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Article

Chromatographic analysis and antiproliferative potential of aqueous extracts of *Punica granatum* fruit peels using the *Allium cepa* test

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Punica granatum L., locally known as romanzeira, is native to Asia but found throughout Brazil. *P. granatum* is used for treating inflammatory, infectious and respiratory diseases. The aim of this study was to evaluate the chromatography and genotoxicity of an aqueous extract of *P. granatum* (pomegranate) fruit peel using the *Allium cepa* L. test. The experiment set-up entailed 7 treatments: T1-distilled water, T2-tea 5 $g.L^{-1}$, T3-tea 10 $g.L^{-1}$, T4-glyphosate at 9.6%, T5-glyphosate with subsequent recovery in distilled water, T6-glyphosate with subsequent recovery in tea 5 $g.L^{-1}$ and T7–glyphosate with subsequent recovery in tea 10 $g.L^{-1}$. The rootlets were collected and fixed in ethanol:acetic acid (3:1) for 24 hours, then stored in 70% ethanol under refrigeration. Analysis was performed using high performance liquid chromatography for the quantification of the extracted phenolic compounds. Gallic acid, catechin, caffeic acid, and rutin were abundant in the extracts of *P. granatum*. The extracts were found to exhibit antiproliferative potential but not antimutagenic or genotoxic activity.

Uniterms: *Punica granatum* L./phytochemistry. *Punica granatum* L./genotoxicity *Punica granatum* L./chromatography analysis. Medicinal plants. Pomegranate.

Punica granatum L., conhecida como romanzeira, é originária da Ásia e encontra-se distribuída por todo Brasil. É usada para o tratamento de doenças inflamatórias, infecciosas e respiratórias. Em decorrência da grande utilização de recursos fitoterápicos, é necessário esclarecer à população sobre a grande quantidade de substâncias existentes nas plantas e sobre os benefícios e prejuízos de tais substâncias à saúde. O presente trabalho objetivou realizar a análise cromatográfica e o estudo da genotoxicidade dos extratos aquosos das cascas dos frutos de *P. granatum* através do teste de *Allium cepa* L. Para a montagem do experimento, foram utilizados 7 tratamentos: T1-água destilada, T2-chá 5 $g.L^{-1}$, T3-chá 10 $g.L^{-1}$, T4-glifosato a 9,6%, T5-glifosato para recuperação em água destilada, T6-glifosato para recuperação em chá 5 $g.L^{-1}$ e T7-glifosato para recuperação em chá 10 $g.L^{-1}$. As radículas foram coletadas e fixadas em etanol:ácido acético (3:1) por 24 horas, e armazenadas em álcool 70%, sob refrigeração. Realizou-se análise por cromatografia líquida de alta eficiência para quantificação dos compostos fenólicos. Nos extratos de *P. granatum* foram observados em maior quantidade: ácido gálico, catequina, ácido cafeico e rutina. Além disso, os extratos demonstraram potencial antiproliferativo, sem apresentar atividade antimutagênica e genotóxica.

Unitermos: *Punica granatum* L./fitoquímica. *Punica granatum* L./genotoxicidade. *Punica granatum* L./análise cromatográfica. Plantas medicinais. Romã.

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INTRODUCTION

Punica granatum L. belongs to the family Punicaceae and is commonly known as pomegranate. It originates from Asia but can be found growing throughout Brazil (Braga, 1961) and is cultivated around the world in regions with tropical and subtropical climates. It is used as an ornamental plant, as a fruit, and for its medicinal properties (Corrêa, 1978).

According to Garcia (1992), the species has anti-inflammatory and antibacterial properties. Naqyi *et al*. (1991) evaluated the antimicrobial activity of an extract of *P. granatum* on gram-positive bacteria and yeast, finding antimicrobial active ingredients in the fruit peels. In addition, *Streptococcus mutans*, *S. mitis*, and *S. sanguis* were highly sensitive to pomegranate extract in terms of growth and capacity to adhere to dental surfaces (Pereira, 1998).

The Middle East, India and China have used *P. granatum* for centuries in folk medicine to treat a number of ailments, such as: inflammation, rheumatism, as well as throat pain. However, the most widespread use of this plant is as a vermifugal or taenicidal agent (Zhicen, 1987; Kapoor, 1990), i.e. it expels intestinal worms (Arun & Singh, 2012). According to Arun and Singh (2012), Chinese and South Africans use the pericarp to treat diarrhea, metrorrhagia, metrostaxis, and stomachache. Besides, its flowers are used as a food supplement for the treatment of diabetes mellitus in Unani medicine.

