Antimutagenic Effect of the Ellagic Acid and Curcumin Combinations

Zoubková H*, Šmerák P and Polívková Z

Institute of General Biology and Genetics of the 3rd Faculty of Medicine, Charles University, Prague, Czech Republic

Abstract

Introduction: There is an evidence to support the health benefits of diets rich in fruits, vegetables, legumes, whole grains and nuts. Plant-based foods are complex mixtures of bioactive phytochemicals. The potential health effects of individual phytochemicals, their combinations or combinations of phytochemicals and others drugs is studied in detail. Ellagic acid belongs to the group of bioactive polyphenols in fruit: strawberries, raspberries, grapes, black currant and walnuts. Curcumin is a natural compound extracted from the root of Curcuma longa plant.

Methods: We perform in vitro Ames test and in vivo micronucleus test toward three mutagens/carcinogens, aflatoxin B1 and 2-amino-3-methylimidazo[4,5-]quinoline and N-nitroso-N-methylurea to prove an antimutagenic effect of ellagic acid, curcumin and their combinations.

Results: We verified the dose dependent antimutagenic effect of ellagic acid, curcumin and their combinations in both tests. The significantly increased effect of some combinations on the mutagenicity of indirect mutagens in the Ames test and on the direct mutagenicity of MNU in the micronucleus test, as compared with effect of ellagic acid or curcumin used separately, was also ascertained.

Keywords: Phytochemical; Ames; Micronucleus; Carcinogenesis

Abbreviations: DNA: Deoxyribonucleic acid; NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet-Derived Growth Factor; EA: Ellagic Acid; CRC: Curcumin; TNF: Tumor Necrosis Factor; AP-1: Activator Protein 1; IQ: 2-amino-3-methylimidazol(4,5-)$f$-quinoline; AFB; Aflatoxin B1; MNU: N-nitroso-N-methyleurea; DMSO: Dimethyl Sulfoxide; b.w.: Body Weight

Introduction: Exogenous factors, such as radiation and xenobiotics, can play an important role in carcinogenesis due to their mutagenic/promoting/co-carcinogenic effects [1]. They induce damage either directly by interacting with the macromolecules or indirectly by the creation of free radicals [2]. If oxidative stress is prolonged, reactive oxygen and nitrogen species are produced and carry out the process of damage. They exacerbate the oxidation of intracellular proteins, lipids, and nucleic acids [3-5]. DNA damage, if left unrepaired, can lead to base mutation, DNA cross-links, single and double-strand breaks, chromosomal breakage and rearrangement [6], genomic instability, neoplastic transformation and, ultimately, carcinogenesis [7]. The covalent interaction of carcinogen-induced reactive species with DNA may result in genotoxic damage during the initiation stage of chemical carcinogenesis [8-9].

This oxidative damage may be prevented or limited by dietary antioxidants (phytochemicals) found in fruits and vegetables [10]. A large number of phytochemicals possess antioxidant and free-radical scavenging properties and are known to modulate important cellular signaling pathways associated with carcinogenesis [7-8]. The epidemiologic studies evaluating associations between intake of a variety of plant-based foods indicate a protective effect, both on cardiovascular diseases and certain cancers. There is appreciable epidemiologic evidence that demonstrates a protective role in diets high in fruits and vegetable, legumes, whole grains and fish on different cancers and cardiovascular diseases [11].

Ellagic acid (EA) belongs to the group of bioactive polyphenols in fruit (strawberries, raspberries, grapes, black currant, walnuts). EA is found in plants in the form of hydrosolable tannins called ellagitannins as the structural components of cell wall and cell membrane. EA demonstrates antimutagenic, antioxidant, anti-inflammatory and anticancer activity [12]. Anticancer activity is manifested by blocking initiation of carcinogenesis, suppressing progression and proliferation of tumors [13,14]. EA decreases the metabolic activation of carcinoigenic substances by inhibition of cytochrome P450 and by induction of phase II enzymes of metabolic transformation [15]. EA also interferes with multiple cell signaling pathways, including the decrease of NF-κB, cyclooxygenase 2, cyclin D1, growth factors VEGF and PDGF and the increase of p21/WAF1 and p53 [14,16,17]. The antiproliferative and proapoptotic activities of EA are proved in cancer cell lines [18].

