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Cyto-genotoxicity of *Verbena officinalis* and *V. bonariensis* using *Allium cepa* bioassay

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ABSTRACT

The genus *Verbena* is an important source of bioactive phytochemicals; however, information on the cytotoxic and genotoxic effects of its different cytotypes and solvent extracts remains limited. The present study evaluated the cytogenotoxic effects of aqueous, acetone, and methanolic extracts of *Verbena officinalis* and *V. bonariensis*, with particular emphasis on cytotype- and solvent-associated variation, using the *Allium cepa* root-tip assay. Root meristems were exposed to the respective extracts for 24 h, while distilled water and sodium azide served as negative and positive controls, respectively. Cytotoxicity and genotoxicity were assessed through mitotic index (MI) and the frequency of chromosomal and mitotic abnormalities. The negative control exhibited the highest MI ($64.20 \pm 0.12\%$), whereas the positive control showed a pronounced reduction ($22.53 \pm 0.41\%$). Among the plant treatments, MI ranged from $45.80 \pm 0.31\%$ to $58.73 \pm 0.18\%$. Aqueous extracts generally maintained higher mitotic activity, with the octoploid *V. officinalis* aqueous extract exhibiting the highest MI ($58.73 \pm 0.18\%$), whereas methanolic and acetone extracts produced greater mitotic inhibition. Extract exposure also induced cytological abnormalities, including chromosome stickiness, bridges, laggards, polar deviations, and variant chromosomes. Aberration frequencies ranged from 2.67 to 11.82% among the *Verbena* treatments, compared with 0.73% in the negative control and 31.60% in the positive control. A distinct solvent-dependent response was evident, with methanolic extracts generally producing the highest aberration frequencies, followed by acetone and aqueous extracts. *V. officinalis* exhibited particularly pronounced responses to methanolic extraction, whereas *V. bonariensis* showed comparatively lower aberration frequencies. Collectively, the findings demonstrate that *Verbena* extracts can modulate mitotic activity and chromosome behaviour in *A. cepa*, with the magnitude of the response varying according to solvent, species/accession, and cytotype. The comparatively stronger cytological effects of organic-solvent extracts highlight the importance of evaluating the cytogenetic safety of phytochemical-rich plant preparations alongside their potential biological activities.

Keywords: *Verbena officinalis*, *Verbena bonariensis*, *Allium cepa* assay, Cytotoxicity, Genotoxicity, Mitotic index, Chromosomal aberrations, Polyploidy

1. Introduction

Plants and plant-derived products form an important part of the human diet and have long been used in traditional medicine for the prevention and management of various diseases. Despite their natural origin and widespread consumption, plant products cannot always be assumed to be completely safe. Certain plant constituents and their metabolic by-products may exhibit toxic, mutagenic, teratogenic, or even carcinogenic effects under particular conditions or at specific concentrations (Plewa *et al.*, 1993; Zink *et al.*, 1998) [1, 2]. However, toxicological information on many edible and medicinal plants remains limited, particularly regarding the consequences of prolonged or repeated exposure (Basaran *et al.*, 1996; Soetan *et al.*, 2009) [3, 4]. This highlights the need to evaluate not only the therapeutic potential of plant products but also their possible cytotoxic and genotoxic effects.

Members of the family Cucurbitaceae are widely consumed as vegetables and fruits and are also valued for their nutritional and medicinal properties. Several cucurbits have been reported to possess anti-inflammatory, antitumor, hepatoprotective, cardiovascular, and immunoregulatory activities (Abdelwahab *et al.*, 2011; Dhiman *et al.*, 2012) [5, 6]. In addition to providing important nutrients, including proteins, cucurbits contain a diverse range of secondary metabolites such as alkaloids, cucurbitacins, sterols, and triterpenes (Dhiman *et al.*, 2012) [6]. These phytochemicals contribute substantially to their biological activities; however, some bioactive constituents may also interfere with normal cellular processes when present at sufficiently high concentrations. Therefore, evaluating their effects on actively dividing cells is important for establishing a more complete understanding of their biological safety.

