

Antimutagenicity protection of *Ginkgo biloba* extract (Egb 761) against mitomycin C and cyclophosphamide in mouse bone marrow

J.B. Vilar, K.R. Leite and L. Chen Chen

Departamento de Biologia Geral, Instituto de Ciências Biológicas,
Universidade Federal de Goiás, Goiânia, GO, Brasil

Corresponding author: L. Chen Chen
E-mail: chenleego@yahoo.com.br

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ABSTRACT. *Ginkgo biloba* (Egb 761) extract, the most prescribed phytomedicine in Europe for the treatment of cerebral insufficiency and vascular diseases, was tested for its possible protective effects against mitomycin C (MMC)- and cyclophosphamide (CP)-induced mutagenicity using the micronucleus test in mouse bone marrow. The extract was co-administered to mice at doses of 50, 100 and 200 mg/kg (*po*) with 4 mg/kg (*ip*) MMC or 24 mg/kg (*ip*) CP. All doses of Egb 761 were significantly ($P < 0.05$) effective in reducing the frequency of micronucleated polychromatic erythrocytes, when compared with MMC or CP alone. Based on these results, we suggest that Egb 761 possesses both direct and indirect antimutagenic potential.

Key words: *Ginkgo biloba*; Egb 761; Antimutagenicity; Mitomycin C; Cyclophosphamide; Micronucleus test

INTRODUCTION

The development of means to prevent cancer and other diseases related to the incidence of mutations, such as atherosclerosis, emphysema, cardiomyopathies, neurodegenerative diseases, are of enormous practical interest, primarily devoted to the detection of anti-risk factors for humans in a variety of test systems (De Flora and Ramel, 1988; De Flora, 1998; De Flora et al., 2001). Some studies in short-term tests have demonstrated that certain compounds are capable, at nontoxic concentrations, of lowering the rate of spontaneous or induced mutations (De Flora et al., 1994; Waters et al., 1996).

These substances are commonly found in plants and can act as natural antimutagens or anticarcinogens, blocking or scavenging the final electrophile moieties in a nucleophilic reaction, generating innocuous products; in this manner, the exposure to these substances would protect against DNA damage (Kaur et al., 2002). Due to the various roles plants can play, for centuries people have used them for healing. Plant products - as parts of food or botanical potions and powders - have been used with varying success to cure and prevent diseases, throughout history. Until recently, plants were an important source for the discovery of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants (Raskin et al., 2002).

Ginkgo biloba (Ginkgoaceae) is a native tree from China that has been exhaustively cultivated in Europe, Australia, Japan, Korea, and the USA, because of its health-promoting properties (Shinozuka et al., 2002). It has been used for 5000 years in traditional Chinese medicine. The standardized extract of this plant, called *Ginkgo biloba* extract - Egb 761 - was until recently the most prescribed phytomedicine in England and France, for the treatment of cerebral insufficiency and vascular diseases (Le Bars et al., 1997). In general, Egb 761 is composed of 24% flavonoids (glycoside derivatives of quercetin, kaempferol, isohamentin and mycetin, in addition to some aglycone), 6% terpenoids (ginkgolides A, B, C, J, M, and bilobalides), and 0.5 to 1% organic acids (acetic, shikimic, p-hydroxybenzoic, vanillic, kynurinic, and ascorbic acids), besides other constituents (Goh and Barlow, 2002).

In view of the widespread use of Egb 761, the objective of the current study was to investigate the antimutagenic potential of multiple doses of Egb 761 on mouse bone marrow cells, to provide some clues about the preferential way of action that could occur when co-administered with mitomycin C (MMC), a direct-acting agent that does not require any molecular activation, or cyclophosphamide (CP), an indirect-acting agent, which needs to be converted enzymatically to its active metabolites.

MATERIAL AND METHODS

Extract sample

Commercially available Egb 761 was purchased at the "Farmácia-Escola" of the "Faculdade de Farmácia" of the Universidade Federal de Goiás.

Chemical and drugs

The chemical and drugs used in the experiment were the following: MMC ($C_{15}H_{18}N_4O_5$;

Bristol-Myers Squibb, Lot No. 217ACB), CP ($C_7H_{15}Cl_2N_2O_2P$; Sigma, Lot No. 87H0207), dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$; Merck, Lot No. 127H0168), monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$; Merck, Lot No. 47H1445), Giemsa (Doles, Lot No. 1081), methanol (CH_4O ; Synth, Lot No. 39107), fetal bovine serum (Laborclin, Lot No. 30721063).

