VEDIC RESEARCH INTERNATIONAL
Phytomedicine
JOURNAL HOME PAGE AT WWW.VEDICJOURNALS.COM
eISSN 2330-0280

RESEARCH ARTICLE
DOI: http://dx.doi.org/10.14259/pm.v2i1.96

Mutagenic And Antimutagenic Effects Of Crude Hydroalcoholic Extract Of Rosemary (*Rosmarinus Officinalis* L.) On Cultured Meristematic Cells *Allium Cepa*.

INGRID FELICIDADE[1,2] JULIANA DIAS LIMA[2], JOÃO RENATO PESARINI[3], ANTÔNIO CARLOS DUENHAS MONREAL[4], MÁRIO SÉRGIO MANTOVANI[5], LÚCIA REGINA RIBEIRO[1], RODRIGO JULIANO OLIVEIRA[3,6,7*]

[1]*Graduate Program in Pathology, Botucatu School of Medicine, São Paulo State University, Botucatu, SP, Brazil.*
[2]*Center for the Studies on Nutrition and Toxicological Genetics, Filadélfia University Centre, Londrina, PR, Brazil.*
[3]*Center for the Studies on Stem Cells, Cellular Therapy and Toxicological Genetics, University Hospital Nucleus, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil*
[4]*Center for Biological and Health Sciences, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil*
[5]*Department of General Biology, Biological Sciences Center, State University of Londrina, Londrina, PR, Brazil.*
[6]*Masters program in Pharmacy, Center for Biological and Health Sciences, Federal University of Mato Grosso do Sul, Campo Grande, MS, Brazil*
[7]*Graduate Program in Health and Development in the Midwest Region, "Dr. Hélio Mandetta" School of Medicine, Campo Grande, MS, Brazil*

Article Info: Received: *December 11th, 2013*; **Accepted:** *December 16th, 2013*

ABSTRACT

Experimental evidence suggests that rosemary has a wide range of biological and pharmacological activities. This study aimed at evaluating the effect of a crude hydroalcoholic extract of rosemary (CHER) on chromosomal aberrations induced by methyl methanesulfonate (MMS) in cultured meristematic cells of *Allium cepa*. The experiments were conducted at three different concentrations of CHER and none showed mutagenic activity. The damage reduction percentage was 65.07, 66.03, and 89.95% for the pre-treatment; 88.04, 93.30, and 84.69% for the simultaneous simple; 75.12, 73.21, and 75.55% for the simultaneous with pre-incubation; and 84.21, 86.60, and 92.82% for the post-treatment. The results indicated that in the antimutagenicity experiments, a combination of these concentrations of CHER with the mutagenic agent MMS indicate that CHER had a chemopreventive effect in cultured *A. cepa* meristematic cells. CHER was effective in reducing DNA damage and is therefore considered chemopreventive and antimutagenic.

Keywords: *Rosemary, crude hydroalcoholic extract; antimutagenic; chemoprevention; methyl methanesulfonate.*

Introduction

Mutations are events that cause alterations in the DNA sequence of an organism. These alterations must be fixed in the genome to be replicated to another cell, by cellular division. Also, mutations can vary from a simple alteration in the sequence of nucleotides to changes in the chromosomal structure/number. All these alterations in DNA correlate with oncogenesis, and thus, low cost trials for preventing mutational events are important.

The first attempt to evaluate tests for mutagenicity in higher plants was conducted by the *U.S. Environmental Protection Agency Genetic Toxicology Program* created in 1982 [1]. In 1991, The *International Programme on Chemical Safety* (IPCS) published the results of the first phase of plant test studies [2]. In 1994, results from the second phase of studies of the IPCS and employees, which evaluated the four most common tests in plants to detect mutagenic effects of environmental pollutants [3], demonstrated that tests in plant systems are reliable and efficient for the quick detection of mutagenic and clastogenic chemical agents [4].

*Corresponding Author
Dr. Rodrigo Juliano Oliveira
Medicine College, Federal University of Mato Grosso do Sul. Cidade Universitária, S/N. Campo Grande, MS – Brasil. CEP: 79070-900. Tele: +55 (67) 3345-3068.
Email: rodrigo.oliveira@ufms.br

Rank & Nielsen [5] recommended that the third phase of testing of studies in plants would be with chemical agents with antimutagenic and anticarcinogenic properties.

The daily use of antimutagenics and anticarcinogenics is the most efficient procedure for the prevention of cancer and genetic diseases. There are several ways in which the actions of mutagenic agents can be reduced or prevented. Thus, chemical agents that influence DNA repair mechanisms or the mutagen's metabolism may be effective antimutagenic substances [6].

Methyl methanesulfonate (MMS) is classified as an alkylating agent, and the damage caused by it involves the transfer of methyl groups to the nitrogenous bases of DNA. This leads to altered base pairing that could introduce transitions, transversions and reading frame changes, in addition to inducing chromosomal breaks, since some alkylating agents, particularly the bifunctional ones, have the capacity to cause intra- and interstrand DNA breaks [7]. Also, according to Rank and Nielsen [5], MMS is an effective chemical for use as a positive control in tests with *Allium cepa*.

Recently, studies have emphasized the beneficial effects of phytochemicals and plant extracts. A wide range of polyphenolic compounds with antioxidant activity were identified in *Rosmarinus officinalis* L., popularly known as rosemary, including diterpene phenolics, in addition to various flavonoids and phenolic compounds [8]. The antioxidants present in rosemary were found to be more potent than α-tocopherol and dibutyl-hydroxytoluene [9], and it has been suggested that these compounds in rosemary have anti-inflammatory properties and chemopreventive action [10, 11], anti-tumor activity [11], and protective effects against H_2O_2 in CaCO-2 cells [12]. Hsieh et al. [13] believe that rosemary is a therapeutic herb with multifunctional properties and that it may be a good adjuvant in the prevention and treatment of diabetes, cardiovascular and neurodegenerative diseases.

