

Genotoxicity of Different *tert*-Butylcalix[4]crowns

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The ability of two calix[4]arene derivatives, namely 25,27-*p*-*tert*-butylcalix[4]dithiooxabenzocrown (**1**) and 25,27-*p*-*tert*-butylcalix[4]trithiooxabenzocrown (**2**), to produce chromosomal aberrations in root meristematic cells of *Allium cepa* and micronuclei (MN) in normochromatic erythrocytes (NCE) of Balb/c mice was investigated. NCE are normal mature red blood cells with a full complement of hemoglobin but lack ribosomes. In the first test, the root tips were treated with a series of concentrations of the two test chemicals ranging from 10^{-7} to 10^{-4} M for 24 or 48 h. Both compounds caused concentration-dependent increases in the percentage of aberrant cells and reductions in the mitotic index. These effects depended, to some extent, on the duration of the treatment. The most conspicuous chromosomal abnormalities were c-mitosis, chromosome bridges, chromosome breaks, chromosome lags as well as micronuclei and multinuclei. In the second test, acridine orange fluorescent staining was applied to evaluate the incidence of MN in NCE of mice intraperitoneally injected with varying contents of the two test chemicals (0.02–0.08 mg/mouse). The two chemicals induced dose-dependent MN formation as compared to the negative control. The second compound had more pronounced cytogenetic influence than the first one. Mitomycin C (MMC, 14 mg/kg body weight), employed as positive control, produced more obvious effects on the parameters investigated.

Key words: Calixcrowns, Chromosome Aberrations, Micronuclei

Introduction

Calixarenes are metacyclopphanes synthesized via the condensation of phenol and formaldehyde under different reaction conditions (Mandolini and Ungaro, 2000). The phenol units are linked, via their *ortho* positions, by methylene bridges (Lamartine *et al.*, 2002). They can exist in different conformations; among them is the “cone” conformation, which has defined upper and lower rims of the cavity. This cavity enables calixarenes to act as hosts for different molecules (Mandolini and Ungaro, 2000). Calixarenes are used as host-guest in commercial applications such as sodium selective electrodes for the measurement of the sodium level in blood. Because some of these molecules are efficient sodium ionophores they are used as chemical sensors. Calixarenes also form complexes with cadmium, lead, lanthanoides and actinoides (Gutsche, 2008). Studies on calixarenes initially intended to employ them as enzyme mimics, such as heme and aldolase mimics (Chung and Kim, 1998). These compounds are

used as new biometric catalysts because of their advantage to provide pre-organization of the catalytic group. Macrocyclic receptors meet some of the requirements for designing artificial receptors like cavities and clefts of appropriate sizes and shapes, reactive binding sites, branches and bridges. Such enzyme models can provide information on mechanistic aspects of enzyme action and may find future application as catalyst in industrial synthesis (Agrawal and Bhatt, 2004). Mecca *et al.* (2004) have designed basic amino acid calix[8]-arene receptors that behave as competitive inhibitors of recombinant human trypsin, probably binding the intended region of Asp residues near the active sites of the tetrameric protein. Francese *et al.* (2005) have recently demonstrated the surface recognition of transglutaminase by their peptidocalix[4]arene diversomers.

Calixcrowns, which are macromolecular derivatives of calix[*n*]arenes that contain crown ethers, are known to form complexes with a wide variety of neutral, cationic and anionic guests. Calixcrowns have been widely used as molecular platforms for

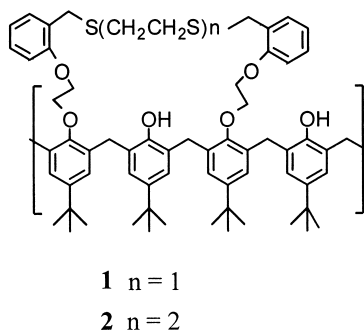


Fig. 1. Chemical structures of calix[4]crowns **1** and **2**.

the synthesis of selective molecular receptors for cations, anions and neutral molecules, in order to develop applications in the fields of separation purification, recovery of metals, pollution control, membranes for electronic devices, and phase transport agents (Lamartine *et al.*, 2002).

