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ORIGINAL ARTICLE

Protective effect of apigenin against hydrogen peroxide induced genotoxic damage on cultured human peripheral blood lymphocytes

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Summary
Apigenin is a member of the flavone family of flavonoids and possesses anti-inflammatory, free radical scavenging and anti-carcinogenic properties. Hydrogen peroxide, which is generated during oxidative stress, is known to damage proteins, nucleic acids and cell membranes and also has been implicated in cancer, ageing and several chronic neurogenerative diseases. The present study focuses on the protective effect of apigenin against genotoxic doses of hydrogen peroxide (H_2O_2) using sister chromatid exchanges (SCEs) and cytokinesis blocked micronucleus (CBMN) assay. The treatment with 50, 100 and 150 μM of H_2O_2 results in a significant dose dependent increase in the frequency of SCEs and MN. The treatment with 100 μM of H_2O_2 along with 5, 10 and 20 μM of apigenin results in a dose dependent significant decrease in the frequency of SCEs and MN on cultured human lymphocytes. A similar result was obtained with treatment with 150 μM of H_2O_2 along with 5, 10 and 20 μM of apigenin. The results of the present study suggest a protective effect of apigenin against hydrogen peroxide induced genotoxic damage on cultured human lymphocytes.

Key words: apigenin; hydrogen peroxide; sister chromatid exchanges; micronucleus, human lymphocytes

INTRODUCTION

Apigenin, a flavonoid, is one of the several active ingredients found naturally in many fruits and vegetables (Peterson and Dwyer 1998). Apigenin is recognized in traditional or alternative medicine for its pharmacological activity (Hoftman 2000). It possesses anti-inflammatory, free radical scavenging and anti-carcinogenic effects (Kim et al. 1998). It has been shown to possess growth inhibitory properties in several cancer lines including breast, colon, skin, thyroid, leukemia cells and pancreatic (Wang et al. 2000, Yin et al. 2001, Ujiki et al. 2006), tumor inhibition (Li et al. 1996) and enzyme inhibitory properties (Jeong et al. 1992). It also possesses antigenotoxic (Siddique et al. 2008) and anticarcinogenic properties (Khan et al. 2006).

Hydrogen peroxide (Reactive oxygen species) is generated during oxidative stress and is known to damage proteins, nucleic acids, cell membranes and has been implicated in cancer, ageing, and also in several chronic neurogenerative diseases (Daroui et al. 2004). Hydrogen peroxide is known to cause

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oxidative DNA damage primarily through the hydroxyl radical that results from the Fenton reaction as shown in the following reaction (Imlay et al. 1988, Halliwell and Gutteridge 1992, Josephson et al. 1991).

$$H_2O_2 + 2Fe^{3+} \rightarrow 2Fe^{2+} + O_2 + 2H^+ \quad (1)$$

$$2Fe^{2+} + H_2O_2 \rightarrow {}^{\circ}OH + Fe^{3+} + OH^- \quad (2)$$

$$\text{Sum} \quad 3H_2O_2 \rightarrow 2\ {}^{\circ}OH + O_2 + 2H_2O$$

H_2O_2 has been associated with the induction of cancer in animals (Shamberger 1972), and is mutagenic and carcinogenic (Pryor 1986); it is believed to be involved in the initiation and promotion of carcinogenesis (Birnboim 1986). It has also been reported to cause DNA damage in the form of chromosomal aberrations (Sofni and Ishidate 1984), sister chromatid exchanges (Tsuda 1981), single strand breaks (Prise et al. 1989) and double strand breaks (Thibodeau and Paquette 1999). The present study was aimed at studying the protective effect of apigenin against hydrogen peroxide induced genotoxic damage on human lymphocyte culture, using sister chromatid exchanges and a cytokinesis blocked micronucleus assay.

MATERIALS AND METHODS

Chemicals

H_2O_2 (CAS 7722-84-1), Apigenin (CAS 520-36-5), Hoechst 33258, Cytochalasin-B (CytB) (Sigma, St. Louis, USA); RPMI 1640, Phytohaemagglutinin-M, antibiotic antimycotic mixture (Gibco, InVitrogen, USA); Dimethyl sulphoxide (DMSO), Giemsa strain (E-Merck Ltd, Mumbai, India); Colchicine and 5-Bromodeoxyuridine (SRL, India).

