqueous extracts of *I. viscosa* exhibit antifungal activity in vitro [21, 22] and it has been demonstrated that some of its organic solvent extracts are antibacterial [23]. Cohen et al. [24] provided evidence for the antifungal activity in plant extracts made with organic solvents, including methanol, ethanol, ethyl acetate, acetone, chloroform, and n-hexane. In addition, this herb has been used in Spanish folk medicine for treating gastroduodenal disorders [25]. *I. viscosa* has antiulcerogenic effects [26], causes abortion [16, 27, 28], prevents zygote implantation in mammals [16], prevents growth of pathogenic fungi [29],

has a strong antioxidant activity [30]. There is published evidence that *I. viscosa* has also nematicidal/antihelmynth properties [31].

I. viscosa contains some pharmacologically active compounds [32, 33] including sesquiterpenes, sesquiterpenes acids [34], azulenes, lactones, flavonoids, and essential oils [18] which are isolated and identified in its leaves. Currently, there is no published data on the cytotoxicity and genotoxicity of *I. viscosa* leaf extracts. The purpose of this study is to investigate cytotoxic and genotoxic effects of *I. viscosa* leaf extracts using the Allium Test.

2. Materials and Methods

2.1. Chemicals. Ethyl methanesulfonate (EMS) (CAS No: M-0880) was purchased from Sigma (Sigma Chemical Co., St Louis, MO).

2.2. Collection of Inula viscosa (L) Ait. I. viscosa specimens were collected from vicinities of Söke-Kuşadası/Aydın (Turkey) during flowering (November 2007). The plant was identified by Dr. Özkan EREN, botanist, Department of Biology; University of Adnan Menderes and voucher specimen was deposited at the Herbarium of Department of Biology, Adnan Menderes University.

2.3. Preparation of the Aqueous Extracts of I. viscosa Leaves. The extracts were prepared according to the traditional use in Turkey (decoction) and we used in this study crude extracts of *I. viscosa* leaves. Studying with crude extracts is appropriate because traditional medicinal herbs are generally used as crude extracts. The *I. viscosa* leaves were rinsed with water, dried in a ventilated oven at 55°C for 24 h and subsequently milled to a fine powder by ground into fine powder using a kitchen blender. The powder was placed in small plastic bags (100 g each) and stored at 4°C until use. The extract was prepared by boiling 20 g powdered plant material mixed with 200 ml distilled water for covered beaker (10% stock solution) for 5 min and, cooled to room temperature for 10 min. Thereafter, the extract was filtered through a filter paper (ISO Lab. Quantitative Filter Paper) to remove particulate matter. Stock solution was diluted with distilled water to 2.5 mg/ml, 5 mg/ml, and 10 mg/ml concentrations. Fresh extract was prepared daily for each experiment, just before administration.

2.4. Allium Test. Small bulbs (1.5–2.0 cm in diameter) of the common onion, *A. cepa,* ($2n = 16$) were purchased at a local supermarket. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. For each extract sample, a series of six bulbs were placed in tap water (pH 7.3) for 48 h and then onion roots were treated with the leaves extracts at 2.5 mg/ml, 5 mg/ml, and 10 mg/ml concentrations of *I. viscosa.* The test tubes were kept in an incubator at 22 ± 1°C and the test samples were changed daily at the same time. Several of the newly formed root tips were then cut from each bulb and examined for any visible morphological abnormalities. The bulbs with satisfactory root lengths (2–2.5 cm) were used in the study, while those with exceptionally long or short roots were discarded (on average 2-3 bulbs). Therefore, individual sets of five bulbs were used for each extract sample. Tap water (pH 7.3) was used as a negative control [35, 36] and Ethyl methanesulfonate (EMS, 2.10^{-2} M) used as a positive control mutagen. EMS has been used in a wide variety of biological test systems in studies of mutation effects [37–39]. EMS induces DNA damage by a direct mechanism, acting at various sites as a monofunctional ethylating agent of nucleotides [40]. After 24 h of exposure, several root tips were removed from the bulbs, fixed in 3 : 1 (v/v) ethanol : glacial acetic acid and stored overnight at 4°C. The next day they were placed in 70% (v/v) aqueous alcohol and refrigerated until used. An average of five slides was made for each bulb using five roottips which hydrolyzed in 1 N hydrochloric acid (HCl) for 3 min and microscope slides were prepared by squashing the stained root tips in 2% (w/v) acetic orcein. Five slide was prepared per bulb, and each slide was examined using Olympus BX51 at a total magnification of 40×10.

