

RESEARCH ARTICLE

Genotoxic and Anti-Genotoxic Effects of Vanillic Acid Against Mitomycin C-Induced Genomic Damage in Human Lymphocytes *In Vitro*

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Abstract

Vanillic acid, a vegetable phenolic compound, is a strong antioxidant. The aim of the present study was to determine its effects on mitomycin C-induced DNA damage in human blood lymphocyte cultures *in vitro*, both alone and in combination with mitomycin C (MMC). The cytokinesis block micronucleus test and alkaline comet assay were used to determine genotoxic damage and anti-genotoxic effects of vanillic acid at the DNA and chromosome levels. MMC induced genotoxicity at a dose of 0.25 µg/ml. Vanillic acid (1 µg/ml) significantly reduced both the rates of DNA damaged cells and the frequency of micronucleated cells. A high dose of vanillic acid (2 µg/ml) itself had genotoxic effects on DNA. In addition, both test systems showed similar results when tested with the negative control, consisting of dimethyl sulfoxide (DMSO) in combination with vanillic acid (1 µg/ml)+MMC. In conclusion, vanillic acid could prevent oxidative damage to DNA and chromosomes when used at an appropriately low dose.

Keywords: Vanillic acid - anti-genotoxic - mitomycin-C - genotoxicity - micronucleus - comet assay

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Introduction

The hydroxylated derivatives of benzoic and cinnamic acids are known as phenolic acids. Vanillic acid (4-hydroxy-3-methoxybenzoic acid, Figure 1) is a dietary phenolic compound found in plants and fruits (Duke, 1992) and an intermediate in the production of vanillin from ferulic acid (Lesage-Meessen, 1996; Civolani, 2000). Additionally, vanillic acid is a metabolic product of caffeic acid that is found in the urine of humans after consuming coffee, chocolate or green tea (Falconnier et al., 1994) and it can exhibit antioxidant, antimicrobial and antimalarial activity (Rechner et al., 2001; Yemis et al., 2011). It is a key component of the vanilla plant and in recent years, it has been used as a preservative and antiseptic agent in the food, pharmaceutical and cosmetic industries (Boyce et al., 2003). Despite its wide application, the effects of vanillic acid on human health are not yet fully understood, particularly from the genotoxicity perspective. Mitomycin C (MMC) is an antibiotic drug from the DNA alkylating agent group. It has potent DNA cross-linking activity and creates oxidative damage within the cell (Gresolia, 2002; Pawar et al., 2009). MMC induces clastogenesis and mutagenesis and inhibits DNA synthesis, therefore showing strong genotoxic effects (Sontakke and Fulzele, 2009). Due to these properties, MMC has been used as

a positive control for *in vitro* genotoxicity test systems (Pawar et al., 2009; Sontakke and Fulzele, 2009).

Our research aimed to examine vanillic acid from two perspectives. First, we determined the genotoxic effect of vanillic acid in human lymphocytes. Experimental genotoxicity tests, such as the cytokinesis block micronucleus (CBMN) and the alkaline comet assay, are well-known models used to study the effects of different physical and chemical agents on DNA. Second, the anti-genotoxic effect of vanillic acid on MMC-induced genetic damage was tested using both *in vitro* test systems.

Materials and Methods

Chemicals

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA): vanillic acid, MMC, low melting agarose (LMA), dimethyl sulfoxide (DMSO), triton X-100, cytochalasin-B, histopaque 1077, sodium

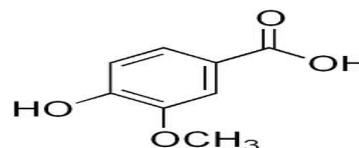


Figure 1. The Chemical Structure of Vanillic Acid

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sarcosinate, trypan blue, ethidium bromide, RPMI 1640 medium, penicillin-streptomycin, fetal calf serum (FCS), phytohemagglutinin-A, L-glutamine, Dulbecco's PBS and Na₂EDTA. Normal melting agarose (NMA) and Giemsa were obtained from E-Merck (Germany). All the other chemicals used in the study were of analytical grade and obtained from local commercial sources.