Human lymphocyte culture

Duplicate peripheral blood cultures were treated according to Carballo et al. (1993). Briefly, about 0.5 ml of heparinized blood samples were obtained from a healthy female donor and were placed in sterile glass culture vials containing 5 ml of RPMI-1640, supplemented with 1.5 of ml fetal calf serum, 0.1 ml of antibiotic-antimycotic mixture and 0.1 ml of phytohaemaglutinin. The vials were placed in an incubator at 37 °C for 72 h. Untreated, negative and positive controls were also run simultaneously.

Sister chromatid exchange analysis

For sister chromatid exchange analysis, bromodeoxyuridine (BrdU, 10 µg/ml) was added at the beginning of the culture. After 24 h, the hydrogen peroxide at final concentrations of 50, 100 and 150 µM was given separately and kept for another 48 h at 37 °C in an incubator. Methylmethane sulphonate at 200 µM and dimethyl sulphoxide (5 µl/ml) were taken as positive and negative control respectively. Simultaneously the activity of 100 and 150 µM of H_2O_2 was examined in the presence of 5, 10 and 20 µM of apigenin (dissolved in DMSO) separately and respectively to see the effect on the sister chromatid exchanges. Duplicate cultures for sister chromatid exchanges were set similarly as described earlier in this text. Mitotic arrest was achieved 1 h prior to harvesting by adding 0.2 ml of colchicine (0.2 µg/ml). Cells were centrifuged at 800 g for 10 min. The supernatant was removed and 5 ml of prewarmed (37 °C) 0.075M KCl (hypotonic solution) was added. Cells were resuspended and incubated at 37 °C for 15 min. The supernatant was removed by centrifugation, and 5 ml of chilled fixative (methanol:glacial acetic acid, 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice.

The slides were processed according to Perry and Wolff (1974). The sister chromatid exchange induction was analysed from 50 plates of second division mitoses per dose.

Cytokinesis – blocked micronucleus assay (CBMN)

The presence of MN in a binucleated cell (BNC) was assayed by blocking the cells at the cytokinesis stage by the method of Fenech and Morley (1985). Lymphocyte culture was set as described earlier in the text. Cyt-B (3 µg/5 ml culture) was added to the culture at 44 h after initiation. The treatments were as described earlier in the text for SCEs analysis.

After a total of 72 h incubation the cells were centrifuged at 800 g for 5 min. Supernatant was discarded and the cell pellets were treated with a hypotonic solution. Cells were fixed with freshly prepared methanol:acetic acid (3:1) for 10 min and then centrifuged at approximately 800 g for 5 min. After air drying, the slides were stained with 5% Giemsa stain in phosphate buffer for 10 min. BNCs surrounded by well preserved cytoplasm were scored for the presence of MN. At least 2000 cytokinesis-blocked (CB) binucleated human lymphocytes with preserved cytoplasm were scored. Micronuclei were identified with the criteria followed by Fenech et al. (2003).

Statistical analysis

Statistical analysis was performed by the χ^2 test for micronucleus and the Student's t-test was performed for sister chromatid exchange using Statistica Soft Inc. SCE was also complemented by ANOVA at the

significance level $2\alpha=0.05$. A regression analysis was also performed to establish the dose effect of apigenin on H_2O_2 induced SCEs and micronucleus using Statistica Soft Inc.

RESULTS

Sister chromatid exchange analysis

The results of the investigation reveal that apigenin is potent enough to reduce the genotoxic damage caused by H_2O_2 on cultured human peripheral blood lymphocytes. The treatments of 50, 100 and 150 μM of H_2O_2 were associated with mean SCEs/cell values of 8.02±0.68, 9.52±0.84 and 10.24±0.94 respectively, which were significant compared with the untreated (1.42±0.16) (Table 1). Regression analysis was also performed to see the dose effect on SCEs/cell by H_2O_2 treatment. An increase in the slope of the linear regression line was observed ($P<0.043$, $r=0.98$) as the dose of H_2O_2 treatment was increased (Fig. 1).

