EVALUATION OF MUTAGENICITY AND ANTIMUTAGENICITY OF *Ilex paraguariensis* A. ST.-HIL.: AQUIFOLIACEAE INFUSION ON *Allium cepa* ASSAY

Juliana Dourado Grzesiuk[1]*
Marilesia Ferreira de Souza[2]
Luciana Paula Grégio d'Arce[3]

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ABSTRACT: Yerba mate (*Ilex paraguariensis* A. St.-Hil.: Aquifoliaceae) is the basis for a hot drink very common in southern Brazil and other countries such as Argentina, Paraguay and Uruguay: the mate. However, the available data about its effect on DNA are still controversial. In this research, we evaluated the mutagenic and antimutagenic activity of mate with the *Allium cepa* assay, analyzing the frequency of micronucleus (MN), cromossomal aberrations (CA) and the mitotic index (MI) from treatments with mate only and treatments with mate and the positive control Methyl Methanesulfonate (MMS) being administered previously, simultaneously and subsequently. Mate itself did not show mutagenic potential. However, the protocols which MMS was administered simultaneously and subsequently, it potentiated the mutagenic effect of the drug. There was no significant increase in the previous treatment with MMS, indicating that there wasn't a positive or negative influence of the mate on the DNA repair system and other mechanisms for reversing damage on meristematic cells of *Allium cepa*. Therefore, our results suggests that substances present in mate can act potentiating mutagenic and carcinogenic agents present in other compounds or perhaps damaging cells barriers from certain substances that harm the genetic material.
KEYWORDS: Inductors; Promoters; Enhancers; Carcinogens.

AVALIAÇÃO DE MUTAGENICIDADE E DE ANTIMUTAGENICIDADE DA INFUSÃO DE *Ilex paraguariensis* A. ST.-HIL.: AQUIFOLIACEAE NO SISTEMA-TESTE *Allium cepa*

RESUMO: A erva-mate (*Ilex paraguariensis* A. St.-Hil.: Aquifoliaceae) é a base de uma bebida quente muito comum no sul do Brasil e em outros países tais como Argentina, Paraguai e Uruguai. No entanto, os dados disponíveis sobre seu efeito no DNA são ainda controversos. Nesta pesquisa, nós avaliamos a atividade mutagênica e antimutagênica do mate no sistema teste de *Allium cepa,* analisando a frequência de micronúcleos (MN), aberrações cromossômicas (CA) e o índice mitótico (MI) de tratamentos somente com mate e tratamentos com mate e o controle positivo Metil Metano Sulfonase (MMS) sendo administrado anteriormente, simultaneamente e posteriormente. O mate sozinho não apresentou potencial mutagênico. Porém, nos protocolos com MMS sendo administrados simultaneamente e posteriormente, o mate potencializou o efeito mutagênico da droga. Não houve aumentos significativos nos protocolos de tratamento anterior com MMS, indicando que não houve uma influência positiva ou negativa do mate no sistema de reparo de DNA ou outros mecanismos de reversão dos danos nas células meristemáticas da cebola. Desta forma, nossos resultados sugerem que substâncias presentes no mate podem agir potencializando agentes mutagênicos e carcinogênicos presentes em outros compostos ou talvez possam danificar as barreiras celulares a certos compostos que agridem o material genético.
PALAVRAS-CHAVE: Indutores; Promotores; Potenciadores; Agentes carcinogênicos.

Introduction

Ilex paraguariensis A. St.-Hil.: Aquifoliaceae*,* known as yerba mate, has a wide variety of industrial applications and is popular for its consumption as mate – hot drink made from crushed leaves of the plant, which is an old custom in the South Brazil and other countries such as Argentina, Paraguay and Uruguay (GORTARI, 1997). About 80% of the original distribution of mate is in Brazil (WINGE, 1997), and it is the fourth product of non-timber extraction plant with higher income for the country (IBGE, 2006).

Although many of the substances identified in mate suggest beneficial properties – such as stimulant, diuretic, anti-obesity, anti-inflammatory, antioxidant and anti-carcinogenic – the literature brings a lot of controversies about its real effect on DNA (HECK; MEJIA, 2007). Several studies have found, in individuals who usually consume mate, an increase in cases not only of oral, pharyngeal (DE STEFANI et al., 1988), laryngeal (DE STEFANI et al., 1987) and esophageal cancer (DE STEFANI et al., 1990; CASTELLSAGUÉ et al., 2000), but also lung (DE STEFANI et al., 1996), renal (DE STEFANI et al., 1998) and urinary bladder cancer (BATES et al., 2007), what does not allow relation to the high temperature at which the drink is consumed.

