



## Assessment of two medicinal plants, *Psidium guajava* L. and *Achillea millefolium* L., in *in vitro* and *in vivo* assays

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### Abstract

The use of medicinal plants by the general population is an old and still widespread practice, which makes studies of their genotoxicity essential. *Psidium guajava* L. and *Achillea millefolium* L. are examples of plants commonly used in popular medicine. *P. guajava* L. is indicated for diarrhea and also as an antiseptic, while *A. millefolium* L. is indicated as an analgesic, antispasmodic, digestive, diuretic, antiseptic, astringent, emollient, wound healer and hemorrhoid medication. The aim of this study was to determine the effects of the infusions of these two plant species on chromosomes and the cell cycle. Leaves from the plants were used to prepare infusions, in the same manner as teas, but at two different concentrations. *Allium cepa* L. root-tip cells (*P. guajava* L. - 2.62 and 26.2 mg/mL, and *A. millefolium* L. - 3.5 and 35.0 mg/mL) and Wistar rat bone marrow cells (*P. guajava* L. - 2.62 and 26.2 mg/100g body weight, and *A. millefolium* L. - 3.5 and 35.0 mg/100g body weight) were used as *in vivo* plant and animal test systems, respectively. Human peripheral blood lymphocytes (*P. guajava* L. - 0.262 and 2.62 µg/mL culture medium, and *A. millefolium* L. - 0.35 and 3.5 µg/mL culture medium) were used as *in vitro* test system. The *P. guajava* L. infusion at the higher concentration caused a statistically significant inhibition of cellular division in the onion root-tip cells, not observed in onion root-tip cells treated with *A. millefolium* L. No statistically significant alterations were found, as compared to untreated controls, in either the cell cycle or the number of chromosome alterations, after treatments with either plant, in rat cells or in cultured human lymphocytes. These results regarding the cytotoxicity and mutagenicity of these plants provide valuable information about the safety of using them as therapeutic agents.

**Keywords:** medicinal herbs, chromosome damage, mutagenicity test.

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### Introduction

The medicinal use of plants is probably as old as Human kind itself. More than 150,000 plant species have been studied, and many of them contain therapeutic substances (Ishii *et al.*, 1984; Hoyos *et al.*, 1992). These substances can be extracted and used in the preparation of drugs, or the plant itself can be used directly as a medication, a practice that is particularly popular in developing countries. However, medicinal plants, and indeed plants in general, synthesize toxic substances, which in nature act as a defense against infections, insects and herbivores, but which often affect the organisms that feed on them. Thus, an assessment of their cytotoxic and mutagenic potential is necessary to ensure a relatively safe use of medicinal plants.

The species *Psidium guajava* L. and *Achillea millefolium* L. are examples of plants commonly used in popular medicine. *P. guajava* L. belongs to the Myrtaceae family and is a twisted trunk. It has green leaves and white flowers. It contains essential oils, rutin, tannins, flavonoids, sesquiterpenic alcohol and triterpenoid acids. Tea made from its leaves or buds is indicated for diarrhea and also as an antiseptic. *A. millefolium* L. belongs to the Asteraceae family and contains aquileic acid, essential oils, tannins, flavonoids and acids. The infusion made from its leaves is indicated as an analgesic, antispasmodic, digestive, diuretic, antiseptic, astringent, emollient, and healing agent, as well as for hemorrhoid treatment (Silva *et al.*, 1995).

In this study, infusions from the leaves of these two plant species, prepared at two different concentrations, were tested for acute mutagenicity *in vivo* in meristematic cells of *Allium cepa* L. root tips and Wistar rat bone marrow cells, and *in vitro* in lymphocytes from human peripheral blood.

## Material and Methods

### Infusions

*Psidium guajava* L. and *Achillea millefolium* L. plants were obtained from the "Irenice Silva" medicinal plant garden of the Maringá State University. The infusions were prepared in the same way as they are usually made, in the quantities indicated below. Fresh *Psidium guajava* L. leaves were placed in a receptacle, covered with boiling water, and left to stand for 10 to 15 min. The infusion was then strained and allowed to cool. The same procedure was carried out for *Achillea millefolium* L. Test solutions of the two plants were prepared at two different concentrations, one like the normally used in teas by the population, and the other ten times more concentrated. The infusions were utilized directly for experiments with *Allium cepa* L. root-tip cells, 1 mL of the infusion per 100 g body weight was administered in the experiments with Wistar rat bone marrow cells, and 0.1 mL of infusion per 10 mL culture medium was utilized in the tests with human lymphocytes. The following final concentrations were used for the treatment of onions: *Psidium guajava* L. - 2.62 and 26.2 mg/mL, and *Achillea millefolium* L. - 3.5 and 35.0 mg/mL; for rats: *Psidium guajava* L. - 2.62 and 26.2 mg/100g, and *Achillea millefolium* L. - 3.5 and 35.0 mg/100g body weight; and for lymphocytes: *Psidium guajava* L. - 0.262 and 2.62 µg/mL, and *Achillea millefolium* L. - 0.35 and 3.5 µg/mL culture medium.

