Cytotoxic and genotoxic action of *Tagetes patula* flower methanol extract and patuletin using the *Allium* test

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Abstract: *Tagetes patula* is used to treat cancer patients in alternative healthcare systems. However, its cytotoxic and genotoxic effects have not been reported. Therefore, the methanol extract of *T. patula* flower, the ethyl acetate fraction, and the pure compound patuletin were evaluated using the *Allium* test. The methanol extract and fraction contained ~3% and ~36% patuletin, respectively, with ~98% purity. The methanol extract caused inhibition of *Allium* root growth displaying an IC_{50} value of ~500 µg/mL, while the fraction and patuletin were more potent by ~2 and ~5 times, respectively. The *Allium* root tips demonstrated a decline in prophase, metaphase, anaphase, and telophase stages with concomitant decrease in percent mitotic index in the methanol extract (~5.64), fraction, and patuletin (~4) as compared to the control (~7.61). However, in only methanol extract-treated root tips, an increase in metaphase stage was noted. In addition, the methanol extract predominantly induced c-type, misaligned, and multipolar chromosomal abnormalities while the fraction and patuletin displayed fragments and sticky chromosomes. The fraction and patuletin also produced micronuclei (~2%). In conclusion, *T. patula* flower methanol extract and ethyl acetate fraction are cytotoxic and genotoxic, which most likely could be due to the patuletin. Further preclinical and clinical studies are required to justify its clinical use.

Key words: *Tagetes patula*, patuletin, *Allium* test, cytotoxic, genotoxic, chromosomal abnormalities

1. Introduction

Tagetes patula belongs to the family Asteraceae. Commonly known as French marigold, it is found abundantly in tropical regions and is also cultivated in Pakistan (Priyanka et al., 2013). *T. patula* has long been used in relieving the complaints of cough, dysentery, liver ailments, stomach ailments, and rheumatism (Vasilenko et al., 1990). Commercially, it is used for the production of high-quality perfumes (Rajalakshmi and Jose, 2004).

Pharmacologically, the methanol extract (ME) of *T. patula* exhibited antiinflammatory activity (15 and 50 mg/mL) in mice due to lipoxygenase enzyme inhibition, which has been linked to its isolated flavonoidal compound, patuletin (Yasukawa and Kasahara, 2013). Its leaves are useful in relieving muscle- and kidney-associated pain (Rahman et al., 2013). The antioxidant effects of patuletin in neuronal cells (Khan et al., 2010) and in rats have also been reported (Abdel-Wahhab et al., 2005). ME is

toxic to phytopathogenic fungi including *Botrytis cinera*, *Fusarium moniliforme*, and *Pythium ultimum* (Mares et al., 2004), while patuletin is also antibacterial, displaying toxicity towards *Micrococcus luteus*, *Corynebacterium*, *Staphylococcus*, and *Streptococcus* species (Faizi et al., 2008). In Pakistan, the powder of *T. patula* flower is also prescribed for the management of cancer patients (Azeemi, 1999). In our previous work, ME and the ethyl acetate (EA) fraction derived from ME demonstrated antiproliferative activity against cervical (HeLa), lung (NCI-H460), and skin (HT-144) cancer cells. In addition, patuletin was also found to be cytotoxic against the cervical (HeLa) cell line (Kashif et al., 2015).

Genotoxic anticancer agents interact either directly with DNA (clastogenic, e.g., bleomycin) or with a mitotic apparatus, leading to unequal distribution of genetic material in daughter cells (aneugenic, e.g., vinblastine). These genetic damages appear as small spherical particles

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outside the nuclear membrane in the form of micronuclei formation and/or as chromosomal abnormalities in the cells. In many laboratories around the world, the *Allium* test is widely used for genotoxic assessment due to its sensitivity, reliability, easy culturing, well-explored cell cycle kinetics (24 h), fast-dividing root tip cells, and correlation with mammalian test systems (Abdel Migid et al., 2007). The root tip cells of *Allium* species (*A. cepa* and *A. sativum*) have large-sized chromosomes (2n = 16) (Konuk et al., 2007), making them popular among researchers. Inhibition in root length accompanied by alterations in mitotic division and chromosomal structures of root tip cells are good indicators of cytotoxicity as well as genotoxicity (Hoshina and Marin-Morales, 2009). *A. cepa* was first used to determine the mutagenicity of inorganic salts (Levan, 1945). However, *A. sativum* has the advantage that each of its cloves can serve as a separate entity, thus reducing the amount of test samples required to perform experiments.

The ME, EA, and pure compound patuletin were evaluated for their growth inhibitory activities to justify this plant's traditional use in cancer. Growth inhibitory and cytotoxic effects were assessed by determining root length inhibition of *A. cepa* and/or *A. sativum* and the mitotic index of their root tip cells. In addition, their genotoxic actions as a mode of anticancer activity were assessed by analyzing the type of chromosomal abnormalities (CAs) and micronuclei formation in *Allium* root tip cells.

2. Materials and methods

Onion and garlic were purchased from a local market and other items were purchased from as follows: bleomycin hydrochloride (Bleocin, Nippon Kayaku Co. Ltd., Japan); vinblastine sulfate (Vinblas, Pharmedic Laboratories Pvt. Ltd., Pakistan); glass slides, glass cover slips (22 × 22 mm, Nr. 1., Citoglas, the Netherlands); acetic acid, orcein powder (Sigma, USA).

2.1. Collection and identification of *T. patula*

The flowers of *T. patula* were identified by Dr. Rubina Dawar and collected and deposited in Herbarium of the Department of Botany, University of Karachi, Pakistan, as voucher specimen KUH GH No. 67280. The flowers were extracted and fractionated to obtain the pure compound patuletin as described earlier (Kashif et al., 2015).

2.2. HPLC fingerprinting of *T. patula* methanol extract, ethyl acetate fraction, and patuletin

High-performance liquid chromatography (HPLC) fingerprinting of ME and EA was conducted to detect the presence of patuletin. The purity of patuletin was also determined as described earlier (Li et al., 2010). Briefly, both samples and standards including patuletin, patulitrin, and methyl protocatechuate were prepared in the mobile

phase. An HPLC-Prominence System Controller CBM-20A (Shimadzu) equipped with a degasser (DGU-20A5), quaternary pump (LC-20AT), autosampler (SIL-20A), and diode array detector (SPD-M20A) and software LC Solution were used for analysis.

2.3. Solutions

The solutions of ME (25–1000 µg/mL), EA (1–100 µg/mL), and patuletin (5–100 µg/mL) were prepared in DMSO with final concentrations not exceeding 2%. Bleomycin (1–100 µg/mL) and vinblastine (1–50 µg/mL) were prepared in distilled water.

2.4. Cytotoxic studies

The cytotoxic effects of ME, EA, and patuletin were determined by measuring root lengths as described earlier (Sehgal et al., 2006) with slight modifications, i.e. the root inhibition was determined at 48 h, as described below.

2.4.1. *A. cepa*

Conical and round onion bulbs weighing 50 ± 10 g with diameters of 17–19 cm were scratched until the root buds were visible without damaging the primordial root. The onions (n = 5) were grouped as: 1) control (d/w), 2) vehicle control (DMSO, 2%), and 3) treated with different concentrations of ME, bleomycin, and vinblastine. Only 5 healthy roots (2–3 cm) per onion were kept intact while the remaining roots were excised. After 48 h, the roots length were noted and photographed.

2.4.2. *A. sativum*

Garlic cloves (n = 30, ~2.0 g) were placed in glass vials for 3 days at 25 °C. On the 3rd day, 5 cloves/group were organized as follows: 1) control (d/w), 2) vehicle control (DMSO, 2%), and 3) treated with various concentrations of ME, EA, patuletin, bleomycin, and vinblastine. After 48 h, the roots length were recorded and photographed.

2.5. Data handling for root length inhibition

The *Allium* root inhibition was determined by two methods, as described below.

2.5.1. Method-1

A. cepa root length inhibition was determined by a commonly used method (Sehgal et al., 2006):

Root length inhibition (%) = Root length (in cm) [(Control – Treated) / Control] × 100.

2.5.2. Method-2

The mean root growth of *A. cepa* and *A. sativum* in the control and treated groups was determined as described earlier (Ali et al., 2017). Root growth was determined as follows: Root growth (cm) = Root length (cm) at (48 h – 0 h). Root growth inhibition (%) = Obtained root growth (cm) [(Control – Treated) / Control] × 100. The graph was plotted between various concentrations of test agents (x-axis) vs. percent root growth inhibitions (y-axis) and corresponding IC_{50} values were determined.