Lymphocyte treatment with vanillic acid and MMC

The vanillic acid was dissolved in 2% DMSO under sterile conditions. Treatment with vanillic acid at doses higher than 2 µg/ml was shown to be highly cytotoxic by trypan blue viability tests, so we used two non-toxic but effective doses of vanillic acid. The MMC was dissolved in sterile distilled water at a concentration of 0.25 µg/ml, as suggested by published literature. All solutions were prepared immediately before performing the experiments to prevent degradation. The cultured lymphocytes were divided into six groups as follows. Group 1: Negative (DMSO) control (2%). Group 2: Vanillic acid (1 µg/ml) Group 3: Vanillic acid (2 µg/ml). Group 4: MMC (0.25 µg/ml). Group 5: MMC (0.25 µg/ml)+vanillic acid (1 µg/ml) Group 6: MMC (0.25 µg/ml)+vanillic acid (2 µg/ml).

Blood samples

Blood samples (a total volume of 6 ml) were taken with heparinized syringes from four healthy, non-smoking donors who were not exposed to radiation or drugs (two males and two females, ages 22-31). Informed consent was obtained from all participants; the study was performed in accordance with the Declaration of Helsinki and with the approval of the local ethics committee.

CBMN assay

The presence of micronuclei (MN) in a binucleated cell was assayed by blocking cytokinesis, as described by Fenech (1991). Duplicated cell cultures were prepared for a MN test. Briefly, heparin-treated whole blood samples (1 ml) were added to a sterile culture tube containing RPMI 1640 medium supplemented with 15% FCS, antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin), 200 mMol L-glutamine and 2.5% phytohemagglutinin. The tubes were incubated at 37°C for 72h. Cytochalasin B was added, at 6 µg/ml, 44 h after the start of incubation. The MMC and vanillic acid treatments were performed 48 h after the beginning of the incubation period and continued until the end of the culture. At the end of the incubation, the cells were harvested by centrifugation. The cells were harvested, cast on a pre-cooled slide and stained with Giemsa.

Slide scoring

One thousand binucleated cells from each donor's blood sample were scored for the presence of micronuclei (MN) under a light microscope at 400x magnification. The MN frequency was expressed as the number of MN per 1000 binucleated cells scored. In addition, the nuclear division index (NDI) was taken into account by examining the number of nuclei in 2000 observed cells. NDI was calculated according to the formula proposed by Eastmond and Tucker (1989) as follows. $NDI = (1 \times N1 + 2 \times N2 + 3 \times N3$

$+ 4 \times N4) / N$, where N1 to N4 represent the number of cells with one to four nuclei and N is the total number of cells scored.

Alkaline comet assay

The assay was performed essentially as described by Singh et al. (1988). Briefly, blood samples were diluted 1:1 with phosphate-buffered saline (PBS) and then layered onto the Ficoll-Histopaque using a 4:3 ratio of (blood+PBS) to Histopaque. The samples were centrifuged at 400 g for 35 min; then, the lymphocyte-enriched layer was removed. The samples were washed twice more with PBS and centrifuged at 350 g for 10 min; then, a final wash with RPMI-1640 medium was performed. The number of viable cells was assessed by staining the cells with trypan blue and counting the cells by a hemocytometer. Viable cells were suspended in RPMI-1640 media supplemented with 15% FCS, 200 mM of L-glutamine, penicillin (100 units/mL) and streptomycin (100 µg/mL). Phytohemagglutinin (0.2 mL) was added to cultured lymphocytes to initiate cell division. The cells were incubated at 37°C in a humidified incubator maintained with 5% CO₂.