Calixarenes are receiving increasing wide attention (Gutsche, 2008). However, their applications are hampered by the lack of information regarding their biological activities; only very few publications appeared in the literature on these activities. In August 2000, a communication on the cellular toxicity of calixarenes stated that calix[6]arene and calix[8]arene sulfonates displayed the same level of toxicity, while slight toxicity was associated with the calix[4]arene sulfonate (Lamartine *et al.*, 2002).

Compounds **1** and **2** (Fig. 1) have different cavity sizes and different donor atoms, which make them potential hosts for the complexation of metal ions, neutral guests and the formation of charge transfer complexes. The ability of these compounds to form complexes with I_2 and C_{60} in addition to their stability constants and thermodynamic properties were reported by Mizyed *et al.* (2005). The present work aimed to investigate the genotoxicity of two of these compounds; 25,27-*p-tert*-butylcalix[4]dithiooxabenzocrown (**1**) and 25,27-*p-tert*-butylcalix[4]trithiooxabenzocrown (**2**) (Fig. 1) administered intraperitoneally to mice or applied *in situ* to onion roots.

Materials and Methods

General

The two test chemicals were synthesized according to the published procedure (Ashram, 2002). All other chemicals were of analytical grade.

Root tip cells of *Allium cepa* as well as normochromatic erythrocytes from Balb/c mice were used as test objects in this study.

Root tip cells

After removing the loose outer scales, the onion bulbs (15–30 g) were grown in the dark at a constant temperature of $(25 \pm 0.5)^\circ\text{C}$. The bases of the bulbs were submerged in tap water. The water was changed daily and aerated by continuous bubbling. When the root tips reached a length of 1–1.5 cm, while intact on the bulb, they were placed in a vial containing the test chemicals. The lethal concentrations and the threshold were first determined and a number of concentrations (10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} M) intermediate between these two limiting doses were selected for further experiments (2–3 bulbs were used for each treatment). The test chemicals were dissolved in 0.5% DMSO. Roots were treated for 24 and 48 h in 5 ml of each test solution; 0.5% DMSO was used as a negative control. During the treatment period, the bulbs and the roots were exposed to light at $(25 \pm 0.5)^\circ\text{C}$. The root tips were harvested in the late morning to obtain a good mitotic index and transferred to Petri plates for a 2.5-h pretreatment in the dark at $(25 \pm 0.5)^\circ\text{C}$. The pretreatment solution was 0.05% colchicine. After that, the root tips were transferred to a freshly prepared Carnoy fixative (absolute methanol/glacial acetic acid, 3:1) for 24 h. The roots were rinsed in distilled water and hydrolyzed in 1 M HCl at 60°C for 5 to 10 min. After hydrolysis, root tips were rinsed in distilled water and stained in the dark in 2% aceto-orcein at 25°C . A standard squash technique (Hanson *et al.*, 2001) was used. Three slides were prepared from each treatment. Duplicate experiments were made for each concentration of the two test chemicals and the matching controls.

Cytogenetic analysis

At least 50 metaphases were analyzed from each treatment, unless otherwise indicated. In each cell, all aberrations that could be recognized were recorded, using a high power (100X) light microscope. The main types of aberrations were: polyploidy, c-mitosis, chromosome bridges, chromosome breaks and micronuclei. Both the percentage of aberrant cells(% Abc) and the mitotic index (% Mi) were calculated. To determine mitotic indices, the percentage of dividing cells

was calculated from 2000 nuclei from each treatment.

Micronucleus assay

Animals

8- to 9-week-old Balb/c male mice (25–30 g) were used for the micronucleus assay. The mice were kept in the animal house unit at Yarmouk University, Irbid, Jordan. The mice were placed in cages (8 each) at 20–22 °C, 60–80% relative humidity, 12 h light-dark cycle and on diet *ad libitum* and water.

Cell preparation

The mice were given the following doses of the test chemicals: 0.667, 1.333, 2.000 and 2.667 mg/kg. The compound was dissolved in 50% DMSO and the solution was administered intraperitoneally at 6.67 ml/kg body weight. Negative and positive controls were used according to Borroto *et al.* (2003) and Mengs *et al.* (1999). For the negative control, 50% DMSO was used, while mitomycin C (MMC), at a dose of 14 mg/kg body weight, was used as a positive control which was dissolved in 50% DMSO. Peripheral blood was then obtained in heparinized capillaries from the retro-orbital vein 36 h after injection.

