African Journal of Biotechnology Vol. 10(36), pp. 6863-6873, 18 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.579
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Assessment of anticlastogenic activity of cinnamic acid: Anticlastogenic index (ACI) and model simulation

Ahmed M. El-Shehawi[1,2]*, Mostafa A. Eldakak[2] and Mohamed A. Seehy[2]

[1]Department of Biotechnology, Taif University, Taif, Saudi Arabia.
[2]Department of Genetics, University of Alexandria, Elshatby, Alexandria, Egypt.

Accepted 9 June, 2011

Cinnamic acid and its derivatives are an important member of the phenolic compound used in food supplements. They usually occur in various conjugated forms, more frequently esters and glycosides. Mice (*Mus musculus*, 2n = 40) were employed as an experimental mammalian system to assess the anticlastogenic activity of cinnamic acid (CA) induced by Endoxan. Four doses; 1/32, 1/16, 1/8 and 1/4 of the LD_{50} of CA (5, 10, 20 and 40 mg/kg, respectively) in combination with five administration times of CA were tested. Micronucleated polychromatic erythrocytes (PCE) and mitotic index (MI) were used as a sensitive short term genotoxic bioassays. The results obtained showed that low doses (1/32 LD_{50} and 1/16 LD_{50}) decreased the percentage of PCE significantly compared with that of the positive control and closer to that of the negative control. The data of this study were used to calculate a new index called anticlastogenic index (ACI). The new index measures the anticlastogenic activity of a compound or an extract. The ACI beside the percentage of PCE can give a deeper look of the anticlastogenic activity of compounds. It also makes it easy to draw conclusions from the genotoxicity data. The maximum ACI of CA was achieved when both CA and Endoxan were given concurrently. On the other hand, the higher doses of CA (1/8 and 1/4 LD_{50}) caused a significant increase in the percentage of PCE. This gives evidence that CA at high doses (20 and 40 mg/kg) would be considered as a positive clastogen itself. CA significantly decreased the mitotic index compared with the negative control. In addition, high doses showed sharp decrease in mitotic index. Direct significant correlation coefficient was found between the ACI and the mitotic index. The data also were used for the simulation of a model to predict the ACI of doses with different relative timing to a treatment of the clastogen that were not conducted experimentally in the limit of experiment data range.

Key words: Anticlastogenic, cinnamic acid, anticlastogenic index, ACI, mitotic index, mice, model simulation of ACI.

INTRODUCTION

Phenolic compounds have become increasingly important in food industry as additive for flavor, taste, color and prevention of oxidative deterioration. In particular, many phenolic compounds are attracting the attention of food and medical scientists because of their anti-inflammatory, antimutagenic and anticarcinogenic properties and their ability to modulate some key enzyme functions (Francis, 1989; Chi-Tang et al., 1991). Hydroxycinnamic acid and its derivatives present a group of phenolic compound used in food that are derived from *p*-coumaric, caffeic, ferulic acid ((Chi-Tang et al., 1991; Hermann, 1989). They usually occur in various conjugated forms, more frequently esters and glycosides. Flavonoids consist mainly of catechins, proanthcyanins anthocyanins, flavones and flavonols. It has been estimated that human consuming high fruit and vegetable, diets ingest up to 1 g of these compounds daily (Chi- Tang

*Corresponding author. E-mail: elshehawi@hotmail.com. Tel: 0554904625.Fax: 0966027367409.

Abbreviations: CA, Cinnamic acid; **PCE,** polychromatic erythrocytes; **MI,** mitotic index; **ACI,** anticlastogenic index; **CMA,** chlormadinone acetate; **NDGA,** nordihydroguaiaretic acid.

et al., 1992).

Antimutagenic effects of phenolic compounds

Many plant phenols including ferulic, caffeic, chlorogenic, ellagic acids, tannic acid, several hydroxylated anthraxquinone and CA derivatives inhibit the mutagenicity and cytotoxicity of benzo[*a*]pyrene and its carcinogenic metabolite (Alexander et al., 1982; Mou-Tuan et al., 1985). Isoflavones fremontin and fremontone isolated from roots of *Psorothamnus fremontii* (Fabaceae) were found to be nontoxic to *Salmonella typhimurium* and were highly active in the inhibition of mutagenicity of ethyl methanesulfonate (EMS) at all concentrations tested (Manikumar et al., 1989). The lioflavones genstein and diazedien were reported to have antimutagenic activity through their suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against the mutagen 3-amino-1,4-dimethyl-5*H*-pyrido [4,3*b*]indole (Trp-P-1) which requires liver metabolizing enzymes (Mitsuo et al., 1999). Vanillin is able to inhibit mutation at the CD59 locus and modify toxicity in a mutagen-specific manner. Possible mechanisms to explain the action of vanillin include inhibition of a DNA repair process that leads to the death of mutants or enhancement of DNA repair pathways that provide protection from mutation, but create lethal DNA lesions during the repair process (Daniel et al., 2000).

The antimutagenic effect of *Cinnamomum cassia* against two mutagens; benzo[*a*]pyrene (B[*a*]P) and Endoxan was examined using the Ames test, *in vivo* chromosomal aberration and micronucleus test. Changes in liver cytochrome P450 (Cyt P450), glutathione content (GSH), glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPX) were evaluated in pretreated animals. Pretreatment decreased Cyt P450 content but increased GSH content and the activity of glutathione-dependent antioxidant enzymes; GST, GR and GPX. The antimutagenic effect of *C. cassia* could be attributed to its modulatory effect on the xenobiotic bioactivation and detoxification processes (Sharma et al., 2001). The antigenotoxic effect of nordihydroguaiaretic acid (NDGA), a phenolic lignan, was studied against chlormadinone acetate (CMA) in mice bone-marrow cells. It reduced the sister chromatid exchanges and chromosomal aberrations induced by CMA. Earlier studies showed that CMA generates reactive oxygen species which are responsible for genotoxic damage. The free radical-scavenging property of NDGA is responsible for the reduction of genotoxic damage induced by CMA in mice bone-marrow cells (Siddique et al., 2008). Curcumin has been reported to have antimutagenic effect against sodium azide induced chromosomal aberrations in *Allium cepa* root meristem, but showed low levels of cytotoxicity indicated in reducing the percentage of mitotic index in all curcumin treated groups. The antimutagenic activity of curcumin is effective at 5 µg/ml in *A. cepa* root meristem cells (Irulappan and Natarajan, 2007). Vannilic and CA were found to have antimutagenic activity against spontaneous mutations in mammalian (human) cells (Audrey et al., 2007).

A correlation was found between the anticlastogenic effect and the antioxidant activity of various flavonoids. This suggests that the effect of flavonoids may be attributed to the hydroxyl radical scavenging activity in a direct or an endogenous enzyme mediated manner (Kayoko et al., 1994; Wei, 1995). CA was reported to be antioxidant in lipid peroxidation and hydroxyl radicals in the NADPH (Árka et al., 2003). Some flavonoids, especially quercetin are strong antioxidants which raised the possibility that quercetin and its related substances might reduce risks of cancer, cardiovascular disease and stroke in human (Sampson et al., 2002).