In vitro studies also bring discordant results. Wnuk et al. (2009), for example, observed a cytotoxic and genotoxic effect in human lymphocytes treated with yerba mate. On the other hand, Schineller et al. (2000) obtained contrasting results by using in their study a system that produces free radicals. The authors suggest that the extract of Ilex paraguariensis could contribute to the increase in antioxidant defenses of the body. Given the important socio-economic role of mate in the region and the major contradiction of the literature concerning its influence on the stability of genetic material, this study applied the *Allium cepa* assay to evaluate its mutagenic and antimutagenic potential.

This assay is being recognized because of benefits such as good correlation when compared to other systems, including mammals, as well as low cost, easy handling and storage, high sensitivity and excellent conditions for cyto-

[1]Bacharel em Biologia pela Universidade do Oeste do Paraná (UNIOESTE) Departamento de Genética, Laboratório de mutagênese.
[2]Bacharel em Biologia pela Universidade do Oeste do Paraná (UNIOESTE) Departamento de Genética, Laboratório de mutagênese.
[3]Professora na Universidade Estadual do Oeste do Paraná (UNIOESTE) Departamento de Genética, Laboratório de mutagênese.
*Endereço para correspondência: Colegiado de Ciências Biológicas. Rua Universitária, 2069, Bairro Jardim Universitário. Cep. 85819110. Cascavel, PR, Brasil. Phone: +55 045 3035-5596. Fax: +55 045 32203142. E-mail: juli_dourado@hotmail.com

logical evaluation (BAGATINI; SILVA; TEDESCO, 2007). Besides, the method was validated by the International Program on Chemical Safety (IPCS, WHO) and has been recognized internationally as efficient in situ analysis of genotoxicity of environmental substances (CABRERA; RODRIGUEZ, 1999).

Material and Method

Seeds of *Allium cepa* were germinated at room temperature (25º C) in petri dishes with filter paper soaked in distilled water and, after growth of 1 cm, the roots were submitted to different treatments, each of which was performed in triplicate.

In order to compare the results, there were two control groups: negative and positive, respectively treated with distilled water (for 24, 72 and 96 hours) and Methyl Methanesulfonate (MMS, ACROS, Geel, Belgium) for 72 hours at a concentration of 4.10^{-4}M. This is a highly chemotherapy drugs that can interact with DNA, alkyling nitrogen bases.

For the mutagenicity evaluation, the roots were submitted for 24 hours to the mate only, in three different concentrations. On the preparation of the mate was tried to reach the greatest similarity possible to the habit of the beverage. Initially, 40ml of hot water (around 70 º C) was added to 20g of the commercial yerba mate most locally consumed placed on a funnel lined with cheesecloth. Then, 5 portions of 30ml of hot water were added successively and the products obtained from the odd filtering were used in treatments, representing the first, third and fifth bowl of the beverage. This process was repeated for each day of treatment.

The experiments that evaluate the antimutagenic potential occurred through three different protocols:

- Pretreatment: when the root were treated with MMS (24h) before the mate (72h), to evaluate the bio-antimutagenic potential;

- Simultaneous treatment: on this treatment the roots were simultaneously submitted to MMS (72h) and the mate (72h), to evaluate the desmutagenic potential.

- Posterior treatment: also to evaluate the desmutagenic effects of mate, the treatment with MMS (72h) was made only after exposure to the mate (24h).

The mate was also tested at three different concentrations for all treatments described above

After the completion of the treatments, the evaluation procedure was followed and, therefore, slides were prepared for viewing under light microscopy (LM) from the protocol published by Grant (1982) with some modifications adapted by our laboratory.

After fixation for 24 hours in Carnoy (3 ethanol: 1 acetic acid), the roots of *Allium cepa* were washed with distilled water in a petri dish and then they were submitted to hydrolysis with 1N HCL at 60º C for 8 minutes. Again, the roots were washed in a petri dish with distilled water for 5 minutes, then transferred to a dark glass with Schiff and left to react for about 40 minutes, isolated from light. The roots were then washed again with distilled water in petri dishes until the complete withdrawal of the dye. So the meristematic region was cut with a razor blade and over the court, was added a drop of 2% acetic carmine. The cover-slip was placed over the macerated meristem, which is spread mechanically and by heating. Excess of dye was removed with filter paper and then the material could be observed on a microscope at a magnification of 400X.

In the analysis, 5000 cells were counted per treatment, classifying them as normal, carriers of chromosomal aberrations (CA) or carriers of micronuclei (MN). It was also evaluated the mitotic index (MI) for each treatment.