Curcumin (diferuloyl methane) (CRC) is a natural compound found extracted from the root of Curcuma longa plant. CRC is an anticancer, antioxidant, antiinflammatory and antiangiogenic agent, capable of inducing apoptosis of cancer cells [19-24]. The protective effect is detected in many in vitro and in vivo studies [22,25,26]. Prevalent evidence suggests that it may be useful for the chemoprevention of colon cancer in humans [27]. Preclinical studies of healthy individuals and patients with premalignant conditions or tumors are reviewed by Thomaset 2006 [28] and Von Löw 2007 [29]. The studies of molecular
targets of curcumin reveal that curcumin modulates the expression of many transcription factors such as TNF-α, AP-1 and NF-κB, cell cycle proteins, signal transducing kinases [14,21,30-32]. CRC enhances the expression of cell cycle inhibitors p21 and p27 as well as tumor suppressor protein p53, but suppress expression of the Rb protein [33]. CRC also exerts the immunomodulatory effect [26,34].

We have already published the antimutagenic activity of ellagic acid and curcumin as single agents in Ames test, micronucleus test and comet assay [26,35]. To prove the presumption about synergism or antagonism of antimutagenic activity of ellagic acid and curcumin, we have studied the activity of their combinations in the Ames test and the micronucleus test and we compared it with the activity of single agents. We used two indirect mutagens/carcinogens aflatoxin B, and 2-amino-3-methylimidazo(4,5-f)quinoxine and the direct mutagen/carcinogen N-nitroso-N-methylurea.

Material and Methods

Ames test

The antimutagenic activity of ellagic acid, curcumin and their combinations in vitro was detected using the Ames test with Salmonella typhimurium TA98 and TA100 strains [36-39]. Ellagic acid (Sigma-Aldrich) and curcumin (Sigma-Aldrich) were used at the following concentrations: 0.3 µg, 3 µg, 30 µg, and 300 µg/plate individually and in combinations. Mutagenic substances were used at the following concentrations: AFB1 (Alexis Biochemicals, San Diego, CA, USA) in 10 µg and 1 µg per plate in both bacterial strains, IQ (ICN Biomedicals, Eschwege, Germany) in 0.1 µg and 0.01 µg per plate in the strain TA98, IQ in 10 µg and 1 µg per plate in the strain TA100. Direct mutagen MNU (Sigma-Aldrich, St. Louis, MO, USA) was used at concentrations of 100 µg and 10 µg per plate only in the strain TA100, as these concentrations had no effect in the strain TA98 [26,35]. Each concentration of mutagen was combined with four different concentrations (0.3 µg, 3 µg, 30 µg, and 300 µg/plate) of phytochemical substances individually and also in the following mixtures: 0.3 µg of EA+0.3 µg of CRC, 3 µg of EA+3 µg of CRC, 30 µg of EA+30 µg of CRC and 300 µg of EA+300 µg of CRC per plate. All chemicals were diluted in dimethyl sulfoxide (Sigma-Aldrich Co, Louisiana, USA). The 59 fraction of the liver homogenate from the laboratory rats induced by a mixture of polychlorinated biphenyls (Delor) was used for metabolic activation of indirect mutagens [38]. Percentages of inhibition of mutagenicity was calculated by the formula:

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\text{No. of revertants of mutagen-No. of revertants of mixture of mutagen and antimutagen(s) } \times 100 \\
\text{No. of revertants of mutagen}
\]