When 100 μM of H_2O_2 treatment was given along with 5, 10 and 20 μM of apigenin separately, a significant dose dependent decrease in SCEs/cell was observed i.e. 6.34±0.47, 5.22±0.42 and 4.18±0.40 respectively **(**Table 1). Similar results were obtained when 150 μM of H_2O_2 treatment was given along with 5, 10 and 20 μM of apigenin separately for SCEs/cell i.e. 7.74±0.58, 6.36±0.48 and 5.24±0.44 respectively (Table 1). The negative and positive controls were associated with 1.62±0.24 and 11.28±0.96 SCEs/cell respectively (Table 1). Regression analysis was also performed to study the effect of different doses of apigenin on sister chromatid exchanges induced by 100 and 150 μM of H_2O_2. A decrease in the slope of linear regression line was observed when 100 μM (statistically significant, $r=0.97$) and 150 μM (statistically significant, $r = 0.96$) was treated with 5, 10 and 20 μM of apigenin respectively (Figs 2 and 3).

Micronuclei induction

The treatments of 50, 100 and 150 μM of H_2O_2 were associated with 46, 51 and 57 micronuclei/10^3 cells respectively, and were significantly different from the untreated (10.5 MN/10^3 cells) (Table 2). Regression analysis was also performed to see the dose effect of H_2O_2 treatment on the frequency of MN. An increase in the slope of the linear regression line was observed (statistically significant; $r = 0.99$) (Fig. 4). When 100 μM of H_2O_2 treatment was given along with 5, 10 and 20 μM of apigenin separately, a significant dose dependent decrease in MN/10^3 cells was observed i.e. 36, 30 and 27 MN/10^3 cells respectively (Table 2). Similar results were obtained when 150 μM of H_2O_2 treatment was given along with 5, 10 and 20 μM of apigenin separately i.e. 40, 36 and 31 MN/10^3 cells respectively(Table 2). The positive and negative controls were associated with 68.5 and 14 MN/10^3 cells respectively (Table 2). Regression analysis was also performed to see the effect of different doses of apigenin on micronuclei induction by 100 and 150 μM of H_2O_2. A decrease in the slope of linear regression line was observed when 100 μM (statistically significant 9; $r = 0.92$) and 150 μM (statistically significant; $r = 0.99$) was treated with 5, 10 and 20 μM of apigenin respectively (Figs 5 and 6).

DISCUSSION

The results of the present investigation reveal that apigenin is potent enough to reduce the genotoxic damage caused by H_2O_2 on cultured human peripheral blood lymphocytes. The selected doses of apigenin were not genotoxic, however, at 93 μM, an increase in micronucleus frequency was reported in human lymphocytes *in vitro* (Rithidech et al. 2005). A study conducted by Snyder and Gillies (2002) also showed the clastogenic activity of apigenin at 100 μM in Chinese hamster V79 cells, and proposed that the clastogenic activity of apigenin was due to the ability of apigenin to intercalate DNA molecules. In *in vivo* conditions H_2O_2 may be generated directly by a divalent reduction of O_2 or indirectly by a univalent reduction of $O_2^{\circ-}$. H_2O_2 is the primary product of the reduction of O_2 by numerous oxidases, such as xanthine oxidase, uricase, D-amino acid oxidase, and α-hydroxy acid oxidase localized in peroxisones (Ray and Hussain 2002). H_2O_2 is decomposed to H_2O and O_2 by a reaction catalysed by redox active metal complexes of which catalase and peroxidase are the most effective (Ray and Hussain 2002). H_2O_2 also reacts with $O_2^{\circ-}$ to initiate a Haber-Weiss reaction producing $^{\circ}OH$ in the presence of Fe^{2+}.

$$O_2^{\circ-} + H_2O_2 \xrightarrow{Fe^{2+}} O_2 + {}^{\circ}OH + OH^-$$

°OH is responsible for DNA damage and a high frequency of SCEs (Tsuda 1981). In our present study a dose dependent increase in DNA damage was observed after H_2O_2 treatment. Genotoxicity testing provides a human risk assessment. An increase in the genotoxic damage is associated with an increased overall risk of cancer (Hagmar et al. 1994, 1998). The sister chromatid exchanges and micronucleus are well known markers of genotoxicity and any reduction in the

