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Toxicological evaluation of polar and nonpolar components of *Isodon coetsa* (Lamiaceae)

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Abstract: *Isodon coetsa* (Buch.-Ham. ex D.Don) Kudo, a perennial herb used in traditional Chinese folk medicines as an antibacterial, antiinflammatory, and antitumour agent, was subjected to toxicity analysis. Cytotoxic screening using the *Allium* L. assay was done with extracts containing polar and nonpolar compounds as well as with polar compounds alone. Root tips were treated with different concentrations of both the extracts, 0.005%, 0.01%, 0.05%, and 0.1%, for varying time durations (30 min, 1 h, 2 h, and 3 h). Extremely significant levels of chromosome abnormality ($P < 0.001$) were observed with the extracts when compared with the positive control (0.01% methyl parathion), but the extract with polar and nonpolar compounds showed higher abnormality percentage. Not much variation was observed in the mitotic index when compared to the negative control (distilled water). Studies revealed many clastogenic and nonclastogenic abnormalities. The major abnormalities included chromosome fragments, stickiness, ring chromosomes, chromosome bridges, pulverization, binucleate cell, micronucleus, ball metaphase, chromosome laggards, and shift in microtubule organizing center. The results showed that the nonpolar components possessed more clastogenic activity, which can be specifically targeted in order to destroy cancer cells. The toxic activity of extracts also suggests the need of judicious use of *I. coetsa* in folk medicines.

Key words: *Allium* assay, chromosome abnormalities, clastogenic, cytotoxicity, nonclastogenic, *Isodon coetsa*

1. Introduction

Studies on the cytotoxic activity of plants used in traditional folk medicines have immense relevance in today's disease-engulfed world as they are targeted in modern research to overcome various diseases. Cytotoxic activities of crude extract are analyzed due to the fact that traditional medicines do not have purified active components of the plant. Studies concerning toxicity and mutagenicity are needed to verify the efficacy and safety of use of plants for the treatment of diseases (Macêdo et al., 2008; Ferreira et al., 2009). Investigation of traditional medicinal plants is thus valuable as a source of potential chemotherapeutic drugs and as a measure of safety for the continued use of medicinal plants (Verschaeve et al., 2004).

Plants of the genus *Isodon* (Schrad. ex Benth.) Spach (Rabdosia) in the family Labiatae are a rich natural source of terpenoids. They are cosmopolitan in distribution. A number of those isolated terpenoids have been found to have potent antitumor activities with low toxicity. They are therefore being studied as candidates for anticancer drugs (Sun et al., 2001). Many plants of this genus have been used in traditional Chinese folk medicine for the treatment of respiratory and gastrointestinal bacterial infections and cancer (Sun et al., 2001, 2006; Fujita and Node, 1984) and as antibacterial, antiinflammatory, and antitumor agents. Perusal of the literature revealed that studies on the cytotoxic activity of diterpenoids isolated from several species of *Isodon* have been attempted, but only a few of them exhibited significant activity (He et al., 2009; Zhan et al., 2011; Zhou et al., 2013). Zhao et al. (2011) isolated xerophilusin B from aerial parts of *Isodon coetsa* (Buch.-Ham. ex D.Don) Kudo and it was found to possess cytotoxic activity against HT-29, BEL-7402, and SK-OV-3 human tumor cell lines. Studies on *I. coetsa* collected from India have not been attempted yet. Hence, the plant was selected for cytotoxic studies.

The test system employed for testing the cytotoxic activity of *Isodon coetsa* leaf extract was *Allium cepa* L. According to Rank and Nielsen (1993) and Rank (2003), one of the advantages of the *A. cepa* test is the possibility to expose the test organism directly to complex mixtures without previous treatment of the test sample. Another advantage is the presence of an oxidase enzyme system, which is essential for promutagen evaluations (Fiskesjo, 1985). Thus, *A. cepa* presents metabolic capacity for activating promutagens into mutagens without addition of an exogenous metabolic system to assess the contaminant class (Fatima and Ahmad, 2006). Moreover, the plant tests in some ways are more sensitive than both the microscreen assay and the Ames test. They can even detect some

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carcinogenic substances that are negative in the Ames test (Rank and Nielsen, 1994). The results of higher plant bioassays should not be discarded, considering that a chemical able to induce chromosome damages in plants can also offer risks to other groups of living organisms, since the damaged material is the DNA, which is common to all organisms (Leme and Marin-Morales, 2009).

