crude extract of Cigarette butts caused genotoxic and cytotoxic effects in *Allium cepa*

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Abstract
Allium cepa root meristem cells were used to evaluate cytogenetic effects of cigarette butts crude extract. The effective concentration (EC50) was determined in *Allium* root growth as approximately 0.5 mg/ml. Cytological experiments were carried out using crude extract concentrations 0.25 (EC_{50}/2), 0.5 (EC_{50}) and 1 mg/ml (EC_{50}X2) at 24, 48, 72 and 96h, with control for each combination. Mitotic index decreased with increasing concentration of cigarette butts crud extract at each exposure time. Micronucleated cells were observed at interphase. The frequency of the micronuclei was markedly higher at 1 mg/ml at 48h compared to the other test concentrations. In anaphase-telophase cells, the total percentages of bridges, vagrant chromosomes, c-anaphase and fragments according to total cells with chromosome aberrations calculated as 28.42, 16.67, 14.10 and 0.64% respectively. Total chromosome aberrations increased with an increasing in the cigarette butts crude extract concentration

key words: Cigarette butts; *Allium cepa*; chromosome aberration, mitotic index; micronucleus.

Introduction:
Cigarette butts are undoubtedly an environmental problem causing blight on beaches, streets, sidewalks, waterways, and public spaces [1]. cigarette filters pose a serious litter and toxic waste disposal problem. Cellulose acetate is photodegradable but not bio-degradable. Although ultraviolet rays from the sun will eventually break the filter into smaller pieces under ideal environmental conditions, the source material never disappears; it essentially becomes diluted in water or soil [2,3]. While the environmental impact of a single disposed cigarette filter is minimal, there were 1.35 trillion filtered cigarettes manufactured in the United States in 2007, and of these, more than 360 billion were consumed here [4]. Discarded cigarette butts are not only unsightly; they are also toxic. Environmental groups have expressed concern for marine creatures that ingest littered filters [5,6]. cigarette butts were found to be acutely toxic to a freshwater cladoceran organism and a marine bacteria (microtox) and that the main cause of toxicity was attributed to nicotine and ethylphenol in the leachates from cigarette butts [7]. Even if properly disposed, cigarette butts are hazardous solid waste. It is unknown as to how many must be consumed to cause adverse health effects in marine animals such as birds or mammals. Higher plants provide valuable genetic assay systems for screening and monitoring environmental pollutants. For this purpose, the *A.cepa* is one of the most frequently used higher plant species [8]. The *Allium* test for genotoxicity was introduced by LEVAN [9] and has been used on pesticides in other studies[10, 11, 12]. The *Allium* test was simple and just as reliable as the method were chromosome aberrations were recorded to all types of mitotic cells [13]. The test can used to measure both toxicity (effective concentration, EC_{50}, where root bundles are half the length of the control) and genotoxicity. The rate of the root growth cane be correlated with the mitotic index [14]. The chromosome aberration and micronucleus assays have been shown to be highly reliable in genotoxicity testing [15].

The aims of this study are to test three different concentration of cigarette butts crude extract in context of genotoxic and cytotoxic effects in *A. cepa* root tip cells trough micronucleus test and chromosome aberrations in anaphase-telophase .

Material and Methods:
Test organism/ Grouth conditions- Equal-sized bulbs (25-30 mm in diameter) of commercial variety of *A. cepa* L. (2n=16) were chosen. The onion were kept cool and dry until cytotoxicity testing. Just before use, the outer scales of the bulbs were carfully removed and the brownish bottom plates were scraped away without destroying the root primordial. The experiments were maintioned in laboratory conditions at 22± 2 ºC. The roots were protected from direct sunlight in order to minimize fluctuation of the rate of cell division [16].

Cigarette butts extract- 500 ml of Hexanol were added to 50 gm of cigarette butts and maintained 48 hour on hot plate magnetic stirrer. After that the solid material were separated by sieving through three layer of cotton cloth, the supernated were filtered using watt man's filter paper, the solvent evaporated by using hot plat at 75 ºC, the powder extract were collected and determining of its weight by electrical balance. The yield was 1.8 gm of butts crud extract. DMSO were used to make all concentrations for the experiments**.**