Table 1. **Frequency of sister chromatid exchanges on hydrogen peroxide and apigenin in treatment.**

Treatment	Cells scored	SCE / Cell ± SE
H_20_2 (μM)		
50	50	8.02 ± 0.68[a]
100	50	9.52 ± 0.84[a]
150	50	10.24 ± 0.94[a]
H_20_2 (μM) + Apigenin (μM)		
100 + 5	50	6.34 ± 0.47[b]
100 + 10	50	5.22 ± 0.42[b]
100 + 20	50	4.18 ± 0.40[b]
150 + 5	50	7.74 ± 0.58[b]
150 + 10	50	6.36 ± 0.48[b]
150 + 20	50	5.24 ± 0.44[b]
Apigenin (μM)		
5	50	1.64 ± 0.28
10	50	1.68 ± 0.30
20	50	1.78 ± 0.36
Untreated	50	1.42 ± 0.24
Negative control (DMSO, 5 μl/ml)	50	1.62 ± 0.24
Positive control (MMS, 200 μM)	50	11.28 ±0.96[a]

[a] significant as compared to untreated, [b] significant as compared to H_2O_2 treatment

Table 2. **Evaluation of the effects of apigenin on hydrogen peroxide induced micronucleus on human peripheral blood lymphocytes.**

Treatment	BN Cells scored	Distribution of BN cells according to numer of MN				MN / 10^3 cells
		0	1	2	3	
H_20_2 (μM)						
50	2000	1920	69	10	1	46[a]
100	2000	1911	78	9	2	51[a]
150	2000	1901	86	11	2	57[a]
H_20_2 (μM) + Apigenin (μM)						
100 + 5	2000	1933	62	5	0	36[b]
100 + 10	2000	1943	54	3	0	30[b]
100 + 20	2000	1948	50	2	0	27[b]
150 + 5	2000	1922	72	6	0	40[b]
150 + 10	2000	1931	66	3	0	36[b]
150 + 20	2000	1939	60	1	0	31[b]
Apigenin (μM)						
5	2000	1969	29	2	0	16.5
10	2000	1968	30	2	0	17
20	2000	1966	32	2	0	18
Untreated	2000	1980	19	1	0	10.5
Negative control (DMSO, 5 μl/ml)	2000	1974	24	2	0	14
Positive control (MMS, 200 μM)	2000	1987	92	18	3	68.5[a]