Anticlastogenic effect of polyphenols

Polyphenols reduced significantly the frequency of micronucleated cells among bone marrow cells and peripheral blood cells. They are effective in preventing DNA damage and one of the mechanisms of action might involve scavenging of active oxygen radicals (Yamagishi et al., 2001). Phenolics of olive oil are the only substance that showed a significant anticlastogenic activity before and after x-ray irradiation treatments. They have free oxygen radicals and lipoperoxyradicals scavenging activities (Benavente et al., 2002). The flavonoids quercetin and its glucoside isoquercitrin, administered orally reduced the number of PCE mice bone marrow (Edenharder et al., 2003). Citrus extract of (*Citrus aurantium* var. *amara*) significantly reduced the frequencies of PCE. The optimum dose for protection in mouse was 250 mg/kg to protect mice bone marrow from the side effects of γ-irradiation with respect to the non-drug-treated irradiated control. Therefore, fruits and vegetables contain flavonoids that have protective effects under such stress conditions as irradiation (Hosseinimehr et al., 2003).

Correlation of ACI of polyphenols with anticarcinogenic potential

Curcumin has been reported as a signal transduction modulator and inhibitor of transcription factors such as NF-κB. Curcumin increased the cytotoxicity in cultures of cells isolated from the bone marrow of a patient with non-Hodgkin's lymphoma (NHL). Curcumin is characterized by low toxicity and was described to have a chemoprotective activity. Therefore, the level of reduced glutathione (GSH) was measured and a concentration-dependent increase of GSH levels was recorded in AR-230 and SKW-3 cells (concentration range 5 to 25 µM). Experiments with mice showed significant protection against chromosomal

Table 1. Distribution of mice groups on treatments.

Treatment		Administration time of CA relative to Endoxan				
		24 hB*	3 hB**	Concurrent	3 hA**	24 hA*
NC	NC	+				
	NC		+			
	NC			+		
PC	PC	+				
	PC		+			
	PC			+		
CA	¼ LD_{50}	+	+	+	+	+
	1/8 LD_{50}	+	+	+	+	+
	1/16 LD_{50}	+	+	+	+	+
	1/32 LD_{50}	+	+	+	+	+

*24 h before or after concurrent treatment; **3 h before or after concurrent treatment.
NC, Negative control; PC, positive control; CA, cinnamic acid.

aberrations (clastogenic effect) and inhibition of mitosis in bone marrow cells. Curcumin alone caused reduction of the mitotic index. In addition, curcumin has protective and anticlastogenic activity by enhancing the scavenging of free radicals (Tzvetan et al., 2007). In this study, the focus was to study the administration time of one important member of flavonoids, CA, which is present in one of the famous natural spices namely "cinnamon" using micronucleus test to measure the anticlastogenic activity of Endoxan as well as its effect upon mitotic index. The obtained data were employed to develop a new index called ACI. The ACI simplify the analysis of anticlastogenic data and can be used to predict the ACI of the tested compound in a concentration range including some concentrations that have not been experimentally carried out.

MATERIALS AND METHODS

Chemicals

HPLC grade of CA was purchased from Sigma-Aldrich Company. Endoxan (cyclophosphamide) was obtained from Asta Medica AG, Frankfurt, Germany.

Animals and doses

Eight week old Swiss albino mice (*Mus musculus*, 2n = 40, 25 g per animal) were maintained under conventional laboratory conditions at room temperature for two weeks. Commercial pellet diet and tap water were provided during the experiment. CA was dissolved in DMSO at four different concentrations (50, 25, 12.5 and 6.25 mg per 10 ml DMSO) for preparations of 1/4 LD_{50} (40 mg/kg), 1/8 LD_{50} (20 mg/kg), 1/16 LD_{50} (10 mg/kg) and 1/32 LD_{50} (5 mg/kg) stocks, respectively (http://msds.chem.ox.ac.uk/CI/cinnamic_acid.html). The four doses were administered by giving each animal 0.1 ml from the corresponding stock. Endoxan was dissolved in DMSO to make a stock giving 0.1 ml per mouse (0.4 mg/kg). The negative control group received 0.1 ml of DMSO.

Treatments

Experiment 1: Effect of administration time and dose of CA.

This experiment was designed to estimate the administration time and dose of CA on the anticlastogenic activity of CA. Five administration times were chosen; 24 h before and after giving the Endoxan, 3 h before and after giving the Endoxan and the concurrent time with Endoxan. Seventy eight (78) mice were used in this experiment. They were randomly distributed into 26 groups (three animals per group). Three negative controls (NC) and three positive controls (PC) were used for the treatments at 24 h before and after, 3 h before and after and the concurrent time with Endoxan. Twenty (20) groups were used for the four levels of CA (¼ LD_{50}, 1/8 LD_{50}, 1/16 LD_{50}, 1/32 LD_{50}) and five times of CA administrations (Table 1). Distributions of mice into 26 groups over the various treatments, CA was given eleven hours before sacrificing animals.

Experiment 2: CA effect on cell proliferation

This experiment was designed to test the effect of CA on the cell proliferation rate (mitotic index). Forty five (45) mice were randomly distributed on 15 groups (3 animals each). Three NC groups were used for the three administration times of CA (35, 14 and 11 h). The remaining twelve groups were distributed on the four levels of CA (¼ LD_{50}, 1/8 LD_{50}, 1/16 LD_{50}, 1/32 LD_{50}) and the three administration times (35, 14 and 11 h). Negative control group received equal volume of DMSO. Mice were killed after 11 h (1/2 mitotic cycle) of the last injection either with Endoxan or CA.

Harvesting of bone marrow cells

Animals were killed, lower abdomen, limbs were incised and femora were cleaned and separated from the hip joint. The ends of the femur were trimmed and a blunt needle was pushed to pierce the marrow cavity. Bone marrow was flushed out with isotonic solution (137 mM NaCl, 15 mM Na_2HPO_4, 2.5 KCl, 1 mM K_2HPO_4). The suspension was made up to 5 ml and centrifuged at 1200 rpm for 10 min. The clear supernatant was discarded. Hypotonic solution (75 mM KCl) was added and the pellet was mixed thoroughly over a

Table 2. Means (%) of PCE of CA dose and administration time combination and their interaction.