2.6. Microscopic studies of *Allium* species' root tip cells

The healthy *A. cepa* bulbs (n = 9) and *A. sativum* cloves (n = 9) were treated with respective IC_{50} values of ME, EA, patuletin, bleomycin, and vinblastine. Their roots were processed as described below.

2.6.1. Harvesting, fixation, and hydrolysis of root tips

The root tips (~2–3 mm) from the control and respective treated groups were removed between 11:00 AM and 1:00 PM. These root tips (n = 27) were fixed (ethanol:acetic acid, 3:1, 24 h), hydrolyzed (1 N HCl solution, 5–15 min, to disengage the spatial arrangement between the cells), and rinsed with distilled water.

2.6.2. Preparation and staining of slides

The hydrolyzed root tips were dipped in aceto-orcein stain (2%, 30 min), placed onto a glass slide, smeared, and observed for mitotic stages under a microscope (Nikon TE-2000, Japan) at 400×. The photographs were further processed by Windows Photo Gallery and the relevant parameters were noted, as described below.

2.6.3. Mitotic index

In the control and treated groups, 459–749 cells/root tip were identified for the following stages. A) Interphase stage: the appearance of red, round/circular structure (nucleus) within the cell membrane. B) Mitotic stages: i) Prophase stage: thread-like chromosomes, ii) Metaphase stage: aligned chromosomes at the center, iii) Anaphase stage: chromatids move towards opposite poles, and iv) Telophase stage: two separate bundles of chromatids at opposite poles observed. The mitotic index was calculated as follows (Sehgal et al., 2006):

Mitotic index (%) = (Cells in mitotic stages) / [Total cells (mitotic + interphase)] × 100.

2.6.4. Chromosomal abnormalities (CAs)

The structural CAs in the control and treated groups were classified as i) bridging, ii) c-type chromosomes, iii) fragments, iv) lagging, v) misalignment, vi) multipolar, and vii) sticky chromosomes:

CAs (%) = Structural changes in mitotic stages / Total cell count × 100.

2.6.5. Micronuclei formation

The interphase stages of *A. cepa* and *A. sativum* root tip cells (at least ~14,000/group) were observed for the presence of micronuclei. These were recognized as tiny spherical nonrefractive nuclear particles visible as a dark red color, similar to that of the main nucleus in the interphase stage.

2.7. Statistical analysis

The data for mitotic index, CAs, and micronuclei formation were transformed to minimize the variances in mean values by square root transformation method, i.e. ($\sqrt{x + 1}$), where x is the mean value of the data. In all the experiments, the comparisons between controls and treated groups were made by one-way ANOVA and post hoc test (DMR, Dunnett's multiple comparison test) using SPSS 12.0. Data with $P < 0.05$ were considered significant (Rebouças et al., 2013; Eke and Çelik, 2008).

3. Results

3.1. HPLC fingerprinting of ME, EA, and patuletin

In Figure 1, ME displays three peaks demonstrating the presence of patuletin (~2.97%). However, in EA, it was ~36%. The purity of isolated patuletin was noted to be ~98.32%.

3.2. Root length and growth inhibition in *A. cepa* and *A. sativum*

In *A. cepa* (n = 9) at 48 h, ME significantly reduced percent root inhibition (~8%–39%). However, the IC_{50} value could not be determined (Table 1). On the contrary, using root growth inhibition Method-2, the IC_{50} value was noted. In the case of EA-treated *A. sativum* roots, an IC_{50} value for root growth inhibition of ~225 µg/mL was observed, which was reduced by ~2.5 times in the presence of patuletin (Figures 2 and 3).

Similar to ME, bleomycin failed to display an IC_{50} value for *A. cepa* root length inhibition while vinblastine showed inhibition (data not shown). However, using Method-2, root growth inhibition in the presence of both bleomycin and vinblastine was noted (Figures 2 and 3).

3.3. Mitotic index

3.3.1. *A. cepa*

In Table 2, it is evident that ME reduced cells in telophase stage by ~4.02 times as compared to the control. However, no changes in other mitotic stages were observed. In the presence of a clastogenic agent, i.e. bleomycin, all mitotic stages were significantly decreased. An aneugenic agent, i.e. vinblastine, caused decreases in only anaphase and telophase stages. The reduction in the mitotic index in the presence of ME was similar to vinblastine while it was 2 times less than that of bleomycin.

3.3.2. *A. sativum*

In Table 3, ME demonstrates a significant decrease in prophase (~1.50×), anaphase (~1.31×), and telophase (~3.84×) stages with an increase in metaphase (~1.36×) as compared to the control. In the presence of EA and patuletin, a significant reduction in prophase (~2.36×), metaphase (~1.51×), anaphase (~1.45×), and telophase (~1.97×) was observed, which was also evident in bleomycin-treated cells. The mitotic index was significantly reduced by ~1.4 times in ME- and vinblastine-treated cells while it was ~2 times in the case of EA, patuletin, and bleomycin.

3.4. CAs

Various CAs induced by test agents at their respective IC_{50} values in *Allium* root tip cells are presented in Figure 4.
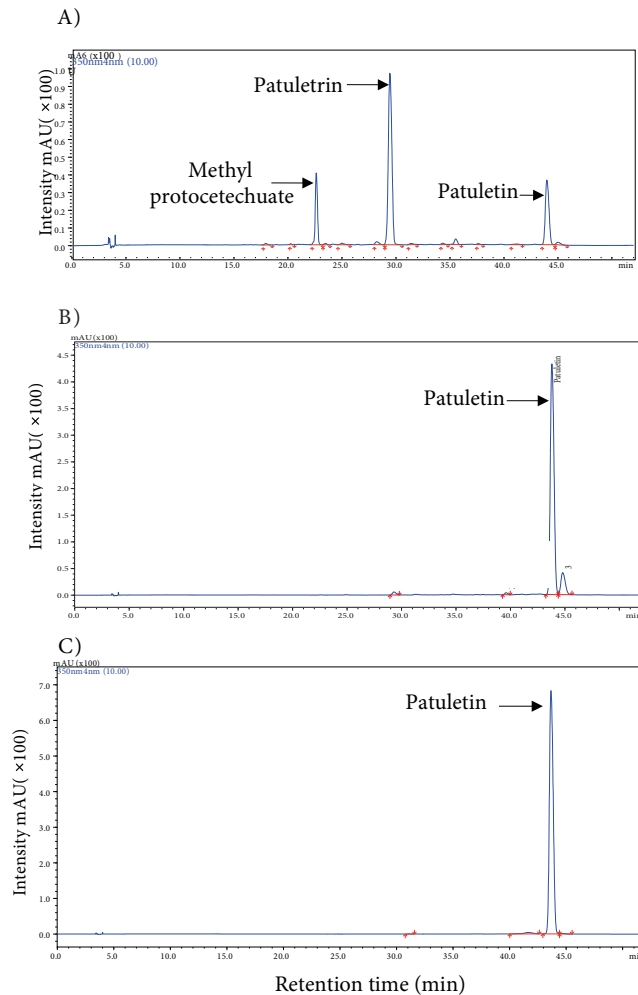
A)

B)

C)

Figure 1. HPLC chromatograms of samples obtained from *T. patula* for the presence of patuletin. A) Methanol extract of *T. patula* flower, B) ethyl acetate fraction, and C) patuletin. Peaks are marked with arrows to signify the compound retention times. Detection wavelength: 350 nm. The unmarked minor peaks are unidentified and insignificant in the genotoxic results. Samples were eluted using mobile phases composed of aqueous phosphoric acid (0.1%, v/v) and acetonitrile. A gradient elution of acetonitrile, 5%–12% (0–12 min), 12%–16% (12–14 min), 16% B (14–21 min), 16%–20% (21–27 min), 20%–25% (27–37 min), was used. The reequilibration time of gradient elution was 15 min. The flow rate was 1.0 mL/min through a Hypersil Gold C8 column with diameter 250 × 46.6 mm and particle size 5 μm (Thermo Electron Corporation). The column temperature was maintained at 30 °C.