Symbols as in the Table 1

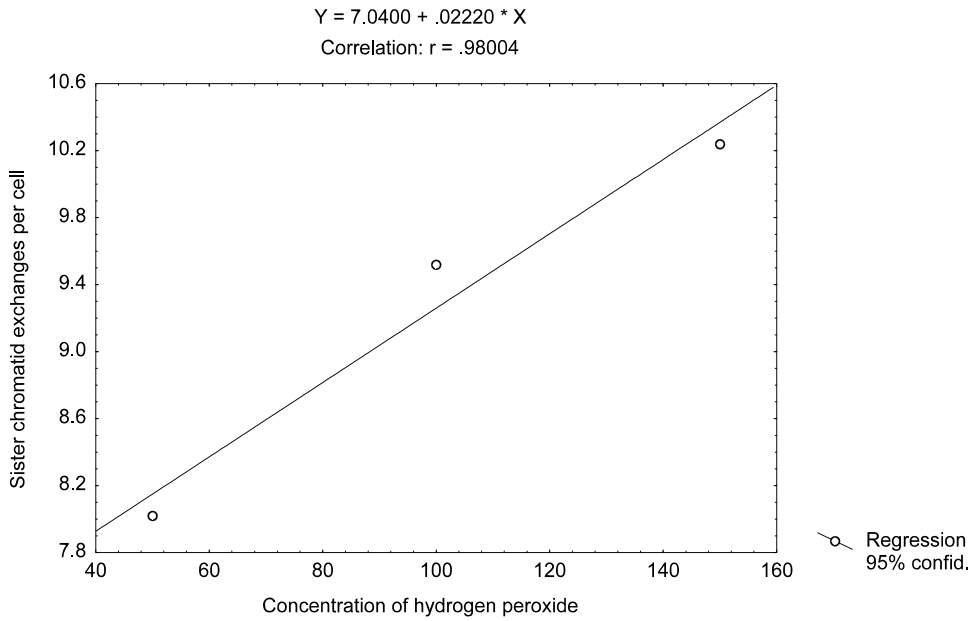

Fig. 1. **Sister chromatid exchange induction by the treatment of different doses of hydrogen peroxide.**

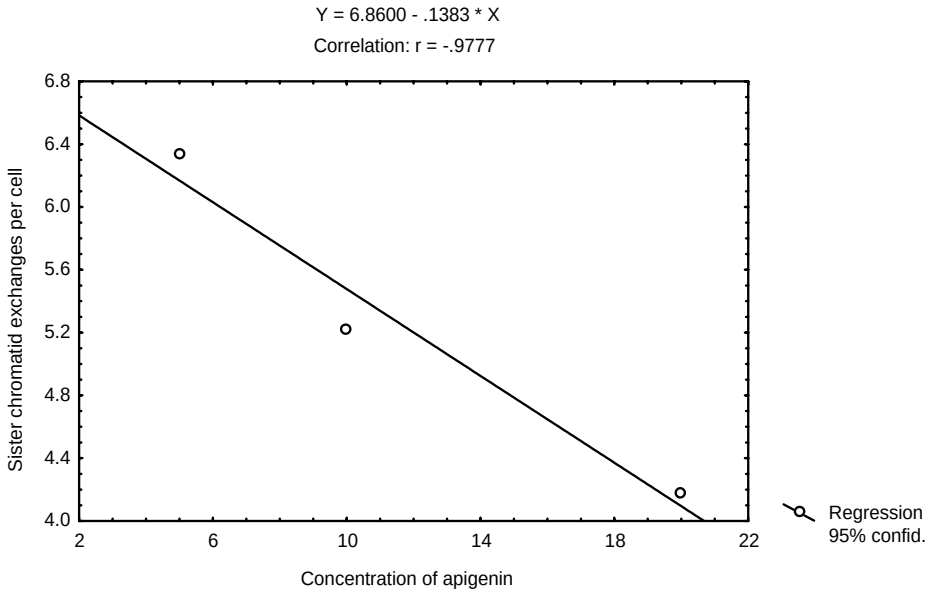

Fig. 2. **Effect of apigenin on sister chromatid exchanges induced by 100 μM of hydrogen peroxide**.

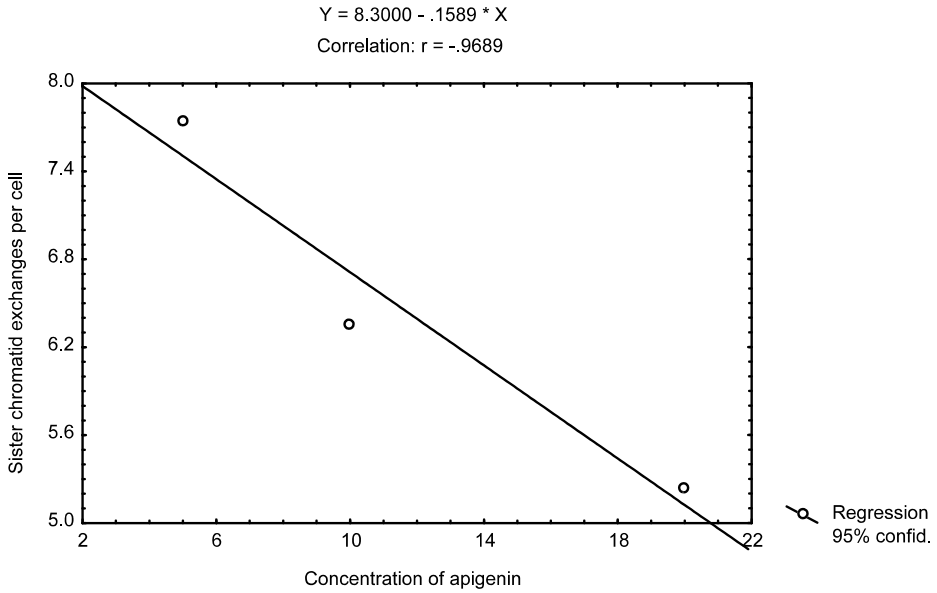

Fig. 3. **Effect of apigenin on sister chromatid exchanges induced by 150 µM of hydrogen peroxide.**