The *Allium cepa* L. root-tip assay provides a simple and sensitive biological system for investigating the cytotoxic and genotoxic effects of plant extracts and other test substances. Young onion roots contain rapidly

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dividing meristematic cells in which disturbances in cell division and chromosome behaviour can be readily detected. Consequently, the assay has been extensively employed to identify antimitotic effects and cytological abnormalities induced by herbal extracts and environmental contaminants (Grant, 1982; Barbério *et al.*, 2009; Roa *et al.*, 2012) [8, 7, 9]. Among the parameters evaluated, the mitotic index (MI) is particularly informative because it reflects the proportion of actively dividing cells and provides an indication of normal cell proliferation or treatment-induced mitotic inhibition (Gadano *et al.*, 2002) [10]. Chromosomal abnormalities observed alongside changes in MI can further provide evidence of potential genotoxic effects.

Plant-based *in vivo* cytogenetic assays therefore offer a practical approach for the preliminary screening of substances that may adversely affect cellular and genetic integrity. Such systems have been recognized as useful tools for toxicological assessment and for detecting potentially harmful environmental agents (El-Shahaby *et al.*, 2003) [11]. Despite the widespread dietary and medicinal use of cucurbits, information concerning their cytotoxic and genotoxic effects remains comparatively limited. Therefore, the present study was undertaken to evaluate the cytotoxic and genotoxic potential of fruit extracts from selected members of the Cucurbitaceae using the *A. cepa* root-tip assay, with particular emphasis on changes in mitotic activity and the occurrence of treatment-induced chromosomal abnormalities.

2. Materials and Methods

2.1 Plant material and cytotype characterization

The experimental material comprised accessions of *Verbena officinalis* and *V. bonariensis* representing different cytotypes. The comparative dataset included a diploid accession of *V. officinalis* (VO-I, 2x), an octoploid accession (VO-II, 8x), and two accessions of *V. bonariensis* (VB-I and VB-II). Aerial parts of the plants were collected and used for preparation of the extracts. Prior to experimentation, the plant material was cleaned to remove adhering debris and unhealthy or damaged tissues.

For taxonomic and cytological traceability, the final manuscript should provide the collection locality, geographical coordinates where available, collection date, voucher specimen number, chromosome number, ploidy level, and method used for cytotype confirmation for each accession. These details should preferably be presented in a separate plant-material table.

2.2 Preparation of plant extracts

The collected aerial parts were thoroughly washed, and healthy plant material was separated, cut into small pieces, and air-dried under suitable conditions. The dried material was subsequently pulverized to a fine powder using a mechanical grinder and stored in airtight containers until extraction.

For extraction, 5 g of dried plant powder was mixed with 100 mL of the respective solvent, corresponding to a plant material-to-solvent ratio of 1:20 (w/v). For aqueous extraction, the plant material was extracted at 60 °C for 6 h. Following extraction, the suspension was filtered through Whatman No. 1 filter paper and centrifuged at 6000 rpm for 30 min to remove residual particulate matter. The resulting extract was concentrated under reduced pressure at 40–45 °C using

a rotary evaporator. The dried extract was subsequently reconstituted as required for the bioassay and stored at 4 °C until further use. Acetone (Ace) and methanolic (MeOH) extracts were prepared following the same general extraction procedure. Before final submission, the original experimental records should be consulted to confirm whether the same plant material-to-solvent ratio, extraction duration, and temperature were applied to all three solvents. Any solvent-specific modifications should be reported explicitly.

2.3 *Allium cepa* cytogenotoxicity assay

The cytogenotoxic potential of the *Verbena* extracts was evaluated using the *Allium cepa* root-tip assay. Healthy and approximately equal-sized *A. cepa* bulbs were selected and initially placed in water to promote the development of actively growing roots. Bulbs exhibiting uniform root growth were subsequently exposed to the respective plant extracts for 24 h.

Following treatment, root tips approximately 1 cm in length were excised and immediately fixed in a Carnoy-type fixative consisting of absolute ethanol, chloroform, and glacial acetic acid in a ratio of 6:3:1 (v/v/v). After fixation, the root-tip material was transferred to 90% ethanol and stored at 4 °C until cytological preparation.

For microscopic examination, the root tips were subjected to the prescribed hydrolysis/staining procedure using 2% acetocarmine and 1 N HCl in a 9:1 ratio. The meristematic region was subsequently squashed in acetocarmine on clean microscope slides. Prepared slides were examined microscopically for mitotic activity, distribution of mitotic phases, and the occurrence of cytological abnormalities.