Dose	Administration time relative to Endoxan*					Mean
	24 hB	3 hB	Concurrent	3 hA	24 hA	
1/32 LD_{50}	29.33	15.66	13.33	14.93	18.43	18.34
1/16 LD_{50}	27.76	20.13	11.5	15.3	18.1	18.56
1/8 LD_{50}	39.6	43.1	44.63	43.67	39.03	42.00
¼ LD_{50}	40.77	43.1	46.33	43.47	43.1	43.35
(-) Control	11.5	11.5	12.43	11.5	11.5	11.68
(+) Control	38.06	38.23	38.43	38.43	38.03	38.24
Mean	31.17	28.62	27.77	27.88	28.03	

*Data were transformed by angular transformation; $LSD_{0.05}$ for timing of treatment relative to Endoxan means =1.5; $LSD_{0.05}$ for means of CA doses =1.4; $LSD_{0.05}$ for the interaction of administration time and doses of CA =3.3; hrB: hours before concurrent treatment; hrA: hours after concurrent treatment.

vortex and then incubated at 37°C for 20 min. The suspension was centrifuged at 1200 rpm for 10 min and the clear supernatant was discarded. Fixation solution (3 absolute methanol: 1 glacial acetic acid) was added drop by drop on the pellet with vortexing and left for 10 min. The fixation step was repeated twice and samples were kept at 4°C for 24 h (Schmid, 1975; Adler, 1988).

Slides preparation and staining

Cell suspensions were dropped by Pasteur pipette on clean sterilized slides (rinsed in methanol for 24 h) at a distance of 70 cm. Slides were stained in 4% Giemsa stain solution for 10 min (Adler, 1988). Slides were photographed using Olympus microscope BH-2 attached to automatic camera unit.

Micronucleus (MN) scoring

The MN in PCE cells were examined microscopically according to the method of Schmid (1975). The frequency of MN was counted for every 1000 cells in each mouse and the percentage of MN was calculated.

Mitotic index

The cell proliferation rate was estimated as the percentage of dividing cells to the total number of cells in stained slides. One thousand cells per mouse were examined (Brusick, 1987).

Anticlastogenic index (ACI)

A new index was introduced in this study, it is named anticlastogenic index (ACI). It is calculated for certain dose and duration of time for the tested compound used before or after treatment of the clastogen to test whether it has anticlasogenic or repair activity of a clastogenic compound. The ACI is defined as the percentage of decrease or increase of PCE induced by the clastogen used as positive control according to the following formula:

$\%ACI_{x,y,z}$= 100 – {(% MN of tested compound) – (%MN of NC)/ (% MN of PC clastogen) – (%MN of NC)} X 100

Where, x is the dose of the tested compound for anticlastogenicity (mg/kg); y is the duration of administration of the compound before or after treatment of the clastogen; z can be designated as one of two forms: before (B) or after (A). ACI = 0 when there is no anticlastogenic or clastogenic activity. ACI has positive values when the tested compound has anticlastogenic activity and has negative values when the tested compound has no ACI, but rather has clastogenic activity. In an experimental design where a positive clastogen is not used and the purpose is to estimate the natural ACI of a compound or natural product whether it has protective or repairing activity. The formula will be changed as follows:

$\%ACI_{x,y,z}$= 100 – {(% MN of NC) – (%MN of tested compound)/ (% MN of PC)} X 100

In this case (no positive clastogen), the ACI of the tested compound is designated protective and repairing.

Statistical analysis and model simulation

The data of PCE, ACI and MI for different doses and different administration time combinations were transformed by angular transformation. Data were analyzed as a factorial arrangement of dose of CA and its administration time relative to Endoxan in complete randomized design (CRD). Comparison between means was made via the least significant difference (LSD). Correlation between mitotic index and ACI was done using statistical analysis modules of Microsoft excel 2007 (Richard et al., 2001). Forecasting module of Microsoft excel 2007 was employed to conclude and /or predict the ACI for the untested doses of CA relative to Endoxan (Richard et al., 2001).

RESULTS AND DISCUSSION

Effect of CA on the percentage of PCE

Lower dose of 1/32 LD_{50} and 1/16 LD_{50} of CA induced percentages of PCEs near to the average of negative control (11.68%). On the other hand, the high doses 1/8 LD_{50} and 1/4 LD_{50} of CA induced percentages of PCE near to that of the positive control (38.24%) (Table 2).

Table 3. Mean of ACI percentages of CA dose, administration time and their interactions.

Dose	Administration time relative to Endoxan*					Mean
	24 hB	3 hB	Concurrent	3 hA	24 hA	
1/32 LD_{50}	39.84	72.9	84.77	75.07	65.67	67.65
1/16 LD_{50}	42.9	77.13	90	74.47	66.4	70.18
1/8 LD_{50}	-16.6	-21.07	-24.47	-23.6	-10.1	-19.17
¼ LD_{50}	-23.47	-28.57	-39.2	-29.73	-30.23	-30.24
Mean	10.67	25.1	27.78	24.05	22.93	

*Data were transformed by angular transformation; $LSD_{0.05}$ for timing of treatment relative to Endoxan means = 3.09; $LSD_{0.05}$ for means of CA doses = 3.45; $LSD_{0.05}$ for the interaction of administration time and doses of CA = 6.91; hrB: hours before concurrent treatment; hrA: hours after concurrent treatment.

They resulted in very close overall averages of PCEs, 18.34 and 18.56% at all various timings, respectively. The higher doses 1/8 LD_{50} and 1/4 LD_{50} of CA gave higher percentages of PCE, 42 and 43.35%, respectively at all administration times (Table 2). It seems CA itself induces PCEs at higher concentrations and has anti-clasotgenic activity as well. Various administration times of CA did not show significant differences. There were no significant differences between the higher doses of CA, 1/8 LD_{50} and 1/4 LD_{50}. The negative control and the positive control groups were two separate groups using LSD of means. For this reason, it is clear that the best doses capable to decrease the percentage of PCE were 1/32 LD_{50} and 1/16 LD_{50} of CA. Although, they scored low percentage of PCEs that made them differ significantly from that of the positive control and that of the higher doses, they differed significantly from the negative control. On the contrary, the higher doses of CA were rather toxic and their toxicity appeared as a combination of Endoxan toxicity because PCE percentages exceeded that of the positive control, lower doses and negative control significantly. Best interaction of administration time and dose of CA was obtained with dose 1/16 LD_{50} at concurrent administration, whereas worst interaction was obtained with dose 1/8 LD_{50} at concurrent administration.

Effect of administration time of CA relative to Endoxan

Table 2 shows that the administration time of CA to Endoxan had effect upon decreasing the percentages of PCE. At the concurrent administration of CA and Endoxan, the percentages of PCE were 13.33 and 11.5% for 1/32 LD_{50} and 1/16 LD_{50} of CA, respectively. For the CA administration time, 3 h before and after Endoxan, the percentage of PCE was not significantly higher than that of concurrent administration time of CA to Endoxan as shown in Table 2.

The case was different for the toxic doses 1/8 LD_{50} and 1/4 LD_{50} of CA. The highest percentage of PCEs was scored at the concurrent treatment of CA and Endoxan. This may be due to their combined clastogenic activity in these treatments. They gave 44.63 and 46.33% PCE for 1/8 LD_{50} and 1/4 LD_{50} of CA, respectively. The percentages of PCE at 3 h before and after Endoxan treatment were less than that of 24 h before and after Endoxan (Table 2). Although, the percentage of PCEs were lower in a dose that corresponds to 1/16 LD_{50} of CA compared with that of 1/32 LD_{50} of CA, the decrease was not significant (Table 2).