The following parameters were used for determination of cytotoxicity and genotoxicity: (i) the mitotic index (MI) was calculated as the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage and (ii) chromatin aberrations (stickiness, breaks and polar deviation) were used as endpoints for determination of cytogenetic effects and micronuclei (MNC) were scored in interphase cells per 1000 cells (‰ MNC) [41]. The most frequent abnormalities are shown in microphotographs.

After 72 h of exposure to the *I. viscosa* leaf extract samples, the root lengths were measured and used as an index of general toxicity. The results for mitotic index and root length are expressed as percent of the negative and positive control. Visible morphological modifications, such as changes in root consistency and color as well as the presence of swelling (c-tumors), hooks or twists in the roots were also observed.

2.5. Statistical Analysis. Statistical analyses were performed using the SPSS 11.5 software package programme. Data on physicochemical parameters, root length, root growth, and mitotic index and chromosomal aberrations were compared using analysis of variance (ANOVA) to confirm the variability of the data and validity of results. Differences between corresponding controls and exposure treatments were considered statistically significant at $P < .05$.

3. Results

3.1. Physicochemical Characterization. The levels of the physicochemical parameters (root number and root length) are presented in Table 1. This results show that all tested concentrations of *I. viscosa* leaf extracts caused significant inhibition in the growth of roots in comparison to negative control and positive control. The inhibition of root number and root length was greater with increasing concentrations