Animals

Healthy young adult (8-12 weeks) males of *Mus musculus* (Swiss Webster out bred mice), weighing 25-30 g, obtained from the Central Animal House of Universidade Federal de Goiás, were used in the studies. All animals were brought to the laboratory 5 days before the experiments. They were housed in plastic cages (40 x 30 x 16 cm) at $24 \pm 2^\circ C$ and $55 \pm 10\%$ humidity, with a natural light-dark cycle of 12 h. Food (appropriate commercial ration; Labina, Ecibra Ltda.) and water were provided *ad libitum*.

Experimental procedure (micronucleus test)

Five animal groups were co-treated by gavage with 50, 100, and 200 mg/kg Egb 761 and 4 mg/kg MMC (*ip*) or 24 mg/kg CP (*ip*) (Salamone et al., 1980). The positive (MMC = 4 mg/kg and CP = 24 mg/kg) and negative control groups (0.1 mL/10 g body weight sterilized distilled H_2O) were also included. After the periods of treatments (36 h for MMC and 48 h for CP), the animals were sacrificed. The femurs were exposed and cleaned, and the epiphysis sectioned. The bone marrow was gently flushed out using fetal bovine serum. After centrifugation (1500 rpm, 5 min), bone marrow cells were smeared on glass slides, coded for blind analysis, air-dried and placed in absolute methanol for 5 min. The smears were stained with Giemsa for detecting micronucleated polychromatic erythrocytes (MNPCE) (Schmid, 1975). For each animal, three slides were prepared and 1000 polychromatic erythrocytes (PCE) were counted to determine the frequency of MNPCE. To determine the cytotoxicity of the plant extract, 1000 normochromatic erythrocytes (NCE) were counted, as well as the frequency of PCE within the same microscope fields. The PCE/NCE ratio was then calculated. The slides were analyzed using an Olympus BH-2 microscope (1000X).

Statistical analysis

The results of the antimutagenicity tests were tabulated and experimental values are reported as means \pm standard deviation. One-way independent sample ANOVA was used to detect differences between the doses of Egb 761 in the same co-treatment (MMC or CP). The Tukey *post hoc* test was then used when necessary. Pearson correlation was used to verify the association between the results obtained with both co-administrations. The percentage of inhibition (%I) was measured by the complement of the rate between the average frequency of MNPCE of the test treatment and the average frequency of MNPCE of the positive control times 100 (%I = $1 - (\text{MNPCE (Egb 761 and MMC or CP)} / \text{MNPCE (MMC or CP)}) \times 100$). The cytotoxicity data were expressed as the PCE/NCE ratio, and the analyses were performed using the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test, for the difference against positive controls.

RESULTS AND DISCUSSION

The antimutagenic analyses are shown in Table 1. Our results demonstrate that Egb 761 was able to significantly protect ($P < 0.05$) DNA from the mutagens studied when compared with each positive control (MMC or CP), at all doses tested. The determination coefficient (R^2) between the two antimutagenic profiles was 53.29% ($P < 0.0003$).

Table 1. Micronucleated polychromatic erythrocyte (MNPCE) frequencies of controls and co-treatments with Egb 761 and mitomycin C (MMC) or cyclophosphamide (CP), values of means \pm SD, percentage of inhibition (%I) and polychromatic/normochromatic erythrocyte (PCE/NCE) ratio (means \pm SD).

Treatment	Individual data MNPCE/1000	Means \pm SD	%I	PCE/NCE
C(-): distilled H ₂ O ¹	2-1-3-3-1	2 \pm 1 ^A	-	0.99 \pm 0.254
C(+): MMC 4 mg/kg ²	30-36-31-42-33	34.4 \pm 4.8 ^B	0.00	0.49 \pm 0.110
50 mg/kg Egb 761 + 4 mg/kg MMC	15-17-14-15-12	14.6 \pm 1.81 ^C	57.56	0.56 \pm 0.06
100 mg/kg Egb 761 + 4 mg/kg MMC	14-16-17-18-18	16.6 \pm 1.67 ^C	51.74	0.57 \pm 0.04
200 mg/kg Egb 761 + 4 mg/kg MMC	16-18-19-15-16	16.8 \pm 1.64 ^C	51.16	0.54 \pm 0.04
C(+): CP 24 mg/kg ³	27-26-24-23-30	26.0 \pm 2.74 ^D	0.00	0.42 \pm 0.09
50 mg/kg Egb 761 + 24 mg/kg CP	16-17-15-15-18	16.2 \pm 1.30 ^E	37.69	1.44 \pm 0.2*
100 mg/kg Egb 761 + 24 mg/kg CP	17-14-13-16-16	15.2 \pm 1.64 ^E	41.54	1.28 \pm 0.11*
200 mg/kg Egb 761 + 24 mg/kg CP	7-6-9-7-8	7.4 \pm 1.14 ^F	71.54	1.43 \pm 0.14*