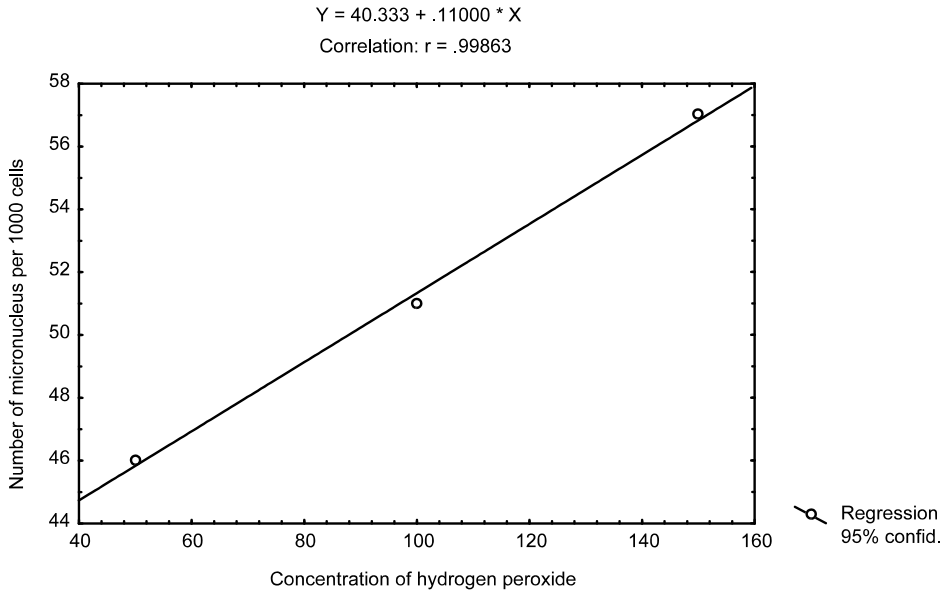

Fig. 4. **Micronuclei induction by the treatment of different doses of hydrogen peroxide**.

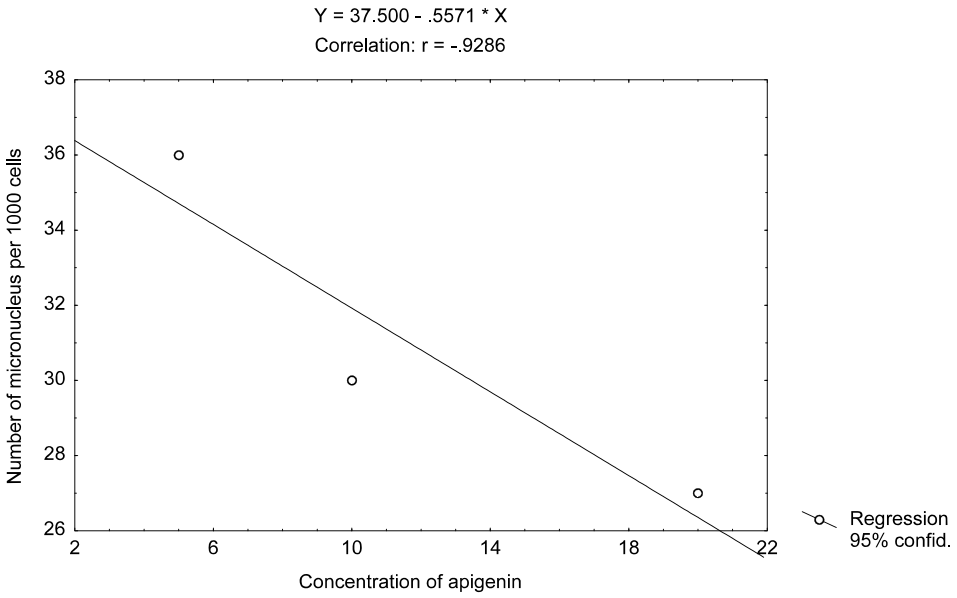

Fig. 5. **Effect of apigenin on micronuclei induction by 100 μM of hydrogen peroxide**.