### *Allium cepa* L. root-tip cells

Onions were placed in flasks with aerated water at room temperature to root. The control group was considered as time zero (0 h) until the first root sample was obtained, which served as control for the bulb. This root sample was then placed for 24 h in the treatment solutions, consisting of two concentrations for each of the two plants, while a control group was left in water for the same 24 h. After this time period, a few more roots were removed, and the bulbs were returned to the water, for a further 24 h, to observe if there was recovery from possible damage. The roots were fixed and stained by the Feulgen reaction and mounted on permanent slides.

The slides were analyzed in a blind test, using an optical microscope with 40X objective lens. A thousand cells per bulb were analyzed, totaling 6,000 for control, treatment, and respective recovery. Cells were examined for morphological and structural alterations, and the mitotic index (MI) was determined.

### Wistar rat bone marrow cells

Wistar rats, *Rattus norvegicus*, weighing approximately 100 g b.w. were obtained from the Central Animal House of the Maringá State University. Six animals were used, three males and three females, for each control and treatment group.

The rats were treated *in vivo* by intraperitoneal injection with 1 mL/100 g b.w. of the treatment solutions, prepared at the two concentrations described for each of the two plants. The positive control animals were treated with 1.5 mg /100 g b.w. cyclophosphamide (CP). The animals were sacrificed 24 h later, with colchicine (0.16%) injected intraperitoneally (0.5 mL/100g b.w.) an hour and a half previously. Bone marrow cells were obtained from the rats using the technique described by Ford and Hamerton (1956) and Preston *et al.* (1981; 1987a). Cytogenetic analysis of the slides was performed with a light microscope using a 100x oil immersion lens. One hundred metaphases were analyzed per animal in a blind test, making up a total of 600 metaphases per control and treatment group. The MI was calculated for 5,000 cells per sex, totaling 10,000 cells per group.

### Human lymphocytes

Human peripheral blood lymphocytes were used to prepare cultures for *in vitro* testing. Lymphocytes were obtained from five healthy, male and female individuals, about 20 years of age, apparently free from infection by pathogenic agents, and who had not been under any treatment for the last six months. The cells were incubated for 8 h in 10 mL of culture medium consisting of 80% RPMI 1640 medium (4% phytohemagglutinin) and 20% fetal calf serum, at 37 °C. The cultures were then treated with 0.1 mL of the test infusions, prepared at the two concentrations for each of the two plants. The positive controls were treated with 0.03 µg/mL mitomycin-C. Cultures were incubated for another 64 h and harvested. To each culture 0.05 mL of colchicine solution (0.016 mg/mL) were added 105 min before fixation.

Macrocultures of peripheral human blood lymphocytes were prepared according to Moorhead *et al.* (1960) and Preston *et al.* (1981, 1987b), and used to determine the frequency and distribution of chromosome alterations. One hundred metaphases were analyzed in a blind test from each control and treatment culture, from the five individuals assessed, totaling 500 cells per group. Two thousand cells were calculated for the MI, totaling 10,000 cells per group.

### Statistical analysis

The data were statistically analyzed by the conditional test for the detection of rare events (Pereira, 1991), with the level of significance set at  $\alpha = 0.05$ .

## Results

### *Allium cepa* L. root-tip cells

Table 1 shows the results obtained with the infusion of *Psidium guajava* L.: the total mean MI, the total number of analyzed cells, and the number of cells in each one of the different phases of the cell cycle (interphase, prophase,

**Table 1** - Treatment of *Allium cepa* L. root-tip cells with two different concentrations of *Psidium guajava* L. (*Pg*).

Treatment (mg/mL)	Group	Cell total	MI (%) mean	Number of cells				
				I	P	M	A	T
Control	Co	6000	8.4	5497	236	104	95	68
	Tr	6000	7.8	5528	176	134	95	67
	Re	6000	7.8	5532	253	104	71	40
<i>Pg</i> (2.62)	Co	6000	9.7	5417	341	120	99	23
	Tr	6000	6.1	5635	181	92	68	24
	Re	6000	5.6	5665	140	98	62	35
<i>Pg</i> (26.2)	Co	6000	7.6	5539	272	84	60	45
	Tr	6000	1.1*	5937	24	28	7	4
	Re	6000	5.3	5681	176	70	50	23

I: Interphase; P: Prophase; M: Metaphase; A: Anaphase; and T: Telophase. Co - Control-0 h; Tr - Treatment-24 h; and Re - Recovery-24 h. \*Statistically significant.

metaphase, anaphase and telophase), for each group of six onions, the controls and the treated plants. Table 2 shows the results obtained with *Achillea millefolium* L. plants, for the two concentrations and the recovery.