2. Materials and methods

2.1. Plant material

Isodon coetsa (Buch.-Ham. ex D.Don) Kudo was collected from Munnar in the Idukki district of Kerala (10°6′0″N, 77°4′0″E, 1602 m) and identified taxonomically, and the voucher specimen was stored (CALI 123726) in the Calicut University Herbarium.

2.2. Preparation of the extract and control

Fresh aqueous extracts containing both polar and nonpolar compounds as well as polar compounds alone were prepared by grinding the leaves in distilled water. The nonpolar compounds were selectively removed from the extract using diethyl ether to obtain extract with polar compounds alone. The lowest concentrations of the extract, 0.1%, 0.05%, 0.01%, and 0.005% (w/v; Ex_1, Ex_2, Ex_3, Ex_4; PEx_1, PEx_2, PEx_3, PEx_4), were chosen after preliminary toxicity analysis.

Distilled water and an organophosphorus pesticide, methyl parathion (0.01%), were taken as the negative control (NC) and the positive control (PC), respectively.

2.3. Cytotoxic assay

Uniformly sized bulbs of *Allium cepa* were sorted and planted in sterilized sandy soil without manure to prevent cellular alterations. Germinated bulbs with healthy roots (1–2 cm) were collected at the peak mitotic period (0900–1000 hours), washed in distilled water, and kept in different concentrations of the extract. Root tips cut from the samples at different time intervals of 30 min, 1 h, 2 h, and 3 h were washed in distilled water and immediately fixed in modified Carnoy's fluid for 1 h. Mitotic squash preparation was done with the help of improved techniques (Sharma and Sharma, 1990). Hydrolysis with 1 N HCl and staining with 2% acetocarmine was carried out. Mitotic index and abnormality percentage were calculated by counting the mitotic cells and aberrant cells, respectively, out of the total cells scored. All the slides were scanned and tabulated, and photomicrographs were taken with a Leica ICC 50 digital camera attached to a Leica DM 500 research microscope.

2.4. Statistical analysis

Data obtained on mitotic index and abnormality percentage were subjected to statistical analysis. Duncan's multiple range test and one-way ANOVA were performed to determine mean separation and significance of treatments using SPSS 20 (SPSS Inc., Chicago, IL, USA).

3. Results

The cytotoxic assay was done using extracts containing polar and nonpolar compounds as well as extract having polar compounds alone, which revealed that the former possess more clastogenic activity when compared to the latter (Table). Studies showed many clastogenic and nonclastogenic abnormalities (Figures 1 and 2). The extracts exhibited high levels of clastogenicity. The major clastogenic abnormalities observed included chromosome fragments, stickiness, ring chromosomes, chromosome bridges, and pulverization, while the nonclastogenic abnormalities observed were binucleate cell, micronucleus, ball metaphase, chromosome laggards, and shift in the microtubule organizing center (MTOC). Some rare abnormalities like ghost cell, nuclear emergence, hyperchromasia, nuclear intrusion, chromosome gaps, chromosome rosette, comet anaphase, nonsynchronized division in binucleate cell, centromere attraction, unilateral anaphase, unipolar anaphase, and disappearance of nucleus in one cell after cytokinesis could also be observed. Not much variation was observed in the mitotic index when compared to the negative control ($P < 0.001$), but high levels of chromosomal abnormalities ($P < 0.001$) with increasing concentration and time duration were found, indicating the clastogenic effect of the extract. The extract with polar and nonpolar compounds showed extremely significant chromosome abnormality ($P < 0.001$) when compared to the control as well as the extract with polar compounds alone. Thus, it may be noted that the clastogenic activity observed was mainly due to the effect of nonpolar components present in the extract.