2.4 Experimental controls and treatment groups

Root tips maintained in distilled water were used as the negative control (NC), whereas sodium azide was employed as the positive control (PC). An inconsistency was identified in the available experimental documentation regarding the concentration of sodium azide. The methodological narrative reports a concentration of 0.5 mg L⁻¹, whereas the corresponding table note indicates 0.05 g L⁻¹ (equivalent to 50 mg L⁻¹). Because these concentrations differ substantially, the actual concentration used experimentally should be verified from the original laboratory records before manuscript submission. No concentration should be selected or harmonized solely on the basis of assumption.

The experimental treatments consisted of aqueous (Aq.), acetone (Ace), and methanolic (MeOH) extracts prepared from the investigated *Verbena* accessions. The supplied treatment dataset includes VO-I, VO-II, VO-III, VO-IV, VB-I, and VB-II. The identity and cytotype status of VO-III and VO-IV should therefore be reconciled with the plant-material description before final submission to ensure consistency between the Materials and Methods and Results sections.

2.5 Cytological assessment and calculation of endpoints

For each treatment, microscopic observations were made on root-tip meristematic cells, and the total number of cells examined, number of dividing cells, distribution of cells among different mitotic phases, and number of cells exhibiting chromosomal or mitotic abnormalities were recorded.

The mitotic index (MI) was calculated as:

$$\text{MI (\%)} = \left(\frac{\text{Number of dividing cells}}{\text{Total number of cells examined}} \right) \times 100$$

The frequency of cells in an individual mitotic phase was calculated as:

$$\text{Mitotic phase (\%)} = \left(\frac{\text{Number of cells in the respective mitotic phase}}{\text{Total number of cells examined}} \right) \times 100$$

The overall frequency of cytological abnormalities was calculated as:

$$\text{Aberration frequency (\%)} = (\text{Number of abnormal cells} / \text{Total number of cells examined}) \times 100$$

Where applicable, individual abnormalities, including chromosome stickiness, disturbed metaphase, chromosome bridges, laggards, fragments, multipolarity, and other aberrant configurations, were recorded separately.

The methodological protocol additionally defines micronucleus frequency as:

$$\text{Micronucleus frequency (\%)} = (\text{Number of cells containing micronuclei} / \text{Total number of cells examined}) \times 100$$

However, micronucleus observations are not included in the currently available dataset. Consequently, micronucleus frequency was not included in the present analysis or interpretation and should only be incorporated if the corresponding primary observations are available.

2.6 Statistical analysis

Quantitative data were expressed as mean $\pm$ standard error (SE). Differences among control and treatment groups were evaluated using analysis of variance (ANOVA), followed by an appropriate post hoc multiple-comparison test.

The available statistical documentation, however, contains inconsistencies that should be resolved before final submission. One dataset reports statistically significant differences at $p \leq 0.05$ and denotes homogeneous groups using superscript letters, whereas another table specifies Duncan's Multiple Range Test (DMRT) with a significance level of $p < 0.001$. The original statistical output should therefore be consulted to confirm the number of independent replicates, exact ANOVA model employed, post hoc multiple-comparison procedure, and prespecified significance threshold.

Following verification, a single consistent statistical approach and significance criterion should be reported throughout the Materials and Methods, Results, tables, figures, and corresponding legends.

3. Results

The *Verbena* extracts reduced mitotic activity in *Allium cepa* root-tip cells compared with the negative control (Figure 1, Table 1). The negative control exhibited the highest mitotic index (MI; $64.20 \pm 0.12\%$),

whereas the positive control showed the lowest ($22.53 \pm 0.41\%$). Among the extract treatments, MI ranged from $45.80 \pm 0.31\%$ to $58.73 \pm 0.18\%$. Aqueous extracts consistently maintained higher mitotic activity than methanolic and acetone extracts. The octaploid *V. officinalis* aqueous treatment exhibited the highest MI ($58.73 \pm 0.18\%$) among the plant treatments, followed by diploid *V. officinalis* ($55.93 \pm 0.29\%$) and *V. bonariensis* (54.00%). Methanolic and acetone extracts showed lower MI values (45.80 – 47.60%).