Micronucleus test

The experiment in vivo, bone marrow micronucleus test, was carried out on male Balb/C mice each weighing 20-24 g (VELAZ s.r.o., Unîtice, Czech Republic). The animals were housed under standard conditions and divided into groups of 10 mice for treatment. EA and CRC were tested individually and in combinations. They were applied to mice by gavage three days sequentially, ellagic acid at the doses of 1 and 2 g/kg b.w. and curcumin at the doses of 0.25 and 0.5 g/kg b.w. The combinations of them were used in concentrations: 1 g of EA/kg+0.25 g of CRC/kg and 2 g of EA/kg+0.5 g of CRC/kg b.w. Mutagens (AFB1, IQ, and MNU) were applied individually and also in the mixtures with phytochemicals. AFB1, was used in the concentration of 1 mg/kg b.w. IQ was used in the concentration of 20 mg/kg b.w. and MNU in the concentration of 50 mg/kg b.w. Mutagens were applied in the single dose on the third day. All substances (diluted in DMSO) were applied in volumes of 100 µl/10 g b.w. The control group of mice received 7% solution of DMSO orally. The micronucleus test on mouse bone marrow was carried out according to Schmid, 1975 [40]. A total number of 1000 polychromatophilic erythrocytes were scored per animal for an evaluation of frequencies of micronuclei.

All groups of samples were tested in two separate experiments and each sample was tested in three plates in the Ames test. In the micronucleus test, all samples were tested in three separate experiments. For statistical analysis we used Student’s t-test.

Results

Results of Ames test

The results of the Ames test are presented in Tables 1-4. They are expressed as a number of revertants and also as a percentage of inhibition of mutagenic activity. The samples of EA, CRC and their combinations were tested separately without mutagen (Table 1), and in combination with mutagen AFB1 (Table 2), with mutagen IQ (Table 3) and with mutagen MNU (Table 4). An activity of mixtures of EA and CRC were compared to the results of phytochemicals used separately all in combination with mutagens (Tables 2-4). Neither ellagic acid and curcumin, nor their combinations revealed any mutagenicity in both bacterial strains TA98 and TA100 (Table 1). Significant dose dependent antimutagenic activity was detected at two highest concentrations (30 and 300 µg/plate) of EA, CRC and their combinations on mutagenicity of both concentrations of AFB1, (10 and 1 µg/plate) (Table 2). The only exception of significance was in the decrease of mutagenicity of the combinations of 30 µg of EA/plate mixed with 10 µg of AFB1 in both bacterial strains and 30 µg of EA mixed with 1 µg of AFB1, in the strain TA 100 (Table 2). In both strains, the antimutagenic activity of two combinations (3 µg of EA+3 µg of CRC and 30 µg of EA+30 µg of CRC mixed with 1 µg of AFB1) was significantly higher than the activity of the same concentrations of EA or CRC used separately.

The dose dependent inhibition effect of phytochemicals and their combinations were detected on mutagenicity of both concentrations of IQ (0.1 and 0.01 µg/plate in the strain TA98 and 10 and 1 µg/plate in the strain TA100) (Table 3). There was significant difference between combinations with concentrations of 30 µg of EA+30 µg of CRC combined with 0.1 µg or 0.01 µg of IQ in the strain TA98 and in the concentrations of 3 µg of EA+3 µg of CRC and 30 µg of EA+30 µg of CRC both combined with 1 µg of IQ in the strain TA100 than EA or CRC of the same concentrations used separately (Table 3).

The activity of phytochemicals against the direct mutagen MNU, used at concentration of 100 µg/plate was significant only in the combinations of phytochemicals with higher concentrations (30 µg of EA+30 µg of CRC, 300 µg of EA+300 µg of CRC) (Table 4). The differences between the activity of combinations and separate phytochemicals were not significant. The antimutagenic activity against lower concentration of MNU (10 µg/plate) was more obvious, but the effect of phytochemicals combinations similarly did not differ from the effect of EA or CRC used separately (Table 4).