4. Discussion

Cytotoxic chemicals act on mitotic cells in 3 different manners (Ray and Barman, 1987): preprophase inhibition, inhibition of mitotic spindle formation and orientation (mitoclastic agents), and inhibition of cell plate and cell wall formation between daughter nuclei resulting in binucleate and multinucleate cells. *Allium* has long been used in investigating physical and chemical mutagenesis, pollutant agents, plant extracts, and similar active materials' cytogenetic effects in mitotic cell division (İlbaş et al., 2012). The leaf extracts at different concentrations act differentially by disturbing nucleic acid metabolism, leading to hazards in DNA and protein synthesis, which results in an array of abnormalities both at the nuclear and chromosome levels on *Allium cepa* root tip meristem (George and Geethamma, 1990).

The leaf extracts of *Isodon coetsa* showed clastogenic activity with increasing concentrations and time durations. The chromosome abnormalities observed were both clastogenic aberrations attributable to the direct action in chromosomes and nonclastogenic/physiological

Table. Mitotic index and abnormality percentage in control and various treatments.

Extract	Time duration (h)	Total cells ± SE	Dividing cells ± SE	Chromosomal aberrations ± SE		Mitotic index ± SE	Abnormality % ± SE
				Clastogenic	Nonclastogenic		
	½	699 ± 1.87^{a}	553 ± 2.20^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	79.1 ± 0.90^{b}	0.00 ± 0.00^{a}
NC	1	720 ± 1.39^{a}	668 ± 1.05^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	78.9 ± 0.50^{b}	0.00 ± 0.00^{a}
	2	682 ± 2.92^{a}	536 ± 1.94^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	78.6 ± 0.54^{b}	0.00 ± 0.00^{a}
	3	713 ± 1.99^{a}	612 ± 1.12^{b,c}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	85.8 ± 0.63^{c,d}	0.00 ± 0.00^{a}