Preparation of blood smears

Clean prewashed glass slides were used for the blood smears preparation; at least four slides were prepared from each group. Then the blood films were air-dried and fixed in methanol for about 3 min (Schmid, 1975; Heddle *et al.*, 1983). The experiment was repeated twice for each treatment.

Staining and micronucleus evaluation

The smeared preparations were stained with acridine orange (A.O.) according to the method of Stockert and Lisanti (1972) with some modifications according to Hayashi *et al.* (1983). The A.O. stock solution was prepared as a 0.1% aqueous solution that was available for several weeks at 4 °C. The A.O., at 0.24 mM, was prepared in 1/15 M Sørensen's phosphate buffer. The fixed slides were stained in this solution for 3 min at room temperature. The slides were rinsed in the buffer three times for 2–3 min each. Whenever the nuclei emitted a reddish fluorescence, the slides were further rinsed for another several minutes until the nuclei were emitting green fluorescence. The

preparations were mounted with the same buffer, and sealed with Canada balsam. Observations were made within 1 h using a NIKON microscope ECLIPSE E400 with a 40X objective and Y-FL EPI-FLUORESCENCE attachment that had a 420–490 nm excitation filter and a 520 nm barrier filter for observation and microphotography (Hayashi *et al.*, 1983). From each treatment, 2000 NCE from each one of the three animals were screened for micronuclei formation. Therefore, 12000 normochromatic erythrocytes were analyzed from the two experiments for each treatment.

Results

Chromosomal abnormalities

Microscopic examination of the squashes of *Allium cepa* root tip meristem cells showed that there are several observable types of chromo-

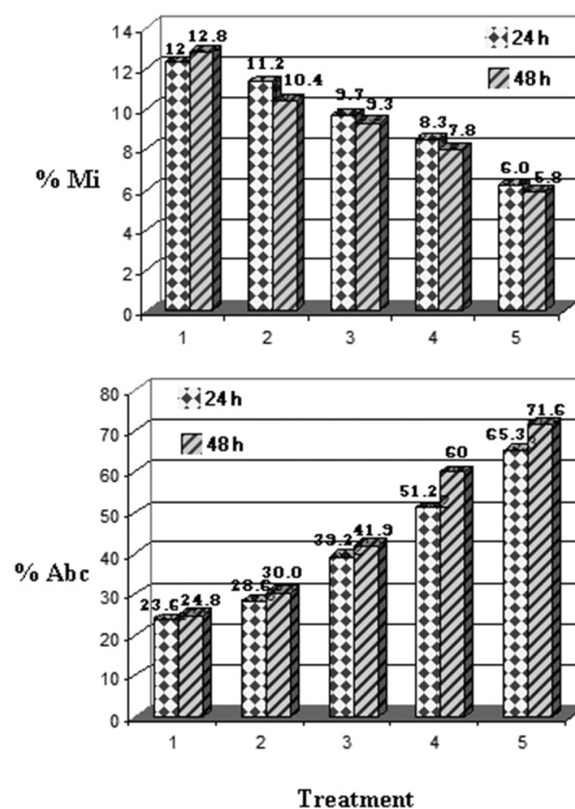


Fig. 2. The effect of **1** at different concentrations on mitosis (Mi) and aberrant cells (Abc) of *A. cepa* root tip cells at different times. 1, Control; 2, 10^{-6} M; 3, 10^{-5} M; 4, 10^{-4} M; 5, 10^{-3} M.

Table I. Percentage of aberrant cells and mitosis in *A. cepa* meristems following incubation with various concentrations of **1** for 24 and 48 h.