Results of micronucleus test

All three mutagens revealed significant mutagenic activity in the micronucleus test. The number of micronuclei in animals, which received phytochemicals and their combinations without mutagen, did not differ from those of the control group (Table 5). EA, CRC and their combinations significantly reduced the number of micronuclei, which was high by the mutagenic activity of AFB1, IQ and MNU. The decrease of micronuclei numbers was dose dependent (Figures 1-3). The activity
of the combinations of EA and CRC on the mutagenicity of indirect mutagens AFB, and IQ did not differ from the activity of phytochemicals used separately (Figures 1 and 2). Only the mutagenicity of 50 mg/kg of MNU was significantly more reduced by the combination of EA and CRC at concentrations of 2 g/kg of EA+0.5 mg/kg of CRC in three daily doses in comparison with the same doses of individual phytochemicals (Figure 3).

Discussion

Antimutagenesis, a prevention of genotoxic damage is a part of chemoprevention and could be considered as a major mechanism to inhibit carcinogenesis in the initiation stage [9]. Chemoprevention, as a defense anti-cancer mechanism provided by phytochemicals, was defined in 1966 by Wattenberg [41]. In our department, we studied the antimutagenic and immuno-modulatory effects of individual phytochemicals of natural origin both in vitro and in vivo conditions. We confirmed that the phytochemicals in the pure forms

| EA+CRC dose (µg/plate) | S. typhimurium TA98+S9 | S. typhimurium TA100+S9 |
|------------------------|------------------------|------------------------|
|                        | No of revertants ± SD  | No of revertants ± SD  |
| 0.3EA                  | 20 ± 5                 | 79 ± 3                 |
| 3EA                    | 24 ± 5                 | 78 ± 6                 |
| 30EA                   | 20 ± 3                 | 69 ± 10                |
| 300EA                  | 17 ± 2                 | 67 ± 7                 |
| 0.3CRC                 | 18 ± 5                 | 81 ± 9                 |
| 3CRC                   | 19 ± 4                 | 71 ± 5                 |
| 30CRC                  | 19 ± 5                 | 70 ± 7                 |
| 0.3EA+0.3CRC           | 18 ± 2                 | 74 ± 8                 |
| 3EA+3CRC               | 20 ± 6                 | 73 ± 9                 |
| 30EA+30CRC             | 16 ± 4                 | 70 ± 8                 |
| 300EA+300CRC           | 20 ± 7                 | 68 ± 3                 |
| control - DMSO         | 22 ± 4                 | 79 ± 8                 |

SD: standard deviation

Table 1: Ellagic acid, curcumin and their combinations in Ames test.

| AFB • antimutagen(s) dose (µg/plate) | S. typhimurium TA98+S9 | S. typhimurium TA100+S9 |
|--------------------------------------|------------------------|------------------------|
|                                      | No of revertants ± SD  | % of inhibition        | No of revertants ± SD  | % of inhibition        |
| 10+0                                 | 471 ± 94               | -                        | 762 ± 90               | -                        |
| 1+0                                  | 559 ± 100              | -                        | 703 ± 72               | -                        |
| 10+0.3EA                             | 446 ± 67               | -5                       | 729 ± 114              | -4                       |
| 10+3EA                               | 467 ± 81               | -1                       | 701 ± 129              | -8                       |
| 10+30EA                              | 406 ± 119              | -14                      | 685 ± 131              | -10                      |
| 10+300EA                             | 127 ± 42               | -73                      | 501* ± 134             | -34                      |
| 10+0.3CRC                            | 446 ± 117              | -5                       | 666 ± 141              | -13                      |
| 10+3CRC                              | 456 ± 99               | -3                       | 641 ± 138              | -16                      |
| 10+30CRC                             | 67 ± 25                | -86                      | 522* ± 212             | -32                      |
| 10+300CRC                            | 9 ± 5                  | -98                      | 226* ± 108             | -70                      |
| 10+0.3EA+0.3CRC                      | 434 ± 41               | -8                       | 605* ± 168             | -21                      |
| 10+3CRC+3CRC                         | 474 ± 38               | +1                       | 601* ± 174             | -21                      |
| 10+30CRC+30CRC                       | 73 ± 30                | -85                      | 480* ± 193             | -37                      |
| 10+300CRC+300CRC                     | 12 ± 7                 | -98                      | 146* ± 67              | -81                      |
| 1+0.3EA                              | 548 ± 56               | -2                       | 703 ± 100              | 0                        |
| 1+3EA                                | 573 ± 26               | +3                       | 681 ± 88               | -3                       |
| 1+30EA                               | 449 ± 88               | -20                      | 634 ± 158              | -10                      |
| 1+300EA                              | 54 ± 18                | -90                      | 244* ± 109             | -65                      |
| 1+0.3CRC                             | 591 ± 39               | +6                       | 644 ± 108              | -8                       |
| 1+3CRC                               | 575 ± 22               | +3                       | 663 ± 80               | -6                       |
| 1+30CRC                              | 103 ± 31               | -82                      | 532* ± 129             | -24                      |
| 1+300CRC                             | 36 ± 22                | -94                      | 147* ± 41              | -79                      |
and also in the form of juices of natural plants might have an important role in the prevention of carcinogenesis by their antimutagenic effect [26,35,42-47]. In addition, it was presented by other research groups that CRC and EA were able to activate or inhibit many cellular molecules of signaling pathways and became involved in the regulation of cancer cell division [14].