Only the treatment with *Psidium guajava* L. infusion at the higher concentration gave a result showing a statistically significant difference as compared to the control. Similar data were found when the results obtained with this treatment were compared with the recovery results, indicating that there was a recovery of cell division after 24 h in water. *Achillea millefolium* L. infusion did not produce any statistically significant difference when the results from the treatments were compared with the results of the control and the respective recovery.

Thus, infusions of *Psidium guajava* L. at the lower concentration and of *Achillea millefolium* L. at either concentration did not cause significant suppression of cell division in the *Allium cepa* L. root-tip cells, within the 24-h treatment period. There was a strong, statistically signifi-

cant inhibition of cell division with the higher concentration of *Psidium guajava* L. infusion. However, this anti-mitotic effect cannot have been too drastic, as cell growth recovered after 24 h in water.

#### Wistar rat bone marrow cells

Table 3 shows the results obtained in male and female Wistar rats treated with infusions of *Psidium guajava* L. and *Achillea millefolium* L., at both concentrations: the total mean MI, the total of analyzed metaphases, and the number and types of chromosome alterations, for untreated controls and treated animals.

Infusions of *Psidium guajava* L. or *Achillea millefolium* L., at either of the two concentrations, did not induce a statistically significant increase in the number of chromosome alterations, nor did they alter cell division rates in Wistar rat bone marrow cells, as compared to the results obtained from untreated controls.

**Table 2** - Treatment of *Allium cepa* L. root-tip cells with two concentrations of *Achillea millefolium* L. (*Am*).

Treatment (mg/mL)	Group	Cell total	MI (%) mean	Number of cells				
				I	P	M	A	T
Control	Co	6000	4.4	5736	155	50	27	32
	Tr	6000	3.0	5820	109	43	13	15
	Re	6000	3.5	5791	109	67	17	16
<i>Am</i> (3.5)	Co	6000	3.7	5777	100	48	35	40
	Tr	6000	2.0	5880	57	36	14	13
	Re	6000	2.3	5862	82	33	12	11
<i>Am</i> (35.0)	Co	6000	4.2	5746	130	51	43	30
	Tr	6000	1.2*	5927	47	16	9	1
	Re	6000	3.2	5807	109	45	21	108

I: Interphase; P: Prophase; M: Metaphase; A: Anaphase; and T: Telophase. Co - Control-0h; Tr - Treatment-24 h; and Re - Recovery-24 h. \*Statistically significant.

**Table 3** - Types of cytogenetic alterations in bone marrow cells of Wistar rats treated with two concentrations of *Psidium guajava* L. (*Pg*) and *Achillea millefolium* L. (*Am*).

Treatment (mg/100 g b.w.)	IM (%) mean total	Alteration total (%)	Alteration				Metaphase total
			Gap		Break		
			ct	cr	ct	cr	
Control	1.32	2 (0.3)	0	1	1	0	600
CP	1.32	79 (13.2)*	6	0	46	27	600
<i>Pg</i> (2.62)	1.86	0	0	0	0	0	600
<i>Pg</i> (26.2)	1.99	0	0	0	0	0	600
<i>Am</i> (3.5)	0.90	4 (0.7)	2	0	1	1	600
<i>Am</i> (35.0)	1.40	3 (0.5)	0	0	2	1	600

ct: chromatid; cr: chromosome. CP - 1.5 mg cyclophosphamide/100g body weight (positive control). \*Statistically significant.

### Human lymphocytes

Table 4 shows the results obtained with infusions of *Psidium guajava* L. and *Achillea millefolium* L., at the two concentrations, in human lymphocytes from five individuals, three males and two females: mean total MI values, total of metaphases analyzed, and types and numbers of alterations found in treated cells and in untreated controls.

The infusions of the two plants, at the concentrations studied and within the treatment period used, did not show mutagenic effects nor growth inhibitory effects, as compared to untreated controls. The infusions did not induce chromatid or chromosome breaks or gaps, nor did they significantly increase the incidence of these alterations.

### Discussion

The data obtained in these experiments, two *in vivo* treatments including one animal test system and one *in vitro* treatment with a mammalian cell test system, indicate that the infusions of the *Psidium guajava* L. and *Achillea millefolium* L. plants, at the concentrations tested and used as described above, do not have mutagenic effects on metaphase chromosomes and do not affect cell division. In spite of the strong inhibition of cell division caused by the

higher concentration of both the *Psidium guajava* L. and the *Achillea millefolium* L. infusions in the *Allium cepa* L. root meristem test system, this antiproliferative effect was rather cytostatic than cytotoxic, since there was recovery of cell division after replacement of the plant infusion by water for 24 h.