The final data, resulted of the observation on MO, were statistically analyzed by One Way ANOVA test. When differences were detected at a significance level of 0.05 between the groups, the Tukey test was followed, which allows the comparison of treatments with the negative and positive controls, as well as the treatments with each other. Statistical analysis was performed with the software SigmaStat 3.5 (Systat Software, Inc.).

Results

Some of the genetic alterations found are exemplified in Figure 1.

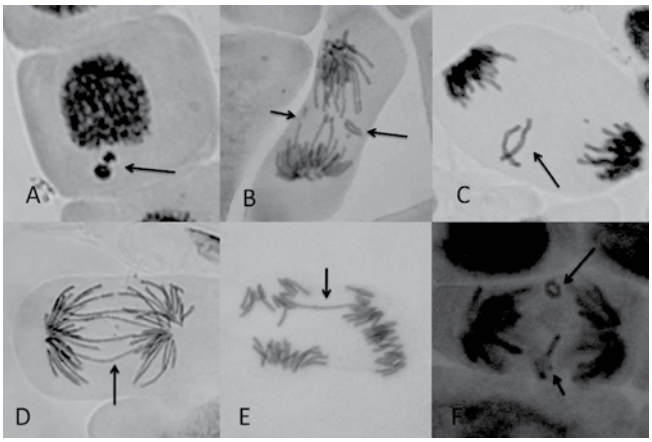

Figure 1: Some of the genetic alterations found on the blades analysis: (A) Micronuclei; (B) Chromosomal break (smaller arrow) and chromosomal loss (bigger arrow); (C) Chromosomal loss; (D) Anaphase bridges; (E) Multi-polar division with anaphase bridge; (F) Chromosomal loss (smaller arrow) and chromosomal ring (bigger arrow).

Analyzing the results of treatments for assessment of mutagenicity, when the roots were exposed only to the mate, there were no significant differences in the frequencies of MN and CA comparing to the negative control (Table 1).

However, the treatments for evaluation of antimutagenicity showed increased frequencies of MN and CA in comparison to the positive control (Table 1). The statistically significant results ($p < 0.05$) from the frequencies of MN was on the posterior treatments - which represented increases of 81.11%, 79.66% and 75.60% respectively to the three concentrations tested (1st, 3rd and 5th bowl) - and simultaneous - which represented increases of 86.88%, 76.81% and 85.11% (Figure 2). Regarding the frequency of AC, only the posterior treatments stood out statistically ($p < 0.05$) with increases of 51.92%, 35.46% and 13.94% respectively to the concentrations (Figure 3).

The mitotic index, in contrast, had a statistically decrease ($p < 0,05$) in all treatments which the MMS was present. The pre-treatments, simultaneous and posteriors obtained similar declines, averaging 47.5%, while in treatments

with only the positive control, the decline was smaller, only 30.48%.

Not in the frequencies of MN and CA, or in the MI, were found statistically significant differences between the concentrations of yerba mate.

Table 1: Averages and standard deviations of the frequencies of micronuclei chromosomal aberrations and cell divisions for 5000 cells analyzed.

Treatments	Micronuclei	Cromossomal aberrations	Cell divisions
Water 24h	0,00067 ± 0,00023[a]	0,00087 ± 0,00011a	0,05713 ± 0,00181a
Water 72h	0,00286 ± 0,00294[a]	0,00113 ± 0,00042a	0,05533 ± 0,01024a
Water 96h	0,00226 ± 0,00202[a]	0,00093 ± 0,00061a	0,05820 ± 0,00592a
1[a] Bowl*	0,00173 ± 0,00080[a]	0,00107 ± 0,00023a	0,04753 ± 0,00528a
3[a] Bowl*	0,00167 ± 0,00090[a]	0,00207 ± 0,00061a	0,05653 ± 0,02022a
5[a] Bowl*	0,00227 ± 0,00080[a]	0,00173 ± 0,00099a	0,04073 ± 0,03117a
MMS	0,10520 ± 0,02390b	0,00527 ± 0,00050b	0,03847 ± 0,00099b
MMS + 1[a] Bowl**	0,19660 ± 0,03121c	0,00580 ± 0,00131b	0,03427 ± 0,01032b
MMS + 3[a] Bowl**	0,18600 ± 0,02259c	0,00527 ± 0,00090b	0,03100 ± 0,00220b
MMS + 5[a] Bowl**	0,19473 ± 0,03881c	0,00527 ± 0,00083b	0,02633 ± 0,00750b
MMS > 1[a] Bowl***	0,12140 ± 0,00360b	0,00587 ± 0,00150b	0,03093 ± 0,00094b
MMS > 3[a] Bowl***	0,14100 ± 0,01720b	0,00393 ± 0,00058b	0,02867 ± 0,00290b
MMS > 5[a] Bowl***	0,12960 ± 0,00840b	0,00507 ± 0,00099b	0,03207 ± 0,00519b
1[a] Bowl > MMS****	0,19053 ± 0,02100c	0,00813 ± 0,00023c	0,03033 ± 0,00234b
3[a] Bowl > MMS****	0,18900 ± 0,02253c	0,00713 ± 0,00076c	0,02900 ± 0,00191b
5[a] Bowl > MMS****	0,18473 ± 0,01763c	0,00600 ± 0,00053c	0,02767 ± 0,00163b