Food phytochemicals provided complex interactions in biological systems [48]. The combination of natural phytochemicals in fruits and vegetables, which provided health benefits, might not be replaced by the effect of single phytochemicals [10,49]. Also, combinations of phytochemicals might have result in significant effect at concentrations, in which the single agents were inactive [50].
Table 5: Ellagic acid, curcumin and their combinations in micronucleus test.

| Substances tested | Dose | No of micronuclei | SD |
|-------------------|------|-------------------|----|
| EA                | 3 × 1 g/kg | 0.4 | 0.5 |
| EA                | 3 × 2 g/kg | 0.2 | 0.4 |
| CRC               | 3 × 0.25 g/kg | 0.6 | 0.9 |
| CRC               | 3 × 0.5 g/kg | 0.4 | 0.5 |
| EA+CRC            | 3 × (1+0.25) g/kg | 0.4 | 0.5 |
| EA+CRC            | 3 × (2+0.5) g/kg | 0.2 | 0.4 |
| control-DMSO      | -    | 0.2 | 0.4 |

SD: Standard Deviation

Table 5: Effect of EA, CRC and their combinations on mutagenicity of MNU in Ames test.

| Substances tested | Dose | MNU+antimutagen(s) (µg/plate) | S. typhimurium TA100 |
|-------------------|------|-------------------------------|----------------------|
|                   |      | No of revertants | ± SD | % of inhibition |
| 100+0             | 1643 | 166 | 0.2 |
| 10+0             | 392  | 26 | 0.2 |
| 100+0.3EA         | 1624 | 164 | 0.2 |
| 100+3EA          | 1658 | 145 | 0.2 |
| 100+30EA         | 1567 | 166 | 0.2 |
| 100+300EA        | 1358 | 223 | 0.2 |
| 100+0.3CRC        | 1619 | 208 | 0.2 |
| 100+3CRC         | 1536 | 263 | 0.2 |
| 100+30CRC        | 1490 | 212 | 0.2 |
| 100+300CRC       | 1243 | 228 | 0.2 |
| 100+0.3EA+0.3CRC  | 1569 | 205 | 0.2 |
| 100+3EA+3CRC     | 1481 | 236 | 0.2 |
| 100+30EA+3CRC    | 1288 | 240 | 0.2 |
| 100+300EA+300CRC | 1015 | 243 | 0.2 |
| 10+0.3EA         | 414  | 35 | 0.2 |
| 10+3EA           | 407  | 89 | 0.2 |
| 10+30EA          | 327  | 126 | 0.2 |
| 10+300EA         | 254* | 32 | 0.2 |
| 10+0.3CRC        | 385  | 47 | 0.2 |
| 10+3CRC          | 422  | 90 | 0.2 |
| 10+30CRC         | 211* | 21 | 0.2 |
| 10+300CRC        | 154* | 9 | 0.2 |
| 10+0.3EA+0.3CRC  | 406  | 89 | 0.2 |
| 10+3EA+3CRC      | 364  | 89 | 0.2 |
| 10+30EA+3CRC     | 187* | 29 | 0.2 |
| 10+300EA+300CRC  | 144* | 16 | 0.2 |
| control-DMSO     | 112  | 9 | 0.2 |