3.4.1. *A. cepa*

In prophase stage, only ME and vinblastine significantly increased sticky and c-type CAs (Figure 5A). In metaphase stage, ME produced a significant increase in c-type CAs (~10×), which was doubled in the presence of vinblastine. Misaligned CAs with both ME and vinblastine (~90×

and ~60×, respectively) were also present (Figure 5B). In anaphase cells, ME demonstrated bridging (~14×) of CAs with no fragments while both ME and vinblastine displayed multipolar (~10×) chromosomes. In contrast, bleomycin exhibited a significant rise in bridging (Figure 5C). In the telophase stage, only bleomycin induced chromosomal fragments (Figure 5D).

Table 1. Effect of methanol extract of *T. patula* flower on *A. cepa* root length.

Concentration (μg/mL)	Root length (cm) ± SEM	
	Time (h)	
	0	48
100	2.41 ± 0.04[1]	4.80 ± 0.05[7] (8 ± 0.47)
200	2.47 ± 0.05[1,2]	4.60 ± 0.06[6] (12 ± 0.5)
300	2.40 ± 0.05[1]	4.35 ± 0.05[5] (17 ± 0.5)
400	2.47 ± 0.05[1,2]	4.17 ± 0.06[4] (20 ± 0.36)
500	2.58 ± 0.05[2]	3.98 ± 0.05[3] (24 ± 0.15)
600	2.49 ± 0.05[1,2]	3.53 ± 0.06[2] (33 ± 0.58)
1000	2.43 ± 0.05[1,2]	3.2 ± 0.05[1] (39 ± 1.04)
IC$_{50}$	–	Not obtained
Control	2.43 ± 0.04[1,2]	5.23 ± 0.07[8]

Onion bulbs used (n = 9–12) and roots per onion (n = 5–7), total number of roots observed: control (distilled water) and methanol extract of *T. patula* (n = 44–60), each value represents mean ± SEM of 3 or 4 independent experiments. Values within parentheses represent the percentage root length inhibition as compared to the control. The inhibitory concentration that decreases the root length by 50% = IC$_{50}$. In columns, dissimilar superscript numerals (1–8) represent significant differences (P < 0.05) while similar numerals represent nonsignificant root lengths compared to each other.

It was also noted that ME- and vinblastine-treated cells had significantly increased cumulative CAs in prophase (~30×), metaphase (~80× and ~59×, respectively), and anaphase (~12× and ~9×, respectively) stages with no CAs in the telophase stage. However, in the case of bleomycin-treated root tip cells, CAs were significantly increased (~30×) only in the telophase stage. The potency order for the induction of CAs was ME > vinblastine > bleomycin.

3.4.2. *A. sativum*
In the prophase stage, only ME- and vinblastine-treated cells caused significant increases in c-type while only ME induced sticky chromosomes (Figure 6A). In the metaphase stage of ME- and vinblastine-treated cells, c-type and misaligned chromosomes were evident. On the other hand, EA, patuletin, and bleomycin displayed significantly higher rates of fragments (Figure 6B). In the anaphase stage of ME-treated cells, an increase in the bridged type of CA (~11×) was noted along with the induction of multipolar chromosomes (~12×), which was observed in vinblastine-treated cells. Conversely, EA, patuletin, and bleomycin caused an increase in fragmented chromosomes (~20%). In addition, EA also displayed the bridged type of CA (Figure 6C). In the telophase stage

of EA-, patuletin-, and bleomycin-treated cells, only the fragment type (~10%) of CAs was noted (Figure 6D).

It was observed that ME-treated cells caused a significant production of CAs in the prophase stage (~30×), while in the presence of EA and patuletin, it was only in telophase phase (~3×). However, both groups presented higher CAs in metaphase (90× and 34×, respectively) and anaphase (~20× and ~39×, respectively) stages as compared to the control. In the case of bleomycin, a significant rise of CAs in metaphase (~20×), anaphase (~30×), and telophase (~2.22×) was evident. However, vinblastine produced significantly more CAs in prophase (~17×), metaphase (~80×), and anaphase (~20×). The potency order for the induction of CAs was ME > vinblastine > EA = patuletin = bleomycin.

3.5. Micronuclei formation
Figure 7 displays that EA, patuletin (in *A. sativum*), and bleomycin (in both *A. cepa* and *A. sativum*) caused a significant rise in micronuclei formation.

4. Discussion
In the present study, the cytotoxic actions of ME, EA, and patuletin (flavonoid) were evaluated by root length inhibition and mitotic index in *A. cepa* and *A. sativum*. In addition, the types of structural CAs as well as micronuclei formations were also noted for the evaluation of aneugenic and/or clastogenic actions.

The *Allium* test resembles (~82%) mammalian test systems (Ray et al., 2013), making this test an efficient tool for the detection of toxicity of unknown chemicals. It utilizes mixed-function oxidases (Grant, 1982) for their metabolic activation. It can also determine genotoxins by identifying CAs and micronuclei formation. The IC$_{50}$ values for root inhibition at 48 h is considered reliable for the assessment of the toxicity, genotoxicity (İlbaş et al., 2012), and cytogenetic actions (Konuk et al., 2007) of the compounds. However, IC$_{50}$ values for *A. cepa* root inhibition in the presence of ME and bleomycin could not be determined using Method-1. The reason could be an unequal length of roots (ranging from 2 to 3 cm) at 0 h among the control as well as the treatment groups. Therefore, *A. cepa* root lengths of all the groups at 48 h were subtracted from their corresponding readings at 0 h to normalize the difference in root length and this was referred to as root growth (Ali et al., 2017). Using this approach, the IC$_{50}$ value of root growth inhibition for ME in *A. cepa* and *A. sativum* was ~500 μg/mL and ~423 μg/mL, respectively, indicating that *A. sativum* is more sensitive than *A. cepa*, as has been observed earlier (Saxena et al., 2010).

The *A. cepa* test was considered unsuitable for the assessment of EA or patuletin due to the requirement of large volumes of solutions (~300 mL). To cope with this
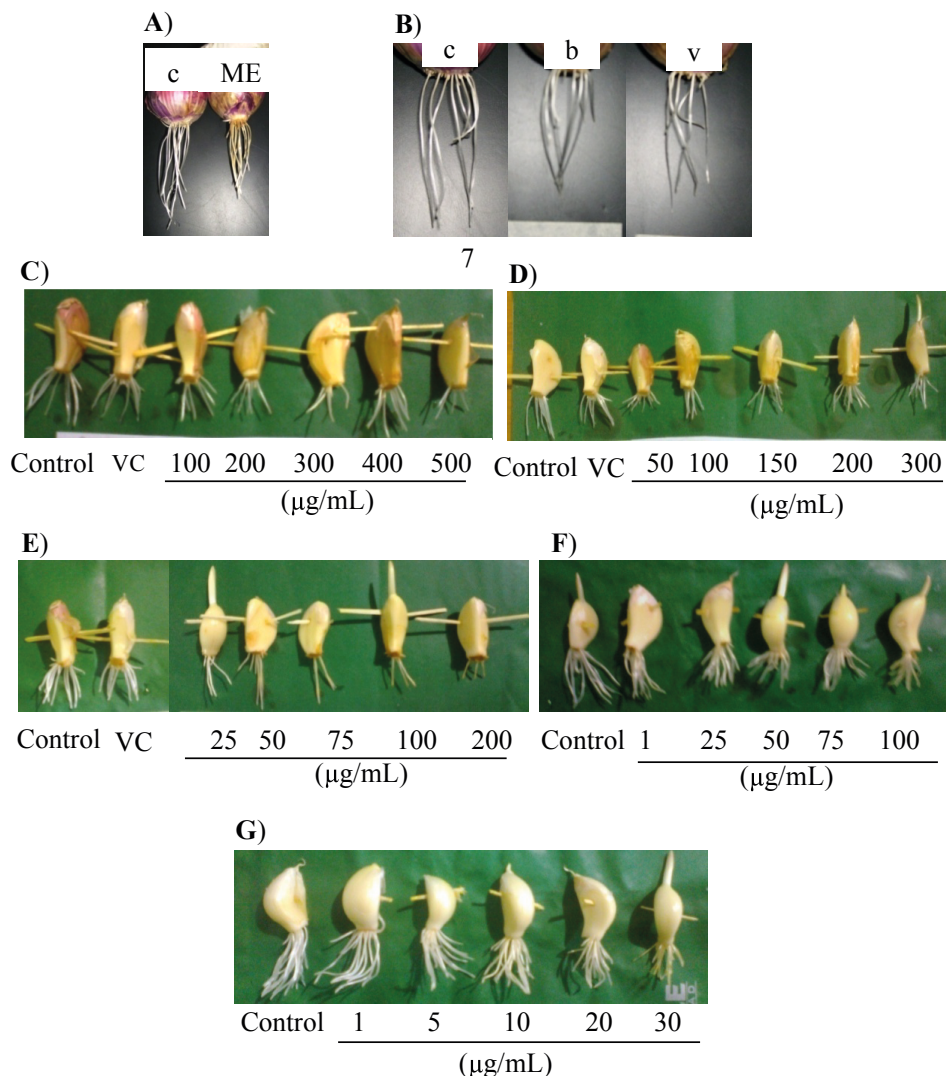
Figure 2. Root growth of *A. cepa* and *A. sativum* at 48 h. *A. cepa* root growth in A) (c) control and (ME) methanol extract of *T. patula*: 500 µg/mL; B) (c) control, (b) bleomycin (50 µg/mL), and (v) vinblastine (15 µg/mL). *A. sativum* root growth: C) methanol extract of *T. patula* flower, D) ethyl acetate fraction, E) patuletin, F) bleomycin, and G) vinblastine. In C, D, and E: (VC) vehicle control is 2% DMSO.