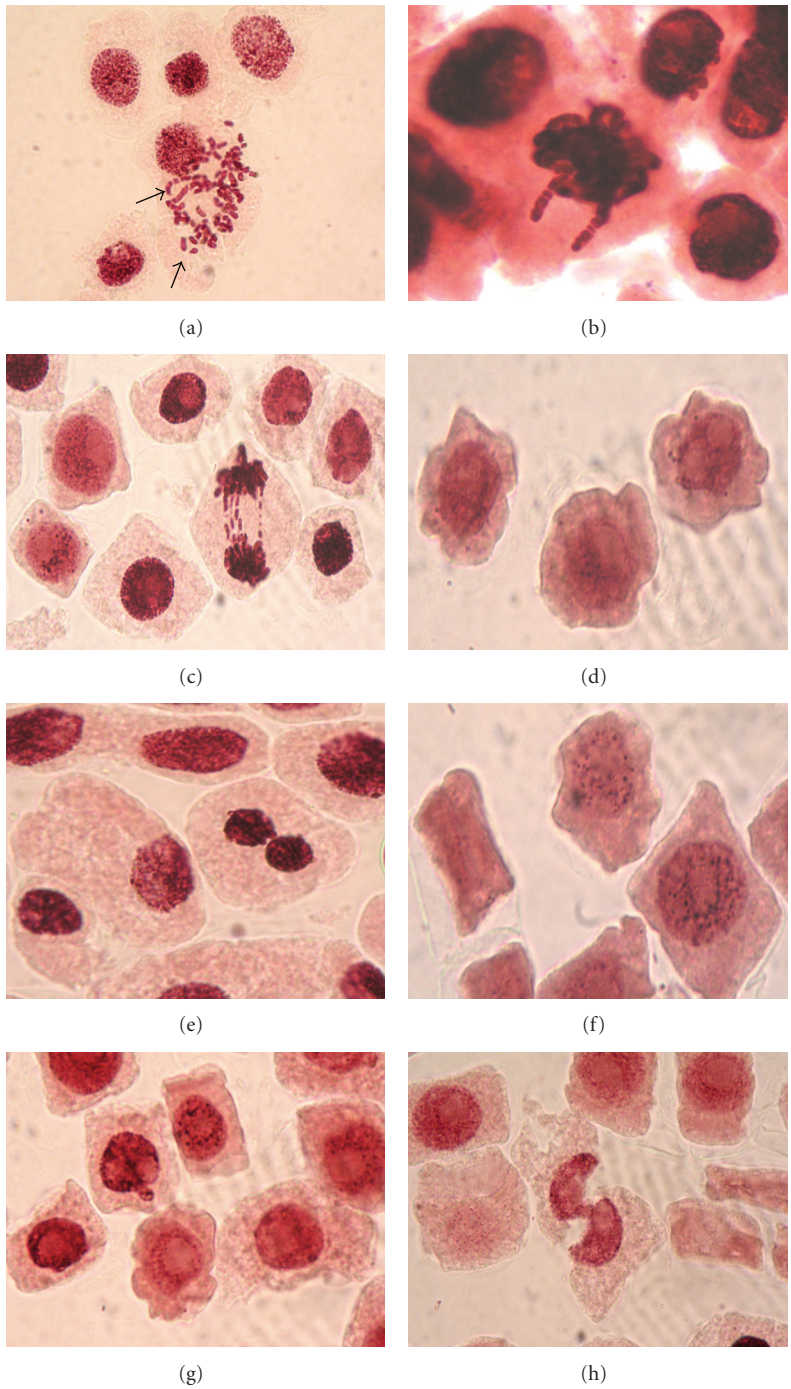

Figure 1: Mitotic and chromosomal aberrations after the *Inula viscosa* leaf extract treatments in *Allium cepa* root tip meristem cells visualized with light microscopy. (a) Fragments; (b) stickiness; (c) polar deviation and chromatid bridges; (d) membrane damage; (e) binucleated cell; (f) apoptotic bodies; (g and h) cells with damaged nucleus.

of *I. viscosa* leaf extracts. Measured average root length is 3.58 cm in negative control and 2.74 cm in positive control. However, average root length in 10 mg/ml treatment group was decreased significantly compared to that of the negative control (2.82 cm) (Table 1). Average root lengths in treatment groups were decreased depending on concentration, significantly. The root morphology was nearly normal during the negative control treatment, but at 2.5 mg/ml *Inula* leaf extract, the roots appeared slightly yellow and at 5 mg/ml *Inula* leaf extract, the roots appeared a slightly brown. At 10 mg/ml *Inula* leaf extract, the roots morphology showed an obvious difference in its appearance in that it turned to brownish in colour.

3.2. Cytogenetic Analysis. With the objective of investigating the possible mechanism involved in root growth inhibition,

Table 1: The average root numbers and root lengths in controls and treatment concentrations.

Treatment groups	Concentrations	Average root number ± SD	Average root lengths (cm) ± SD
Negative control	Tap water	37.6 ± 4.03	3.58 ± 0.61
Positive Control (EMS)	2×10^{-2} M	28.6 ± 4.72*	2.74 ± 0.42*
$Inula_1$	2.5 mg/ml	22.0 ± 5.52*	3.36 ± 0.52
$Inula_2$	5 mg/ml	20.6 ± 2.30*	3.12 ± 0.33
$Inula_3$	10 mg/ml	19.2 ± 5.97*	2.82 ± 0.53*

* $P < .05$ in One Way ANOVA.

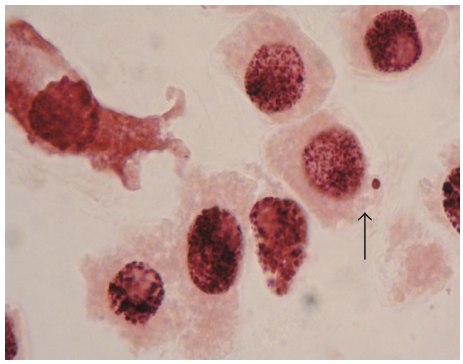

Figure 2: Micronucleus.

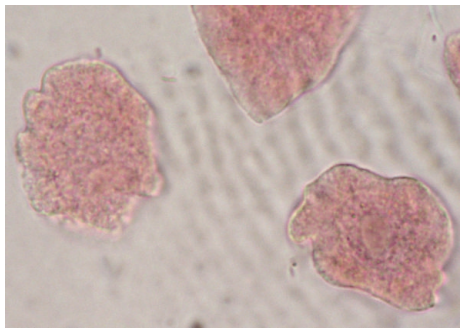

Figure 3: Ghost cells.

cytogenetic analysis was performed. *I. viscosa* leaf extracts provoked strong inhibition of the mitotic index, where a statistically significant difference in relation to the control and the decrease in the mitotic index was positively correlated with increasing concentration of the *I. viscosa* leaf extracts (Table 2).