SD standard deviation

*Statistically significant difference between the sample with mutagen and antimutagen(s) and the sample with specific mutagen p ≤ 0.05

**Statistically significant difference between the sample with mutagen and antimutagen(s) and the sample with specific mutagen p ≤ 0.01

Table 4: Effect of EA, CRC and their combinations on mutagenicity of MNU in Ames test.

Interactions of phytochemicals might be antagonistic, additive and/or synergistic depending on the certain experimental conditions and concentrations [48, 49]. The additive or synergistic effect of combinations of phytochemicals or phytochemicals and synthetic drugs was previously detected in many research projects under both in vitro and in vivo conditions [51-58]. For instance, Verma et al. (1997) described a synergistic inhibition effect of curcumin and genistein on proliferation of MCF-7 breast cells induced by estrogenic pesticides [51]. Lev-Ari et al. (2005) provided that curcumin synergistically potentiated the growth inhibition and the pro-apoptotic effect of celecoxib in pancreatic adenocarcinoma cells [52] or colorectal cancer cells [53]. Also ellagic acid and quercetin interacted synergistically with resveratrol in the induction of apoptosis in human leukemia cells [54]. Resveratrol combinations with ellagic acid and other phytochemicals were very potent inhibitors of skin tumorgenesis [55].

In our research the antimutagenic effect of the combinations of phytochemicals and individual phytochemicals of the same high concentrations was detected on mutagenicity of both concentrations of indirect mutagens, AFB, and IQ, in the Ames test. The increased antimutagenic effect of the combinations was mostly detected in two middle concentrations of phytochemicals (3 and 30 µg of EA and CRC) in the comparison to the effect of individual phytochemicals of the same concentration. The increased significant antimutagenic effect of combinations was limited by concentration. Considering these results, we could not confirm the presumption about effective-low-concentration combinations of tested phytochemicals [50]. The combination of the highest concentration (300 µg of EA and CRC) did not show an
induce GST under understanding their chemical and biological functions and their mechanisms.

The potentiation of effects of phytochemicals was usually detected in the highest concentration (2 g of EA/kg + 0.5 g of CRC/kg) of phytochemicals used individually of the same concentration was compared with the effect of the direct mutagen MNU in the Ames test and in the micronucleus test. The antimutagenic effect of ellagic acid and curcumin was dose dependent. An increased antimutagenic effect against indirect mutagens in the Ames test was proven mainly in the combinations of phytochemicals of two or more compounds. The synergistic effects of dietary phytochemicals should be further explored for additional beneficial and reliable outcomes on the basis of which most current cancer drugs were synthesized [50]. Recent studies showed that phytochemicals were also able to reverse the chemoresistance or radioresistance of tumor cells [66,67]. More information about the combined effects of phytochemicals is needed to avoid the possible unfavorable effects of their unbalanced combinations.

**Conclusion**

In Ames test (*in vitro*) and in micronucleus test (*in vivo*), ellagic acid and curcumin did not show any mutagenic effect to bacteria and mice. We verified the antimutagenic effect of ellagic acid and curcumin against the indirect mutagens IQ and AFB, and against the direct mutagen MNU in the Ames test and in the micronucleus test. The antimutagenic effect of ellagic acid and curcumin was dose dependent. An increased antimutagenic effect of the ellagic acid and curcumin combinations was proved in the Ames test against the indirect mutagens IQ and AFB, and in the micronucleus test against the direct mutagen MNU as compared with effect of ellagic acid or curcumin used separately.

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**Declaration of Interest Statement**

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