problem, *A. cepa* seeds were tested for seed germination and growth of roots (data not shown), but they failed to fulfill the recommended criteria (Wang et al., 2001). Therefore, *A. sativum* cloves were used, which provided the advantage of being individual entities along with reduced requirements (~5-fold) of test solutions for the root inhibition studies. The IC_{50} values of EA and patuletin were ~2 and ~4 times lower than that of ME (~423 µg/mL), indicating that antiproliferative and cytotoxic action was increased in EA and patuletin as compared to ME. The potency order of root inhibition was patuletin > EA

> ME. Earlier studies demonstrated root length inhibition in the presence of anticancer drugs. In the present study, the IC_{50} value obtained in the presence of tubulin inhibitor (vinblastine, IC_{50} ~13 µg/mL) displayed more potent effects in inhibiting root growth than a DNA-damaging agent (bleomycin, IC_{50} ~57 µg/mL).

The inhibitory effect of toxic agents on root length is associated with a reduction in cell division (Ray et al., 2013), which can be determined by suppression of the mitotic index. The mitotic index provides information about the proportion of cells in various mitotic stages (M-phase) of
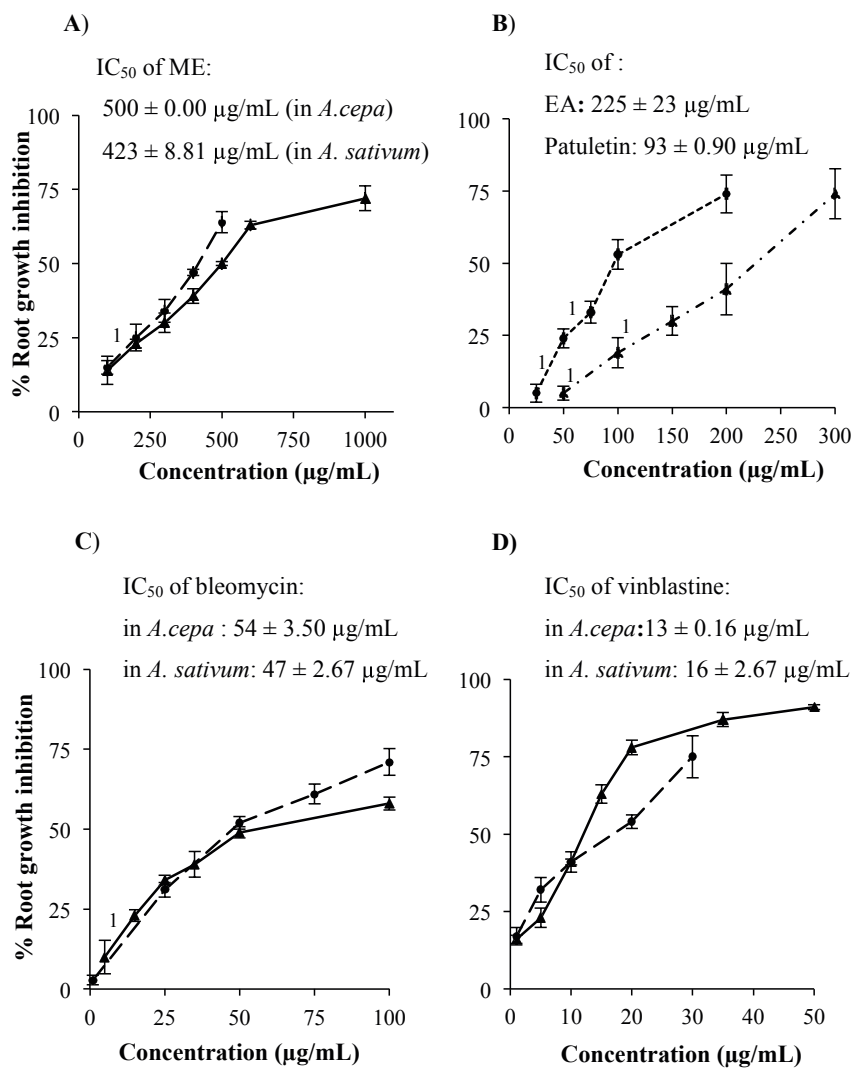
A)

B)

C)

D)

Figure 3. Effect on *Allium* root growth. A) Root growth inhibition of *A. cepa* (▲——) and *A. sativum* (⁻ ⁻●⁻ ⁻) in the presence of methanol extract of *T. patula* flower (ME). Control root growth (cm) in *A. cepa* = 2.80 ± 0.12, and in *A. sativum* = 1.32 ± 0.03. B) Root growth inhibition caused by ethyl acetate fraction (EA, ⁻·⁻▲⁻·⁻) and patuletin (----●----) in *A. sativum*. Control root growth (cm) for ethyl acetate fraction = 1.62 ± 0.09, and patuletin = 1.59 ± 0.04. C) Root growth inhibition of *A. cepa* (▲——) and *A. sativum* (⁻ ⁻●⁻ ⁻) in the presence of bleomycin. Control root growth (cm) in *A. cepa* = 3.94 ± 0.11, and in *A. sativum* = 1.57 ± 0.09. D) Root growth inhibition of *A. cepa* (▲——) and *A. sativum* (⁻ ⁻●⁻ ⁻) in the presence of bleomycin. Control root growth (cm) in *A. cepa* = 3.72 ± 0.11, and in *A. sativum* = 1.65 ± 0.14. n = 52–60 root tips/concentration, percent root growth inhibition with superscript numeral (1) represents nonsignificant while without numerals indicates significant difference (P < 0.05) as compared to control root growth inhibition.

the cell cycle, i.e. prophase, metaphase, anaphase, and telophase. In the present study, *T. patula* samples, vinblastine, and bleomycin reduced the mitotic index as compared to the control (~7.5%), which supported their cytotoxic action. The potency order of suppression of the mitotic index

was EA = patuletin = bleomycin > ME = vinblastine. Furthermore, metaphase arrest in ME-treated *A. sativum* root tip cells was also evident. However, in ME- and vinblastine-treated *A. cepa* root tips cells, it was obtained when the metaphase:anaphase ratio was applied as described

Table 2. Effect on mitotic stages, interphase, and mitotic index of *A. cepa* root tip cells.