LSD means showed that the highest percentages of PCE were caused by doses 1/32 LD_{50} and 1/16 LD_{50} of CA at 24 h before Endoxan. It was clear giving 31.17 and 28.03%, respectively. High percentage of PCE was found at 24 h before concurrent Endoxan compared with that of the 24 h of CA after Endoxan. This may be due to the mechanism of natural recovery and/or excretion of most CA from the body.

ACI for the various doses of CA

Lower doses of CA, 1/32 LD_{50} and 1/16 LD_{50}, averaged an ACI of 67.65 and 70.18%, respectively for all administration times of Endoxan (Table 3, Figure 1). The higher doses, 1/8 LD_{50} and ¼ LD_{50}, of CA showed a clastogenic index (negative ACI) rather than that of ACI with an average of -19.17 and -30.24% for all various administration time of CA. This revealed similar results to that have been obtained for PCEs (Table 2). Also, Figure 1 reveals that both doses of 1/32 LD_{50} and 1/16 LD_{50} of CA showed high and close ACIs. On the other hand, the higher doses, 1/8 LD_{50} and 1/4 LD_{50}, of CA showed close negative ACI, have clastogenic activity, rather than anticlastogenic activity. Table 3 and Figure 1 show that in the case of the lower doses of CA, 1/32 LD_{50} and 1/16 LD_{50}, the best administration time was the concurrent administration of CA and Endoxan. They gave the highest ACI of 84.77 and 90%, respectively. This significantly decreased at the 3 h before and after Endoxan as well as at 24 h before and after Endoxan,

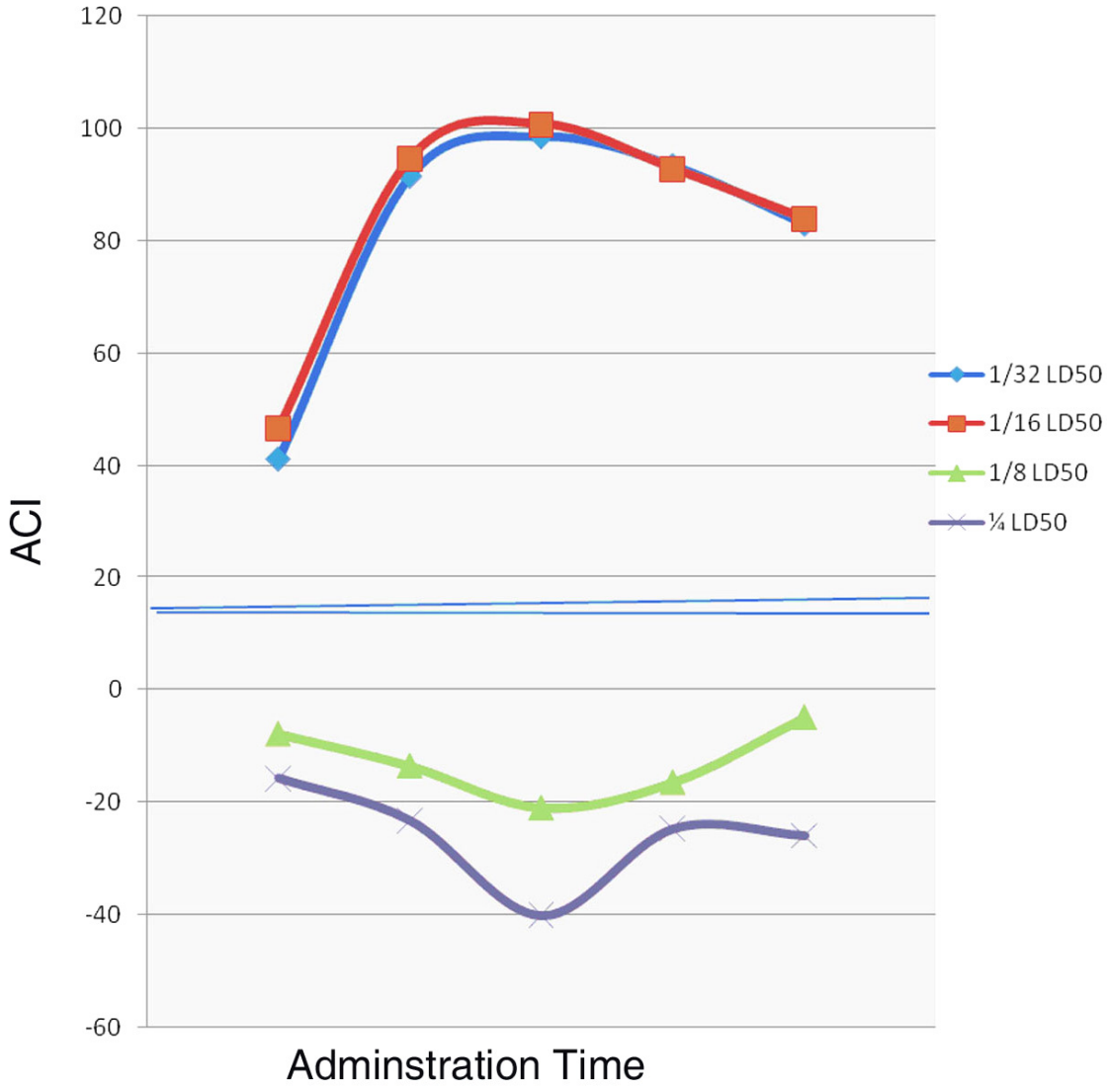

Figure 1. ACI for various doses of CA and various timing.

respectively. On the other hand, higher doses (1/8 LD_{50}, 1/4 LD_{50}) of CA, showed lower ACIs (-24.47 and -39.2%, respectively) when CA and Endoxan were given concurrently. It is clear that all results for higher doses are negative, meaning that they do not have ACI, but they have clastogenic activity (Table 3).

It is easy to categorize the groups easily into anti-clastogenic or clastogenic groups using LSD means (Table 3). No significant difference in ACI between the lower two doses of CA, but the two higher doses form a group with clastogenic index. LSD of means showed that the best administration time of CA is concurrently with Endoxan, while the worst at 24 h before Endoxan. This again may be due partially to the short half life of Endoxan.

The use of the ACI introduced in this study gives the same accurate results and its interpretation. ACI showed parallel data to the data calculated of PCE percentages at lower doses of CA. The best relative administration time for CA is at concurrent time with Endoxan. This could be due to the short half life of Endoxan. Higher doses of CA were clastogenic rather than anticlastogenic and consequently have negative ACI values. The ACI makes it easier in understanding the index of the chemical compounds or extracts used since its value is a function of the percentage of the PCE in the clastogen treatment. Therefore, the deviation from this percentage, positive value of ACI to negative values means that the compound or extract has clastogenic potential. Overall, the high ACI values (Table 3) are associated with lower PCE (Table 2). This strongly supports the validity of the ACI and its use to measure the anticlastogenic activity of clastogens.

The ACI for different doses of CA administration time

Table 4. Means of MI* as affected by dose and duration of exposure to CA (cinnamic acid).