Treatment	Concentration [M]	Time [h]	No. of dividing cells	Chromosome aberrations										No. of cells with aberrant chromosome aberration	% Aberrant cells	% Mitosis
				Polyploidy	c-Mitosis	Bridges	Micronuclei	Condensed mitosis	Lagging	Tripolar	Irregular prophase	Breaks	Others ^a			
Control	0.5% DMSO	24	492	3	17	1	4	13	–	1	7	1	17	116	23.6	12.3
				2	16	3	2	10	–	2	3	–	14			
		48	514	1	21	2	4	11	–	1	9	1	14	128	24.8	12.8
				3	16	1	3	15	1	–	5	–	20			
25,27- <i>p-tert</i> -Butylcalix[4]dithiooxabenzocrown (1)	10^{-7}	24	452	2	18	5	4	16	–	1	6	–	10	128	28.3	11.3
				1	22	4	3	13	1	2	7	1	12			
		48	416	2	16	–	5	12	–	–	7	1	15	126	30.3	10.4
				3	10	2	10	15	–	2	10	–	13			
	10^{-6}	24	388	2	16	3	9	16	2	1	11	1	17	152	39.2	9.7
				3	20	5	7	14	1	–	7	3	14			
		48	372	3	17	5	5	15	–	2	9	1	18	156	41.9	9.3
				5	20	3	7	17	1	3	10	1	14			
	10^{-5}	24	340	5	19	6	10	18	–	2	8	1	14	174	51.2	8.5
				8	17	8	12	17	–	4	5	2	18			
		48	320	5	19	12	16	16	2	1	13	2	15	192	60.0	8.0
				5	17	16	15	14	–	1	10	1	12			
	10^{-4}	24	248	7	15	5	14	10	2	5	8	3	8	162	65.3	6.2
				9	13	7	17	8	1	3	13	1	13			
		48	236	9	15	2	15	10	–	7	16	5	12	169	71.6	5.9
				5	12	5	16	7	1	4	10	3	15			

^a Others like chromosome blending, uncoiling, syncytium, divergence, resident, sticking and loop.

somal abnormalities that occur through their mitotic cycle. Data of Tables I and II as well as of Figs. 2 and 3 show that the two compounds decreased the number of dividing cells of *A. cepa* root tip meristem cells as expressed by % Mi. Furthermore, the data show that the two

test chemicals increased the number of cells carrying chromosome aberrations as indicated by % Abc. For both chemicals, there were statistically significant differences in the means of % Abc and % Mi ($P \leq 0.05$) compared to the control group. There were also statistically sig-

Table II. Percentage of aberrant cells and mitosis in *A. cepa* meristems following incubation with various concentrations of **2** for 24 and 48 h.

Treatment	Concentration [M]	Time [h]	No. of dividing cells	Chromosome aberrations										No. of cells with aberrant chromosome aberration	% Aberrant cells	% Mitosis
				Polyploidy	c-Mitosis	Bridges	Micronuclei	Condensed mitosis	Lagging	Tripolar	Irregular prophase	Breaks	Others ^a			
Control	DMSO	24	492	3	17	1	4	13	–	1	7	1	17	116	23.6	12.3
				2	16	3	2	10	–	2	3	–	14			
		48	514	1	21	2	4	11	–	1	9	1	14	128	24.8	12.8
				3	16	1	3	15	1	–	5	–	20			
25,27- <i>p-tert</i> -Butylcalix[4]trithiooxabenzocrown (2)	10^{-7}	24	316	5	17	5	14	13	1	1	8	1	21	162	51.3	7.9
				3	15	6	16	10	–	2	7	–	17			
		48	304	5	12	1	13	16	–	1	3	2	20	160	52.6	7.6
				9	14	4	16	10	1	2	8	–	13			
	10^{-6}	24	292	6	10	5	14	15	2	3	12	1	21	165	56.5	7.3
				4	12	7	12	13	–	1	10	–	17			
		48	298	4	11	9	15	22	2	3	5	1	19	180	60.4	7.45
				4	13	7	17	20	1	4	4	2	17			
	10^{-5}	24	286	5	16	2	18	13	–	4	11	3	20	206	72.0	7.15
				7	21	10	16	10	2	8	16	1	23			
		48	298	9	12	14	19	26	1	6	5	–	21	214	71.8	7.45
				5	16	13	17	19	–	4	7	4	16			
	10^{-4}	24	264	7	9	11	18	16	2	4	5	4	17	208	78.8	6.6
				9	14	16	20	14	1	8	10	5	18			
		48	236	8	12	11	23	12	1	7	2	7	16	194	82.2	5.9
				5	13	17	20	11	–	5	5	9	10			