T. patula flower	Cells in mitotic stages				Total (a)	Interphase (b)	Total cells (a+b)	Mitotic index (%) a / (a+b)
	Prophase	Metaphase	Anaphase	Telophase				
Methanol extract	346[1,2,3] (2.28 ± 0.15)	313[1] (2.06 ± 0.08)	118[1,2] (0.78 ± 0.09)	54[3] (0.36 ± 0.02)	831	14350[2] (94.53 ± 0.11)	15181	5.47 ± 0.11[2]
Genotoxic drugs (positive control)								
Bleomycin (clastogenic)	172[3] (1.14 ± 0.10)	168[2] (1.12 ± 0.09)	94[2] (0.62 ± 0.07)	89[2,3] (0.59 ± 0.05)	523	14545[1] (96.53 ± 0.14)	15068	3.48 ± 0.15[3]
Vinblastine (aneugenic)	409[1,2] (2.50 ± 0.05)	266[1] (1.61 ± 0.16)	117[2] (0.72 ± 0.07)	121[2] (0.74 ± 0.11)	913	15495[2] (94.43 ± 0.14)	16408	5.57 ± 0.14[2]
Control	646[1] (3.46 ± 0.86)	326[1] (1.72 ± 0.18)	187[1] (1.00 ± 0.11)	273[1] (1.45 ± 0.12)	1432	17374[3] (92.37 ± 1.00)	18806	7.63 ± 1.00[1]

Onion bulbs used (n = 9), roots per onion (n = 3), total number of root tips observed in control and with various concentrations of test samples (n = 27), each value represents mean ± SEM of 3 independent experiments. Values without parentheses represent the number of cells and those within parentheses are mean ± SEM of percentage of cells in various mitotic and interphase stages
Mitotic Index (%) = Total cells in mitotic stages (a) / Total cells [mitotic stages (a) + interphase (b)] × 100.
In columns, dissimilar superscript numerals (1–4) represent significant differences (P < 0.05) and similar numerals represent nonsignificant differences in mitotic stages, interphase, and mitotic index as compared to each other. Test agents used (IC$_{50}$ values): *T. patula* flower methanol extract (500 μg/mL), bleomycin (50 μg/mL), and vinblastine (15 μg/mL).

Table 3. Effect on mitotic stages, interphase, and mitotic index of *A. sativum* root tip cells.

T. patula flower	Cells in mitotic stages				Total (a)	Interphase (b)	Total cells (a+b)	Mitotic index (%) a / (a+b)
	Prophase	Metaphase	Anaphase	Telophase				
Methanol extract	219[2] (2.35 ± 0.34)	198[1] (2.13 ± 0.10)	73[2] (0.78 ± 0.10)	36[3] (0.39 ± 0.08)	526	8780[2] (94.36 ± 0.09)	9306	5.64 ± 0.39[2]
Ethyl acetate fraction	139[4] (1.50 ± 0.08)	95[3] (1.03 ± 0.06)	65[2] (0.70 ± 0.06)	70[2] (0.76 ± 0.05)	369	8870[3] (96.01 ± 0.05)	9239	4.00 ± 0.05[3]
Patuletin	131[4] (1.44 ± 0.17)	91[3] (1.00 ± 0.04)	69[2] (0.76 ± 0.04)	59[2,3] (0.65 ± 0.10)	350	8740[3] (96.15 ± 0.35)	9090	3.85 ± 0.35[3]
Genotoxic drugs (positive control)								
Bleomycin (clastogenic)	120[4] (1.23 ± 0.15)	89[3] (0.91 ± 0.02)	57[2] (0.58 ± 0.05)	57[2,3] (0.58 ± 0.05)	323	9498[3] (96.71 ± 0.10)	9821	3.29 ± 0.10[3]
Vinblastine (aneugenic)	248[2] (2.51 ± 0.30)	176[1,2] (1.78 ± 0.19)	74[2] (0.75 ± 0.08)	74[2] (0.75 ± 0.01)	575	9358[2] (94.18 ± 0.16)	9933	5.82 ± 0.46[2]
Control	365[1] (3.54 ± 0.64)	159[2] (1.56 ± 0.21)	104[1] (1.02 ± 0.17)	153[1] (1.50 ± 0.03)	781	9452[1] (92.40 ± 0.18)	10233	7.61 ± 0.58[1]

Garlic cloves used (n = 9), roots per garlic (n = 2), total number of root tips observed in control and with test samples (n = 18). The test agents used (IC$_{50}$ values): *T. patula* flower methanol extract (500 μg/mL), ethyl acetate fraction (225 μg/mL), patuletin (100 μg/mL), bleomycin (50 μg/mL), and vinblastine (15 μg/mL). For more details see the legend of Table 2.

earlier (Jordan et al., 1992). Therefore, the mitotic data of tubulin disruptors (aneugenic) should be subjected to the metaphase:anaphase ratio to determine metaphase arrest. On the other hand, no metaphase arrest was observed in the *Allium* data for EA, patuletin, and bleomycin (clastogenic). In contrast, cells were arrested in the interphase stage, which might occur due to their direct genotoxic effects, decrease in cell numbers entering mitotic division,

and the suppression of DNA synthesis by blocking the G$_1$, G$_2$, and/or S-phases (Türkoğlu, 2009), leading to alterations in the chromosomal structures.