Dose	Duration of exposure to CA			Mean
	35 h	14 h	11 h	
Negative control	20.3	20.83	20.57	20.57
1/32 LD_{50}	20.3	20	19.4	19.9
1/16 LD_{50}	19.4	19.4	19.4	19.4
1/8 LD_{50}	15.67	16.03	15.67	15.79
1/4 LD_{50}	15.3	14.2	14.57	14.69
Mean	18.19	18.09	17.92	

*Data were transformed by angular transformation; $LSD_{0.05}$ for means of CA doses = 0.29; $LSD_{0.05}$ for means of duration of exposure to CA = 0.37; $LSD_{0.05}$ for the interaction between duration of exposure to and doses off CA = 0.64

relative to Endoxan were calculated according to the formula in materials and methods section. The highest ACI of CA as anticlastogenic was obtained when it was administered concurrently with Endoxan (Table 3). CA was less efficient as anticlastogenic when administered before Endoxan since administration after Endoxan showed higher ACI especially at 24 h after Endoxan. The interaction between the various doses of CA and their relative timing to CA also affects the values of ACI since the highest ACI values were obtained when CA and Endoxan were administered concurrently. The highest ACI was obtained at doses 1/16 LD_{50}, 1/32 LD_{50} and the best administration time was the concurrent administration giving mean of ACI 70.18 and 27.78, respectively (Table 3). For LSD means, there were a significant difference between the lower doses CA and that of negative control. It is clear that ACI differ significantly at higher doses at the various administration time compared with the positive control, negative control, as well as lower doses (Table 3).

CA was reported to have antimutagenic activity and most of the phenolic classes have antioxidant activity (Mou-Tuan et al., 1985; Wei, 1995; Yoshioka et al., 1995; Sampson et al., 2002). The data are in accordance with the fact that CA has short half life of about 7 min (Jinhua et al., 1993; Castillo et al., 2000) and CA has antimutagenic and antioxidant activity (Arka et al., 2003). Also, the ACI presented an evidence to emphasize that our results obtained as ACI are in parallel with anticarcinogen activity of other phenolic classes (Tzvetan et al., 2007). More work needs to be done on this compound to approve its anticarcinogenic activity and caution should be taken since a high dose can make an opposite effect.

Effects of CA on mitotic index

The effect of various doses of CA at different times relative to Endoxan on cell proliferation rate was estimated by mitotic index. Cell proliferation rate decreased at the two lower doses of CA, 1/32 LD_{50} and 1/16 LD_{50}, with a slower rate compared with the sharp decrease in the mitotic index at the higher doses. There was a significant interaction between the dose and the duration of exposure of CA, but the only significant effect on the cell proliferation rate is due to the increase of the dose of CA (Table 4). This was caused by the increase of CA concentration. The results obtained gave an evidence to emphasize other results using other classes of phenolics informing that there is some cellular toxicity of phenolics decreasing the mitotic index significantly (Tzvetan et al., 2007; Juliana et al., 2007; Irulappan and Natarajan, 2007). The obtained data indicate that CA caused a significant decrease in mitotic index and this decrease has high slope at the higher doses, 1/8 and 1/4 LD_{50} of CA, but there were no significant effect of the duration of exposure of CA on the mitotic index (Table 4). LSD values showed that each dose group is independent from the other group, meaning that each group decreases the cell proliferation rate highly significant in a rate different from other groups.

Correlation between ACI and mitotic index

Positive correlation between ACI and MI was found which means that CA works at cellular activity not directly on chromosome structure. The correlation coefficient between the ACI and the mitotic index induced by CA treatment was highly significant (r = 0.95). CA decreases mitotic index significantly due to the increase of the dose of CA, but there were no significant effect of the duration of exposure to CA on the mitotic index (Table 4, Figure 2). Some reports showed that the increase in ACI is accompanied by significant increase of mitotic index. Lower doses of CA (1/32 LD_{50} and 1/16 LD_{50}) showed anticlastogenic activity. This may be one of the mechanisms made by CA to decrease the clastogenic potential through increasing the rate of cell proliferation to substitute aberrant cells. On the other hand, higher doses of CA with negative ACI are accompanied by decrease in mitotic index. This could be as a result of cellular toxicity which confirms that the higher doses of CA, 1/8 LD_{50} and

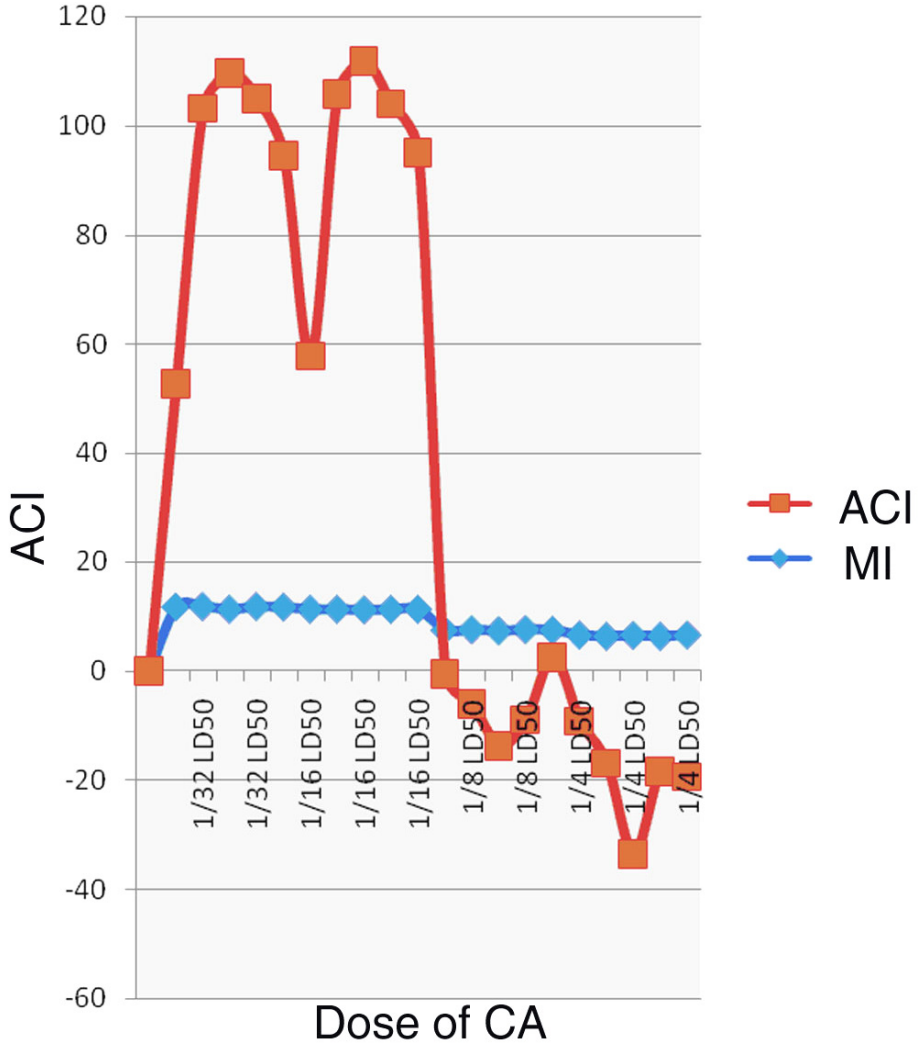

Figure 2. Correlation between ACI and mitotic index.