*Different concentrations of mate .
** Simultaneous treatment
*** Pretreatment with Methyl Methanesulfonate (MMS)
****Posterior treatment with Methyl Methanesulfonate (MMS)
Different letters ([a,b,c]) refer to statistically relevant differences.

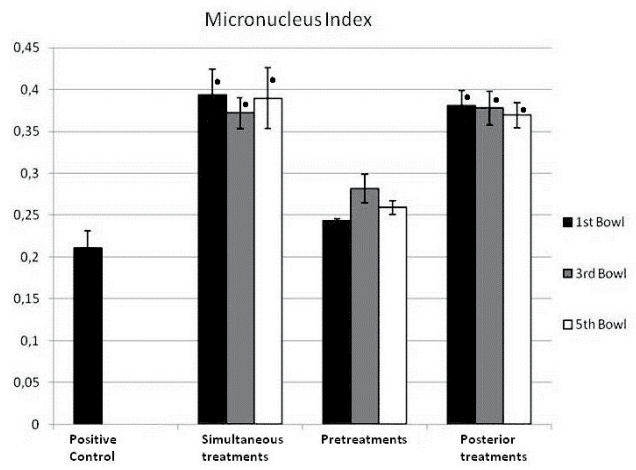

Figure 2: Frequencies of micronuclei observed in the simultaneous treatments, pretreatments and posterior treatments compared to the positive control. • Treatments statistically different from positive control.

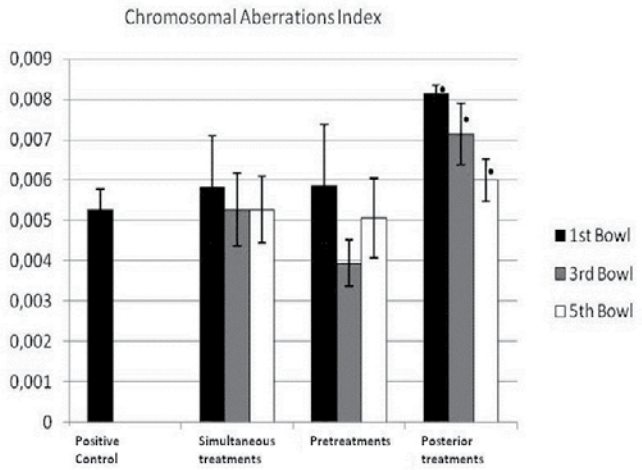

Figure 3: Frequencies of chromosomal aberrations observed in the simultaneous treatments, pretreatments and posterior treatments compared to the positive control. • Treatments statistically different from positive control.

Discussion

The AC and MN have been widely used as markers of DNA damage, since the increase of their frequencies is strong evidence of genotoxicity of the evaluated substance (RIBEIRO, 2003).

Comparing the MN and CA frequencies from the experiments where the roots were only treated with the mate to the treatments with positive and negative controls, there is no evidence of a mutagenic potential. These results agree with the report of Alves et al. (2008) when they used the technique of counting MN in human lymphocytes with their cytokinesis blocked after treatment with yerba mate. The results led them to conclude that mate alone is probably not capable of inducing neither clastogenic nor aneugenic changes on the genetic material, leading to formation of tumors.

In the meantime, the frequencies of MN and CA on the simultaneous and posterior treatments has not decreased compared to the positive control, as expected, but increased. This increase was statistically significant in both treatments, but the increased frequency of AC was statistically significant only in the posterior treatment with MMS. Whereas MN are the manifestations during the interphase of damage to genetic material occurred in the stages of cell division (SALVADORI; RIBEIRO; FECECH, 2003), the high frequency of MN, by itself, indicates indirectly that there was CA on the previous division cycle. However, as the number of mitotic cells observed in this study was much lower compared to cells in interphase, the clearest parameter was the analysis of the frequency of MN. Thus, even if the frequency of AC is not considered as relevant in the simultaneous treatments, they had similar frequencies of MN to the posterior treatments, indicating that there was an influence of the yerba mate above MMS in both treatments.