CA induction is a useful parameter of genotoxicity. In control *Allium* root tip cells, the presence of CAs (~0.80%) might be due to automutagenic effects (Ivanova et al., 2005) of genotype and environmental interactions. The ME, similar to vinblastine, induced higher rates of
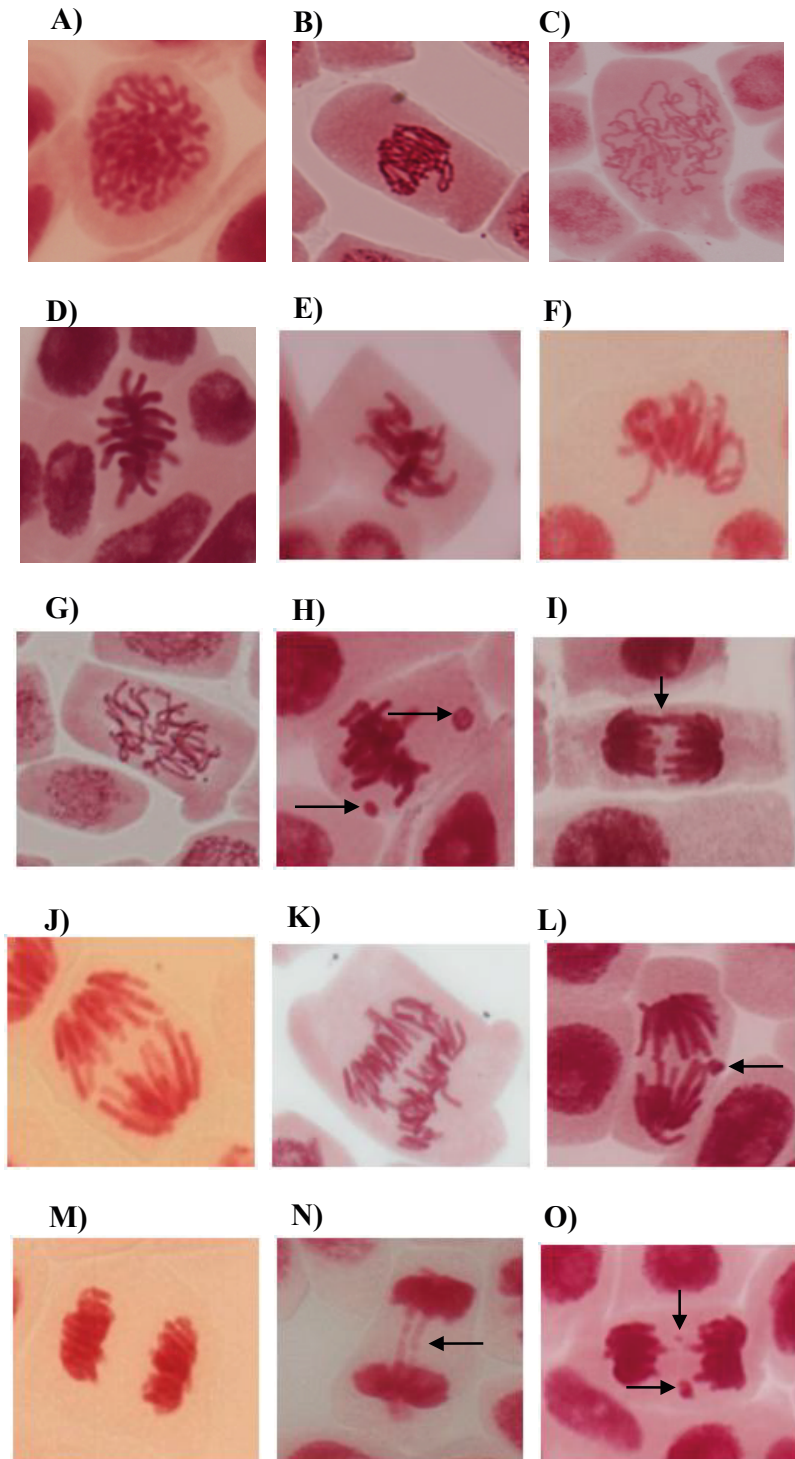
Figure 4. Representative chromosomal abnormalities in prophase, metaphase, anaphase, and telophase mitotic stages of *Allium sativum* root tip cells. Prophase: (A) normal, (B) sticky, and (C) c-type. Metaphase: (D) Normal, (E) misaligned, (F) sticky, (G) c-type, and (H) fragments. Anaphase: (I) Normal, (J) bridging, (K) multipolar, and (L) fragment. Telophase: (M) Normal, (N) bridging, and (O) fragments. In the presence of the methanol extract of *T. patula* flower (ME: 500 µg/mL) and vinblastine (15 µg/mL) predominant chromosomal abnormalities were those shown in B, C, E, F, G, J, K, and N, while in the presence of the ethyl acetate fraction (225 µg/mL), patuletin (100 µg/mL), and bleomycin (50 µg/mL) they were H, L, O. The arrows indicate corresponding chromosomal abnormalities. Magnification: 400×.
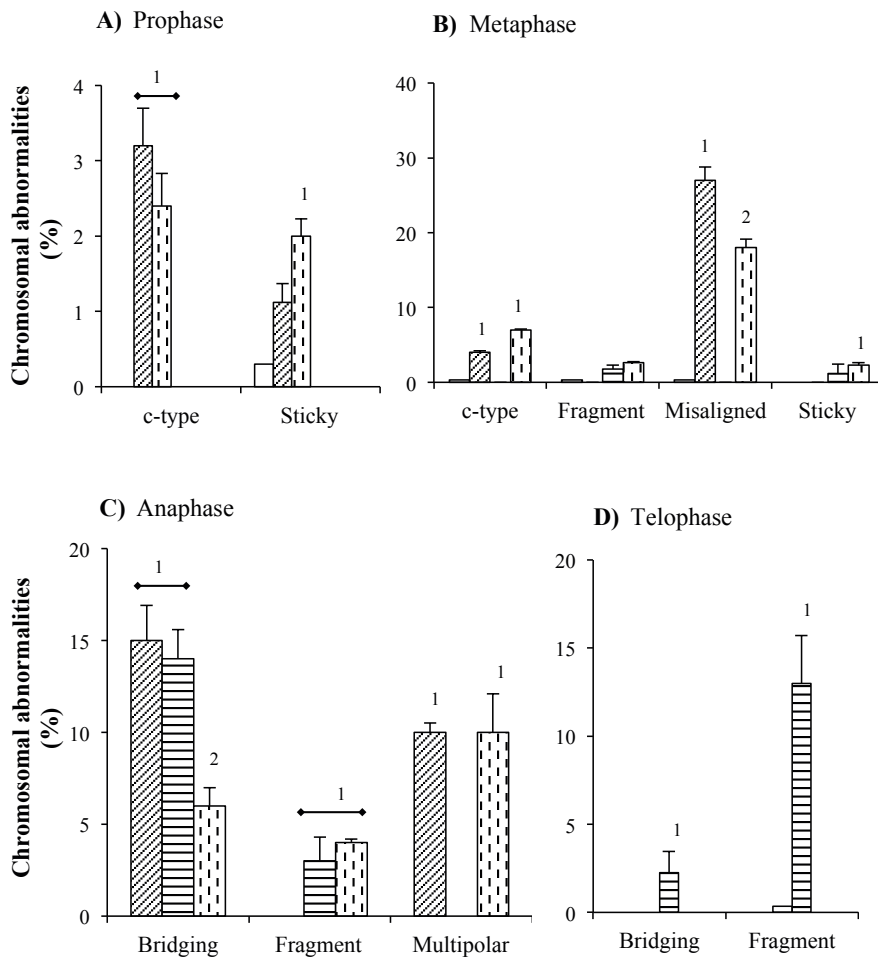
Figure 5. Chromosomal abnormalities in *A. cepa* root tip cells: (☐) control, (▨) methanol extract of *T. patula* flower-ME, (▤) bleomycin, and (▦) vinblastine. The percentages of cells with chromosomal abnormalities were calculated from total cells in respective mitotic stages (presented in Table 2). The bars without superscript numerals represent nonsignificant chromosomal abnormalities as compared to the control. Dissimilar superscript numerals (1–2) represent significant differences ($P < 0.05$) and similar numerals represent nonsignificant percentage values within particular chromosomal abnormalities. The test agents used (IC_{50} values): methanol extract of *T. patula* flower (ME: 500 µg/mL), bleomycin (50 µg/mL), and vinblastine (15 µg/mL).

CAs (~17%–22%) than EA, patuletin, and bleomycin, indicating that all treatments possess genotoxic potential. The lower rates of CAs in EA, patuletin, and bleomycin are related to the reduced availability of cells in mitotic stages as compared to ME- and vinblastine-treated cells. Furthermore, ME- and vinblastine-induced CAs (c-mitosis, misaligned, and multipolar chromosomes) were mostly related to the interference with tubulin and spindle organization, indicating aneugenic action (Fernandes et

al., 2009). The c-type chromosomes are usually formed as a result of microtubule and spindle abnormalities leading to scattered and chaotic chromosomes (Akyil et al., 2012). Misaligned chromosomes arise due to abnormal microtubules causing incomplete tubulin polymerization, which leads to random arrangement of chromosomes at the equatorial plate. Multipolar divisions are the result of problems in spindle fiber depolymerization that occur during polar shifting in metaphase and anaphase stages
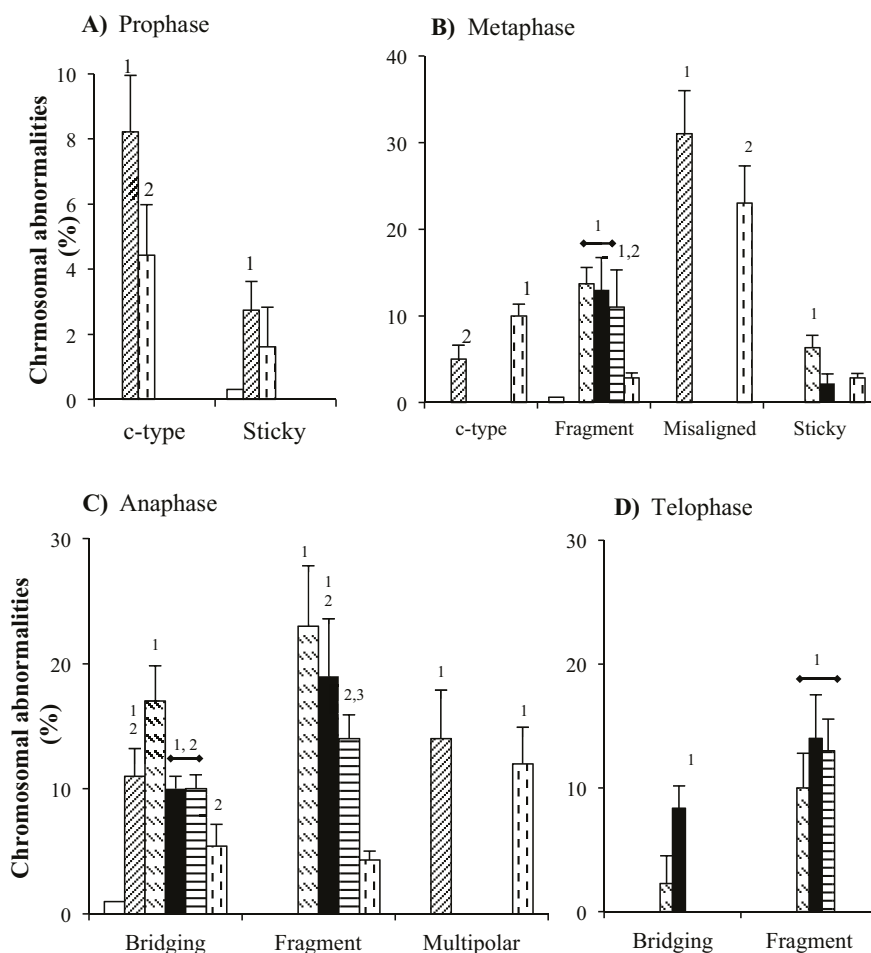
Figure 6. Chromosomal abnormalities in *A. sativum* root tip cells: (⬚) ethyl acetate fraction, (■) patuletin; for others refer to Figure 5. The percentages of cells with chromosomal abnormalities were calculated from total cells in respective mitotic stages (presented in Table 3). The bars without superscript numerals represent nonsignificant chromosomal abnormalities as compared to the control. Dissimilar superscript numerals (1–3) represent significant difference ($P < 0.05$) and similar numerals represent nonsignificant percentage values within particular chromosomal abnormalities. The test agents used (IC_{50} values): ethyl acetate fraction (225 µg/mL), patuletin (90 µg/mL); for others, refer to Figure 5.