^a Others like chromosome blending, uncoiling, syncytium, divergence, resident, sticking and loop.

nificant differences in the means of % Mi and % Abc of *A. cepa* meristem cells that were exposed to 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} M of the two compounds compared to the negative control group. The exception noted was the 10^{-7} M dilution of compound **1** which showed no significant

differences between its mean and the negative control mean in the case of % Abc. The effect in both cases was concentration-dependent. The t-test was used to analyze the effects of time for the two compounds on both % Mi and % Abc. There were no significant differences between

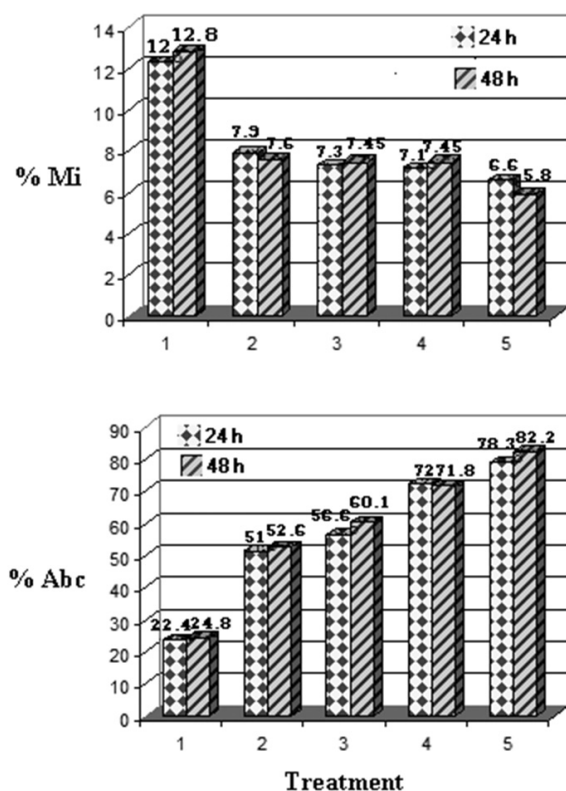


Fig. 3. The effect of **2** at different concentrations on Mi and Abc of *A. cepa* root tip cells at different times. 1, Control; 2, 10^{-7} M; 3, 10^{-6} M; 4, 10^{-5} M; 5, 10^{-4} M.

the means of % Mi and % Abc at the two treatment times of both compounds. However, the % Mi was slightly reduced when the *A. cepa* root tip cells were exposed for 48 h to **1** compared with 24 h. A tendency for a regular increase or decrease in the % Abc was not clear.

Additionally, compound **2** showed higher % MI mean differences than **1** (Fig. 4).

Micronucleus assay

In general, there were no wide variations among the numbers of micronuclei recorded in the blood smears of mice within an experimental group. Therefore, the results of the micronucleus assay were pooled (Table III). Statistically significant differences ($P < 0.05$) were observed between the means of MN formation compared to the negative and positive groups. Significant differences were also observed in the means of

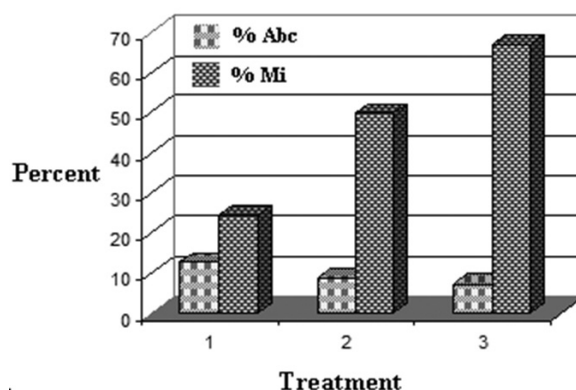


Fig. 4. The effect of compounds **1** and **2** on Mi and Abc of *A. cepa* root tip cells compared with the negative control. 1, Control; 2, compound **1**; 3, compound **2**.