Although there are several studies on the properties of rosemary, there is no report on the biological effects of rosemary in a plant test system and no consideration for its mode of antimutagenic action. This study aimed to evaluate the mutagenic and antimutagenic action, along with mode of action, of a crude hydroalcoholic extract of rosemary (CHER), by determining its effect on chromosomal aberrations induced by MMS in meristematic cells of *Allium cepa*.

Materials and Methods

A. Induction of DNA damage

DNA damage was induced with MMS (Acros Organics®; CAS 156890050) at a concentration of 10 µg/mL. Its chemical structure is $C_2H_6O_3S$. MMS was chosen as the damage inducer because it is an alkylating agent; in other words, it adds alkyl groups at various positions of DNA bases [14]. It is known that alkylating agents are able to induce a variety of deleterious modifications of the DNA strands including adducts, cross-links and breaks, and these can be expressed as chromosomal [15] and micronucleus lesions.

B. Chemopreventive agent

The test material was a crude extract of *Rosmarinus officinalis* L. (rosemary). This bush exerts a potent antioxidant activity which is mainly due to polyphenolic components [16]. The concentrations were 4.5, 9.0 and 18.0 µg/mL, according to pilot experiments conducted (data not shown). The extraction process was carried out in accordance with Dias et al. [17], with modifications. Rosemary leaves were collected from the Horto de Plantas Medicinais of Centro Universitário Filadélfia-UNIFIL, Londrina, PR, Brazil. This material was allowed to dry in circulating air (40°C) and ground for use. At the Centro de Estudos em Nutrição e Genética Toxicológica (CENUGEN - UNIFIL), the resulting powder was submitted to dynamic maceration with 70% ethanol (Biotec®; lot: 25348), where 200 g of powdered rosemary was placed in a beaker along with 200 mL of 70 % ethanol. This suspension was mixed using a Fisatom magnetic stirrer (Brazil; Series: 318946; Model: 752A) for a period of 4 hours. This procedure was repeated three times with the same powder. After filtration, the residue was discarded and the solvent evaporated at 40 °C under vacuum (Rotavapor Tuche; Model: RE111), until the removal of all the solvent and then dried (Labconco Dryer; Model: Freeze dryer 8). The extract was re-suspended with distilled water for further use in *Allium cepa* meristematic cell cultures.

C. Experimental design and analysis

Meristematic cells of *Allium cepa* were used as the plant test system. The seeds (Isla®; Lot: 21332A) were allowed to germinate at room temperature in Petri dishes, where they were covered with filter paper soaked with distilled water [18]. They were then subjected to different treatments and protocols, according to Oliveira et al. [19], with modifications, for the evaluation of mutagenicity and antimutagenicity.

To determine mutagenicity, the seeds were cultivated for 120 hours and the treatments carried out were: (a) Control (b) MMS and (c) CHER at three different concentrations. In the Control treatment, seeds were sown for 120 hours in distilled water (3 mL). In MMS treatment, seeds were grown for 72 hours in distilled water (3 mL), then washed twice in distilled water, and subsequently transferred to plates containing 3 mL of and aqueous solution of 10 µg/mL MMS for 48 hours. In the evaluation of CHER effects, the seeds were cultivated for 72 hours in distilled water (3 mL), washed twice in distilled water, and then transferred to three different plates containing 3 mL of aqueous CHER solution at concentrations of 4.5, 9.0 or 18.0 µg/mL, respectively. The treatments were carried out for 48 hours.

For the determination of antimutagenic effect and the mode of action of CHER, the extract was combined with MMS according to the following protocols:

Pre-treatment - the seeds were sown in distilled water (3 mL) for

24 hours; washed twice in distilled water, and transferred to a plate containing CHER solution (3 mL), where they remained for 48 hours. Afterward, the seeds were washed and allowed to grow for another 48 hours in a plate containing MMS solution (3 mL).

Simple simultaneous - the seeds were sown in distilled water (mL) for 72 hours, washed twice with distilled water, and transferred to a culture plate containing 3 mL solutions of MMS and CHER. These seeds were grown in the presence of both solutions for a period of 48 hours.

Simultaneous with pre-Incubation – the aqueous solutions of MMS and CHER were pre-incubated in a 37°C incubator for 1 hour and then transferred to culture plates. The seeds that were germinated in this solution (3 mL) for 48 hours, were previously grown in another Petri plate for 72 hours in the presence of distilled water (3mL). Before transferring the seeds, they were washed twice with distilled water.

Post-treatment - the seeds were cultivated for 24 hours in distilled water (3 mL), then washed twice and transferred to a plate containing MMS solution (3 mL) for 48 hours. Next, the seeds were again washed twice in distilled water and transferred to a plate containing CHER (3 mL).

All treatments and protocols were performed in triplicate. The MMS solutions were always 10 µg/mL, and the concentrations of CHER solutions tested were 4.5, 9.0 and 18.0 µg/mL. After 120 hours of cultivation, the roots were harvested at noon, because of the higher mitotic index at this time, and then fixed in Carnoy's solution (3 ethanol: 1 glacial acetic acid, 3:1) [20] for at least 6 hours. Subsequently, the seeds were submitted to acid hydrolysis in 1 N HCl at 60 °C for 6 minutes and then submitted to Schiff's reagent for 2 hours in the dark. The tips of the seeds were cut with a blade for the extraction of the meristematic region and placed on a slide. A drop of acetic carmin (2%) was later added to intensify the cytoplasmic staining of meristematic cells [18]. The material was then coverslipped, with slight pressure just to provide a better spread of cells on the slide. That pressure was applied delicately so as not to influence the analysis [21]. The coverslip was removed with the aid of liquid nitrogen. The slide with the material was allowed to dry at room temperature for 24 hours, and then coverslipped with a drop of Permout® placed on the biological material. The slides were analyzed on the next day.