(Bhatta and Sakya, 2008). Additionally, in the presence of ME, lagging chromosomes were also indicated, which are also generated due to defects in spindle apparatus proteins (Liman et al., 2012). Conversely, EA, patuletin, and bleomycin displayed chromosomal fragments (~10%–20%), suggesting their clastogenic action. These are formed from chromosomal cuts, incompetent repair machinery, and interference in the structural organization of chromatin (Asthana et al., 2011). Some CAs were commonly noted in ME-, EA-, and patuletin-treated root tip cells including sticky (~3%–5%) and bridging (~10%–20%) CAs. Sticky chromosomes are clustered

chromosomes associated with incomplete dissolution of the nucleoproteins that direct inappropriate chromosome fiber folding, inhibition of spindle formation, retardation of contraction and/or condensation of chromosomes, and improper depolymerization of DNA. It is an irreversible toxic effect resulting in cell death. Bridged chromosomes originate from sticky chromosomes due to improper chromosomal movement. They can also arise from fusion, breakage, and uneven distribution of chromatids during translocation (El-Ghamery et al., 2000).

Micronuclei formation is another parameter for the determination of genotoxins. In the present study, control

A)

B)

Normal

Micronuclei formation
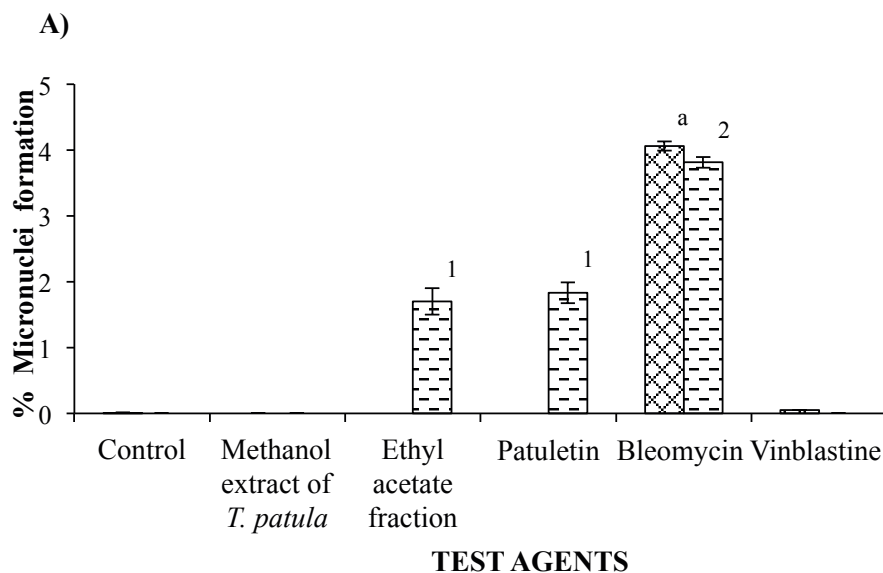
Figure 7. Micronuclei detection in *A. cepa* and *A. sativum* root tip cells. **A)** (▨) *A. cepa*, (▦) *A. sativum*. Total number of bulbs (n = 9), roots per onion (n = 3), total number of root tips observed in control (distilled water) and with various concentrations of test agents (n = 27). *A. sativum*: Total number of cloves (n = 9) and roots per garlic clove (n = 2), total number of root tips in control (distilled water) and with various concentrations of test agents (n = 18). Each value represents mean ± SEM of 3 independent experiments. The values without superscripts (letters or numerals) represent micronuclei formation similar to the control while those with superscript letters (a in *A. cepa*) or numerals (1–2 in *A. sativum*) are significantly (P < 0.05) different as compared to the control. In *A. sativum*, dissimilar superscript numerals (1–2) represent significant (P < 0.05) and similar numerals represent nonsignificant micronuclei formation when compared to each other. The treatments used (IC$_{50}$ values): methanol extract of *T. patula* (500 µg/mL), ethyl acetate fraction (225 µg/mL), patuletin (100 µg/mL), bleomycin (50 µg/mL), and vinblastine (15 µg/mL). B) Representative photos of micronuclei formation in *Allium* root tip interphase cells of *A. cepa*.

A. sativum cells in the interphase stage displayed no micronuclei while a few (~0.11%) were observed in *A. cepa*, similar to earlier reports on automutagenic effects (Ivanova et al., 2005). No micronuclei formation in the presence of ME (500 µg/mL) or vinblastine (15 µg/mL) might possibly be related to the absence of the fragment type of CAs, which leads to the formation of micronuclei (Hoshina and Marin-Morales, 2009). However, in *A.* *sativum* interphase cells, EA (225 µg/mL) and patuletin (90 µg/mL) induced micronuclei formation (~1.8%). This assures us that *T. patula* flower has genotoxic properties. Furthermore, its genotoxic potential should be explored in rodent bone marrow erythrocytes due to additional factors such as pharmacokinetics and repair systems.

The HPLC fingerprinting of ME revealed that it contains ~3% patuletin (a flavonoidal molecule), which

was increased to ~36% in EA. The IC_{50} value of root length inhibition for EA (225 μg/mL) involved patuletin of ~90 μg, which was similar to the IC_{50} of patuletin itself (purity of ~98%), suggesting that its cytotoxic and genotoxic activities are related to patuletin. This finding also corresponds to an earlier work in which the growth inhibitory effect of patuletin was ~6 and ~12 times higher than that of ME and EA, respectively, on cervical cancer (HeLa) cells (Kashif et al., 2015). Flavonoids possess antioxidant properties at concentrations between 5 and 25 μM, protecting cells from oxidative damage (Wätjen et al., 2005; Siddique and Afzal, 2009). However, flavonoids at higher concentrations produce genotoxic effects along with the following essential structural features: 1) double bond between carbons 2 and 3 of the C-ring, 2) OH^- group at carbon 3 of the C-ring, 3) OH^- group at carbon 7 of the A-ring, and 4) catechol or pyrogallol on the B-ring (Silva et al., 2000). The clastogenic action of flavonoids at higher concentrations could be induced by intercalation of DNA, topoisomerase II inhibition, and generation of free radicals (Snyder and Gillies, 2002).

In conclusion, ME, EA, and patuletin possess cytotoxic and genotoxic actions. In both *Allium* species, ME acts as an aneugen (comparatively higher mitotic index with metaphase arrest, formation of c-type and misaligned chromosomes, and no detectable micronuclei formation), while EA and patuletin are clastogenic (low mitotic index, chromosomal fragments, induction of micronuclei) in action. However, further preclinical and clinical studies are required to determine the clinical efficacy of *T. patula* flower against human cancers.

Acknowledgments

References

Abdel Migid HM, Azab YA, Ibrahim WM (2007). Use of plant genotoxicity bioassay for the evaluation of efficiency of algal biofilters in bioremediation of toxic industrial effluent. Ecotoxicology and Environmental Safety 66: 57-64. doi: 10.1016/j.ecoenv.2005.10.011

Abdel-Wahhab MA, Said A, Huefner A (2005). NMR and radical scavenging activities of patuletin from *Urtica urens* against aflatoxin B_1. Pharmaceutical Biology 43: 515-525. doi: 10.1080/13880200500220730

Akyil D, Oktay S, Liman R, Eren Y, Konuk M (2012). Genotoxic and mutagenic effects of aqueous extract from aerial parts of *Achillea teretifolia*. Turkish Journal of Biology 36: 441-448. doi: 10.3906/biy-1112-25

Ali SA, Haque S, Mudassar, Versiani MA, Faizi S et al. (2017). Cytotoxicity and chromosomal aberrations induced by methanolic extract of *Cuscuta reflexa* and its pure compounds on meristematic cells of *Allium* species. Pakistan Journal of Pharmaceutical Sciences 30: 521-529.