***Allium* root growth test/ Determination of EC_{50}**- Clean and healthy onion bulbs were set up and allowed to produce roots in distilled water for 24 h, where after the homogeneously rooted five bulbs transferred to the control (distilled water) and different concentrations of butts crud extract (5- 50, 1- 10 and 1-3 mg/ml) for 96 h. During the experiment, the test solution were changed every 24 h instead of aeration. The root lengths from the control and experimental sets were measured (lengths of ten roots from each bulb) at the end of exposure time. The relative reduction of root length was calculated as the percentage of the deviation from the control (T/C,%). The effective concentration (EC_{50}) value

was determined as approximately 0.5 mg/ml. EC refers to effective concentration and the number 50 indicate the effective concentration for 50% growth inhibition [17]. Experiments were carried out in triplicate**.**

Cytogenetic parameters- The onion were rooted in distilled for 24h. The five bulbs which have approximately same root length were transferred to the control test solutions. Cytological experiments were carried out using butts extract concentrations of 0.25 (EC_{50}/2), 0.5 (EC_{50}) and 1 (2x EC_{50}) at 24, 48, 72 and 96 h, with a control for each combination. The root tips were sampled between 0.7.00 – 0.8.00 h, as the highest mitosis frequency in the onion is recorded between 06.00 – 0.9.00 h (Sharma, 1983). After completion of exposure, roots from 5 bulbs were immediately cut and fixed in solution of ethanol (99%) and glacial acetic acid (3:1) for 24 h. The roots were transferred to 70% alcohol and stored in refrigerator until use. The root tips were macerated in a solution of 1N HCl at 60 ºC for 7 min. Then, the roots were washed with distilled water three times. Chromosomes were stained with Geimsa 5% solution for 20 min followed by squashing in 45% acetic acid. One slide prepared for each bulb.

All slides were coded and examined blindly. The mitotic index, micronucleus in interphase, and chromosome aberrations in anaphase- telophase were investigated in cytogenetic analysis for each concentration and exposure time. The mitotic index was determined by scoring more than 5000 cells (more than 1000 cells per slide). Mitotic index was calculated as the percent ratio of dividing cells and total numbers of cells scored. Micronucleus frequency was determined by examination of more than 1000 interphase cells per slide (totally more than 5000 fore each treatment). In chromosome aberration test, 100 cells in anaphase or telophase were examined for aberrations per slide. Chromosome aberrations were examined in 500 anaphase-telophase cells for each treatment. The chromosome aberrations scored were stickiness, bridges, vagrant chromosomes, c- anaphase, multipolarity and fragments**.**

Statistical analysis- Data were analyzed by SPSS, ver, 17.0. The analysis of variance (ANOVA) was used to assess the significant differences between control and each treatment. If there was a significant differences ($P \leq 0.05$), the experimental data analyzed using Duncan's multiple range test.

Results and Discussion:

Effects on root growth and mitotic index

The growth of roots were decreased with increasing of butts extract concentration($P < 0.05$). Above 1 mg/ml, there were no root growth during 96 h. After 96 h of growth in control, the average length of roots was 5.34± 0.18 cm. Dose- response curves obtained between the concentrations of crud extract and growth of *Allium* roots determined the effective concentration (EC_{50}) value which retards 50% root growth as 0.5 mg/L. The root length after 96 h in EC50 was 2.35 ± 0.06 cm.

The effect of butts crud extract in mitotic index of meristem cells in root tips of *A. cepa* was determined (Table 1). There were significant differences between experimental groups compared to control ($P < 0.05$). Mitotic index decreased significantly with the three concentrations of butts crud extract when compared to control at each experiment. There were no significant differences between extract concentration at 24 h. In contrast Mitotic index was significantly low at 1.5 mg/ml compared to other concentrations at 48, 72 and 96 h (Table 1**).**

Effets in micronucleus formation and chromosome aberrations

Table 1 shows the results of genotoxicity tests in *Allium cepa* root tip cells at anaphase-telophase. The highest butts crud extract 1.5 mg/ml showed high toxicity on root tip cells. At 96 h, it was not possible to score 500 anaphase or telophase cells . The changes in morphology and organization of the chromosomes of exposed root tips were observed (Table2). Four types of chromosome aberrations were recorded in anaphase- telophase cells (Fig. 1). The total percentages of bridges, vagrant chromosomes, c-anaphase and fragments according to total cells with chromosome aberrations were calculated as, 28.42, 16.67, 14.10 and 0.64 respectively. The total chromosome aberrations increased with an increasing of butts crud extract. The total chromosome aberrations (%) were significantly higher at the highest concentration 1.5 mg/ml of butts crud extract. At interphase cells the micronuclei were observed (Fig. 2). The induction of micronucleus formation was generally observed with all treatment except control. Micronucleus formation was markedly higher at 1.5 mg/ml than other concentrations of butts crud extract.

