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In Vitro and In Vivo Study of the Clastogenicity of the Flavone Cirsitakaoside Extracted From *Scoparia dulcis* L. (Scrophulariaceae)

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The mutagenic effect of the flavone cirsitakaoside extracted from the medicinal herb *Scoparia dulcis* was evaluated in vitro by using human peripheral blood cultures treated with doses of 5, 10, and 15 μg of the flavone/ml culture medium for 48 h. The compound proved to be mutagenic at the highest concentration tested (15 μg/ml). Furthermore, the proliferative index was significantly reduced in all cultures treated with the flavone, although the mitotic index was not reduced. However, the clastogenic activity of the flavone cirsitakaoside was not observed when Swiss mice were treated orally with doses of 10, 20, and 30 mg/animal for 24 h. *Teratogenesis Carcinog. Mutagen. 18:293–302, 1998.*

Key words: flavone; clastogenicity; chromosomal aberrations; cirsitakaoside

INTRODUCTION

Scoparia dulcis L. is a widespread tropical herb, widely used in Brazilian folk medicine as an analgesic and antipyretic, in gastric disorders, bronchitis, diabetes, hypertension, and topically to treat haemorrhoids, insect bites, and skin wounds. Extracts of *S. dulcis* were shown to have analgesic and anti-inflammatory properties

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related to the flavonoid and glutinol content of the plant [1], and sympathomimetic activity that may explain its effectiveness upon topical application [2].

Flavonoids belong to the class of low molecular weight phenols that are widely distributed throughout the plant kingdom, with about 3,000 different flavonoids having been described thus far. Their chemical structure consists of a common diphenylpyrane skeleton with two benzene rings linked by a heterocyclic pyrane ring or pyrone. This basic structure permits multiple patterns of substitutions and variations giving origin to subclasses such as flavonoids, flavones, catechins, flavanones, antocyanidines, and isoflavonoids [3].

The modifications occurring in each type of flavonoid, such as hydroxylation, methylation, acylation, glucosylation, or rhamnosylation, result in the enormous diversity of types and characteristic colors of the flavonoids detected in vascular plants [3]. Flavonoids are present in foods usually as glycosides. Flavonoids and flavones, mainly located in the leaves of plants, usually occur as O-β-glycosides, with D-glucose being the most frequent sugar residue [4].

This group of metabolites has received great interest due to their ample occurrence on the tilachoid membranes of higher plants, to their high consumption by humans and animals, and to their broad spectrum of biological functions and medicinal properties, such as anti-inflammatory, antiallergic, antiviral, antibacterial, and antitumoral activities [5,6], in addition to their important role in plant-environment interaction.

The mutagenic activity of certain flavonoids has been recorded in prokaryotic and eukaryotic systems [7,8], whereas other flavonoids have shown antimutagenic activity in *Salmonella typhimurium* [9–11] and eukaryotes [12–14].

The high chemical reactivity of flavonoids is expressed by their ability to bind to biological polymers and to chelate certain transition metals, and also by their efficacy in catalyzing electron transport and scavengering free radicals. Thus, they may act as antioxidants, affect the phosphorylation of cell proteins, and inhibit or activate enzymes [15–18].

The present study was undertaken in order to evaluate the possible clastogenic effect of the flavone cirsitakaoside by determining the frequency of chromosome aberrations in vitro, using the human peripheral blood lymphocyte culture assay and in vivo on bone marrow cells of *Mus musculus* mice.

MATERIAL AND METHODS

Chemical Agent

In the present investigation we studied a flavone extracted from the aerial parts of the medicinal herb *Scoparia dulcis* (Scrophulariaceae). The flavone cirsitakaoside ($C_{23}H_{24}O_{11}$) has a molecular weight of 476.44 and the major modifications in its chemical structure (Fig. 1) are methoxy groups at positions 6 and 7 as well as a hydroxyl at position 5 and a sugar in carbon 4' [19].

The compound was isolated in the Laboratory of Natural Products of the Department of Chemistry, Federal University of Maranhão by routine phytochemical extraction processes using organic solvents, and isolation and purification by crystallization and recrystallization. The compound was identified by physical methods of organic analysis (IR, ^{1}H-NMR, ^{13}C-NMR, DEPT, and ^{1}H-^{13}CNMR COSY) and its degree of purity (96%) was evaluated by hydrogen nuclear magnetic resonance [20].