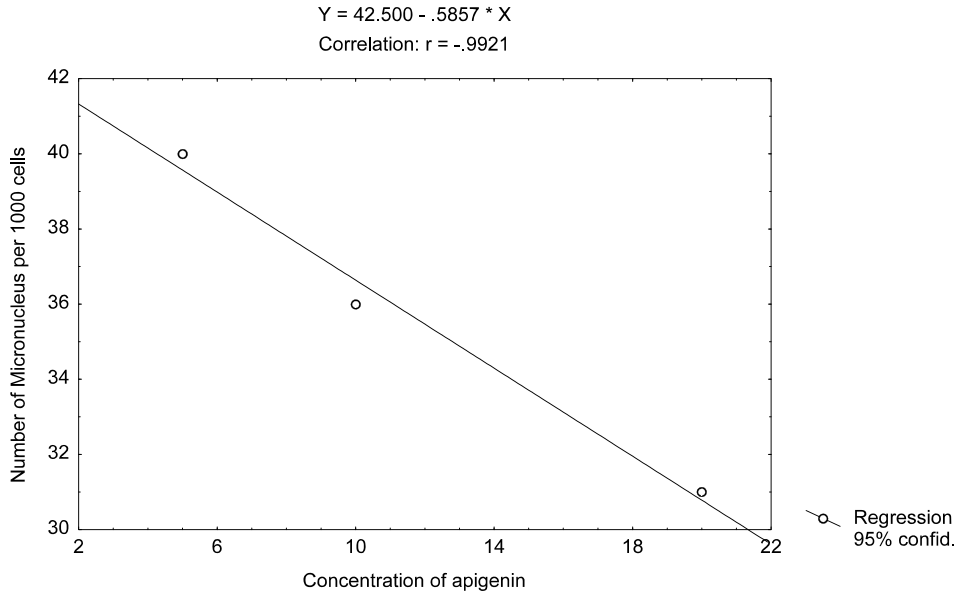

Fig. 6. **Effect of apigenin on micronuclei induction by 150 μM of hydrogen peroxide**.

frequency of these genotoxic endpoints gives an indication of the antigenotoxicity of a particular compound (Albertini et al. 2000). In our present study with H_2O_2, apigenin reduced the genotoxic damage; this is due to the possible scavenging of electrophiles/nucleophiles or by enhancing the DNA repair system or DNA synthesis by apigenin (Noel et al. 2006). The concentrations of apigenin used in the present study were not genotoxic in themselves but reduced the genotoxic effects of H_2O_2. Information on plasma or serum concentrations of apigenin is lacking and studies on *in vivo* absorption, metabolism

and potential genotoxicity of apigenin are warranted (Rithidech et al. 2005). Higher doses of apigenin have been reported to induce micronuclei in cultured human lymphocytes (Noel et al. 2006). The genotoxic damage by flavonoids at higher concentrations are considered to be due to DNA intercalation, poisoning of DNA topoisomerase II, generation of reactive metabolites and inhibition of key enzymes (Stopper et al. 2005). A particular flavonoid may exhibit genotoxic damage in isolation (Noel et al. 2006), but may modulate the genotoxic effect when associated with other compounds as is evident from the present study. Less information is available on the *in vivo* absorption, metabolism and plasma concentration of apigenin (Manach et al. 2004). However it has been reported that apigenin is absorbed and metabolized by humans after intake and its half life is about 12 h (Nielsen et al. 1999). The results of the present study showed that the apigenin is potent enough to reduce the genotoxic damage of H_2O_2 thereby reducing the chances of developing cancers, as the high concentration of H_2O_2 leads to the cancer induction in animals due to the DNA damage (Shamberger 1972).

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