Cytological examination revealed chromosome stickiness, bridges, laggards, polar deviations and vagrants following extract treatment (Table 2). The negative control showed the lowest aberration frequency (0.73%), whereas the positive control exhibited the highest (31.60%). Among *Verbena* treatments, aberration frequencies ranged from 2.67 to 11.82% . A clear solvent-dependent pattern was observed, with methanolic extracts generally producing the highest aberration frequencies, followed by acetone and aqueous extracts. In diploid *V. officinalis*, methanolic extracts produced the highest aberration frequencies (11.64 – 11.82%), whereas aqueous extracts showed substantially lower values (2.93 – 3.46%). In *V. bonariensis*, aberration frequencies ranged from 2.67 – 2.73% for aqueous, 3.53 – 4.53% for acetone and 5.40 – 5.94% for methanolic extracts.

Overall, the results indicate that *Verbena* extracts influenced both mitotic activity and chromosomal behaviour in a solvent-dependent manner, with methanolic extracts showing greater cytological effects and aqueous extracts producing comparatively milder effects.

Table 1. Number of dividing cells and mitotic index in controls and *Verbena* extract treatments.

Category	Treatment	Dividing cells (mean $\pm$ SE)	Mitotic index (%) (mean $\pm$ SE)
Control	NC	321.00 ± 0.58	64.20 ± 0.12
Control	PC	112.67 ± 2.03	22.53 ± 0.41
VO diploid (2x)	VO-I Aq.	279.67 ± 1.45	55.93 ± 0.29
VO diploid (2x)	VO-I MeOH	234.33 ± 0.33	46.87 ± 0.07
VO diploid (2x)	VO-I Ace	236.33 ± 1.33	47.27 ± 0.27
VO octaploid (8x)	VO-II Aq.	293.67 ± 0.88	58.73 ± 0.18
VO octaploid (8x)	VO-II MeOH	238.00 ± 1.58	47.60 ± 0.31
VO octaploid (8x)	VO-II Ace	237.67 ± 1.45	47.53 ± 0.29
VB diploid (2x)	VB-I Aq.	270.00 ± 1.15	54.00 ± 0.23
VB diploid (2x)	VB-I MeOH	230.33 ± 0.88	46.07 ± 0.18
VB diploid (2x)	VB-I Ace	235.00 ± 1.73	47.00 ± 0.35
VB diploid (2x)	VB-II Aq.	270.00 ± 0.57	54.00 ± 0.12
VB diploid (2x)	VB-II MeOH	229.00 ± 1.53	45.80 ± 0.31
VB diploid (2x)	VB-II Ace	237.67 ± 1.45	47.53 ± 0.29

Abbreviations: NC, negative control; PC, positive control; VO, *Verbena officinalis*; VB, *Verbena bonariensis*; Aq., aqueous; MeOH, methanolic; Ace, acetone; 2x, diploid; 8x, octaploid.

The original source uses superscript letter groupings for significance; these should be restored after the statistical model and significance threshold are verified.

NC denotes negative control (distilled water); PC denotes positive control (sodium azide). Aq., Ace and MeOH denote aqueous, acetone and methanolic extracts, respectively. The supplied source states that values are mean $\pm$ SE and reports DMRT at $p < 0.001$ in the table note; however, the displayed aberration table contains single numerical values rather than explicit $\pm$ SE values. This should be checked against the original statistical output.