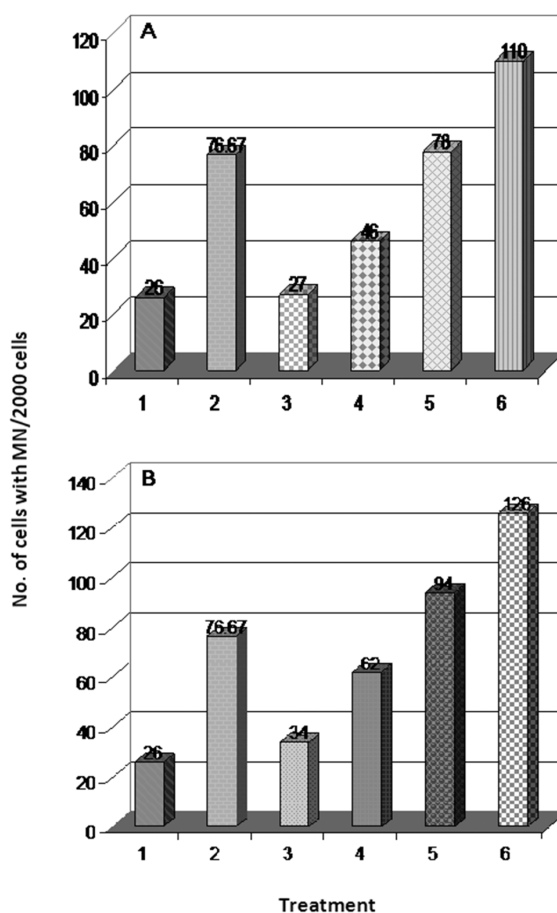


Fig. 5. The effect of different concentrations of compounds **1** and **2** on inducing MN formation in normochromatic erythrocytes of Balb/c mice. 1, Without treatment; 2, MMC; 3, 0.02 mg/mouse; 4, 0.04 mg/mouse; 5, 0.06 mg/mouse; 6, 0.08 mg/mouse.

Table III. Pooled micronuclei (MN) data from the two experiments with **1** and **2** administered intraperitoneally to the mice at 0.04 mg/mouse and 0.08 mg/mouse.

Dose given to each mouse	Animals	Experiment	Total number of cells with MN/2000 normochromatic erythrocytes	Mean $\pm$ SD of the total number of cells with MN/2000 normochromatic erythrocytes
Without treatment (50% DMSO)	1	1	25	26.0 ± 3.59
	2		30	
	3		29	
	1	2	25	
	2		20	
	3		27	
1 0.04 mg/mouse	1	1	48	46.0 ± 6.45
	2		55	
	3		41	
	1	2	45	
	2		39	
	3		48	
1 0.08 mg/mouse	1	1	109	110.0 ± 5.55
	2		111	
	3		116	
	1	2	99	
	2		114	
	3		111	
2 0.04 mg/mouse	1	1	58	62.0 ± 6.45
	2		69	
	3		65	
	1	2	55	
	2		69	
	3		69	
2 0.08 mg/mouse	1	1	132	126.0 ± 5.55
	2		118	
	3		122	
	1	2	129	
	2		131	
	3		124	

MN formation in mice that received different concentrations of both compounds. An exception is noticed in mice that received the lowest concentration (0.02 mg/mouse) of compound **1** where no significant differences were observed compared to the negative group. There were no

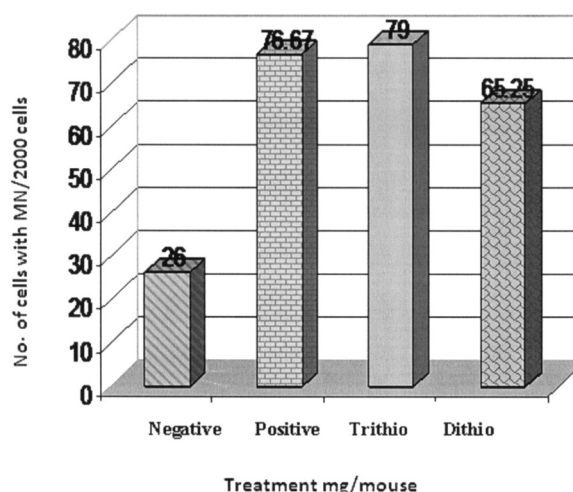


Fig. 6. The effect of compounds **1** and **2** on inducing MN formation in normochromatic erythrocytes of Balb/c mice compared with the negative and positive groups.