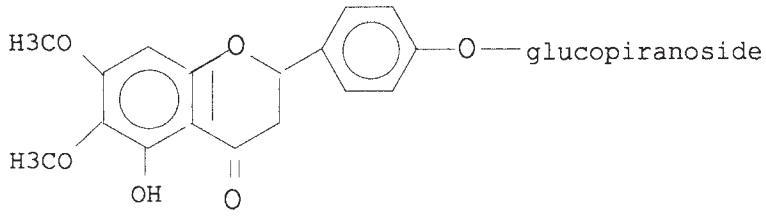

Fig. 1. Chemical structure of 5-hydroxy,6-7dimethoxyflavone 4′-O-D-glucose.

Test Systems

In vitro test with human peripheral blood lymphocytes. Blood samples were collected from healthy individuals (two men and three women), aged 22 to 31 years, who fulfilled the following requirements during the last 6 months before the study: a) no clinically diagnosed diseases, b) no ingestion of medications, c) no viral or bacterial infection, d) no exposure to radiation, e) no exposure to toxic or mutagenic and/or carcinogenic products, and f) being non-smokers.

Cultures were prepared with 1 ml plasma in 10 ml culture medium consisting of 80% RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO), 20% fetal calf serum (Cultilab, Campinas, SP.), 2% phytohemagglutinin (GIBCO BRL, Gaithersburg, MD), 0.01 mg/ml streptomycin (Ceme, Brasília, DF), and 0.005 mg/ml penicillin (Fontoura Wyeth S.A.). Cells were cultured at 37°C for 48 h.

Pilot experiments were conducted in order to determine the doses to be used in the definitive assays. Cultures were treated continuously with different concentrations of the flavone diluted with dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany), starting 9 h after the beginning of culture. Cultures treated with the 20 μg/ml dose showed a significant reduction in mitotic index and those treated with a dose higher than 40 μg/ml showed full inhibition of cell growth. Thus, the doses selected for the definitive experiments were 5, 10, and 15 μg/ml.

A solvent control was used in all experiments, in which the cultures received the same volume of DMSO necessary to obtain the highest dose of the flavone, as well as a positive control in which the cultures were treated with doxorubicin (Farmitalia Carlo Erba, 0.1 μg/ml). Colchicine (Sigma Chemical Co., St. Louis, MO, 0.4 μg/ml) was added to the cultures 90 min before fixation. The material was harvested and fixed [21], and 100 metaphases per culture were analyzed.

The mitotic index was calculated by determining the number of metaphases obtained from 1,000 cells counted per culture. For the determination of the proliferative index, cells were incubated with bromodeoxyuridine (Sigma Chemical Co., St. Louis, MO, 10 μg/ml) for 72 h and treated and harvested as described above. Slides were prepared by the Giemsa fluorescence technique [22] in combination with the technique of Korenberg and Freedlender [23] using bis-benzimide (Sigma Chemical Co., St. Louis, MO, 1.2 μg/ml PBS) and Giemsa (Merck, Darmstadt, Germany, 1 ml/30 ml phosphate buffer). The proliferative index was obtained by analyzing 100 metaphases/culture and using the formula $PI = [(M_3 - M_1) + 1]$, where M^1 and M^3 are percentages of metaphases in the first and third cycles of cell division, respectively [24].

In vivo test with *Mus musculus* bone marrow cells. Thirty Swiss mice (*Mus musculus*) weighing approximately 30 g were obtained from the Central Animal House of the Faculty of Medicine of Ribeirão Preto, USP. The animals were divided into

five experimental groups of six mice each (three males and three females) receiving 0.5 ml of solution by gavage of one of the following treatments: water (negative control), pure corn oil (solvent control), 8 mg cyclophosphamide/kg body weight (positive control), and flavone diluted in corn oil at the concentrations of 10, 20, or 30 mg/animal. The doses were determined by observing the limit of solubility of the drug [25]. The animals were sacrificed 24 h after the treatments. Two hours before sacrifice, they received 0.3 ml of a 1.0% colchicine solution i.p. (Merck, Darmstadt, Germany). Cytologic preparations were obtained [26] and 100 metaphases per animal were analyzed.

Cytotoxicity was determined on the basis of mitotic index, counting the number of metaphase cells in 1,000 cells per animal.