Cytogenetic alterations were investigated and the results can be seen in Table 3. *I. viscosa* leaf extracts induced chromosome and cytological alterations both in treatment and control groups. An analysis of chromosome aberrations showed that most of the fragments detected in the different treatments were of chromosome type (Figure 1(a)). The observation of chromosome breaks showed the clastogenic effect of *I. viscosa* leaf extracts. The occurrence of chromosome fragments allows observation of statistically significant differences at *I. viscosa* leaf extracts.

In addition to the chromosome fragments, sticky metaphase and polar deviations (wrong directions of chromosome movement) were also observed (Figures 1(b) and 1(c)). In general, it was possible to observe an increase of different abnormalities as the *I. viscosa* leaf extracts concentration increased. In *Allium* test, a strong toxic effect of *I. viscosa* leaf extract was observed, supported by great occurrence of sticky metaphases, leading to cellular death (mitotic index decrease). A statistically significant increase in total aberrant cells ($P < .05$) (aberrant cells include chromosome breaks, stickiness and polar deviation) as compared with the negative control (Table 3), however, the highest value of aberrant cells is shown by the positive control.

Statistical analysis showed that the genotoxic activities of the *I. viscosa* leaf extracts induced micronuclei in the root tip meristem cells of *A. cepa.* Micronucleus formation in 1000 cells per slide (‰MNC value) was also increased in extract concentrations compared with negative and positive control, which is statistically significant ($P < .05$) (Figure 3(a)). The increase occurred in the positive control, $Inula_1$ and $Inula_2$ with respect to the negative control, and not in $Inula_3$ which is expected since MI is extremely low.

In addition, cells with membrane damage (Figure 1(d)), binucleated cells (Figure 1(e)), and nucleus damage (Figures 1(g) and 1(h)) were found in various frequencies. Also, apoptotic cells (Figure 1(f)) were detected in the group treated with *I. viscosa* leaf extract. Moreover, ghost cells were detected in 10 mg/ml *I. viscosa* leaf extract treatment (Figure 2(a)).

4. Discussion

In this study, toxic effects of *I. viscosa* leaf extract was evaluated by analyzing root growth and root morphology. The higher *I. viscosa* extracts caused an inhibition of root growth and there was a statistically significant difference between control groups. In addition, the *I. viscosa* extracts induced slightly yellow, slightly brown and brownish in coloration in roots. Cyto- and genotoxicity were estimated by observing cytological parameters such as the mitotic index and number of chromosome abnormalities, including chromosome breaks, stickiness, and polar deviations. The mitotic index (MI) of *A. cepa* meristematic cells treated with the EMS was significantly decreased (1.924% in comparison to negative control). Significant inhibition in the onion roots treated with the *I. viscosa* extracts (3.200%, 1.984% and 0.088% compared to the negative control) (Table 2). A

Table 2: The dividing and total cells counted in microscopic observations and mitotic values in control and in treatment concentrations.

Treatment groups	Concentrations	Total cells	Dividing cells	MI (%) ± SD
Negative control	Tap water	25000	1687	6.748 ± 1.17
Pozitive control (EMS)	2×10^{-2} M	25000	481	1.924 ± 0.91*
$Inula_1$	2.5 mg/ml	25000	800	3.200 ± 0.60*
$Inula_2$	5 mg/ml	25000	518	1.984 ± 0.75*
$Inula_3$	10 mg/ml	25000	22	0.088 ± 0.05*

* $P < .05$ in One Way ANOVA.

Table 3: Chromosome and mitotic aberrations in the root meristem cells of *Allium cepa* after *I. viscosa* leaf extract treatment.