Roughened slides were cleaned with 100% methanol and air-dried. Two solutions, 0.5% normal-melting agarose (NMA) and 0.5% low-melting agarose (LMA), were prepared in Ca²⁺- and Mg²⁺-free PBS. NMA (0.1 ml) was used to create the first layer, upon which 1000 cells suspended in 75 µL LMA+10 µL PBS were used for the second layer. To prevent any additional DNA damage, the remaining steps were conducted in the dark. To lyse the cells and denature the DNA, the slides were immersed in freshly prepared ice-cold lysis solution (1% sodium sarcosinate, 2.5 M of NaCl, 100 mM of Na₂EDTA, 10 mM of Tris-HCl at a pH 10, 1% Triton X-100 and 10% DMSO). The slides were then incubated for 1 h at 4°C in the dark and placed in an ice bath on a horizontal electrophoresis unit. The electrophoresis unit was filled with fresh buffer (1 mM of Na₂EDTA, 300 mM of NaOH; pH 13) to cover the slides, and the slides were maintained in high-pH buffer for 20 min to denature the DNA and expose the alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V (300 mA). Following electrophoresis, the slides were washed gently in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove the alkali and detergents, and then they were stained with 100 µl of ethidium bromide (2 µg/ml).

Microscopic detection of comets

For each sample, two slides were prepared, and 200 cells from each slide were scored. Observations were made using 400x magnification on a fluorescence microscope equipped with a 530-nm excitation filter and a 590-nm barrier filter. The genetic damage index (GDI) was visually determined based on the size and intensity of the comet tail. The tails were divided into five categories (0-4) as follows: Class 0 (no damage), Class 1 (little damage with a tail length that was shorter than the diameter of the nucleus), Class 2 (medium damage with a tail length one to two times the diameter of the nucleus), Class 3 (significant damage with a tail length between two-and-

a-half and three times the diameter of the nucleus) and Class 4 (significant damage with a tail longer than three times the diameter of the nucleus). These categories were based on those established by Collins (2004). We used this categorization to obtain a quantitative measurement of DNA damage based on a score average that was weighted according to the number of cells with each grade of damage. The formula for determining the DNA damage was measured by the Genetic Damage Index (GDI) = $(\text{Class 1} + 2 \times \text{Class 2} + 3 \times \text{Class 3} + 4 \times \text{Class 4}) / (\text{Class 0} + \text{Class 1} + \text{Class 2} + \text{Class 3} + \text{Class 4})$. DNA damage was expressed as the mean percentage of cells with medium, high and complete DNA damage and was calculated as the sum of the cells categorized with Classes 2, 3 and 4 damage (Palus et al., 2003). Percentage of damaged cells (% DC) = $[\text{Class 2} + 3 \times \text{Class 3} + 4 \times \text{Class 4}] / \text{Sum of cells in all classes including 0 and 1} \times 100$.

Statistical analysis

The IBM SPSS Statistics 20 program was used for statistical analyses. Statistical analysis was performed using one-way analysis of variance (ANOVA) and post-hoc Tukey tests. Values are represented as the means \pm S.D. for the samples in each group. P-values of <0.05 were considered significant.

Results

Two different concentrations (1 and 2 $\mu\text{g/ml}$) of vanillic acid and MMC (0.25 $\mu\text{g/ml}$) were evaluated both in combination and alone in two different assays (CBMN and alkaline comet) to determine the genotoxic and cytotoxic effects of vanillic acid on human peripheral lymphocytes *in vitro*. DMSO (2%) was used as a negative control. The frequency of vanillic acid-induced MN and NDI in human lymphocytes is summarized in Table 1.

The MN frequency of all vanillic acid- and MMC-treated groups is shown in Figure 2. Briefly, as shown in Table 1 and Figure 2, the low dose of vanillic acid (1 $\mu\text{g/ml}$) did not show any significant differences in the MN frequency when compared with the DMSO negative control ($p > 0.05$). On the other hand, the high dose of vanillic acid (2 $\mu\text{g/ml}$) increased MN formation significantly when compared with DMSO ($p < 0.05$). The MN frequency of the MMC-treated group was significantly higher than that of the DMSO control ($p < 0.001$); in addition, the MMC+vanillic acid (2 $\mu\text{g/ml}$) combination treatment showed a significantly higher MN formation when compared with the DMSO control

($p < 0.005$). However, MMC+vanillic acid (2 $\mu\text{g/ml}$) in combination significantly decreased the MN frequency when compared with MMC alone ($p < 0.05$). Vanillic acid (1 $\mu\text{g/ml}$) significantly decreased the MN frequency induced by MMC when compared to the MMC+DMSO control levels ($p < 0.001$).