¹Sterilized and distilled water (negative control group). ²MMC (4 mg/kg - positive control group). ³CP (24 mg/kg - positive control group). Values followed by the same letter do not present significant differences ($P > 0.05$). *Significant difference ($P < 0.05$) against its own positive control group.

In the light of these results, it is possible that common mechanisms of action of the extract, such as antioxidant activity, free radical scavenging and even gene regulation, could contribute to its direct and indirect antimutagenic profile. The use of MMC as an antitumor drug is due to its direct action on DNA. At least two different processes are responsible for its biological effects: DNA alkylation (which can lead to cross links) and the generation of free radicals, such as superoxide and hydroxy radicals (which can lead to DNA strand breaks) (Kang et al., 2006). On the other hand, the alkylating agent CP is one of a group of anticancer drugs that are administered as inactive prodrugs and that are activated *in vivo* via one or more metabolic steps. It is known that the initial step in the bioactivation of cyclophosphamide involves cytochrome P-450-mediated hydroxylation at C-4 (LeBlanc and Waxman, 1990).

Until now, the literature has demonstrated that Egb 761 has antioxidant properties, can act as a free radical scavenger and anti-inflammatory agent, can improve cognition, memory and anxiety, and can affect gene regulation (Gaudineau et al., 2004; Chen et al., 2005; Ergun et al., 2005; Krizková et al., 2008). It is also possible that direct interactions between the Egb 761 extract and these mutagens occur, since it has been pointed out that commonly used herbal medicines cause some herb-drug interactions (Izzo and Ernst, 2001; Williamson, 2001). Egb 761 itself has been reported to interact with some drugs, such as aspirin, acetaminophen or warfarin, provoking occurrences of cerebral and subarachnoid hemorrhage (Vaes and Chyka, 2000).

No significant difference ($P > 0.05$) in the frequency of MNPCE was observed among the Egb 761 doses co-administrated with MMC and no significant difference ($P > 0.05$) was observed among the treatments and positive control PCE/NCE ratio (Table 1). The co-administration of Egb 761 and CP, on the other hand, revealed a significant increase in the PCE/NCE ratio and a significant difference ($P < 0.05$) among the MNPCE frequen-

cies (Table 1). The higher dose, 200 mg/kg Egb 761 and CP, caused a substantial augmentation of the %I, compared with the doses of 50 and 100 mg/kg. The %I of the treatment with 200 mg/kg Egb 761 and MMC was 51.16% and %I of the treatment with 200 mg/kg Egb 761 and CP was 71.54%, which suggests that the standardized *Ginkgo* extract was more effective in the modulation of the mutagenic potential and cytotoxicity of the indirect-acting drug CP. Despite the conflicting results obtained when the ability of the standardized *Ginkgo* extract or its constituents to modulate the cytochrome P-450 (CYP) was evaluated (Kuilman et al., 2000; Budzinski et al., 2000; Lautraite et al., 2002; Huynh and Teel, 2002), the effectiveness of the Egb 761 extract in the modulation of CP mutagenicity and cytotoxicity could be related to the inhibition of some CYP enzymes, which was demonstrated by Qualls et al. (1998). In this manner, changes in the level of expression of hepatic P-450s could affect the level of cyclophosphamide metabolism and, accordingly, its efficacy.

Other studies also support the antigenotoxic potential of *Ginkgo biloba*. Nunes (2004) observed its protective effect against doxorubicin (which can act as an intercalating antitumor drug or by the generation of reactive oxygen species) (Tewey et al., 1984) in *D. melanogaster*, and Krizková et al. (2008) related its antimutagenic action against ofloxacin and acridine orange in the flagellate *E. gracilis*. In conclusion, our results suggest for the first time that the standardized extract of this plant shows medicinal potential as an anti-mutagenic agent, modulating the mutagenicity caused by both direct- and indirect-acting chemical mutagens, in a mammalian model.

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