Statistical Analysis

Data were submitted to analysis of variance by the F test and the means were compared by the Tukey test, with the level of significance set at 5%. Data concerning chromosome alteration counts were transformed to $\sqrt{x + 0.5}$ and percentage data (mitotic index and abnormal metaphases) were transformed to arcsin $\sqrt{\%/100}$ [27].

RESULTS

In Vitro Test With Human Peripheral Blood Lymphocytes

Table I presents the types of chromosome alterations observed in peripheral blood lymphocyte cultures from five individuals (two men and three women), which were treated with the flavone cirsitakaoside at concentrations of 5, 10, and 15 μg/ml 9 h after the beginning of culture. The positive control group treated with doxorubicin (0.1 μg/ml) showed a highly significant increase in frequency of chromosome alterations ($F = 19.11$; $P < 0.01$) and of altered cells ($F = 18.73$; $P < 0.01$), thus validating the data for all parameters analyzed since all analyses were done in a blind test. No significant difference was observed between the negative control group and the DMSO group, with the data obtained agreeing with those expected.

The group treated with the highest concentration of the flavone showed a signifi-

TABLE I. Mean Frequency of Chromosomal Aberrations and Abnormal Metaphases, and Mitotic and Proliferative Indices Observed in Peripheral Blood Lymphocyte Cultures From Five Individuals (Two Men and Three Women) That Were Treated Continously With Different Concentrations of the Flavone Cirsitakaoside†

Treatment			Chromosomal aberrations					CA (%)		AM (%)	
(μg/ml)	MI (%)	PI	G´	G´´	Q´	Q´´	AO	Gaps	No-gaps	Gaps	No-gaps
0	5.02	1.24	21	—	5	2	—	5.6	1.4	4.8	1.2
DMSO (0,375%)	6.06	1.14	19	2	5	—	—	5.2	1.0	5.0	1.0
5	5.98	0.92*	38	2	22	4	1^{DC}	13.4	5.4	11.8	4.8
10	4.92	0.71*	49	—	23	5	1^{EX}	15.6	5.8	14.0	5.2
15	3.42	0.68*	63	2	46	5	2^{EX}	23.6*	10.6*	21.2*	10.2*
DXR	4.56	—	111	4	124	12	2^{TR},1^{R}	50.8*	27.8*	38.4*	25.2*

†*, Differ from controls by the Tukey test at the 5% level of probability; CA, chromosomal aberrations; AM, abnormal metaphases; G´, chromatid gap; G´´, chomosome gap; Q´, chromatid break; Q´´, chromosome break; DC, dicentric; R, ring; TR, triradial figure; EX, exchange; OA, others aberrations; DXR, doxorubicin (0.1 μg/ml).

cant increase compared to the negative control both in terms of frequency of chromosomal aberrations (F = 5.94; $P < 0.01$) and of frequency of abnormal metaphases (F = 6.38; $P < 0.01$). These results are the same when gaps were excluded (F = 4.51; $P < 0.05$ and F = 4.59; $P < 0.05$ to chromosomal aberrations and abnormal metaphases, respectively). The most frequently observed chromosome aberration was cromatid type.

The groups treated with the concentrations of 5 and 10 μg/ml did not differ significantly from controls but also did not differ from the group treated with the highest concentration of the flavone. This result suggests that aberrations tended to increase in cultures treated with the two lowest concentrations and that this increase was dose dependent.

The data concerning the proliferative and mitotic indices are also presented in Table I. A highly significant difference in proliferative index (F = 27.58; $P < 0.01$) was observed between the groups treated with the flavone and the negative and solvent controls. The data also show that the two highest doses did not differ from one another but differed significantly from the lowest dose tested. A negative effect on the progression of the cell cycle was more evident in cultures treated with the doses of 10 and 15 μg/ml. The cytotoxicity of cirsitakaoside was evaluated on the basis of the mitotic indices observed in the different treatments. No significant difference was observed when these values were compared among treatments or between treatments and controls (F = 2.58; $P > 0.05$).