The average NDI values of all groups in this study are shown in Figure 3. As shown in Table 1 and Figure 3, there were no differences between the NDI values of DMSO and vanillic acid (1 and 2 $\mu\text{g/ml}$). MMC induced a significant decrease in NDI compared with the DMSO control ($p < 0.001$). The NDI value of the low dose vanillic acid (1 $\mu\text{g/ml}$) and MMC combination was not significantly different from that of the DMSO control ($p > 0.05$). When compared with the MMC group, the low dose vanillic acid and MMC combination showed a significant improvement in NDI values ($p < 0.001$). The NDI values were significantly lower in the vanillic acid (2 $\mu\text{g/ml}$)+MMC combination treatment when compared with the MMC and also DMSO control group ($p < 0.001$);

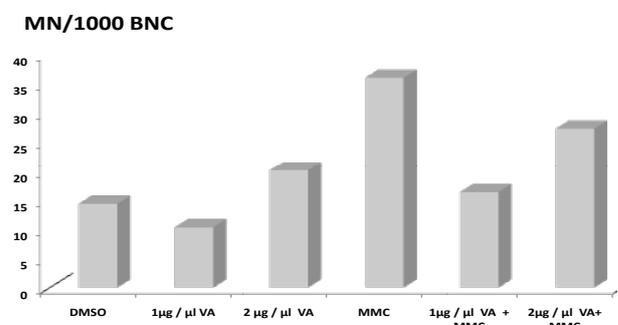


Figure 2. MN Frequency of Vanillic Acid and Vanillic Acid+MMC in the CBMN Assay. VA, Vanillic acid; MMC, Mitomycin-C.

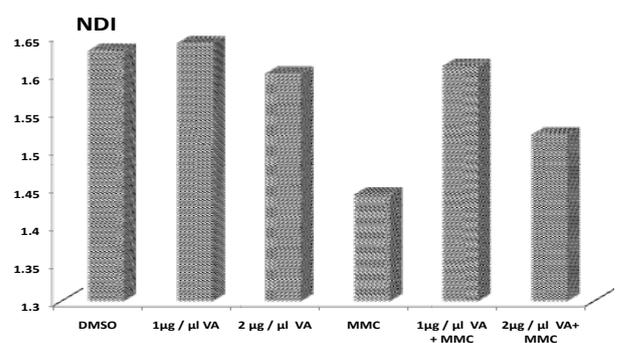


Figure 3. The Average Values of Nuclear Division Index, (NDI) of Vanillic Acid and Vanillic Acid+MMC in the CBMN Assay. VA, Vanillic acid; MMC, Mitomycin-C

Table 1. The Cyto-Genotoxicity of Vanillic Acid and Vanillic Acid+Mitomycin C on CBMN Assay in Human Blood Lymphocytes*

Dose Groups	MN/1000	No. of	No. of	No. of	No. of Polynucleated	No. of
	Binucleated Cells	Mononucleate Cells	Binucleate Cells	Trinucleate Cells	Cells (>4)	NDI
DMSO Control	14.2 \pm 2.75	1036.25 \pm 26.1	781.75 \pm 12.3	89.50 \pm 6.24	92.5 \pm 7.77	1.63 \pm 0.24
1 $\mu\text{g}/\mu\text{l}$ Vanillic acid	10.2 \pm 2.50	1007.50 \pm 34.4	801.00 \pm 19.0	86.50 \pm 5.07	105.0 \pm 11.1	1.64 \pm 0.31
2 $\mu\text{g}/\mu\text{l}$ Vanillic acid	20.0 \pm 2.16	1053.50 \pm 28.3	770.00 \pm 14.4	87.75 \pm 6.85	88.7 \pm 7.77	1.60 \pm 0.25
Mitomycin C	36.0 \pm 3.65	1316.75 \pm 36.1	554.25 \pm 22.9	60.50 \pm 7.05	68.5 \pm 7.23	1.44 \pm 0.29
1 $\mu\text{g}/\mu\text{l}$ Vanillic acid+Mitomycin C	16.2 \pm 2.06	1043.50 \pm 26.1	776.25 \pm 11.7	89.25 \pm 5.79	91.0 \pm 9.20	1.61 \pm 0.25
2 $\mu\text{g}/\mu\text{l}$ Vanillic acid+Mitomycin C	27.2 \pm 4.57	1194.50 \pm 33.9	648.75 \pm 17.5	74.75 \pm 8.77	82.0 \pm 8.29	1.52 \pm 0.29