1/4 LD_{50}, showed cellular toxicity and caused significant decrease in mitotic index which increased CAI by replacing the aberrant cells. The obtained results agree with the results obtained by Tzvetan et al. (2007) on curcumin which showed correlation of ACI with its MI.

Model simulation of ACI

ACI with administration time of CA

***Dose 1/32 LD_{50} and 1/16 LD_{50}*:** In this study, another way of analysis of clastogenic data was introduced which could make an efficient understanding of the anti-clastogenic data. It is the model simulation of the ACI results. It is obvious of the results that the lower doses 1/32 LD_{50}, 1/16 LD_{50} of CA have significant ACI (Table 3). A model simulation of the ACI for administration time of CA relative to Endoxan that was not experimentally carried out, but lies in the timing range tested in the experiment.

For dose 1/32 LD_{50} of CA, the derived equation from the curve of relative timing range from 24 h before treatment of CA to concurrent administration of CA and Endoxan was y = 1.7649X + 81.718; (y = ACI, x = relative timing of CA to Endoxan). The same equation for that of the relative timing range from 24 h after treatment of CA to concurrent administration of CA and Endoxan was y = -0.6675X + 81.208. From these equations, a graph was drawn for the relative timings that were not conducted and simulate their ACI on one hour intervals as shown in Figure 3. The closest two relative timings of treatment of CA to Endoxan, 1 h before and after, to the concurrent administration gave ACI of 80.54%, respectively. The second closest timings, 2 h before and after showed 79.79 and 77.91%, respectively. These two points of

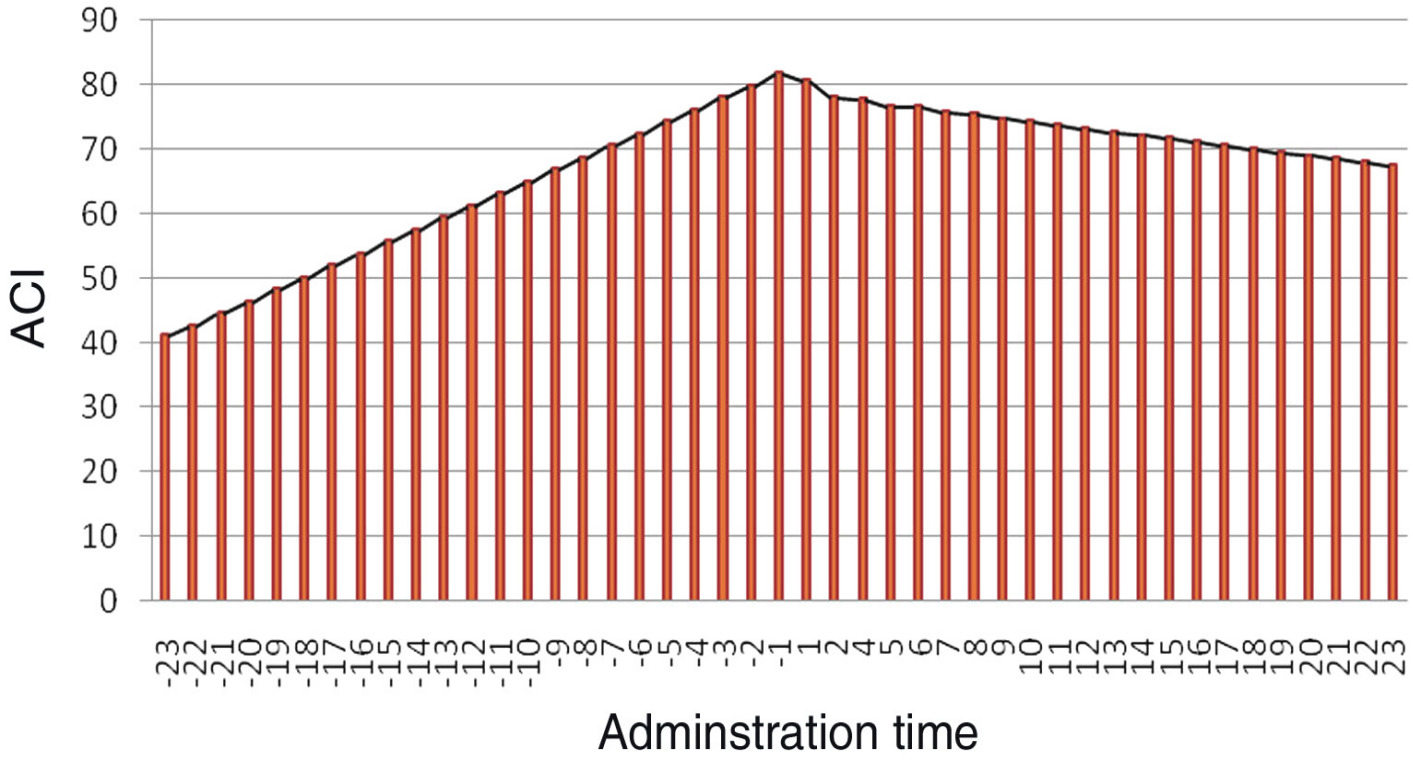

Figure 3. Simulation of ACI of CA at dose 1/32 LD_{50} over 24 h before and after concurrent administration of CA and Endoxan at one hour intervals.

relative timings showed high ACI of CA which emphases the short term anticlastogenic activity of CA. For dose 1/16 LD_{50} of CA, the derived equation from the curve of relative timing range from 24 h before treatment of CA to concurrent administration of CA and Endoxan was y = 1.8395X + 86.555. The same equation for that of the relative timing range from 24 h after treatment of CA to concurrent administration of CA and Endoxan was y = -0.7632x + 83.835. From these equations, a graph can be drawn for the relative timings that were not carried out. Simulation of their ACI on one hour intervals (Figure 4) showed that the closest two relative timings, 1 h before and after concurrent administration of CA to Endoxan showed ACI of 86.651 and 83.07%, respectively. The second closest two relative timings, 2 h before and after concurrent administration of CA to Endoxan showed ACI of 84.67 and 78.95%, respectively. These two points of relative timings showed high ACI of CA which emphases the short term anticlastogenic activity of AC (Figure 4).