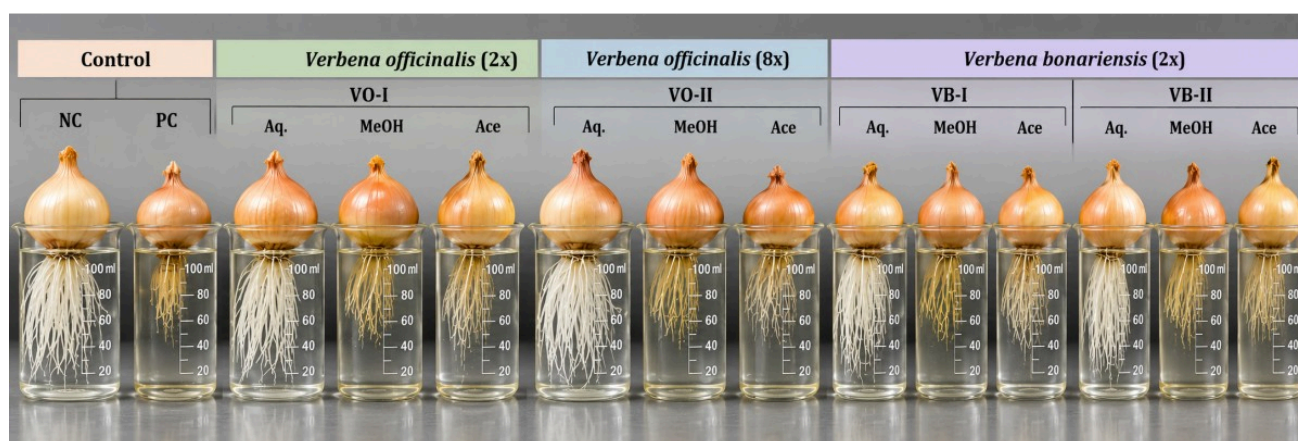


Figure 1. Representative *Allium cepa* root growth following exposure to aqueous (Aq.), methanolic (MeOH) and acetone (Ace) extracts of diploid (2x) and octaploid (8x) *Verbena officinalis* (VO) and diploid *V. bonariensis* (VB). NC, negative control (distilled water); PC, positive control (sodium azide). The image illustrates treatment-associated variation in root growth corresponding to the cytotoxicity assessment.