A total of 15,000 cells / treatment (1000 cells/slide; 3 replications) were analyzed using a light microscope (Microscope DBG) at 400X magnification. To obtain the mitotic index, the number of cells in the different phases of mitosis (prophase, metaphase, anaphase and telophase) was divided by the total number of cells. To obtain the total frequency of aberrations, the total number of aberrations was divided by the total number of cells. The percent reduction in DNA damage (%RD), suggested by Waters et al. [22], was obtained using the following calculation:

$$\%RD = \frac{\text{Mean of positive control} - \text{Mean of associated group}}{\text{Mean of positive control} - \text{Mean of negative control}} \times 100$$

Statistical analysis was performed by ANOVA/Tukey. The differences were significant when $p < 0.05$.

Results

The main aberrations caused by MMS and observed in this study were anaphase and telophase bridges, breaks, gaps and chromosomalic bridges in prophase, metaphase, anaphase and telophase, metaphase adhesions, nuclear buds, disruptions in anaphase and telophase bridges, mini cells and micronucleus.

The means and standard error referring to the total number of chromosomal aberrations, total frequency of aberrations, mitotic index and percent reduction in DNA damage were determined in the analysis of *Allium cepa* cells exposed to MMS and/or CHER and are shown in Tables 1 to 5.

Statistical analysis of the mutagenicity test results (Table 1) showed that the three concentrations of CHER tested had no mutagenic effect because the mean numbers of chromosomal aberrations found with the three concentrations of CHER were statistically similar to that of Control. The frequency of cells with chromosomal changes ranged from 3.67 to 8.67.

In the protocol of pre-treatment (Table 2), it could be seen that co-treatment with CHER had a chemopreventive effect for all extract concentrations tested and the frequency of damaged cells ranged from 10.67 to 28.00 while percent reduction in DNA damage was 65.07, 66.03 and 89.95% for doses of 4.5, 9.0 and 18.0 µg/mL, respectively. These results demonstrate a dose-response relationship. It was found in simple simultaneous treatment (Table 3) that the three concentrations of CHER were efficient in preventing damage to DNA. The frequency of damaged cells ranged from 8.33 to 14.33. The percent reduction in DNA damage was 88.04, 93.30 and 84.69% for 4.5, 9.0 and 18.0 µg/mL, respectively, and an antimutagenic effect was demonstrated. Although in the protocol of simultaneous with pre-incubation (Table 4), reduction in DNA damage was lower, ranging from 73.21 to 75.55%, it was still evident that there was statistically significant prevention of DNA damage and that it occurred at the three extract concentrations tested. The frequency of cells with chromosomal changes was 73.33, 21.00, 22.33 and 20.00 for treatments with MMS alone and MMS with the three extract doses in ascending order, respectively.

In the post-treatment protocol (Table 5), there were often damaged cells of 14.67, 13.00 and 8.67 for the CHER at concentrations of 4.5, 9.0 and 18.0 µg/mL, respectively, indicating efficiency in the reduction of DNA damage. The percent reduction in DNA damage ranged from 84.21 to 92.82%.

Table 1: Chromosomal aberrations, means and standard error, total frequency of aberrations, and mitotic index determined in 15,000 meristematic cells of *Allium cepa* exposed to MMS and CHER, using the protocol of mutagenicity.

Treatment	Concentration	Chromosomal aberrations									Mean ± SE	FTA (%)	MI (%)
		B	CB	D	L	A	BU	BC	MC	MN			
Mutagenicity													
Control		2	0	0	2	0	1	0	0	6	3.67 ± 0.33[a]	0.07	**1.41[a]**
MMS	10.0 µg/mL	23	7	3	65	0	4	3	2	113	73.33 ± 4.67[b]	1.41	**1.86[a]**
CHER	4.5 µg/mL	11	0	0	2	0	1	1	1	10	8.67 ± 2.67[a]	0.16	**1.43[a]**
	9.0 µg/mL	6	0	2	3	0	0	1	1	2	5.00 ± 1.73[a]	0.10	**1.97[a]**
	18.0 µg/mL	**5**	**1**	**0**	**14**	**0**	**0**	**0**	**0**	**3**	**7.67 ± 3.71[a]**	**0.15**	**2.36[a]**

CHER: crude hydroalcoholic extract from rosemary; SE: standard error; FTA (%): total frequency of aberrations expressed as a percentage; MI (%): mitotic index expressed as a percentage; B: anaphase and/or in telophase with bridges; CB: chromosome breaks in prophase, metaphase, anaphase and/or telophase; D: delayed prophase, metaphase, anaphase and/or telophase; L: chromosome losses in prophase, metaphase, anaphase and/or telophase; A: adherence in metaphase; BU: nuclear bud; BC: bridge collapse in anaphase and/or telophase; MC: mini cell and MN: micronucleus; MMS: aqueous solution of methyl methanesulfonate; CHER: aqueous solution of crude hydroalcoholic extract of rosemary. [a,b]Letters indicate statistically different results (p <0.05, ANOVA/Tukey).

Table 2: Chromosomal aberrations, means and standard error of mean, total frequency of aberrations, mitotic index and percent reduction in DNA damage, determined in 15,000 meristematic cells of *Allium cepa* exposed to MMS and CHER, in the evaluate of the antimutagenicity referring to the protocol of pre-treatment.

Treatment	Concentration	Chromosomal aberrations									Mean ± SE	FTA (%)	MI (%)	%RD
		B	CB	D	L	A	BU	BC	MC	MN				
Antimutagenicity														
Controle		2	0	0	2	0	1	0	0	6	3.67 ± 0.33[a]	0.07	1.41[a]	**NA**
MMS	10.0 µg/mL	23	7	3	65	0	4	3	2	113	73.33 ± 4.67[b]	1.41	1.86[a]	**NA**
CHER+MMS	4.5 µg/mL	12	7	3	32	0	0	3	1	26	28.00 ± 4.04[a]	0.53	3.19[b]	**65.07**
	9.0 µg/mL	15	6	2	28	0	0	2	0	29	27.33 ± 9.35[a]	0.51	4.55[b]	**66.03**
	18.0 µg/mL	**2**	**4**	**1**	**18**	**0**	**0**	**0**	**0**	**8**	**10.67 ± 5.61[a]**	**0.20**	**0.98[a]**	**89.95**

%RD: percent of damage reduction. For abbreviations see legend to Table 1. [a,b]Letters indicate statistically different results (p <0.05, ANOVA/Tukey).