	½	844 ± 6.41^{c,d}	259 ± 1.99^{a}	286 ± 2.40^{e}	76 ± 1.54^{b,c,d}	30.7 ± 0.69^{a}	42.9 ± 0.27^{g}
PC	1	939 ± 9.52^{c,d}	232 ± 3.49^{a}	349 ± 3.05^{f}	101 ± 1.92^{d}	24.7 ± 0.77^{a}	47.9 ± 0.31^{g}
	2	815 ± 5.17^{c,d,e}	182 ± 1.61^{a}	348 ± 1.71^{e}	68 ± 1.14^{d,e}	22.3 ± 0.38^{a}	51.1 ± 0.34^{g}
	3	939 ± 11.43^{d,e}	259 ± 3.07^{a}	487 ± 5.91^{g}	90 ± 1.53^{d,e}	27.6 ± 0.52^{a}	61.4 ± 0.46^{g}
	½	743 ± 2.30^{a,b}	631 ± 2.10^{c}	228 ± 2.31^{d}	67 ± 1.35^{b,c,d}	84.92 ± 0.53^{c}	39.57 ± 1.04^{f}
Ex_1	1	964 ± 4.05^{e}	845 ± 3.55^{e}	297 ± 1.86^{e}	102 ± 1.79^{d}	87.65 ± 0.44^{e}	41.42 ± 0.52^{e}
	2	841 ± 3.65^{d,e}	734 ± 3.95^{d,e}	268 ± 2.62^{d}	106 ± 2.54^{f}	87.20 ± 0.61^{d,e}	44.51 ± 0.73^{e}
	3	693 ± 2.17^{a}	603 ± 2.09^{b}	275 ± 1.89^{d}	89 ± 1.47^{d,e}	87.01 ± 0.73^{d,e}	52.55 ± 0.71^{f}
	½	967 ± 5.53^{e}	840 ± 4.13^{g}	308 ± 1.33^{e}	53 ± 0.87^{b,c}	86.87 ± 0.62^{d}	37.40 ± 0.84^{e}
Ex_2	1	831 ± 9.29^{b,c}	673 ± 7.27^{c}	259 ± 3.51^{d,e}	101 ± 1.60^{d}	80.98 ± 0.44^{c}	43.44 ± 0.89^{f}
	2	839 ± 6.99^{d,e}	715 ± 5.86^{d,e}	278 ± 1.80^{d}	95 ± 0.83^{e,f}	85.22 ± 0.51^{c}	44.62 ± 0.93^{e}
	3	944 ± 4.37^{d,e}	786 ± 4.10^{e}	406 ± 2.35^{f}	68 ± 1.02^{c,d}	83.26 ± 0.38^{b}	50.25 ± 0.42^{e}
	½	728 ± 2.54^{a}	655 ± 2.01^{c,d}	193 ± 1.42^{d}	82 ± 1.08^{c,d}	89.97 ± 0.58^{e}	37.80 ± 0.46^{e}
Ex_3	1	690 ± 2.13^{a}	608 ± 1.94^{c,d}	280 ± 2.19^{d,e}	87 ± 1.41^{c,d}	88.12 ± 0.43^{e}	53.20 ± 0.61^{i}
	2	774 ± 2.82^{b,c,d}	681 ± 1.99^{c}	271 ± 3.40^{d}	100 ± 3.00^{f}	87.98 ± 0.62^{d,e}	47.90 ± 0.49^{f}
	3	1031 ± 4.01^{e}	910 ± 3.21^{f}	387 ± 2.87^{e,f}	100 ± 2.06^{e}	88.26 ± 0.33^{e}	47.27 ± 0.60^{d}
	½	901 ± 4.47^{d}	793 ± 3.87^{f,g}	395 ± 3.75^{f}	98 ± 1.31^{d}	88.01 ± 0.46^{d}	54.58 ± 0.59^{h}
Ex_4	1	706 ± 3.37^{a}	619 ± 2.77^{b,c}	284 ± 2.72^{d,e}	81 ± 1.23^{c,d}	87.68 ± 0.40^{e}	51.70 ± 0.28^{h}