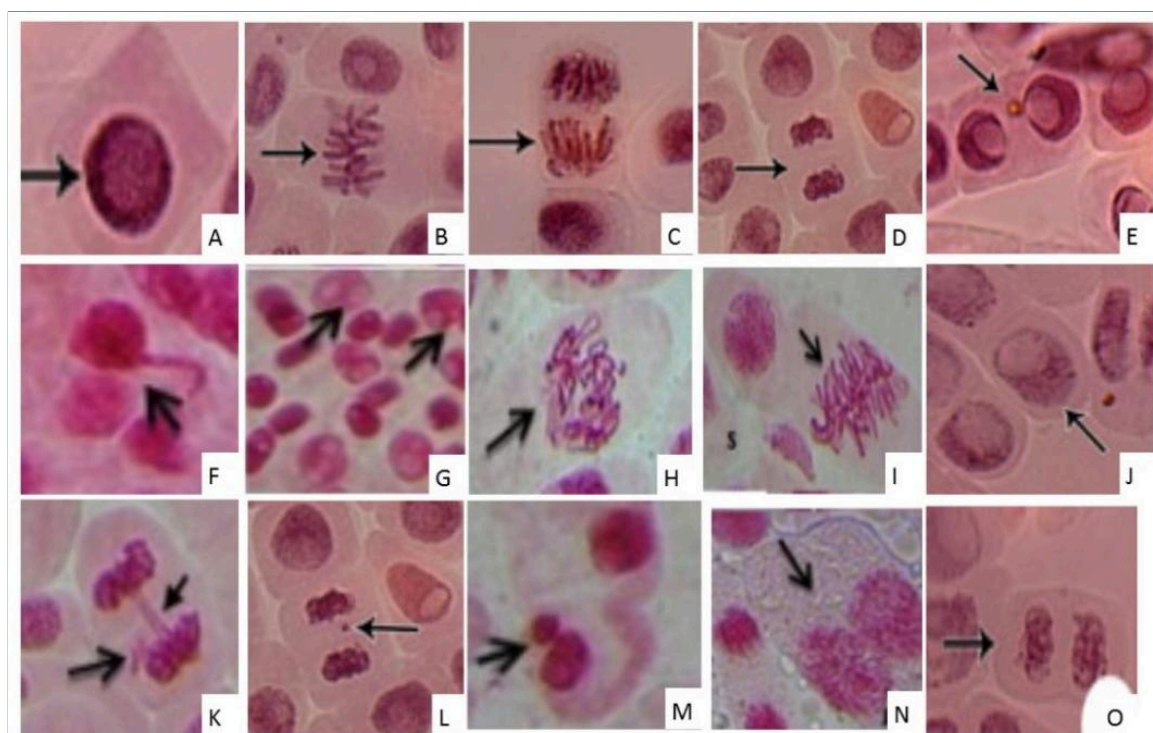


Figure 1. Cytological observations of *Allium cepa* root-tip cells showing normal mitotic stages and chromosomal abnormalities following treatment with sodium azide and *Verbena* extracts. (A–O) Representative photomicrographs of *A. cepa* root meristematic cells illustrating normal mitotic stages and treatment-induced cytological abnormalities (arrows indicate the respective abnormalities) following exposure to distilled water (negative control), sodium azide (positive control), and *Verbena* extracts. **Negative control (A–D):** Normal mitotic stages showing (A) prophase, (B) metaphase, (C) anaphase, and (D) telophase. **Positive control—sodium azide (E–L):** Representative cytological abnormalities showing (E) polyploid prophase, (F) nuclear body formation, (G) pycnotic interphase nucleus, (H) chromosome stickiness during prophase, (I) polyploid cell, (J) lobulated nucleus, (K) chromatin bridge, and (L) laggard chromosome(s). **Plant extract-treated cells (M–O):** (M) Nuclear body formation following treatment with an extract of the diploid cytotype of *Verbena bonariensis*; (N) binucleate prophase following treatment with the methanolic extract of the diploid cytotype of *V. officinalis*; and (O) normal telophase following treatment with the aqueous extract of the octoploid cytotype of *V. officinalis*.

4. Discussion

The *Allium cepa* root-tip assay remains a trusted and sensitive in-vivo tool for checking the cytotoxic and genotoxic effects of chemicals, pollutants and plant extracts (El-Shabbaby *et al.*, 2003; Teixeira *et al.*, 2003; Ventura *et al.*, 2008; Nefic *et al.*, 2013) [11, 12, 13, 14]. Because root meristem cells divide actively, changes in mitotic activity and chromosome structure are easy to see. The mitotic index (MI) simply records the proportion of cells in mitosis and therefore serves as a clear measure of proliferative activity (Smaka-Kinel *et al.*, 1996; Sreeranjini *et al.*, 2011) [15, 16]. A fall in MI after treatment is usually read as a mitodepressive or cytotoxic signal. The same pattern has

been noted with wastewater and assorted plant preparations (Fiskesj, 1997; Babatunde *et al.*, 2006; Olorunfemi *et al.*, 2011) [18, 17, 19], with extracts from many higher plants (Frescura *et al.*, 2013; Khanna *et al.*, 2013) [20, 21], and specifically with cucurbit species (Lamsal *et al.*, 2010) [22]. Even natural phytochemicals can therefore slow cell division in a dose- and composition-dependent way.

How far MI drops also helps gauge severity: a sharp reduction relative to controls often points to strong toxicity, while a milder decline suggests sub-lethal stress (Panda *et al.*, 1985; Antonsiewicz, 1990) [24, 23]. Earlier work has discussed similar cytotoxic thresholds (Sharma, 1983) [25], yet these markers must be interpreted with care because the response changes with the substance, concentration, exposure time and the physiological state of the roots.

Lower MI can arise from blocked DNA synthesis, arrest in G₂, or failure to enter mitosis (Sudhakar *et al.*, 2001) [26]. Disturbances in nucleoprotein synthesis and cellular ATP supply may further hinder chromosome movement, microtubule organisation and spindle elongation (Majewska *et al.*, 2003) [27]. In short, mitotic suppression usually reflects wider metabolic interference rather than a single molecular hit.

Table 2. Cytological effects of aqueous, acetone and methanolic extracts of *Verbena* spp. on *Allium cepa* root-tip cells.

Treatment	Stickiness	Bridges	Laggards	Polar dev.	Vagrants	Total aberrant	Aberrations (%)
NC	1.33	0.67	0.00	0.33	1.33	3.66	0.73
PC	29.33	27.00	39.33	30.33	32.00	158.00	31.60
VO-I (2x) Aq	6.33	3.67	2.33	2.67	2.33	17.33	3.46
VO-I (2x) Ace	7.33	5.33	4.67	5.67	5.67	28.67	5.73
VO-I (2x) MeOH	10.33	11.67	14.83	10.73	10.67	58.23	11.64
VO-II (2x) Aq	5.67	2.67	1.67	2.33	2.33	14.67	2.93
VO-II (2x) Ace	6.67	4.67	3.33	4.00	5.00	23.67	4.73
VO-II (2x) MeOH	12.00	12.67	15.33	9.33	9.79	59.12	11.82
VB-I Aq	5.67	1.67	1.67	2.33	2.33	13.67	2.73
VB-I Ace	5.33	3.67	2.67	3.00	3.00	17.67	3.53
VB-I MeOH	6.67	5.00	4.33	5.33	5.67	27.00	5.40
VB-II Aq	5.00	1.33	2.00	2.00	2.00	13.33	2.67
VB-II Ace	6.33	3.33	4.67	3.67	4.67	22.67	4.53
VB-II MeOH	8.00	4.67	5.67	5.67	5.67	29.68	5.94