Table 3: Chromosomal aberrations, means and standard error of mean, total frequency of aberrations, mitotic index and percent reduction in DNA damage, determined in 15,000 meristematic cells of *Allium cepa* exposed to MMS and CHER, in the evaluate of the antimutagenicity referring to the protocol of simple simultaneous.

Treatment	Concentration	Chromosomal aberrations									Mean ± SE	FTA (%)	MI (%)	%RD
		B	CB	D	L	A	BU	BC	MC	MN				
Antimutagenicity														
Control		2	0	0	2	0	1	0	0	6	3.67 ± 0.33[a]	0.07	1.41[a]	**NA**
MMS	10.0 µg/mL	23	7	3	65	0	4	3	2	113	73.33 ± 4.67[b]	1.41	1.86[a]	**NA**
CHER+MMS	4.5 µg/mL	7	0	1	15	0	0	0	0	13	12.00 ± 6.03[a]	0.23	2.29[a]	**88.04**
	9.0 µg/mL	7	1	2	4	0	0	0	0	11	8.33 ± 2.67[a]	0.16	2.46[a]	**93.30**
	18.0 µg/mL	**16**	**1**	**3**	**10**	**0**	**0**	**1**	**0**	**12**	**14.33 ± 2.85[a]**	**0.27**	**2.82[a]**	**84.69**

%RD: percent of damage reduction. For abbreviations see legend to Table 1. [a,b]Letters indicate statistically different results (p <0.05, ANOVA/Tukey).

The analysis showed that the mitotic index in the protocol pre-treatment (concentrations 4.5 and 9.0 µg/mL) and simultaneous with pre-incubation (concentrations 4.5, 9.0 and 18.0 µg/mL) showed statistically significant differences. However, the treatments did not show cytotoxicity because there was an increase rather than decrease in the mitotic index that could indicate genetic damage and therefore delay of the cell cycle (Tables 1 and 5).

Because of these results and considering the chemopreventive effects in different protocols, there was a need to check the rates of increase and/or decrease in antimutagenic activity related to the simple simultaneous treatment protocol (Table 6). The calculations were made by dividing the percent reduction in DNA damage for the different treatments in the simple simultaneous protocol by the percent reduction in DNA damage for corresponding treatments in the other protocols, multiplying by 100, and subtracting this product from 100 [19]. The analysis of this table showed that there was an increased reduction in DNA damage with 4.5 and 9.0 µg/mL CHER of 35.30 and 41.30 percentage points, respectively, for the pre-treatment protocol. For the highest dose, there was a decrease in preventive activity and this was -5.85 percentage points. In the pre-incubation protocol there was a decrease of -17.20 percentage points for DNA damage reduction at the lowest concentration tested. For the two higher doses, there was an increase in DNA damage reduction of 27.44 and 12.10 percentage points for concentrations of 9.0 and 18.0 µg/mL, respectively. In the post-treatment protocol, there was an increase for the two lower doses and a decrease for the highest dose. The percentage points were 4.55, 7.74, -8.76 for concentrations of 4.5, 9.0 and 18.0 µg/mL, respectively. In view of these findings, it can be inferred that the antimutagenic activity of rosemary involves both desmutagenic and bioantimutagenic effects. In evaluating the increasing and decreasing rates of activity with the different protocols relative to the simple simultaneous one, there was no evident change that could predict a better desmutagenic or bioantimutagenic action.

Discussion

Tests with *Allium cepa* have been used since the 1930s [23], and according to Rank and Nielsen [5], the sensitivity of tests in plants vary from 82 to 100% compared to the test performed with mammals, and it has been reported that if a chemical agent is capable of causing chromosomal damage in a plant system, this may also correlate to mutagenic events in other organisms such as mammals. Similarly, it can be inferred that if an agent is considered antimutagenic in a test plant, this property can be extended to other organisms. Thus, the *Allium cepa* assay can be considered as a screening method for mutagenic and antimutagenic substances.

This study evaluated the CHER, a promising antioxidant and antimutagenic agent containing a large number of phenolic compounds and some flavonoids, against damage induced by MMS.

MMS is an alkylating agent used in studies of mutagenesis. This monofunctional alkylating agent has a direct action and is considered a weak mutagen [24, 25]. MMS reacts with alkyl nucleophilic molecules such as DNA whose bases are known sites for alkylation, specifically nitrogen (N) and oxygen (O). Purine bases such as adenine (at positions N1, N3 and N7) and guanine (at positions N2, N3 and N7 and O6) are more likely to be alkylated than are the pyrimidine bases. The sites more susceptible to alkylation are N7 of guanine (N7G) and N3 of adenine (N3A). MMS induces about 80% of adducts at N7G, 10% at N3A and only 0.3% at O6-guanine [26]. The N-alkylpurines (N7G and N3A) do not cause pairing errors during replication, but they tend to cause the spontaneous formation of apurinic sites due to the weakening of glycosidic links. The apurinic sites can be mutagenic through the reincorporation of a wrong base. In addition, adducts derived from the ring-opened type in N7G can inhibit replication [27] and constitute a potential threat of mutagenesis.

According to Jenkins et al. [15], O^6-alkylguanine (particularly O^6-methylguanine) seems to be the primary site responsible for mutations and chromosomal changes such as sister chromatid exchange and chromosome breakage. The mechanism for the conversion of these adducts into chromosomal damage is still unclear, but there is one possible model.