Asthana M, Kumar A, Sharma S (2011). Cytogenetical effects of *Terminalia bellerica*, Roxb. on root meristem of *Vicia faba*. Advances in Bioresearch 2: 174-177.

Azeemi KS (1999) Rang-o-Roshni se Ilaj. Karachi, Pakistan: Ishtiaq Printing Press (in Urdu).

Bhatta P, Sakya SR (2008). Study of mitotic activity and chromosomal behaviour in root meristem of *Allium cepa* L. treated with magnesium sulphate. Ecological Society 15: 83-88. doi: 10.3126/eco.v15i0.1947

Eke D, Çelik A (2008). Genotoxicity of thimerosal in cultured human lymphocytes with and without metabolic activation sister chromatid exchange analysis proliferation index and mitotic index. Toxicology In Vitro 22: 927-934. doi: 10.1016/j.tiv.2008.01.012

El-Ghamery AA, El-Nahas AI, Mansour MM (2000). The action of atrazine herbicide as an inhibitor of cell division on chromosomes and nucleic acids content in root meristems of *Allium cepa* and *Vicia faba*. Cytologia 65: 277-287. doi: 10.1508/cytologia.65.277

Faizi S, Siddiqi H, Naz A, Bano S, Lubna Mazhar K et al. (2008). Antibacterial and antifungal activities of different parts of *Tagetes patula*: preparation of patuletin derivatives. Pharmaceutical Biology 46: 309-320. doi: 10.1080/13880200801887476

Fernandes TC, Mazzeo DE, Marin-Morales MA (2009). Origin of nuclear and chromosomal alterations derived from the action of an aneugenic agent-Trifluralin herbicide. Ecotoxicology and Environmental Safety 72: 1680-1686. doi: 10.1016/j.ecoenv.2009.03.014

Grant WF (1982). Chromosome aberration assays in *Allium*. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Research 99: 273-291. doi: 10.1016/0165-1110(82)90046-X

Hoshina MM, Marin-Morales MA (2009). Micronucleus and chromosome aberrations induced in onion (*Allium cepa*) by a petroleum refinery effluent and by river water that receives this effluent. Ecotoxicology and Environmental Safety 72: 2090-2095. doi: 10.1016/j.ecoenv.2009.07.002

İlbaş Aİ, Gönen U, Yılmaz S, Dadandı MY (2012). Cytotoxicity of *Aloe vera* gel extracts on *Allium cepa* root tip cells. Turkish Journal of Botany 36: 263-268. doi: 10.3906/bot-1102-5

Ivanova E, Staikova TA, Velcheva I (2005). Cytogenetic testing of heavy metal and cyanide contaminated river waters in a mining region of Southwest Bulgaria. Journal of Cell and Molecular Biology 4: 99-106.

Jordan MA, Thrower D, Wilson L (1992). Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. Journal of Cell Science 102: 401-416.

Kashif M, Bano S, Naqvi S, Faizi S, Lubna et al. (2015). Cytotoxic and antioxidant properties of phenolic compounds from *Tagetes patula* flower. Pharmaceutical Biology 53: 672-681. doi: 10.3109/13880209.2014.936471

Khan AL, Hussain J, Hamayun M, Gilani SA, Ahmad S et al. (2010). Secondary metabolites from *Inula britannica* L. and their biological activities. Molecules 15: 1562-1577. doi: 10.3390/molecules15031562

Konuk M, Liman R, Ciğerci IH (2007). Determination of genotoxic effect of boron on *Allium cepa* root meristematic cells. Pakistan Journal of Botany 39: 73-79.

Levan A (1945). Cytological reactions induced by inorganic salt solutions. Nature 156: 751.

Li L, Li MH, Xu LJ, Guo N, Wu-Lan TN et al. (2010). Distribution of seven polyphenols in several medicinal plants of Boraginaceae in China. Journal of Medicinal Plants Research 4: 1216-1221. doi: 10.5897/JMPR10.095

Liman R, Gökçe UG, Akyıl D, Eren Y, Konuk M (2012). Evaluation of genotoxic and mutagenic effects of aqueous extract from aerial parts of *Linaria genistifolia* subsp. *genistifolia*. Brazilian Journal of Pharmacognosy 22: 541-548. doi: 10.1590/S0102-695X2012005000028

Mares D, Tosi B, Poli F, Andreotti E, Romagnoli C (2004). Antifungal activity of *Tagetes patula* extracts on some phytopathogenic fungi: ultrastructural evidence on *Pythium ultimum*. Microbiological Research 159: 295-304. doi: 10.1016/j.micres.2004.06.001

Priyanka D, Shalini T, Navnnet VK (2013). A brief history of marigold (*Tagetes* species): a review. International Research Journal of Pharmacy 4: 43-48.

Rahman AHMM, Nitu SK, Ferdows Z, Islam AKMR (2013). Medico-botany on herbaceous plants of Rajshahi, Banglabesh. American Journal of Life Sciences 1: 136-144. doi: 10.11648/j.ajls.20130103.20

Rajalakshmi R, Jose J (2004). Karyomorphometrical analysis and chemical polymorphism in *Tagetes erecta* and *Tagetes patula.* Philippine Journal of Science 133: 135-144.

Ray S, Kundu LM, Goswami S, Roy GC, Chatterjee S et al. (2013). Metaphase arrest and delay in cell cycle kinetics of root apical meristems and mouse bone marrow cells treated with leaf aqueous extract of *Clerodendrum viscosum* Vent. Cell Proliferation 46: 109-117. doi: 10.1111/cpr.12011

Rebouças Sde O, da Silva J, Bertoni RS, Decker N, Santos MS et al. (2013). Assessment of the genotoxic and mutagenic properties of *Himatanthus articulatus* bark extracts used as phytotherapeutic drug in the Amazon. Journal of Ethnopharmacology 147: 474-480. doi: 10.1016/j.jep.2013.03.041

Saxena PN, Gupta SK, Murthy RC (2010). Carbofuran induced cytogenetic effects in root meristem cells of *Allium cepa* and *Allium sativum*: a spectroscopic approach for chromosome damage. Pesticide Biochemistry and Physiology 96: 93-100. doi: 10.1016/j.pestbp.2009.09.006

Sehgal R, Roy S, Kumar VL (2006). Evaluation of cytotoxic potential of latex of *Calotropis procera* and Podophyllotoxin in *Allium cepa* root model. Biocell 30: 9-13.

Siddique YH, Afzal M (2009). Protective effect of apigenin against hydrogen peroxide induced genotoxic damage on cultured human peripheral blood Lymphocytes. Journal of Applied Biomedicine 7: 35-43. doi: 10.32725/jab.2009.003

Silva ID, Gaspar J, da Costa GG, Rodrigues AS, Laires A et al. (2000). Chemical features of flavonols affecting their genotoxicity. Potential implications in their use as therapeutical agents. Chemico-Biological Interactions 124:29-51. doi: 10.1016/S0009-2797(99)00139-8

Snyder RD, Gillies PJ (2002). Evaluation of the clastogenic, DNA intercalative, and topoisomerase II-interactive properties of bioflavonoids in Chinese hamster V79 cells. Environmental and Molecular Mutagenesis 40: 266-276. doi: 10.1002/em.10121

Türkoğlu S (2009). Genotoxic effects of mono-, di-, and trisodium phosphate on mitotic activity, DNA content, and nuclear volume in *Allium cepa* L. Caryologia 62: 171-179. doi: 10.1080/00087114.2004.10589683

Vasilenko YuK, Bogdanov AN, Frolova LM, Frolov AV (1990). Hepatoprotective properties of preparations from spreading marigold. Khimiko-Farmatsevticheskii Zhurnal 24: 53-56. doi: 10.1080/00087114.2004.10589683

Wang X, Sun C, Gao S, Wang L, Shuokui H (2001). Validation of germination rate and root elongation as indicator to assess phytotoxicity with *Cucumis sativus*. Chemosphere 44: 1711-1721. doi: 10.1016/S0045-6535(00)00520-8

Wätjen W, Michels G, Steffan B, Niering P, Chovolou Y et al. (2005). Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. Journal of Nutrition 135: 525-531. doi: 10.1093/jn/135.3.525

Yasukawa K, Kasahara Y (2013). Effects of flavonoids from French marigold (florets of *Tagetes patula* L. on acute inflammation model. International Journal of Inflammation 2013: 309493.