significant differences between the MN means of the positive group and the group of mice that were injected with (0.02 mg/mouse) of each compound. Fig. 5 shows dose-dependent increases in the number of micronucleated normochromatic erythrocytes for each compound. By examining the effect of both chemicals on MN formation (Table III), mice which were treated with **2** had a higher MN mean in comparison with mice treated with **1** (Fig. 6). There were no significant differences in the means of MN when compared to the positive group mean.

Discussion

To our knowledge, there are no genotoxic studies on calix[4]crown derivatives. Calix[*n*]arenes functionalized with guanidinium groups at the upper rim and alkyl chains at the lower rim bind to DNA, condense it, and, in some cases, promote cell transfection depending on their structure and lipophilicity (Sansone *et al.*, 2006). The platelet-derived growth factor (PDGF) and its receptor PDGFR are required for tumour growth and angiogenesis; so disruption of the PDGF-PDGFR interaction should lead to starvation of tumours and reduction of tumour growth. Potent PDGF antagonists have been discovered through the synthesis of a series of calix[4]arene-based compounds that are designed to bind to the three-

loop region of PDGF. The effects of lower-rim alkylation, linker and number of interacting head groups on the calix[4]arene scaffold on PDGF affinity and cellular activity have been investigated (Zhou *et al.*, 2006).

In the present study, the frequency of % Abc increased with increasing dose, while % Mi decreased. There were statistically significant differences between the concentrations used for each compound compared to the negative control. Also, all concentrations used were far away from the result of the negative control group, except in case of **1** at the concentration of 10^{-7} M, which showed no significant difference in its % Abc and its result slightly above the control. Alterations in chromosomes and the meristematic cells cycle division of the onion root have been used to warn the population about the consumption of herbal tea (Vicentini *et al.*, 2001). It is exciting that the % Mi decreased while the % Abs increased; this can be explained by the cytotoxic effects of the compounds and suppression of the cell proliferation and disturbance of mitotic functions. The effects of the two compounds on the mitotic index of *A. cepa* root tip cells were dose-dependent. No significant differences were observed by comparing the effects of time for each compound at 24 and 48 h. However, there was a slight increase in the % Abc, and a small decrease in the % Mi at 48 h. Furthermore, it was shown that **2** had greater effects on the % Mi and % Abc of *Allium cepa* root tips than **1**.

The two compounds, at different concentrations, had statistically significant differences in their ability to induce MN as compared to the negative control. With one exception, compound **1** at 0.06 mg/mouse, there was no significant difference between its MN mean and the positive control. Also, the data show that the lowest two

concentrations of each compound were less effective in inducing MN formation in NCE than the positive control.

In contrast, the highest two doses (0.06 and 0.08 mg/mouse) produced effects that exceeded the positive control (MMC 14 mg/kg body weight). The data indicate that the two calixcrown compounds had significant effects on the induction of MN formation in mice; compound **2** had a greater effect in this regard than compound **1** (Table III).

Finally, chromosomal aberrations might be formed by the direct effect of the two calixcrowns on the DNA or through disturbing the synthesis of DNA and protein, or the translation of RNA, so that no materials relating to the chromosome movement could be formed. Another possibility is that **1** and **2** can prevent the re-establishment of the chromosome under normal conditions through interfering with the normal repair of some damages to the new fusions, such as the rearrangement of chromosomal bridges, loops and fragments. Lagging of chromosomes or failure to reach the equatorial plane may result from problems in the formation of spindle fibers or the destruction of their function leading to the interference with regulation of chromosome movement, so that the chromosomes can not reach the equatorial plane in time (Qian, 2004). The inhibition of spindle formation causes sever abnormalities such as sticking, unequal distribution, multipolar, chromosomal bridges and laggards (Ünceer *et al.*, 2003). It is hoped that this work will suggest and invite further research on calixcrowns.

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