O^6-Alkylguanine adducts are not removed before the cell progresses to S-phase, thus the replication of DNA occurs with the transfer of the damage, causing an erroneous pairing, T instead of C. The mismatch repair then attempts to correct this defect by excising the mismatched T, producing DNA strand gaps, which subsequently block the next cycle of DNA replication. The stall that interrupts the replication forks may result in chromosomal aberrations by the induction of double-strand breaks in the DNA helix. With regard to N7G adducts, base excision creates a break in the temporary DNA strand, which tends to lead to double-strand breaks in DNA and chromosome fragmentation. Therefore, these mechanisms may explain some chromosomal aberrations found after exposure to MMS, for example, chromosomal breaks and fragmentation [5]. In view of these reports, there is a need to find chemopreventive agents able to modulate these events and, somehow, prevent the chromosomal changes resulting from exposure to MMS. To assist in the understanding of how this adjustment can occur investigators have used different protocols, which are described in the literature, to understand the antimutagenic action of natural compounds. Thus, any substance able to reduce the frequency of spontaneous or induced mutations, regardless of the mechanism of action, is considered antimutagenic and can thereby be classified as bioantimutagenic or desmutagenic [27, 28].

Bioantimutagens are substances that reduce mutagenesis through effects on the mechanisms of DNA repair or on replication processes. These compounds act after an adduct is formed in DNA, but before the damage results in a mutation. Because these compounds are able to change the process of

Table 4: Chromosomal aberrations, mean, standard deviation of the average, total frequency of aberrations, mitotic index and percentage of reduction of damage to DNA, tested at 15,000 meristematic cells of *Allium cepa* exposed to MMS and the CHER, in the evaluate of the mutagenicity referring to the protocol of simultaneous with pre-incubation.

Treatment	Concentration	Chromosomal aberrations									Mean ± SE	FTA (%)	MI (%)	%RD
		B	CB	D	L	A	BU	BC	MC	MN				
Mutagenicity														
Control		2	0	0	2	0	1	0	0	6	3.67 ± 0.33^{a}	0.07	1.41^{a}	**NA**
MMS	10.0µg/mL	23	7	3	65	0	4	3	2	113	73.33 ± 4.67^{b}	1.41	1.86^{a}	**NA**
CHER+MMS	4.5µg/mL	17	3	3	15	0	0	2	0	23	21.00 ± 7.02^{a}	0.40	2.82^{b}	**75.12**
	9.0µg/mL	16	2	4	20	0	0	0	0	25	22.33 ± 4.67^{a}	0.42	3.19^{b}	**73.21**
	18.0µg/mL	**8**	**4**	**2**	**33**	**0**	**0**	**0**	**0**	**13**	**20.00 ± 3.51^{a}**	**0.37**	**2.90^{b}**	**75.55**

%RD: percent of damage reduction. For abbreviations see legend to Table 1. [a,b] Letters indicate statistically different results (p <0.05, ANOVA/ Tukey).

Table 5: Chromosomal aberrations, mean, standard deviation of the average, total frequency of aberrations, mitotic index and percentage of reduction of damage to DNA, tested at 15,000 meristematic cells of *Allium cepa* exposed to MMS and the CHER, in the evaluate of the mutagenicity referring to the protocol of post-treatment.

Treatment	Concentration	Chromosomal aberrations									Mean ± SE	FTA (%)	MI (%)	%RD
		B	CB	D	L	A	BU	BC	MC	MN				
Mutagenicity														
Control		2	0	0	2	0	1	0	0	6	3.67 ± 0.33^{a}	0.07	1.41^{a}	**NA**
MMS	10.0 µg/mL	23	7	3	65	0	4	3	2	113	73.33 ± 4.67^{b}	1.41	1.86^{a}	**NA**
CHER+MMS	4.5 µg/mL	9	4	2	15	0	0	1	0	13	14.67 ± 1.20^{a}	0.28	2.15^{a}	**84.21**
	9.0 µg/mL	8	3	2	10	1	0	1	0	15	13.00 ± 3.00^{a}	0.16	1.83^{a}	**86.60**
	18.0 µg/mL	**7**	**0**	**0**	**4**	**1**	**0**	**0**	**0**	**14**	**8.67 ± 4.26^{a}**	**0.17**	**1.29^{a}**	**92.82**

%RD: percent of damage reduction. For abbreviations see legend to Table 1. [a,b] Letters indicate statistically different results (p <0.05, ANOVA/ Tukey).

Table 6: Percent increase or decrease in antimutagenic activity of crude hydroalcoholic extract of rosemary for the different treatment protocols compared to protocol simultaneous simple.

Concentration	Protocols		
	Pre-treatment	Simultaneous with pre-incubation	**Post-treatment**
4.5 µg/mL	+35.30	-17.20	**+4.55**
9.0 µg/mL	+41.30	+27.44	**+7.74**
18.0 µg/mL	**-5.85**	**+12.10**	**-8.76**

mutation, they are called true antimutagens [29].

The protective effects of bioantimutagens may occur through (i) increase in DNA replication fidelity, (ii) stimulation of DNA repair [30, 31] and (iii) increase in the repair mechanism's speed of break-induced DNA recombination, thus reducing the number of mutations caused by breaks [32].

On the other hand, desmutagenic substances are able to inactivate a mutagen agent and they are characterized by the action of these compounds directly on the mutagenic agent, or on their precursors, inactivating them chemically or enzymatically [6, 30, 31, 33]. It has also been reported that desmutagenic compounds are all agents that interact with mutagens through mechanisms other than DNA repair or

replication. Focus will be given to the mechanism of desmutagenic activity through enzymatic modulation, because, according to the literature, this mechanism would be more relevant to the substance tested [32].

Enzymatic modulators are agents that prevent the formation of mutagens or their more potent forms [6]. They can act in enzyme systems, through the induction of phase I or II enzymes, or through altering the balance of different enzyme activities [32].

In an attempt to elucidate how the molecules or chemical compounds act on antimutagenicity, it was necessary to use different treatment protocols [34]. In the midst of various protocols proposed in the literature, this work used three: pre-treatment, simultaneous with two variations (simple and with pre-incubation) and post-treatment. Also, Miyasato et al. (2014) [35] and Mauro et al. (2014) [36] have utilized these protocols to demonstrate the mode of action of natural products in the allium cepa assay.