ACI with dose of CA

***Dose 1/32 LD_{50} to 1/8 LD_{50}*:** Model simulation of the ACI for doses range from 1/32 LD_{50} (5 mg/kg) to 1/8 LD_{50} (20 mg/kg) at a rate of 0.4 mg/kg was carried out. The expected function of ACI for doses 1/32 LD_{50} (5 mg/kg) and 1/16 LD_{50} (10 mg/kg) of CA was y = 0.448X+ 96.3 (y = ACI, X = dose of CA, mg/kg). The same function of ACI for dose 1/8 LD_{50} (20 mg/kg) of CA was y = -3.806X + 36.13. As shown from the simulated model (Figure 5), it is clear that the different doses of a given anticlastogenic agent giving concurrently with the clastogenic agent improves the repair mechanism. The simulation model can be useful in informing us about a predicted ACI of using certain dose and this can be used for testing ACI of wide range of chemical compounds.

The results obtained from this study clearly indicate that CA has anticlastogenic effect at lower doses, but has clastogenic activity at higher doses. This was confirmed with parallel positive ACI and negative ACI, respectively. Administration of CA was more efficient when it was concurrent with Endoxan. Also, application of CA before Endoxan was less efficient as anticlastogenic agent. This is far from concurrent administration before or after the less efficient of CA. This is in accordance with the fact that CA has short half life and dissipates fast from biological systems. The proposed ACI gives direct indication of the effect of anticlastogenic agent upon the repair system, since anticlastogenicity gives positive values of ACIs and clastogenicity gives negative ACIs. Mitotic index decreased with increasing ACI giving an evidence of the role of anticlastogen in enhancement of repair system. In addition, according to the proposed

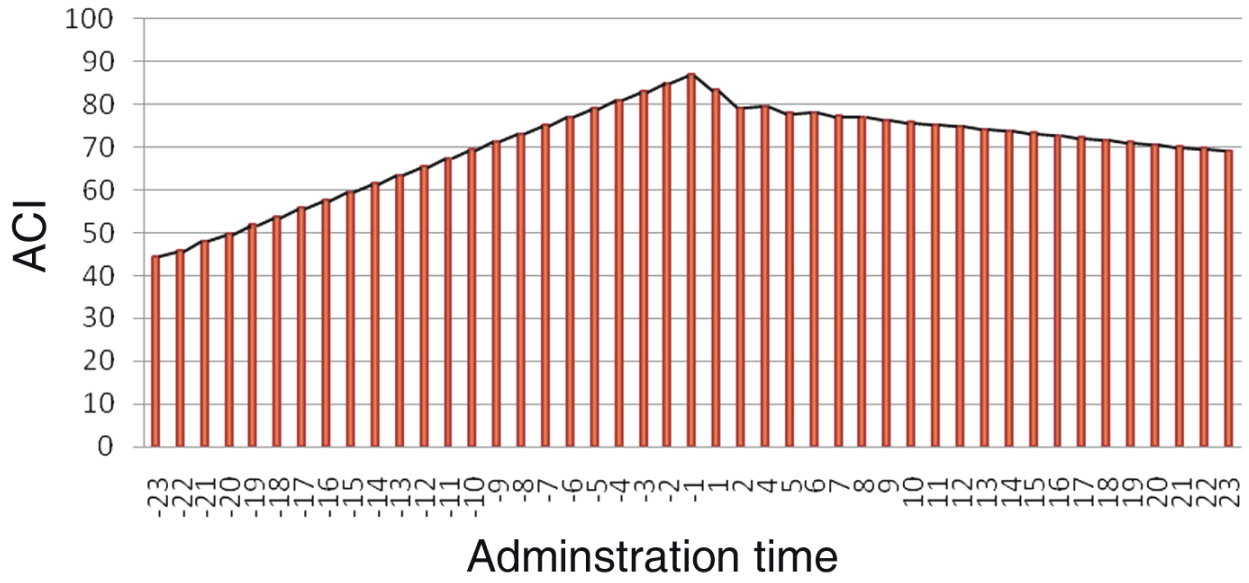

Figure 4. Simulation of ACI of CA at dose 1/16 LD_{50} over 24 h before and after concurrent administration of CA and Endoxan at one hour intervals.

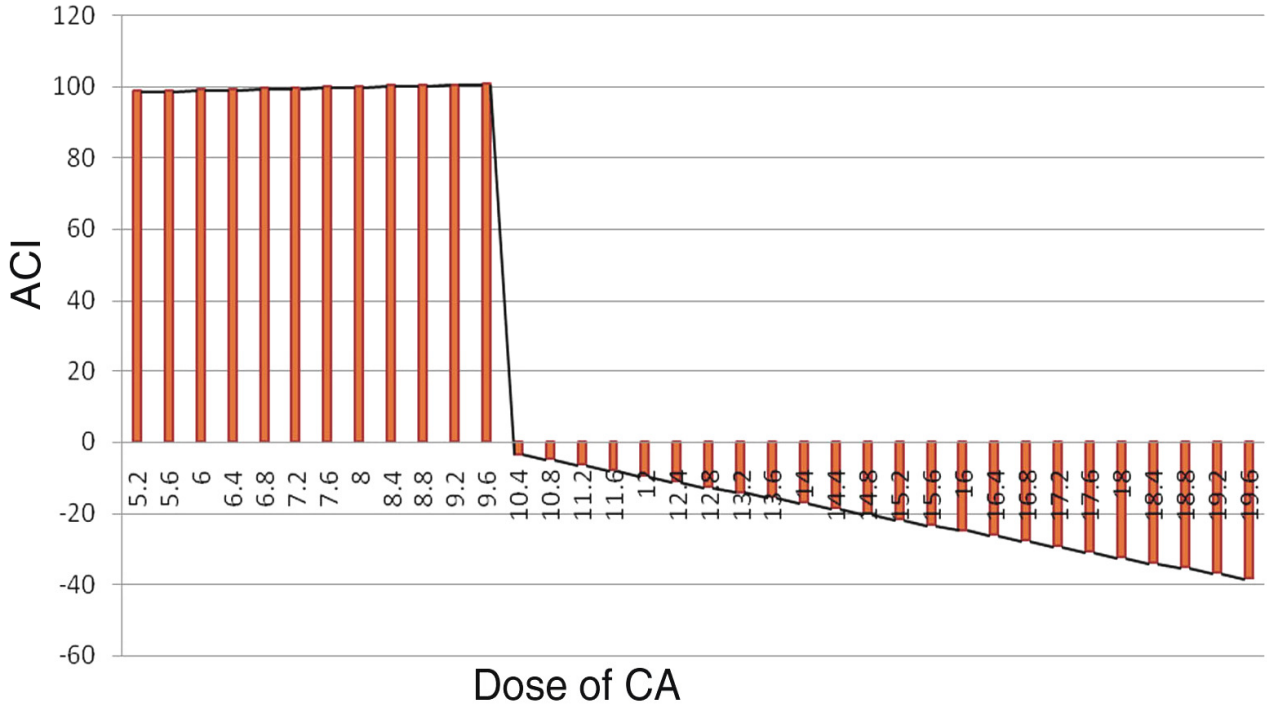

Figure 5. Simulation of ACI of CA and the various doses of CA (mg/kg) ranging from 1/32 LD_{50} to 1/8 LD_{50} based upon 0.4 mg/kg rate of increase.

system previously mentioned that ACI can be predicted for the untested dose for a given clastogenic agent.