*No. of Cells Scored=8000, MN, Micronuclei; NDI, Nuclear Division Index; DMSO, Dimethyl sulphoxide

nevertheless, the NDI value of the high dose vanillic acid and MMC combination was also higher than that of the MMC group ($p < 0.01$).

To evaluate the comet assay, the concentration of viable cells was assessed by a trypan blue dye exclusion test (data not shown). As shown in Figure 4 and Table 2, a statistically significant decrease in the genetic damage index (GDI) was observed in cells treated with vanillic acid (1 $\mu\text{g/ml}$) when compared with the DMSO control ($p < 0.05$). On the other hand, the GDI in the 2 $\mu\text{g/ml}$ of vanillic acid treatment was significantly higher than in the DMSO control ($p < 0.001$). The MMC treatment induced a significant increase in GDI when compared with the DMSO control ($p < 0.001$). Vanillic acid (1 $\mu\text{g/ml}$)+MMC in combination induced GDI at a level equal to the negative control ($p > 0.05$) and this combination attenuated the GDI in comparison with MMC alone ($p < 0.001$). The combination of 2 $\mu\text{g/ml}$ of vanillic acid+MMC also showed a significantly lower GDI compared with the MMC only treatment group ($p < 0.001$).

As shown in Figure 5, the comet assay determined that while a 1 $\mu\text{g/ml}$ dose of vanillic acid decreased the percent of damaged cells, the 2 $\mu\text{g/ml}$ dose of vanillic acid significantly increased the percent of damaged cells

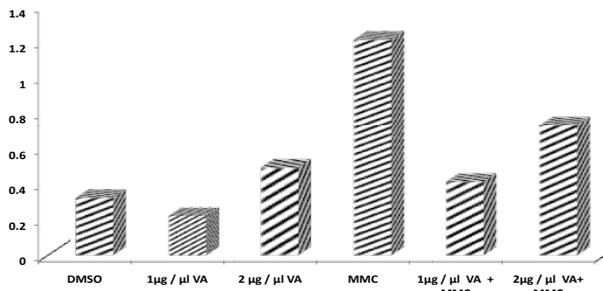


Figure 4. Genetic Damage Index (GDI) of Vanillic Acid and Vanillic Acid+MMC in the Alkaline Comet Assay. VA, Vanillic acid; MMC, Mitomycin-C

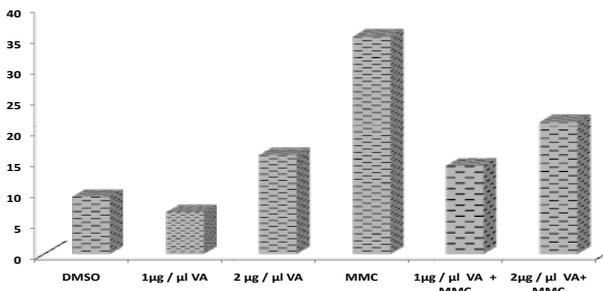


Figure 5. Damaged Cells (%) of Vanillic Acid and Vanillic Acid+MMC in the Alkaline Comet Assay. VA, Vanillic acid; MMC, Mitomycin-C