Considering that the simple simultaneous treatment protocol indicates both activities, desmutagenic and bioantimutagenic, the simultaneous with pre-incubation determines desmutagenic activity, and pre-treatment and post-treatment determines bioantimutagenic activity [19, 37]; the analysis of the results suggests that the mechanism of action of the CHER is both desmutagenic and bioantimutagenic. This finding is consistent with the study of Kohlmeier et al. [32], which claims that the flavonoid compounds act in a desmutagenic manner (modulating enzymes) and that polyphenolic compounds act in a bioantimutagenic manner, and also as desmutagens through enzyme modulation, in addition to the antioxidant activity of both types of compounds.

In a more detailed analysis of the results and different protocols, it can be inferred that for the pre-treatment protocol, where the cultures were first exposed to CHER and then to the MMS, the interaction between the compounds, mutagenic and antimutagenic, occurs inside the cell. After exposure of the meristematic cells with the rosemary extract, the bioactive principles (namely flavonoids and polyphenolic compounds) would enter the cell and remain in the cytoplasm, or act on the nucleus, and the interactions between the plant compounds and MMS would then occur in the intracellular milieu, after the treatment with MMS and its uptake by the cell. Thus, for this protocol, two types of changes can be considered: (i) the bioactive principles in CHER can interact directly with MMS, preventing its mutagenic action, and therefore, it would have a desmutagenic activity and/or (ii) the same bioactive principles could modulate the enzyme system by improving the ability to repair the damage induced by MMS already mentioned in this discussion. It is believed that this mechanism of action is the most likely one.

The simple simultaneous treatment can be considered as being very similar to the pre-incubation protocol, except that in this case, the compounds could react in the extracellular as well as intracellular milieu, allowing the influence of desmutagenic activities. However, as some compounds could reach the intracellular environment, MMS could cause DNA damage, while the compounds in rosemary's crude hydroalcoholic extract could modulate the repair system and reverse the damage. Again, these possibilities could explain a bioantimutagenic effect.

In simultaneous treatment with pre-incubation, interaction between the test substances prior to the treatment of root tips, would allow the inactivation of MMS by the bioactive principles in rosemary extracts. Thus, in subjecting the root tips to this exposure, the antimutagenic activity observed is due to a desmutagenic effect, since MMS would have already had its mutagenic activity decreased due to inactivation caused by the rosemary extract compounds.

Lastly, in the post-treatment protocol, it was demonstrated that after DNA damage by MMS exposure, the treatment with the rosemary extract seemed to modulate the repair system so that the percent reduction in DNA damage remained high.

The methodology used here to study mutagenic and antimutagenic mechanisms using different protocols, was also reported by Oliveira et al. [19]. In their study, the authors used the same treatment protocols in mammalian cells, CHO-K1 and HTC, to determine the mechanism of action -glucan, and they found that this compound shows antimutagenic activity as a desmutagen as well as a bioantimutagen. In another study, conducted by the same research group, the authors confirmed the suggestion of the action mechanism using ovary cells from wild-type and in repair-deficient Chinese hamster cells, CHO-K1 and CHO-xrs5, respectively, and thus, they were able to demonstrate that the protocols are truly efficient in determining -glucan's mode of action in view of the damage caused by mutagenic agents, among them being MMS [37].

Another important fact to be reported is that the results suggest a better activity by desmutagen regarding bioantimutagen. This can be better understood when it was analyzed the percentages of damage reduction. In the meantime, it appears that the smaller variations were observed for the post-treatment protocol, and these variations are not very substantial. When analyzing the percentages of reduced damage in the pre-incubation protocol, there is an increase of around 20 percentage points, which indicates that there is a better desmutagenic activity since this protocol is specific for evaluating this mode of action. Even though the pre-treatment protocol can indicate the two modes of action, it produced high rates of increase in the order of up to 40 percentage points and this rate may identify a preferably desmutagenic action. Thus, it is still considered that the protocol with greater efficiency was the pre-treatment one which produced the highest percentages of damage reduction and increased percentage points in antimutagenic activity.

According to Samak-Kincl et al. [38], a decrease in mitotic index by 50%, compared with the control, may be considered

cytotoxic. However, in this search there was no statistically significant decrease relative to the control. On the other hand, what could be observed was an increase in the mitotic index, which besides indicating cytotoxicity could demonstrate a possible action of the rosemary extract stimulating cell division. However, new studies are needed to better clarify this finding.

It is known that oxidative stress may induce the formation of free radicals that cause damage to DNA, and when this damage is not repaired it can lead to mutations and to single- and double-strand breaks in DNA, cross-linking, chromosomal breaks and rearrangements. This potential induction of cancer due to oxidative stress can be prevented or limited by a diet rich in antioxidants [39].

According to Kohmeier et al. [32], polyphenolic and flavonoid compounds have antioxidant properties. The antioxidant activity of the polyphenolic compounds in rosemary is mainly due to the presence of *o*-dihydroxyl groups in their structures and the ability of these groups to donate hydrogen atoms and to neutralize the reactivity of free radicals. [40, 41].

According to some authors, rosemary has anticarcinogenic and antimutagenic activity. The antimutagenic activity of rosemary is attributed, according to Fahin et al. [41], to the presence of phenolic compounds with high antioxidant activity. These authors studied the effect of rosemary on mouse bone marrow cells, using cyclophosphamide as the mutagen. Slamenova et al. [42] also demonstrated rosemary's antimutagenic activity.

In that study, CaCO-2 colon cancer cells and hamster V79 lung cells were used as models for the protective effect of rosemary against oxidative damage to DNA induced by hydrogen peroxide and methylene blue with visible light excitation. DNA damage was assessed using the comet assay, and the results showed that rosemary exhibited a protective effect against DNA oxidative damage.