Treatment groups	Concentrations	Chromosome breaks (%) ± SD	Stickiness (%) ± SD	Polar deviations (%) ± SD	Aberrant cell (%) ± SD	MNC (‰) ± SD
Negative control	Tap water	—	0.69 ± 0.91	7.65 ± 1.97	8.34 ± 1.85	0.28 ± 0.18
Positive control (EMS)	2×10^{-2} M	—	31.63 ± 12.88*	8.97 ± 6.18	40.60 ± 9,94*	0.68 ± 0.18
$Inula_1$	2.5 mg/ml	7.22 ± 2.61	17.32 ± 2.52*	6.09 ± 1.36	30.63 ± 5.03*	0.64 ± 0.17
$Inula_2$	5 mg/ml	0.95 ± 0.91	28.74 ± 8.18*	10.14 ± 1.33	39.83 ± 7.10*	0.48 ± 0.41
$Inula_3$	10 mg/ml	—	8.89 ± 1.44*	9.44 ± 12.96	18.33 ± 6.93*	0.04 ± 0.08*

* $P < .05$ in One Way ANOVA.

positive correlation was found between inhibition of root growth and decrease of MI. The decline of MI below 22% in comparison to negative control can have lethal impact on the organism [42], while a decrease below 50% usually has sublethal effects [43] and is called cytotoxic limit value [44]. MI measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be interpreted as cellular death or a delay in the cell proliferation kinetics [45]. Reduction in the mitotic activity could be due to inhibition of DNA synthesis or a blocking in the G2 phase of the cell cycle, preventing the cell from entering mitosis [46]. Mitodepressive effects of some herbal extracts, including the ability to block the synthesis of DNA and nucleus-proteins, were reported earlier [47, 48]. Several other herbal extracts have been reported to inhibit mitosis [7, 49, 50]. The decreased MI in *A. cepa* roots treated with *I. viscosa* leaf extracts is probably due to either disturbances in the cell cycle or chromatin dysfunction induced by an external factor, in this case, *I. viscosa* extracts- DNA interactions. The results herein suggest that the tested *I. viscosa* leaf extracts concentrations have inhibitory, mito-depressive effects on root growth and cell division of *A. cepa* and it can prevent DNA synthesis and the reduction in number of the dividing cells in roots produced by the cytotoxic effects of compounds found in *I. viscosa* leaf extracts.

I. viscosa leaf extracts showed the strongest genotoxic effects in the root meristem cells. The observation of sticky metaphase reinforces the hypothesis of the toxic effect of *I. viscosa* leaf extracts. Metaphases with sticky chromosome, loses their normal appearance, and they are seen with a sticky "surface," causing chromosome agglomeration [51]. Stickiness has been attributed to the effect of pollutants and chemical compounds on the physical-chemical properties of DNA, protein or both, on the formation of complexes with phosphate groups in DNA, on DNA condensation or on formation of inter- and intra chromatid cross links [52–56]. Chromosomal aberrations (CA) are changes in chromosome structure resulting from a break or exchange of chromosomal material. Most of the CA observed in cells are lethal, but there are many related aberrations that are viable and that can cause genetic effects, either somatic or inherited [57]. The presence of chromosome fragments is an indication of chromosome breaks, and can be a consequence of anaphase/telophase bridges [58, 59]. The induction of chromosome breaks, disturbances on microtubule assembly and cellular death can be related. Our results showed induction of chromosome type of aberration in the cells treated with the *I. viscosa* leaf extracts. Somehow the *I. viscosa* leaf extracts not only interfere with the cell cycle, but also affect chromatin organization or DNA replication, causing chromosome breaks. Frequencies of total chromosome aberrations increased significantly upon exposure to *I. viscosa* leaf extracts which indicate clastogenic activity (Table 3). These results are in line with the results of many research groups that examined the effects of different medicinal herbs [7, 60, 61].