Table 2. DNA Damaging Effect of Vanillic Acid and Vanillic Acid+Mitomycin C on Alkaline Comet Assay in Human Lymphocytes*

Dose Groups	No. of Normal Cells (Class 0)	Class 1	Class 2	Class 3	Class 4	GDI	% of Damaged Cells
DMSO Control	84.7±3.09	6.00±1.82	4.50±1.29	2.25±0.96	2.50±1.73	0.32±0.08	9.25±2.63
1 $\mu\text{g/ml}$ Vanillic acid	89.2±2.50	4.00±1.41	3.50±1.00	1.75±0.50	1.50±0.60	0.22±0.04	6.75±1.26
2 $\mu\text{g/ml}$ Vanillic acid	77.5±2.38	6.50±1.73	9.00±1.63	3.00±0.82	3.25±1.26	0.49±0.08	16.0±3.37
Mitomycin C	60.5±2.64	4.25±1.50	7.75±2.22	8.25±2.36	19.3±1.89	1.21±0.05	35.3±1.71
1 $\mu\text{g/ml}$ Vanillic acid+Mitomycin C	81.7±1.26	4.00±2.16	7.75±1.50	3.50±1.29	3.00±0.00	0.41±0.03	14.2±1.89
2 $\mu\text{g/ml}$ Vanillic acid+Mitomycin C	72.5±2.08	6.00±1.41	6.50±2.64	5.75±1.26	9.25±2.50	0.73±0.09	21.5±1.91

*Number of Cells Scored=400, GDI, Genetic Damage Index; DMSO, Dimethyl sulphoxide.

when compared with DMSO treatment alone ($p < 0.05$ and $p < 0.001$, respectively). The MMC treatment alone increased the percent of damaged cells over the DMSO treatment alone ($p < 0.001$). The percent of damaged cells was significantly higher in the combination of vanillic acid (1 and 2 $\mu\text{g/ml}$) with MMC than in the DMSO control ($p < 0.01$) and these combinations also significantly attenuated the percent of damaged cells when compared with the MMC treatment alone ($p < 0.001$).

Discussion

Initially, the genotoxic and cytotoxic effects of vanillic acid, a dietary phenolic compound, were investigated in cultured human blood lymphocytes by both a CBMN and an alkaline comet assay. A 1 $\mu\text{g/ml}$ dose of vanillic acid induced no significant cell damage, whereas a 2 $\mu\text{g/ml}$ dose of vanillic acid significantly increased the frequency of micronuclei compared with the DMSO control in the CBMN test. Neither dose of vanillic acid (1 or 2 $\mu\text{g/ml}$) displayed cytotoxic effects with regards to the NDI measurements from the CBMN test. In the comet assay, a low dose of vanillic acid (1 $\mu\text{g/ml}$) decreased the GDI, but a 2 $\mu\text{g/ml}$ dose of vanillic acid treatment elevated the GDI ratio compared with the DMSO control values. The percentage of damaged cells, as measured by the comet assay, decreased with the 1 $\mu\text{g/ml}$ and increased with the 2 $\mu\text{g/ml}$ dose of vanillic acid compared with the DMSO control. Next, we examined the potential ameliorative effects of vanillic acid on MMC-induced genotoxicity. To determine these levels, the MMC-treated human lymphocytes were examined. High MN frequency, low NDI, increased GDI and a higher degree of cell damage were found in the MMC-treated lymphocytes. These results demonstrated the genotoxic effect of MMC *in vitro*. MMC alone and in combination with vanillic acid treatment were used to evaluate the possible ameliorative activity of vanillic acid on MMC-induced genotoxic effects. As expected, both doses of vanillic acid reduced the genotoxic and cytotoxic effects of MMC in human lymphocytes for both cytogenetic endpoints. This effect may arise from the antioxidant properties of vanillic acid, which have been shown in recent studies (Tai et al., 2011; 2012). Possibly contributing to this activity is the hydroxyl group, which places vanillic acid in the category of phenolic antioxidants (Tai et al., 2012). It has been reported that vanillic acid has both antimicrobial and anti-mutagenic activities and can exhibit a chemopreventive effect in experimentally induced carcinogenesis in rats