In this study, the effects of *Psidium guajava* L. and *Achillea millefolium* L. infusions were assessed in three test systems, one plant, one rodent and one human, taking into account that any particular substance may produce different results in different test systems. However, most of the negative results were coincident in the three systems tested. There was also a concern to test extracts prepared in the same way and at the same concentrations as the therapeutic teas used by the population at large, so as to assess the effects of the medicinal plants in the form they are actually administered. Thus, the commonly used concentration was tested and another one, ten times stronger, to assess whether this higher concentration had any toxic or mutagenic effects. An antimetabolic effect was only observed with the higher concentration of both *Psidium guajava* L. and *Achillea millefolium* L. in *Allium cepa* L., and this effect was not irreversible, as cell division recovered within 24 h.

**Table 4** - Types of cytogenetic alterations in human lymphocytes treated with two concentrations of *Psidium guajava* L. (*Pg*) and *Achillea millefolium* L. (*Am*).

Treatment (µg/mL culture medium)	IM (%) mean total	Alteration total (%)	Alteration				Metaphase total
			Gap		Break		
			ct	cr	ct	cr	
Control	2.54	0	0	0	0	0	500
MMC	2.33	72 (14.4)*	6	0	40	26	500
<i>Pg</i> (0.262)	2.63	0	0	0	0	0	500
<i>Pg</i> (2.62)	2.26	1 (0.2)	0	0	1	0	500
<i>Am</i> (0.35)	2.24	1 (0.2)	0	0	1	0	500
<i>Am</i> (3.5)	2.43	2 (0.4)	1	0	1	0	500

ct: chromatid; cr: chromosome. MMC: mitomycin-C 0.03 µg/mL (positive control). \*Statistically significant.

The *Psidium guajava* L. and *Achillea millefolium* L. plants contain tannins, complex chemical substances which are distributed throughout the plants. These tannins protect plants against herbivores and the action of nitrogen-fixing bacteria, besides inhibiting seed germination. They have the property of precipitating proteins, and are used for the tanning of leather and skins. At high doses, tannins can irritate the mucous tissues, and at low doses they can make them impermeable, as they precipitate small quantities of proteins. This can prevent the penetration of damaged mucous tissues by noxious agents, thereby explaining their anti-diarrheal and healing properties. In addition, they are good enzyme inhibitors and act as antidotes for certain types of alkaloids (Martins *et al.*, 1995). Horikawa *et al.* (1994) evaluated the activity of six Chinese medicinal herbs against benzo[*a*]pyrene and found that tannin compounds and catechins were responsible for the inhibition of mutagenicity in *Salmonella*.

The *Psidium guajava* L. and *Achillea millefolium* L. plants contain tannins, which, as effective enzyme inhibitors, may be the compounds in the infusion responsible for the inhibition of cell division in the *Allium cepa* L. root meristem. It is also known that the inhibitory effect of tannins requires continuous exposure of the cells or tissues in question to these substances. This could explain the recovery of cell division after discontinuation of the treatment and the returning of the roots to water for 24 h, thus removing them from the continuous exposure to the tannin-containing test solutions.

*Psidium guajava* L. also contains flavonoids, and, according to Bu-Abbas *et al.* (1996), the high concentration of these substances in green tea, as compared to black tea, may indicate that this component is among those responsible for the antimutagenic, and probably anticarcinogenic, properties attributed to green tea or its fermented products. Ohtsuka *et al.* (1995) examined the effects of nine active compounds of the Chinese medicinal herb sho-saiko-to on the mutagenic effect of AF-2, and found the main active groups to be saponins and flavonoids.

The presence of catequinic components and flavonoids in *Psidium guajava* L. and of tannins found in both *Psidium guajava* L. and *Achillea millefolium* L. could account for the lack of mutagenic effects caused by infusions of these plants at the concentrations studied and in the test systems used here. These compounds have been shown by other investigators to possess antimutagenic effects in a different test system, a property that was not specifically addressed in the present study.

Thus, the results of the present study do not recommend the consumption of tea made of these plants. Should it however be consumed, it is recommended that the tea should be prepared exactly according to the recipe and taken in the prescribed doses, bearing in mind that its prolonged use is not advisable. A long period of rest is recommended between uses of the plants, to allow the person to

recover or to lose the habit, so that the plant can act more effectively without being toxic. Therefore, caution regarding the indiscriminate use of medicinal plants by the population continues to be extremely necessary.

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