Chromosomal abnormalities supply complementary evidence of genotoxic stress. Breakage, faulty repair or exchange, and spindle or segregation problems can all generate structural and numerical aberrations (Khanna *et al.*, 2013; Timothy *et al.*, 2014) [21, 28]. Stickiness, chromatin bridges, laggards, polyploid cells, nuclear irregularities and binucleate cells therefore indicate interference with both chromosome integrity and the orderly progress of mitosis.

Stickiness itself is a strong sign of disturbed chromatin organisation; it has been linked to changes in chromosomal proteins and to the sticky (st) gene (Pagliarini *et al.*, 2008; Risso-Pascotto *et al.*, 2009) [29, 30]. Sticky chromosomes frequently fail to separate at anaphase, favouring the formation of bridges and other segregation errors (Ranjbar *et al.*, 2011) [31]. Bridges may result from persistent associations, breakage-and-reunion events or incomplete chromatid separation, while laggards typically signal spindle malfunction or poor attachment to spindle fibres.

Cytomixis—the transfer of chromatin between neighbouring cells through specialised cytoplasmic channels (Mursalimov *et al.*, 2013) [32]—is another noteworthy response. It has been recorded in onion roots treated with sodium azide (Siddiqui *et al.*, 2007) [33] and with methanolic plant extracts. Physical factors, genetic determinants and interactions between genetic control and environmental stress have all been implicated (Narain, 1976; Ghanima *et al.*, 2003; Haroun *et al.*, 2004) [35, 34, 36]. Chromatin transfer can alter nuclear content and is associated with micronuclei, bridges, binucleate cells, enucleated cells and abnormal chromosome numbers (Mursalimov *et al.*, 2011; Mursalimov *et al.*, 2013; Negron-Ortiz, 2007) [37, 32, 38]. Nuclear bodies or binucleate cells seen after extract treatment may therefore be downstream signs of disrupted nuclear organisation, segregation or cytokinesis.

These cytological effects are closely tied to the complex mix of alkaloids, flavonoids, polyphenols, tannins and other secondary metabolites present in plant extracts (Hayakawa *et al.*, 1999; Perez-Carreón *et al.*, 2002; Prajitha and Thoppil, 2016) [39, 40]. Depending on concentration and chemical interactions, such compounds can upset oxidative balance, DNA integrity, spindle formation, cell-cycle checkpoints or intracellular signalling. Biological activity should therefore not be assumed beneficial—or harmful—simply because the material is plant-derived.

The joint appearance of mitodepressive effects and chromosomal or nuclear abnormalities is especially relevant when medicinal plants are

intended for repeated or prolonged use. Certain bioactive constituents can influence intercellular signalling, gene expression and epigenetic mechanisms, including DNA and histone modifications (Trosko and Upham, 2005; Prajitha and Thoppil, 2016) [41]. Measuring both MI and chromosomal abnormalities together therefore gives a fuller picture of cytotoxicity and genotoxicity than either endpoint alone.

In summary, the *A. cepa* assay shows that plant extracts can affect cell proliferation and chromosome behaviour differently according to their phytochemical profile and extraction solvent. Reduced mitotic activity signals a mitodepressive response, while abnormalities such as stickiness, bridges, laggards, polyploid cells, nuclear bodies, lobulated or pycnotic nuclei and binucleate cells point to problems in chromosome segregation, nuclear organisation or cytokinesis. These observations underline the value of including cytogenetic safety checks in studies of medicinal plant extracts. At the same time, findings from the *A. cepa* system should be treated as an initial screening step rather than direct proof of human toxicity. Concentration–response experiments, oxidative-stress and DNA-damage markers, mammalian cell systems and suitable in-vivo models are still needed to clarify mechanisms and establish true toxicological relevance.

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