(Tsuda et al., 1994; Rajaand, 2010). Except for these few reports, there is limited number of studies investigating the genotoxic/anti-genotoxic properties of vanillic acid *in vitro*. However, the anti-mutagenicity, antioxidant activity and anti-carcinogenicity of vanillin are well studied in the literature (Tai et al., 2011; Kumar et al., 2012). Vanillic acid is an oxidized form of vanillin and exhibits more free radical scavenging activity than vanillin (Sasaki et al., 1990). In our study, high doses of vanillic acid (2 µg/ml) combined with MMC did not result in a greater anti-mutagenic effect than that of the low dose vanillic acid+MMC combination. This might be due to the genotoxicity of a high dose of vanillic acid. In the current study, 1 µg/ml of vanillic acid did not show any cytotoxic or genotoxic effects in cultured human blood lymphocytes. However, although a high dose of vanillic acid did not develop cytotoxic effects, it did create genotoxicity in human lymphocytes. This result is compatible with other reports that show pro-oxidant and oxidative properties of some antioxidant phenolics (Childs et al., 2001; Sakihama et al., 2002; Kessler et al., 2003). It could be suggested that flavonoids should not be considered pure antioxidants because under certain reaction conditions, they can also display pro-oxidant activity. This unexpected behavior of vanillic acid might be explained by its pro-oxidant abilities.

MMC is a chemotherapeutic agent that is still used in the treatment of different cancers in some countries Sontakke and Fulzele (2009). MMC is a highly genotoxic agent that induces chromosomal aberrations and sister chromatid exchanges in cultured human blood lymphocytes (Krishnaja and Sharma, 2008), increases MN frequency (Fauth et al., 2000) and induces higher MN formation in human liver fibroblast cultures (Nesti et al., 2000). In our study, MMC generated a high MN and a low NDI in the CBMN assay and a high GDI and an increased percentage of damaged cells in the alkaline comet assay. These findings agree with other studies that showed mutagenic and clastogenic activity for MMC (Fauth et al., 2000; Krishnaja and Sharma, 2008; Sontakke and Fulzele, 2009). It has been reported that *in vivo* vanillin treatment decreased the MMC-induced MN ratio in the bone marrow of mice (Inouye et al., 1988). Additional research demonstrated that vanillin reduced the DNA damage induced by H₂O₂ in V79 cells *in vitro* (Tamai et al., 1992). In addition, the anti-mutagenic activity of vanillin was shown by an *in vitro* Ames test with the TA104 strain of salmonella (Shaughnesy et al., 2001). Previous reports suggested that despite the reduced DNA damage *in vitro* (Tamai et al., 1992), vanillin increased the toxicity induced by N-methyl-N-nitrosoguanidine, MMC and H₂O₂ in human-hamster hybrid A1 cells (Gustafson et al., 2000). The results of our study revealed that the higher dose of vanillic acid in combination with MMC showed a higher MN frequency, GDI, proportion of damaged cells and lower NDI than the lower dose combination treatment. These findings show that a higher dose of vanillic acid may exert DNA-damaging activity in human lymphocytes. We did not observe any signs of synergistic effects between MMC and the higher dose of vanillic acid. Given the significant decrease in MMC-induced genotoxicity levels

in all parameters with a higher dose of vanillic acid, both doses of vanillic acid showed protective effects on the genotoxicity of the antineoplastic drug MMC.

We can conclude that the dietary, phenolic compound vanillic acid shows an anti-genotoxic effect on MMC-induced genetic damage in healthy human lymphocytes, as measured by the CBMN and alkaline comet assays. Moreover, vanillic acid at higher doses may exert *in vitro* genotoxic effects. Despite the anti-oxidant and anti-genotoxic capabilities of vanillic acid, care should be taken with regard to its concentration in the daily diet. More intensive research, both *in vitro* and *in vivo*, is required to clarify the genotoxic and anti-genotoxic effects of vanillic acid.

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