On the other hand, anticarcinogenic activity was reported by Steiner et al. [43], who indicated that rosemary compounds have an antiproliferative effect against leukemic cells and can cooperate with other anticarcinogenic natural compounds in the inhibition of tumor growth and differentiation of neoplastic cells. Offord et al. [44] studied the molecular mechanism involved in the chemopreventive action of rosemary polyphenolic components using human liver cells and bronchial cell models. These investigators found two mechanisms involved with the anticarcinogenic action of these polyphenolic compounds: (i) inhibition of the metabolic activation of pro-carcinogens catalyzed by P450 cytochrome enzymes in phase I induction of detoxification and (ii) catalyzed by phase II enzymes such as glutathione S-transferase.

Based on the studies discussed above and the results of this research, it can be inferred that the antioxidant activity of rosemary compounds may have favored the potent reduction in DNA damage in all antimutagenic protocols of this study. Therefore, *Allium cepa* meristematic cells could have overcome oxidative stress, caused by the donation of methyl radicals from MMS which might have resulted in the formation of free radicals.

Studies on the anticarcinogenic and antimutagenic effects of rosemary are still recent.

However, the literature mentions that this plant has proven activity in mammalian systems, which suggests that perhaps in the near future rosemary can be used as a functional food capable of preventing DNA damage related to the onset of cancer. Another interesting fact is that if the reduction in DNA damage demonstrated in this plant test system were to be confirmed in mammalian test systems, the former method could have advantages with regard to the speed and low cost for the screening of antimutagenic compounds.

Acknowledgements

This study was supported by the Pro-Rector's Office of Research of Centro Universitário Filadélfia - UNIFIL, Londrina, Paraná state, Brazil. Thanks also go to Miss Isabel Florentino for her help with the extract.

References

1. Constantin MJ, Owens ET: **Introduction and perspectives of plant genetics and cytogenetic assays**. A report of the U.S. environmental Protection Agency Gene-Tox Program. *Mutation Research* 1982, **99**:1-12.
2. Sandhu SS, De Serres FJ, Gopalan HNB, Grant WF, Valemínsky J, Becking GC: **Status report of the International Programme on Chemical Safety's Colaborative Study on plant test systems**. *Mutation Research* 1991, **257**:19-25.
3. Sandhu SS, De Serres FJ, Gopalan HNB, Grant WF, Valemínsky J, Becking GC: **An introduction and study design**. *Mutation Research* 1994, **310**:169-173.
4. Sandhu SS, De Serres FJ, Gopalan HNB, Grant WF, Svendsgaard D, Valemínsky J, Becking GC: **Results and recommendations**. *Mutation Research* 1994, **310**:257-263.
5. Rank J, Nielsen MH: *Allium cepa* **anaphase-telephase root tip chromosome aberration assay on N-methyl-N-nitrosourea, maleic hydrazide, sodium azide, and ethyl methanesulfonate**. *Mutation Research* 1997, **390**:121-127.
6. Ferguson LR: **Antimutagens as cancer chemopreventive agents in the diet**. *Mutation Research* 1994, **307**:395-410.
7. Snustad DP, Simmons MJ: *Fundamentos de Genética* edn 4th. Vila Mariana: Guanabara Koogan; 2008.
8. Del Baño MJ, Castillo J, Benavente-García O, Lorente J,

Martín-Gil R, Acevedo C, Alcaraz M: **Radioprotective-antimutagenic effects of rosemary phenolics against chromosomal damage induced in human lymphocytes by γ-rays**. *Journal of Agricultural and Food Chemistry* 2006, **54**:2064-2068.