I. viscosa leaf extracts significantly induced the formation of MNC in *A. cepa* root cells at 2.5–10 mg/ml concentrations. Frequencies of MNC increased in 2.5 mg/ml and 5 mg/ml *I. viscosa* leaf extract. However, MNC frequency decreased in *A. cepa* roots treatment at the highest *I. viscosa* leaf extract concentration (10 mg/ml), due to high cytotoxicity. The frequency of cells with micronuclei is a good indicator of the cytogenetic effects of tested chemicals. Micronuclei (MN) often results from the acentric fragments or lagging chromosomes that fail to incorporate into the daughter nuclei during telophase of the mitotic cells and can cause cellular death due to the deletion of primary genes [62, 63]. Recent studies have suggested MNC-induced effect of various plant extracts. In our recent study, we report MNC-induced effect of

Lavandula stoechas aqueous extract and *Ecballium elaterium* fruit juices on *A. cepa* root tip meristematic cells [7, 64]. Furthermore, Soliman [60] reported MNC formation by *Azadirachta indica* A. Juss. aqueous extract treatment on *A. cepa* root tip meristematic cells.Akinboro and Bakare [50] reported MNC formation by treatment of some *Psychotria* species extracts on *A. cepa* root tip meristematic cells.

In this study, membrane damage cells was observed in groups treated with 5 mg/ml and 10 mg/ml *I. viscosa* leaf extracts. These results show that *I. viscosa* leaf extracts over certain concentrations may cause cytotoxicity as they cause membrane damage. Maoz and Neeman [29] evaluated effects of *I. viscosa* extract on chitin synthesis in dermatophytes and *Candida albicans*. They demonstrated that *I. viscosa* extract inhibited the growth of dermatophytes and *C. albicans* and caused a significant decline in chitin content. Chemically speaking, chitin is closely related to chitosan (a more water soluble derivative of chitin). It is also closely related to cellulose in that it is a long unbranched chain of glucose derivates which replaces chitin in plants. In addition, it is needed to clarify whether the decline of cellulose is due to direct inhibition of the membrane enzyme, cellulose synthase, or due to damage of the whole membrane. Binucleated cells have been observed in 5 mg/ml extract treatment group. The occurrence of binucleated cells is the result of prevention of cytokinesis or cell plate formation. Microtubules have been implicated in cell plate formation and *I. viscosa* leaf extracts can be one of the involved factors, resulting in inhibition of cytokinesis. These results are in accordance with the literature data. Similar inhibition of cytokinesis cells were also reported by Kaushik [65], Borah and Talukdar [66], and Gömürgen et al. [67].

On the other hand, ghost cells have been observed in various frequencies in this study for the first time (Figure 2). The ghost cell induced effect of *I. viscosa* was not reported by other authors. Ghost cell is a dead cell in which the outline remains visible, but whose nucleus and cytoplasmic structures are not stainable [68]. It is a possibility that substances in the high concentrations (10 mg/ml) of *I. viscosa* leaf extract leading to nucleus damage and prevention of cytoplasmic structures resulted in ghost cells.

In addition, *I. viscosa* leaf extracts induced DNA damage and cell death and/or apoptosis in various frequencies in this study. This study shows for the first time the induction of cell death and/or apoptosis caused by high concentrations (5 mg/ml and 10 mg/ml). Cell death is a basic biological process of living organism. The cell death was induced by high concentrations of such as toxin, stress, heavy metals, chemicals and other. The authors suggested that cells undergo death after moderate stress. In this way, in this paper demonstrated that *I. viscosa* leaf extract induced cytogenetic alterations (cytoplasmic shrinkage, nuclear condensation, DNA fragmentation, membrane blebbing, cytoskeleton alterations and appearance of apoptotic bodies) aid mainly cell death in root tips of *A. cepa* (Figures 1(d), 1(f), 1(g), and 1(h)). *A. cepa* demonstrated to be more sensitive as expected. Considering this, the aim of this study is to determine the features of cell death in root tips of *A. cepa* induced by *I. viscosa* leaf extracts.

Finally, we conclude that when applied in high doses, *I. viscosa* leaf extract shows cytotoxic and genotoxic activity. We used in this study crude extracts of *I. viscosa* leaves. Studying with crude extracts is appropriate because traditional medicinal herbs are generally used as crude extracts. However, working with crude extracts also means working with complex mixtures of biologically active compounds. Some of these compounds can be cytotoxic and/or genotoxic; others can be cytoprotective and/or antigenotoxic. The results of this study suggest that, although *I. viscosa* has beneficial effects as a medicinal herb, it can cause serious problems and damage on cells when used improperly.

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