These results suggest that yerba mate is able to potentiate the effects of certain mutagens, what corroborates with several epidemiological studies, such as the one performed by Bates et al. (2007), who observed a relation between the consumption of yerba mate and the increase of bladder cancer cases only when associated with the smoking habit, concluding that perhaps some substance in the yerba mate acts promoting an exacerbated effect of mutagens present in the cigarette.

Both, tobacco and yerba mate, contribute to the high level of exposure to benzo [a] pyrene in the population of southern Brazil. Fagundes et al. (2006) found in their study increased levels of 1-hydroxypyrene glucuronide in the urine of smokers as well as consumers of mate, which is the main indicator of exposure to polycyclic aromatic hydrocarbons (PAHs). These results suggest that there are factors other than temperature, which are related to an increased risk of esophageal squamous cell carcinoma (EEC), previously found in individuals exposed to mate in southern Brazil. Besides the presence of benzo [a] pyrene, other substances such as tannins and nitrous compounds, have been identified as suspects of potential carcinogens present in yerba mate.

The negative results for increased frequency of MN and CA for the treatments only with yerba mate have shown, however, that in the concentrations studied, their substances are not mutagenic and carcinogenic itself, or are not in sufficient quantity to exert such effects. Furthermore, the non-effect also in the pretreatments indicates no influence, either positively or negatively, from the mate on the reversal of DNA damage on meristematic cells of onion's roots treated with MMS, suggesting that mate doesn't have bio-antimutagenic properties. Thus, our results suggest that substances present in mate can act in the potentiation of mutagenic and carcinogenic agents present in other compounds, or perhaps in the weakening of cells barriers from the entrance of certain substances that harm the genetic material. This hypothesis corroborates with Sewri et al. (2003) studies. The authors report, through epidemiological study, the relation between the increased risk of developing esophageal cancer and consumption of mate alone. However, there was a much higher influence from the mate when its consumption was associated with the smoking habit. So the authors suggest the possibility of yerba mate have in its composition substances that act as a solvent for the carcinogens found in tobacco.

Many other epidemiological studies also found associations between increased frequency of various types of cancer and the consumption of mate alone (DE STEFANI et al., 1990, 1996, 2007). This may be due to the high temperature at which the mate is consumed, perhaps by the factor of exposure of individuals to many other mutagenic agents that may be enhanced by mate, or possibly a combination of these two factors.

Moreover, several other authors observed antioxidant effects of the mate (FILIP et al., 2000; BRACESCO, 2003; BIXBY et al., 2005; BRAVO; GOYA; LECUMBERRI, 2007). Schinella et al. (2000), for example, used a system of free radical production in their study and observed inhibition of enzymatic and nonenzymatic lipid peroxidation in the liver of mice treated with the mate, through a high free radicals scavenging capacity. The authors suggest that this potential is attributed to substances present in the mate, as the alkaloids (caffeine), amino acids, polyphenols (chlorogenic acid) and flavonoids.

Authors such as Da Silva et al. (2009) found a decrease of DNA damage by using as mutagens, substances that produces free radicals. This study, however, used as positive control an alkylating agent of nitrogen bases, MMS, and not a drug capable of generating free radicals. Thus, the effect of sequestration of reactive oxygen metabolites could not be found, which does not exclude the possibility of its existence, even in the work of Bates et al. (2007) and Sewri et al. (2003), which was already mentioned, found relation between the consumption of yerba mate and the increase of cases of urinary bladder and esophagus cancer when associated to smoking habit.

In the smoke produced by burning tobacco are relatively stable free radicals in the particle phase that produces lipid peroxidation and endothelial injury (CHURG; PRYOR, 1985). However, the cigarette is a huge amount of mutagenic and carcinogenic substances that interact with DNA in different ways. Among them are radioactive components, such as lead and polonium, which contribute to the carcinogenic potential of tobacco (CROSS, 1984). In the study by Bates et al. (2007), perhaps the potentiation from mate on the damage caused by mutagens present in cigarette may wad been predominant over the effect of the sequestration of free radicals produced by smoking. Or, in the smoke itself, other types of DNA damage than oxidative stress predominates.

Conclusion

In conclusion, this study found that yerba mate in the form of mate and at similar concentrations to everyday use, interfered in order to enhance the action of the mutagen used (MMS), perhaps by weakening the cells barriers to this mutagen. Because the mate is a complex, composed of various substances, the result obtained in this study does not exclude that it also acts as a potent radical scavenging in the presence of other types of mutagens. However, for clarification on which effect prevails on the systems, more laboratory and epidemiological studies are needed.

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