9. Leal PF, Braga MEM, Sato DN, Carvalho JE, Marques MOM, Meireles MAA: **Functional properties of spice extracts obtained via supercritical fluid extraction**. *Journal of Agricultural and Food Chemistry* 2003, **51**:2520-2525.
10. Lo AH, Liang YC, Shoie-Yn LS, Ho CT, Lin JK: **Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-kB in mouse macrophages**. *Carcinogenesis* 2002, **23**:983-991.
11. Peng CH, Su JD, Chyau CC, Sung TY, Ho SS, Peng CC, Peng RY: **Supercritical fluid extracts of rosemary leaves exhibit potent anti-inflammation and anti-tumor effects**. *Bioscience, Biotechnology, and Biochemistry* 2007, **71**:2223-2232.
12. Aherne SA, Kerry JP, O'Brien NM: **Effects of plant extracts on antioxidant status and oxidant-induced stress in Caco-2 cells**. British *Journal of Nutrition* 2007, **91**:321-328.
13. Hsieh CL, Peng CH, Chyau CC, Lin YC, Wang HE, Peng RY: **Low-density lipoprotein, collagen, and thrombin models reveal that** *Rosemarinus officinalis* **L. exhibits potent antiglycative effects**. *Journal of Agricultural and Food Chemistry* 2007, **55**:2884-2891.
14. Oga S, Camargo MMA, Batistuzzo JAO: *Fundamentos de toxicologia* edn 3rd. São Paulo: Atheneu; 2008.
15. Jenkins GJS, Doak SH, Johnson GE, Quick E, Waters EM, Parryl JM: **Do dose response thresholds exist for genotoxic alkylating agents?**. *Mutagenesis* 2005, **20**:389-398.
16. Huang MT, Ho CT, Wang ZY, Ferraro T, Lou YR, Stauber K, Ma W, Georgiadis C, Laskin JD, Conney AH: **Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid**. *Cancer Research* 1994, **54**:701-708.
17. Dias PA, Foglio MA, Possenti A, Carvalho JE: **Antiulcerogenic activity of crude hydroalcoholic extract of** *Rosmarinus offinalis* **L.**. *Journal of Ethnopharmacology* 2000, **69:**57-62.
18. Fernandes TCC, Mazzeo DEC, Morales MAM: **Mechanism of micronuclei formation in polyploidizated cells of** *Allium cepa* **exposed to trifluralin herbicide**. *Pesticide Biochemistry & Physiology* 2007, **88:**252-259.
19. Oliveira RJ, Ribeiro LR, Silva AF, Matuo R, Mantovani MS: **Evaluation of antimutagenic activity and mechanisms of action of β-glucan from barley, in CHO-k1 and HTC cell lines using the micronucleus test**. *Toxicology in Vitro* 2006, **20**:1225-1233.
20. Matsumoto ST, Mantovani MS, Malagutti MIA, Dias AL, Fonseca IC, Marin-Morales MA: **Genotoxicity and mutagenicity of water contaminated with tannery effluents,as evaluated by the micronucleus test and comet assay using the fish** *Oreochromis niloticus* **and chromosome aberrations in onion root-tips**. *Genetics and Molecular Biology* 2006, **29**:148-158.
21. Correia TFVBC, Maschio LR, Marin-Morales MA, Azeredo-Oliveira MTV. Indução de aberrações cromossômicas em sistema-teste de Allium cepa pela ação do Cádmio (Cd). http://web2.sbg.org.br/congress/sbg2008/pdfs2008/23998.pdf (Accessed on November 29 2013).
22. Waters MD, Brady AL, Stack HF, Brockman HE: **Antimutagenicity profiles for some model compounds**. *Mutation Research* 1990, **238**:57-85.
23. Levan A: **Effect of colchicine on root mitosis in** *Allium*. *Hereditas* 1938, **118**:49-53.
24. Moore MM, Parker L, Huston J, Harrington-Brock K, Dearfield KL: **Comparison of mutagenicity results for 9 compounds evaluated by HGPRT locus in standard and suspension CHO assays**. *Mutagenesis* 1991, **6**:77-85.
25. Tao KS, Urlando C, Heddle JA: **Mutagenicity of methylmethanesulfonate** *in vivo* **at the dlb-1 locus and a lac I transgene**. *Environmental and Molecular Mutagenesis* 1993, **22:**293-296.
26. Beranek DT: **Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents**. *Mutation Research* 1990, **231**:11-30.
27. O'Connor TR, Boiteax S, Laval J: **Ring-opened 7-methylguanine residues are a block to** *in vitro* **DNA synthesis**. *Nucleic Acids Research* 1988, **16**:5879-5894.
28. Grüter A, Friederich U, Würgler FE: **Antimutagenic effects of mushrooms**. *Mutation Research* 1990, **231**:243-249.
29. Clarke CH, Shankel DM: **Antimutagenesis in microbial systems**. *Bacteriological Reviews* 1975, **39**:33-53.
30. De Flora S, Ramel C: **Mechanisms of inhibitors of mutagenesis and carcinogenesis. Classification and overview**. *Mutation Research* 1988, **202**:285-306.
31. Hartman PE, Shankel DM: **Anti-mutagens and anticarcinogens: a survey of putative interceptor molecules**. Environmental and Molecular Mutagenesis 1990, **15**:145-182.
32. Kohlmeier L, Simonsen N, Mottus K: **Dietary modifiers of carcinogenesis**. *Environmental Health Perspectives* 1995, **103**:77-184.
33. Kada T, Shimoi K: Desmutagens and bio-antimutagens – their modes of action. *Bioessays* 1987, **3**:113-116.
34. Flagg EW, Coates RJ, Greenberg RS: **Epidemiologic studies of antioxidants and cancer in humans**.

Journal of the American College of Nutrition 1995, **14**:419-427.

35. Miyasato LESF, Formagio AS, Auharek AS, Kassuya CAL, Navarro SD, Cunha-Laura AL, Monreal ACD, Vieira MC, Oliveira RJ: **Antigenotoxic and antimutagenic effects of Schinus terebinthifolius Raddi in Allium cepa and Swiss mice: a comparative study**. *Genetics and Molecular Research* 2014, *in press*.
36. Mauro MO, Pesarini JR, Marin-Morales MA, Monreal MTFD, Monreal ACD, Oliveira RJ: **Evaluation of antimutagenic activity and mode of action of fructooligosaccharide inulin in meristematic cells of *Allium cepa* culture**. *Genetics and Molecular Research* 2014, *in press*.
37. Oliveira RJ, Matuo R, Silva AF, Matiazi HJ, Mantovani MS, Ribeiro LR: **Protective effect of β-glucan extracted from *Saccharomyces cerevisiae*, against DNA damage and cytotoxicity in wild-type (k1) and repair-deficient (xrs5) CHO cells**. *Toxicology in Vitro* 2007, **21**:41-52.
38. Smaka-Klincl V, Stegnar P, Lovka M, Toman MJ. **The evaluation of wastes, surface and ground water quality using the *Allium* test procedure**. *Mutation Research* 1996, **368**:171-179.
39. Masuda T, Inaba Y, Takeda Y: **Antioxidant mechanism of carnosic acid: Structural identification of two oxidation products**. *Journal of Agricultural and Food Chemistry* 2001, **49**:5560-5565.
40. Zhang HY: **Selection of theoretical parameter characterizing scavenging activity of antioxidants on free radicals**. *Journal of the American Oil Chemists' Society* 1998, **75**:1705-1709.
41. Fahim FA, Esmat AY, Fadel HM, Hassan KF: **Alied studies on the effect of *Rosmarinus officinalis* L. on experimental hepatotoxicity and mutagenesis**. *International Journal of Food Sciences and Nutrition* 1999, **50**:413-427.
42. Slamenova D, Kuboskova K, Horvathova E, Robichova S: **Rosemary-stimulated reduction of DNA strand breaks and FPG-sensitive sites in mammalian cells treated with H_2O_2 or visible light-excited Methylene Blue**. *Cancer Letters* 2002, **177**:145-153.
43. Steiner M, Priel I, Giat J, Levy J, Sharoni Y, Danilenko M: **Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D3 and retinoic acid**. *Nutrition and cancer* 2001, **41**:135-144.
44. Offord EA, Macé K, Avanti O, Pfeifer AM: **Mechanisms involved in the chemoprotective effects of rosemary extract studied in human liver and bronchial cells**. *Cancer Letters* 1997, **114**:275-281.

Visit us @ www.vedicjournals.com : DOI: http://dx.doi.org/10.14259/pm.v2i1.96

Authors Column