	2	852 ± 4.87^{e}	751 ± 4.65^{e}	347 ± 3.53^{e}	103 ± 1.74^{f}	88.14 ± 0.52^{e}	52.73 ± 0.54^{h}
	3	812 ± 5.75^{b,c}	709 ± 4.89^{d}	345 ± 2.43^{e}	85 ± 1.01^{d,e}	87.31 ± 0.66^{d,e}	53.03 ± 0.67^{f}
	½	768 ± 2.98^{a,b}	698 ± 2.44^{d}	66 ± 2.48^{b}	63 ± 2.54^{b,c}	90.91 ± 0.56^{e}	16.81 ± 0.44^{b}
PEx_1	1	752 ± 3.69^{a,b}	659 ± 3.13^{c}	129 ± 2.94^{b}	34 ± 2.48^{b}	87.64 ± 0.40^{e}	21.63 ± 0.39^{b}
	2	745 ± 2.40^{a,b,c}	645 ± 2.06^{c}	147 ± 0.72^{b}	11 ± 0.83^{a,b}	86.57 ± 0.19^{c,d,e}	21.19 ± 0.49^{b}
	3	742 ± 3.34^{a,b}	654 ± 3.10^{b,c,d}	161 ± 1.72^{b}	21 ± 1.36^{a,b}	88.12 ± 0.42^{e}	24.60 ± 0.77^{b}
	½	807 ± 2.79^{b,c}	704 ± 2.60^{d,e}	117 ± 1.15^{c}	45 ± 1.48^{b}	87.22 ± 0.41^{d}	20.06 ± 0.51^{c}
PEx_2	1	857 ± 3.65^{c,d}	747 ± 3.17^{d}	158 ± 2.94^{b,c}	63 ± 2.55^{b,c}	87.16 ± 0.19^{e}	25.78 ± 0.38^{c}
	2	724 ± 2.51^{a,b}	625 ± 2.20^{c}	183 ± 1.33^{b,c}	45 ± 1.18^{c,d}	86.32 ± 0.29^{c,d}	31.50 ± 0.33^{d}
	3	891 ± 5.54^{c,d}	771 ± 4.74^{e}	248 ± 3.52^{c,d}	43 ± 2.57^{b,c}	86.54 ± 0.47^{d,e}	32.71 ± 0.46^{c}
	½	861 ± 3.41^{c,d}	751 ± 3.14^{e,f}	127 ± 2.64^{c}	50 ± 2.09^{b}	87.20 ± 0.34^{d}	20.57 ± 0.55^{c}
PEx_3	1	927 ± 4.01^{d,e}	790 ± 3.64^{d,e}	245 ± 2.21^{d}	43 ± 1.58^{b}	85.20 ± 0.22^{d}	31.21 ± 0.58^{d}
	2	841 ± 3.20^{d,e}	730 ± 3.26^{d,e}	214 ± 1.96^{c}	40 ± 1.63^{b,c,d}	86.76 ± 0.60^{c,d,e}	29.97 ± 0.49^{c}
	3	835 ± 2.72^{b,c}	709 ± 3.03^{d}	252 ± 1.57^{c,d}	24 ± 1.18^{a,b}	84.85 ± 0.60^{c}	33.04 ± 0.16^{c}
	½	756 ± 2.41^{a,b}	658 ± 2.14^{c,d}	127 ± 2.39^{c}	98 ± 2.44^{d}	87.03 ± 0.37^{d}	29.78 ± 0.44^{d}
PEx_4	1	761 ± 2.34^{a,b}	653 ± 1.89^{c}	176 ± 1.67^{c}	56 ± 1.99^{b,c}	85.81 ± 0.38^{d}	30.46 ± 0.37^{d}
	2	774 ± 2.59^{b,c,d}	682 ± 1.71^{c,d}	211 ± 1.30^{c}	36 ± 1.15^{b,c}	88.15 ± 0.62^{e}	31.89 ± 0.26^{d}
	3	779 ± 2.75^{a,b}	670 ± 2.51^{c,d}	207 ± 1.84^{b,c}	40 ± 1.60^{b}	85.99 ± 0.32^{c,d}	31.68 ± 0.40^{c}