In Vivo Test With *Mus musculus* Bone Marrow Cells

The data concerning the in vivo assays are presented in Table II. The frequencies of chromosomal aberrations and of abnormal metaphases observed in the different treatments did not differ significantly from control when gaps were not considered (F = 1.09; $P > 0.05$ and F = 0.85; $P > 0.05$, respectively), except for the positive control group (F = 31.55; $P < 0.01$). With respect to the number of abnormal metaphases, there was a significant increase (F = 3.02; $P < 0.05$) only for the dose of 20 mg/animal when the gaps were computed. However, the Tukey test revealed that this dose only differed from the solvent control. The absence of cytotoxicity was revealed when statistical analysis demonstrated that the animals submitted to different treatments did not present significantly different indices when compared to controls (F = 1.28; $P > 0.05$).

TABLE II. Mean Frequencies of Chromosomal Aberrations, Abnormal Metaphases, and Mitotic Indice, With and Without Gaps, Observed in the Different Groups of Animals (Three Males and Three Females) Treated Orally for 24 h With the Flavone Cirsitakaoside and in Their Respective Controls[†]

Treatment		Chromosomal aberrations		CA (%)		AM (%)	
(mg/animal)	MI (%)	Gaps	Breaks	Gaps	No-gaps	Gaps	No-gaps
0	1.48	4	4	1.3	0.67	1.2	0.6
Oil	1.38	6	5	1.8	0.83	1.8	0.8
10	1.00	6	7	2.2	1.17	2.2	1.2
20	1.33	11	12	3.8*	2.00	3.7*	1.8
30	1.35	7	10	2.8	1.67	2.5	1.3
CP	1.15	35	74	18.2*	12.3*	13.5*	10.0*

[†]*, differ from controls by the Tukey test at the 5% level of probability; CA, chromosomal aberrations; AM, abnormal metaphases; CP, cyclophosphamide (8 mg/kg b.w.)

DISCUSSION

In the present study we evaluated the flavone cirsitakaoside (5-hydroxy,6-7-dimethoxy-4-glucopyranoside flavone) in terms of its ability to induce DNA damage using two tests systems, in vitro and in vivo.

The data obtained in the in vitro assay showed that cirsitakaoside significantly increased the frequency of chromosome alterations, indicating the mutagenic potential of this flavonoid. However, this clastogenic activity was not observed in the in vivo system.

The mutagenicity of some flavonoids has been well established in prokaryote and eukaryote systems. In a study of the genetic effects of three flavonoids on Chinese hamster ovary cells, the clastogenic effect of quercetin was observed in the absence of metabolic activation and of kaempferol and galangin with and without activation [28]. Some flavonoids such as quercetin, galangin, morine, and myricetin, in addition to having mutagenic effects, also have comutagenic effects, potentiating the mutagenic effect of aromatic amides and amines [29].

The molecular mechanism underlying the mutagenic and carcinogenic effects of flavonoids is little understood. It has been suggested that the ability of some flavonoids to damage DNA may be based on oxidation catalyzed by metals, with the subsequent production of reactive oxygen species such as hydrogen peroxide and hydroxyl radicals [30,31].

However, damage is not restricted to DNA. Studies related that the pro-oxidant activity of quercetin enhances lipid peroxidation, particularly in the presence of Fe^{+3} ions. Formica and Regelson [5] suggested that the pro-oxidant or antioxidant activity of quercetin depended on the redox state of its biological environment. Since in the present study the lymphocytes were cultured under aerobic conditions in medium containing metal ions, we suggest that the chromosome damage observed may have originated from a pro-oxidant effect.

However, it is generally accepted that flavonoids are not potent genotoxic agents in higher organisms and that flavonoids, particularly flavonols and flavones, may have an important antimutagenic and anticarcinogenic activity. Many experimental studies have been conducted on their protective effects [32].

The antimutagenic activity of flavonoids frequently increases with increasing lipophilia by methylation of hydroxyl phenol groups. In addition, it has been established that flavonoids are excellent substrates for the enzyme catechol-O-methyltransferase. Thus, quercetin usually shows mutagenicity in vitro but not in vivo since it is rapidly detoxified by methylation [33].

In addition to the mutagenic effect observed in vitro, a significant reduction in proliferative index was observed in the cultures treated with the flavone cirsitakaoside, although this flavone did not reduce the mitotic index at the concentrations tested in both systems. Other authors also observed a considerable dose-dependent mitotic delay in CHO cultures treated with three flavonoids [28].