Table 1- Mitotic index and micronucleus frequency of control and experimental groups of *Allium cepa* treated with different concentrations of butts crud extract for different times.

Treatment		No. of Dividing cells	Mitotic index (% ± SE)	No. of Interphase cells	Micronuclei (%)
Time (h)	Conc. mg/ml				
24	0	500	9.70 ± 0.37[a]	5120	0
	0.25	500	8.09 ± 0.41[ab]	5145	0.10
	0.5	500	7.86 ± 0.33[b]	5522	0.06
	1	500	3.48 ± 0.86[c]	5463	0.26
48	0	500	10.37 ± 0.92[a]	5134	0.02
	0.25	500	7.27± 0.32[b]	5086	0
	0.5	500	7.52 ± 0.36[b]	5281	0.09
	1	500	7.01 ± 0.26[b]	5217	0.21
72	0	500	9.12 ± 0.46[a]	5060	0
	0.25	500	8.66 ± 0.43[a]	5115	0.06
	0.5	500	5.15 ± 0.44[b]	5156	0.04
	1	500	3.35 ± 0.51[c]	5198	0.12
96	0	500	8.43 ± 0.26[a]	5528	0.02
	0.25	500	5.84 ± 0.22[b]	5170	0.06
	0.5	500	5.04 ± 0.47[c]	5101	0.02
	1	500	2.19 ± 0.16[c]	5193	0.08

** P< 0.05 in Duncan multiple range test. SE. slandered error. Similar letters mean non significant differences. Different letters mean significant differences.

table 2- Numbers and types of chromosome aberrations in control and experimental groups of *A. cepa* treated with different concentrations of butts crud extract for different times.

Treatment		No. of cells	Anaphase-telophase chromosome aberrations				
Time (h)	Conc. mg/ml		Bridge	Vagrant	C Anaphase	Fragment	Total aberrations (% ± SE)
24	0	500	11	3	2	0	3.20 ± 0.37[a]
	0.25	500	18	8	11	0	10.60 ± 1.96[b]
	0.5	500	16	20	9	1	13.60 ± 0.75[b]
	1	500	26	22	23	1	20.60 ± 2.1[c]
48	0	500	8	0	3	1	2.60 ± 0.51[a]
	0.25	500	27	6	6	0	13.00 ± 0.45[b]
	0.5	500	19	7	17	0	13.80 ± 1.39[b]
	1	500	21	13	13	0	20.00 ± 1.22[c]
72	0	500	9	1	2	0	2.40 ± 0.40[a]
	0.25	500	12	12	7	0	11.40 ± 0.51[b]
	0.5	500	13	13	13	0	13.80 ± 0.86[b]
	1	500	11	11	2	1	27.16 ± 1.35[c]
						1	
96	0	500	6	4	2	1	2.80 ± 0.37[a]
	0.25	500	33	15	4	0	16.60 ± 0.87[b]
	0.5	500	32	21	16	0	19.00 ± 1.14[b]
	1	500	2	0	2		40.58 ± 1.59[c]
Percent of aberrant cells (%)			28.42	16.67	14.10	0.64	

Duncan multiple range test. SE. slandered error. Similar letters mean non significant differences. Different letters mean significant differences.

A b c

Figure 1 ***A. cepa*** **anaphase cells arrows a- Vagrant chromosome, b- Cytoblasmic bridge and c- Fragment. G**

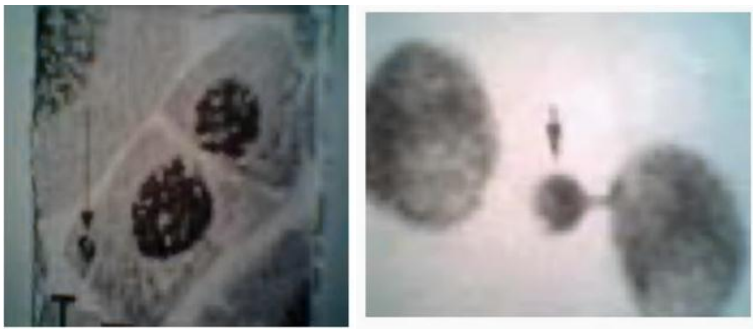

Figure 2 ***A. cepa*** **telophase cell with micronucleus (arrow) Giemsa 1000X.**

Toxic effects of environmental poiiutants may be evaluated by analyzing microscopic (root growth decrease as well as cytological parameters [18].