Abbreviations: NC - negative control (distilled water); PC - positive control (0.01% methyl parathion); extracts with polar and nonpolar compounds of *I. coetsa*: Ex_1 - 0.005%, Ex_2 - 0.01%, Ex_3 - 0.05%, Ex_4 - 0.1%; extracts with polar compounds alone of *I. coetsa*: PEx1 - 0.005%, PEx_2 - 0.01%, PEx_3 - 0.05%, PEx_4 - 0.1%; SE - standard error. Means within a column followed by the same letters are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test.

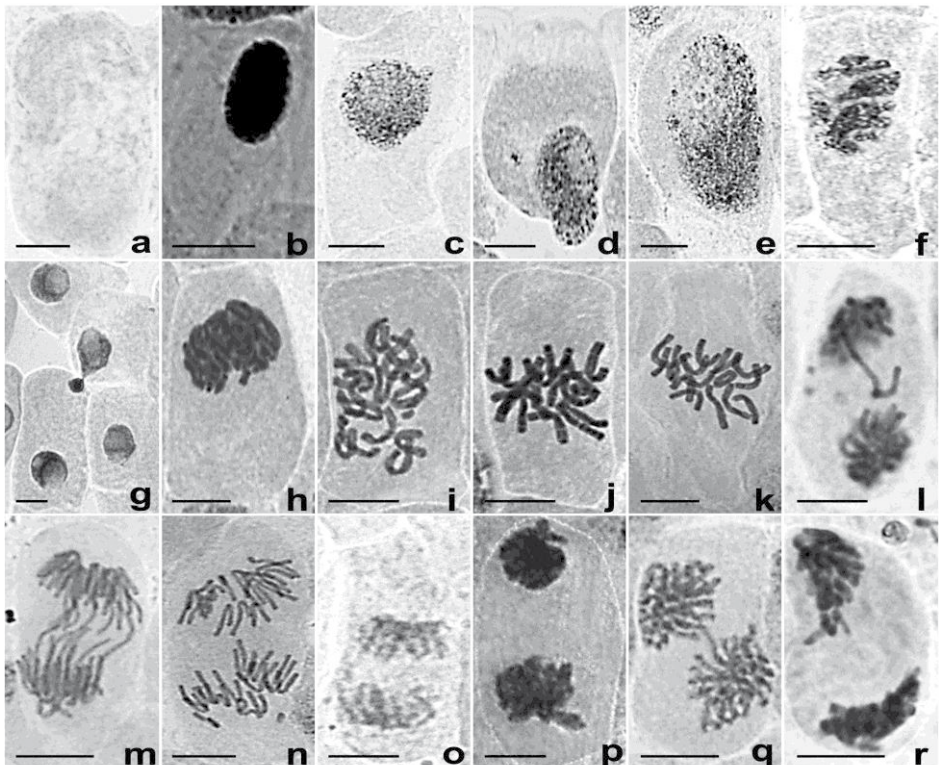

Figure 1. Clastogenic aberrations in treated *Allium cepa* root tip cells. a- ghost cell, b- hyperchromasia, c- nuclear budding, d- nuclear emergence, e- nuclear enlargement, f- karyorrhexis, g- nuclear intrusion, h- coagulated chromosomes at metaphase, i- chromosome fragment at disturbed metaphase, j- chromosome gaps, k- ring chromosome, l- broken chromosome bridge, m- chromosome bridges, n- chromosome fragments, o- chromosome pulverization, p- chromosome rosette at anaphase, q- diagonal stellate anaphase with bridge, r- sticky comet anaphase. Scale bar = 5 µm.

aberrations attributable to spindle abnormalities. Mitotic index did not show much variation, but a slight increase was observed for certain concentrations and time periods due to the c-mitotic activity of the leaf extract (Neelamkavil and Thoppil, 2013). Structural chromosomal alterations may be induced by several factors such as DNA breaks, inhibition of DNA synthesis, and replication of altered DNA. The numeric chromosomal abnormalities, e.g., aneuploidy and polyploidy, are consequences of abnormal segregation of chromosomes, which can occur either spontaneously or by the action of aneugenic agents (Albertini et al., 2000). Numerical abnormalities are characterized by morphological alterations in the interphasic nuclei, as a result of the action of the agent tested. Numerical and chromosome aberration evaluation has been shown to be a sensitive type of analysis for making the investigation of test agent actions even more accurate in relation to their effects on the DNA of exposed organisms (Leme and Marin-Morales, 2009).

The abnormalities resulting from the activity of the extract have more or less a fate of apoptosis or cell death. Ghost cells were being produced by the activity of the extract, which showed nuclear and cytoplasmic damage resulting in dead cells (Çelik and Aslantürk, 2010). Changes in cell and nuclear size often correlate with the functionality of cancer-treating agents. Nuclear enlargement was seen, as reported by Kang et al. (2011) as resulting when SW 480 human colon cancer cells was treated with arctigenin, a natural plant lignin. Karyorrhexis causes a characteristic change in the nuclear morphology leading to apoptosis (Golstein and Kroemer, 2006) which was also observed. These effects, when specifically targeted towards cancer cells, can control their proliferative growth.

The other abnormalities also showed destructive effects where the genetic material was lost or altered. Anaphase bridges could happen during the translocation of the unequal chromatid exchange or due to dicentric chromosomes caused by structural chromosome mutations (El-Ghamery et al., 2000). Binucleate cells were accepted as the inhibition of cytokinesis at any control points of the cellular cycle (Ateeq et al., 2002). Disturbances in the nuclear and microtubular cycles seem