REFERENCES

Adler ID (1988). Cytogenetic tests in mammal: Mutagenicity testing. IRL Press., Washington, 2: 278-282.

Alexander WW, Mou-Tuan H, Richard C, Harold N, Roland L, Haruhiko Y, Jane S, Donald J, Allan C (1982). Inhibition of the Mutagenicity of Bay-Region Diol Epoxides of Polycyclic Aromatic Hydrocarbons by Naturally Occurring Plant Phenols: Exceptional Activity of Ellagic Acid. PNAS, 79: 5513-5517.

Árka O, Josef M, Ivan G (2003). Different antioxidant effects of

polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems. Biochem. Pharmacol. 66: 1127-1137.
Audrey AK, Daniel S, Kanae M, Joanna L, William W, David U, Zongli X, Danica D, Jack T, David D, Catherine K (2007). Antimutagenicity of Cinnamaldehyde and Vanillin in Human Cells: Global Gene Expression and Possible Role of DNA Damage and Repair, Mutat. Res. 616: 60–69.
Benavente-García L, Alcaraz M, Redondo A, Ortuño A, DelRio J (2002). Antioxidant Activity and Radioprotective Effects against Chromosomal Damage Induced in Vivo by X-rays of Flavan-3-ols (Procyanidins) from Grape Seeds (*Vitis vinifera*): Comparative Study versus Other Phenolic and Organic Compounds, J. Agric. Food Chem., 48 : 1738-1745.
Brusick D. (1987). Principles of genetic` toxicology. Plenum Press, New York. p. 304.
Castillo J, Benavente-García O, Del Baño MJ, Lorente J, Alcaraz M, Dato MJ. (2001). Radioprotective Effects Against Chromosomal Damage Induced in Human Lymphocytes by gamma-Rays as a Function of Polymerization Grade of Grape Seed Extracts. J. Med. Food: 4(2):117-123.
Chi-Tang H, Lee C, Mou-Tuan H (1991). Phenolic compounds and their effects on health I analysis, occurrence and chemistry. American Social Society, Washington D.C. pp. 2-7.
Chi-Tang H, Lee C, Mou-Tuan H (1992). Phenolic compounds and their effects on health II analysis, occurrence and chemistry. Am. Social Soc., Washington D.C. p. 350.
Daniel L, Gustafson H, Franz R, Akiko U, Carr S, David D, Charles W (2000). Vanillin (3-methoxy-4-hydroxybenzaldehyde) inhibits mutation induced by hydrogen peroxide, *N*-methyl-*N*-nitrosoguanidine and mitomycin C but not ^{137}Cs -radiation at the *CD59* locus in human-hamster hybrid A_L cells. Mutagenesis, 15: 207-213.
Edenharde R, Krieg H, Köttgen V, Platt K (2003). Inhibition of clastogenicity of benzo[a]pyrene and of its trans-7,8-dihydrodiol in mice *in vivo* by fruits, vegetables, and flavonoids. Mutat. Res. 537: 169-181.
Francis FJ (1989). Food colourants: Anthocyanins. Critical. Reviews in Food Sciences and Nutrition. 28: 273:314.
Hermann K (1989). Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. Crit. Rev. Food Sci. Nutria. 28:315-347.
Hosseinimehr SJ, Tavakoli H, Pourheidari G, Aligholi S, Abbas S (2003). Radioprotective Effects of Citrus Extract Against γ-Irradiation in Mouse Bone Marrow Cells. J. Radiation Res. 44: 237-241.
Irulappan R, Natarajan P (2007). Antimutagenic indexof curcumin on chromosomal aberrations in *Allium cepa* . J. Zhejiang Univ. Sci. B8(7): 470-475.
Jinhua Y, Michael P, John R, Jameson C (1993). Application Of Microencapsulation For Toxicology Studies: III. Bioavailability Of Microencapsulated Cinnamaldehyde. Toxicol. Sci. 20: 83-87.
Kayoko S, Shuichi M, Michiyo F, Sachiko E, Naohide K (1994). Radioprotective effect of antioxidative flavonoids in -ray irradiated mice, Carcinogenesis, 15: 2669-2672.
Manikumar G, Gaetano K, Wani M, Taylor H, Hughes T, Warner J, McGivney R, Wall M (1989). Plant antimutagenic agents, Isolation and structure of two new isoflavones fremontin and fremontone from psrothamnus fremontii. J. Nat. Prod. 52: 769-73.
Mitsuo M, Katsuhisa S, Sei-ichi N, Hiroshi K (1999). Antimutagenic Activity of Isoflavones from Soybean Seeds (*Glycine max* Merrill). Agric. Food Chem., 47: 1346-1349.
Mou-Tuan H, Richard C, Alexander W, Harold N, Jane S, Haruhiko Y, Donald J, Allan C (1985). Inhibition of the mutagenicity of bay-region diol-epoxides of polycyclic aromatic hydrocarbons by tannic acid, hydroxylated anthraquinones and hydroxylated CA derivatives. Carcinogenesis, 6: 237-242.
Richard A, Dean W (2001). Applied Multivariate Statistical Analysis (5th Edition). Prentice Hall New Jersey, p. 767.
Sampson L, Rimm E, Hollman P, DeVries J, Katan M (2002). Flavonol and flavone intakes-Fruit and Vegetable Consumption. Nutr. Res. Newslett. J. Am. Diet. Assoc.102(10):1414-20.
Schmid W (1975). The micronucleus test. Mutat. Res. 31: 9-15.
Sharma N, Trikha P, Athar M, Raisuddin S (2001). Inhibition of benzo[a]pyrene- and cyclophoshamide-induced mutagenicity by Cinnamomum cassia. Mutat. Res. 480-481: 179-88.
Siddique YH, Gulshan A, Tanveer B, Afzal M (2008). Antigenotoxic effect of nordihydroguaiaretic acid against chlormadinone acetate-induced genotoxicity in mice bone-marrow cells. J. Natural Med. 62: 52-56.
Tzvetan A, Konstantinov S, Tzanova T, Kyril D, Topashka-Ancheva M, Berger M (2007). Antineoplastic and Anticlastogenic Properties of Curcumin. Ann. N.Y. Acad. Sci. 1095: 355-370.
Wei H (1995). Antioxidant and antipromotional effects of the soybean isoflavone genestein. Proc. Soc. Exp. Biomed. 208: 124-130.
Yamagishi M, Osakabe N, Natsume M, Adachi T, Takizawa T, Kumon H, Osawa T (2001). Anticlastogenic activity of cacao: inhibitory effect of cacao liquor polyphenols against mitomycin C-induced DNA damage. Food Chem. Toxicol., 39: 1279-1283
Yoshioka K, Deng T, Cavigelli M, Karin M (1995). Antitumor promotion by phenolic antioxidants: inhibition of AP-1 activity through induction of Fra expression. Proc. Nat. Acad. Sci. USA. 92: 4972-4976.