The results of current study indicate the utility of root meristem cells of *A. cepa* in bio- monitoring environmental pollutants such as cigarette butts. In the other hand, the effective concentration (EC_{50}) value proved to be useful parameter for selecting the test concentrations for the genotoxicity assays [19]. In this study the EC_{50} value was detected as about 0.5 mg/ml. The highest chosen concentration for the genotoxicity test as 1 mg/ml ($2xEC_{50}$).

In *Allium* root growth test, crud extract of cigarette butts caused inhibition in root growth that is toxic *in A. cepa.* The fact that the root growth decrease by 50% indicates the presence of toxic substances [20]. Which have sub lethal effects on plants [21]. After 96 h of root growth in all concentrations, the root length was shown as a reliable indicator of toxicity of butts crud extract.

Butts crud extract significantly decreased mitotic index (MI) at all treatments periods. These results showed that cigarette butts crud extract has cytotoxic effect at all tested concentrations. Decreasing of the MI or the inhibition of the DNA synthesis might be caused by the decreasing ATP level and the pressure from the functioning of the energy production center [22,23].

Mitotic index is considered a parameter that allows to estimate the frequency of cellular division [24]. Inhibition of mitotic activities is often used for tracing cytotocic substances [25]. If the EC50 value is chosen as the highest concentration for the genotoxicity test, the mitotic index will never be below 50% of the control [26]. In this study, 0.5 mg/ml of butts crud extract caused more than 50% reduction in mitotic index compared to control. The reduction of mitotic activity was more significant when the concentration of the crud extract increased at each exposure time. The concentration-dependant reduction of mitotic index illustrates the cytotoxic potential of cigarette crud extract in *A. cepa*. Many researcher described similar effects on mitotic index following treatment with cypermethrin insecticide [27], mercuric chloride fungicides [28] and maleic hydrazide [24].

The changes in organization and morphology of the chromosome were observed in the root tips exposed to cigarette butts crud extract. Four main types of chromosome aberrations were recorded in anaphase-telophase: Bridges, vagrant chromosomes, c-anaphase, and fragments (Table 2). The percentage of total chromosome aberrations increased with increasing the test concentration at each exposure time. The bridges involving one or more chromosomes were the most prominent and frequent

type of chromosome aberration. The induction of bridges could be attributed to chromosome breaks, stickiness and breakage and reunion of broken ends. The stickiness of chromosomes prevented the separation of daughter-chromosomes and thus they remained connected by bridges [29,30]. The induction of vagrant chromosomes leads to separation of unequal number of chromosomes in the daughter nuclei and subsequently formation of micronuclei.

The induction of micronucleus in root meristem cells of *A.cepa* is the manifestation of fragments or vagrant chromosomes [31].

In conclusion, the test of chromosome aberration on plant systems constitutes a simple and reliable technique to detect the genotoxicity of pollutants.It also point to the importance of mutagenicity testing.

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المستخلص الخام لأعقاب السكائر يسبب تأثيرات سمية وراثية وسمية خلوية في نبات البصل *Allium cepa*

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الملخص

تم استعمال الخلايا المرستيمية لجذر البصل *Allium cepa* لتقييم التأثيرات الوراثية الخلوية للمستخلص الخام لأعقاب السكائر. وجرى تعيين التركيز المؤثر EC_{50} في نمو جذور البصل وكان 0.5 ملغم\ ملل تقريبا. وأجريت التجارب الخلوية باستعمال تراكيز المستخلص الخام 0.25 ويساوي $EC_{50}/2$ و 0.5 ويساوي EC_{50} و 1 ملغم\ ملل ويساوي EC_{50} 2 لمدة 24، 48، 72، و 96 ساعة مع سيطرة مع كل من التوافيق. أظهرت النتائج انخفاضا في دالة الانقسام الميتوزي مع زيادة تركيز المستخلص الخام لأعقاب السكائر وعند كل وقت تعريض. وتمت ملاحظة الخلايا ذات النوى الدقيقة في الطور البيني. وكان تكرار النوى الدقيقة عاليا بشكل مميز مع التركيز 1 ملغم\ ملل من المستخلص عند 48 ساعة مقارنة مع التراكيز الأخرى. وفي خلايا الطور الانفصالي– النهائي وحسبت النسب المئوية للجسور، الكروموسومات الشاردة، وعدم حدوث الانفصال الكروموسومي والشظايا الكروموسومية بالنسبة للعدد الكلي للخلايا ذات الشذوذ الكروموسومي وكانت 28.42 ، 16.67 ، 14.10 و 0.64 % على التوالي. وقد ازداد الشذوذ الكروموسومي الكلي مع زيادة تركيز المستخلص الخام لأعقاب السكائر.