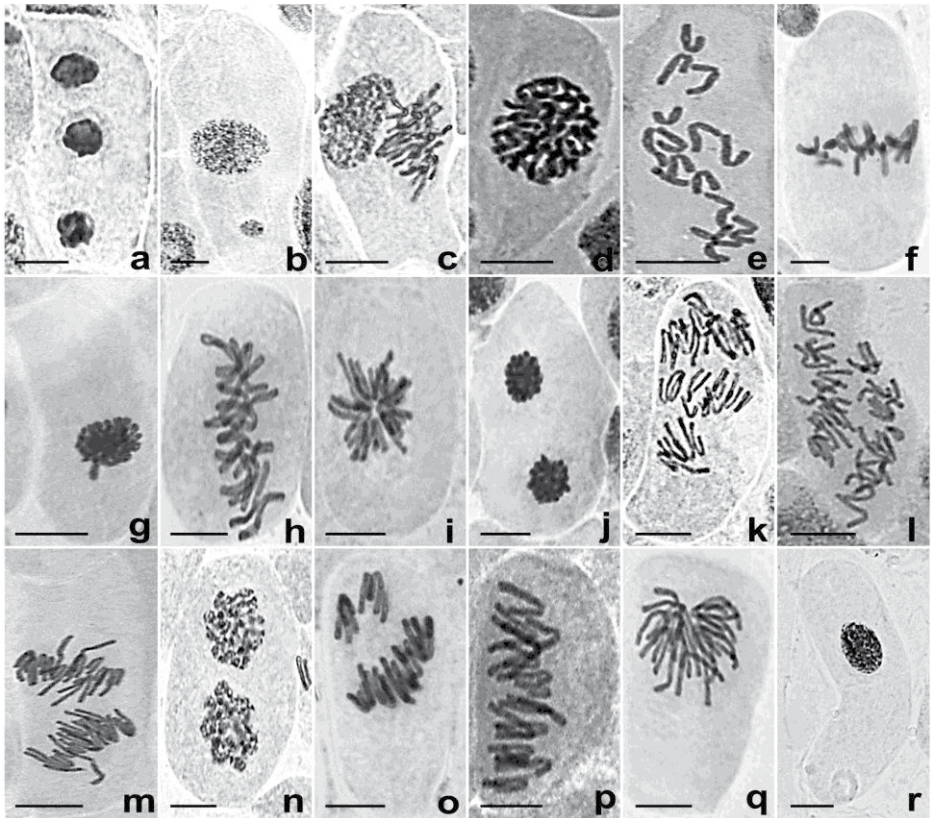

Figure 2. Nonclastogenic aberrations in treated *Allium cepa* root tip cells. a- trinucleate cell, b- micronucleus, c- nonsynchronized division showing prophase and metaphase in binucleate cell, d- ball metaphase, e- centromere attraction, f- hypoploid cell, g- lollypop metaphase, h- pole to pole arrangement at metaphase, i- stellate metaphase, j- ball anaphase, k- chromosome laggards, l- equatorial separation at anaphase, m- shift in MTOC, n- sticky stellate anaphase, o- unequal diagonal separation, p- unilateral anaphase, q- unipolar anaphase, r- disappearance of nucleus in one cell after cytokinesis. Scale bar = 5 µm.

to be associated with the formation of heterophasic bi- or multinucleate cells (Alberts et al., 2008). Micronuclei arise from the development of some chromosomal aberrations, such as, for instance, chromosome breaks and losses. They may also be derived from other processes such as polyploidization, in which they originate from the elimination of excessive DNA of the main nucleus in an attempt to restore the normal conditions of ploidy, which is the same activity observed in the case of nuclear budding (Fernandes et al., 2007). The abnormalities discussed here are hazardous in normal cells, but when specifically aimed, they can aid in the destruction of unwanted cancer cells. This finding is supplemented by the biological activities of the terpenoids present in *Isodon coetsa* (Sun et al., 2006), which emphasizes its potential in anticancer research.

Being popular in folk medicine, the genus *Isodon* is a promising candidate in herbal drug research. Cytotoxic activity of diterpenoids from aerial parts of *Isodon coetsa* against human tumor cell lines was earlier reported by Zhao et al. (2011). The inhibition of mitotic index, induction of chromosome aberration, mitotic aberrations, and micronucleus formation in *Allium cepa* have been considered as indicators of toxicity (Tran and Popova, 2013). Clastogenicity induced in *A. cepa* root meristematic cells was mainly caused by the nonpolar components present in the leaf extracts of *I. coetsa*. This is a warning against the nonjudicious use of the plant in folk medicine. However, the activities shown combined with the biological properties of nonpolar components when specifically targeted can be a boon in anticancer research.

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