The protein tyrosine-kinases play a significant role in the regulation of cell kinetics. Geahlen et al. [34] studied the inhibition of protein tyrosine-kinase activity by flavonoids and reported that the chemical structure of flavones and flavonols has a greater affinity for this protein, thus being more effective in inhibition. Quercetin has been reported to have an anticancer effect on different types of tumors [35–38]. It has been suggested that the antiproliferative effects of quercetin may be due to a specific arrest in the G1 phase of the cell cycle [39].

Certain flavonoids inhibit the proliferation of mitogen-stimulated human peripheral blood lymphocytes. This activity, together with the ability to inhibit lipid peroxidation induced by free radicals and 5-peroxidase activity, may contribute to the antiallergic and anti-inflammatory action of flavonoids [40]. The authors suggested that the antiproliferative action may occur by the inhibition of DNA synthesis.

Thus, the delay in the cell cycle observed in cultures treated with different cirsitakaoside concentrations may be due to the action of the flavone on protein tyrosine-kinase and to inhibition of DNA synthesis. Like quercetin, cirsitakaoside may be used in studies of antitumoral activity due to its ability to delay the cell cycle.

The in vitro effects of cirsitakaoside detected in the present study agree with those obtained in studies on other flavonoids which reported that mutagenic and cytotoxic activity was observed at concentrations of 10 to 60 μg/ml [28]. In the present study, pilot experiments (data not shown) revealed that the dose of 20 μg/ml reduces the mitotic index and that doses above 40 μg/ml fully inhibit cell division.

In the test in vivo, cirsitakaoside was administered by gavage since this is the most common route of human exposure to this compound. According to Preston et al. [25], it is probably most appropriate for predicting effects in humans to utilize a route of exposure that most resembles that anticipated or know to be the route of human exposure. Furthermore, the gastrointestinal tract is one of the most important sites where chemicals are absorbed [41]. Owing to DMSO toxicity observed with the volume necessary to dilute the flavone doses, corn oil was used as solvent.

Evaluation of the in vivo mutagenicity of cirsitakaoside showed that a significant increase occurred only at the dose of 20 mg/animal when gaps were computed. However, the biological meaning of this result is of little relevance since in studies carried out in our laboratory [42] under similar experimental conditions, animals not submitted to any treatment presented a number of breaks close to or even higher than that detected in the present study. In addition, when the means were compared by the Tukey test, the treatment of 20 mg/animal did not differ from the solvent control or from the remaining doses of the flavone, further supporting the absence of an in vivo mutagenic effect of cirsitakaoside.

The absence of mutagenicity and cytotoxicity detected in the in vivo system could be explained by the detoxification occurring during flavonoid metabolism, extensively reported in the literature [43]. In the liver, hydroxyl groups are methylated and glucuronized. In rats, glucoronides are excreted extensively in bile. Glycosides are removed, ring fission by microorganisms occurs in the colon, and the resulting phenolic acids are demethylated and dehydroxylated. Flavonoid residues have been detected in urine and therefore they are not accumulated in the body.

The toxicity of these compounds is very low in animals. In rats, the LD_{50} is 2–10 g/animal for most flavonoids [44]. In the present study, the solubility of the flavone was the limiting factor in the determination of the doses to be tested. Since the animals did not show signs of intoxication or cytotoxicity, the concentrations were increased up to 0.03 g/animal, which was the highest concentration that could be solubilized.

The absorption of flavonoids present in the diet is a problem that has not been fully elucidated. It has been suggested that the flavonoids present in the organism cannot be absorbed by the intestine because they are bound to sugars. Studies reported that there are no enzymes capable of cleaving β-glycoside bonds, with hydrolysis only occurring in the colon, mediated by microorganisms [43,45]. However,

other authors reported that the man absorbs appreciable amounts of quercetin depending on the presence and on the type of sugar in the molecule [4]. However, no data are available about flavonoid metabolism in humans.

Thus, under the conditions tested, cirsitakaoside was able to retard the progression of the cell cycle and showed mutagenicity in the in vitro system but not in the in vivo system at the concentrations tested. We suggest that the absence of mutagenicity of the flavone in mice observed in the present study may have been due to detoxification during metabolism and/or to insufficient absorption to induce clastogenicity. It should also be pointed out that it was impossible to use higher concentrations due to the low solubility of the compound.

Additional information is needed to prevent a possible false negative result, in vivo. In this respect, it should be used other via administration (i.e., intraperitoneal) and multiple exposures of the compound. Further studies are needed to understand the mechanisms involved in the clastogenic action of the flavone cirsitakaoside.

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