Recently, studies have shown cytotoxic activities in extracts from different parts of *P. granatum* in a series of tumor cell subtypes (Jeune *et al*., 2005). Some studies show that the fruit juice, besides retarding oxidation and the synthesis of prostaglandins, can inhibit tumor cell proliferation, reduce tumor invasion, promote apoptosis, and inhibit vessel formation in the *in vitro* model of the chorioallantoic membrane (Toi *et al*., 2003).

Natural health practices, such as herbal medicine, are becoming increasingly widespread, principally due to high costs and frequent collateral effects of traditional allopathic medicine (Alvin, 1997). Factors that drive people to adopt alternative therapies are the high price of medicine and private health care, poor health services, and the search for a more natural treatment without side-affects (Gomes, 1985; Santos, 1984). Therefore, there is a need to inform the population about the large quantity of substances present in plants and about the benefits and damage to human health, since popularly these herbs are regarded as a free source of chemical products that have no side-effects (Bragança, 1996).

P. granatum is generally considered safe since it is widely consumed by numerous populations in many different countries. However, toxic effects have been attributed to consuming this plant, including acute inflammation, internal organ congestion with elevated creatine levels *in vivo* (Vidal *et al*., 2003), allergic reactions, and even death (Gaig *et al*., 1999; Hegde *et al*., 2002; Igea *et al.,* 1991; Lansky, Newman, 2007).

Studies on toxicity and mutagenicity are essential, contributing to the safe and efficient use of medicinal plants. The mitotic index and replication index are used as indicators of adequate cell proliferation (Gadano *et al*., 2002), and can be measured with the *Allium cepa* L. plant test. The chromosomal aberration method in *A. cepa* roots has been validated by the International Programme on Chemical Safety (IPCS, WHO) and the United Nations Environment Programme (UNEP) as an efficient test for the *in situ* monitoring of genotoxic environmental substances (Cabrera, Rodriguez, 1999; Silva *et al*., 2004).

The chromatographic profile of a plant extract can be considered representative of the chemical complexity of the samples, limited by the specific method used and the limitations of the detector. Several studies in the literature cite the use of this chromatographic profile as an analytical method that can evaluate the relationship between chemical information and the characteristics of each plant sample, such as the differentiation among similar botanical species, the variability among plants collected in different geographic locations and under different climatic and culture conditions (Chen *et al*., 2009). Lansky and Newman (2007) found flavonoids (apigenin and narigenin), anthocyanins, tannins (gallic and ellagic acids), alkaloids, ascorbic acid, conjugated fatty acids (punic acids), and ursolic acids to be important substances in *P. granatum*.

The aim of the present study was to analyze the genotoxicity of aqueous extracts of fruit peels of *P. granatum* (pomegranate) using the *A. cepa* test and to identify the chromatographic profile of the extracts studied.

MATERIAL AND METHODS

Preparation of decoctions

Fruit sampling of *P. granatum* was undertaken during April/2011, in Tupanciretã, Rio Grande do Sul, Brazil. A voucher specimen was deposited in the Herbarium SMDB (Santa Maria Dept. of Biology) of the UFSM, under the number 13.964. Fruit peels were placed to dry at room temperature. Tea preparation was carried out during October/2012 in decoction of water for 10 minutes. The teas were used both for the experimental set-up with

A. cepa and for the chromatographic analysis using high performance liquid chromatography (HPLC-DAD).

Allium cepa test

Seven groups of 5 *A. cepa* bulbs were used, all of which were placed to germinate in distilled water. After root emergence, the bulbs were allocated to the respective treatments: T1- distilled water (negative control), T2-tea 5 $g.L^{-1}$, T3-tea 10 $g.L^{-1}$, T4-glyphosate at 9.6% (positive control), T5-glyphosate then recovery in distilled water, T6-glyphosate then recovery in tea 5 $g.L^{-1}$, and T7-glyphosate then recovery in tea 10 $g.L^{-1}$. The teas were prepared at the concentration normally used in Brazil (5 $g.L^{-1}$) and also at twice this concentration (10 $g.L^{-1}$).

The bulbs were placed for 24 hours with roots submerged in the treatments. After 24 hours in glyphosate, treatments T5, T6, and T7 were placed for recovery in distilled water, tea 5 $g.L^{-1}$, and tea 10 $g.L^{-1}$, respectively for a further 24 hours, for recoveries of the possible changes caused by glyphosate. After the end of each treatment, the rootlets were collected and preserved in ethanol: acetic acid (3:1) for 24 hours. Subsequently, the rootlets were placed in ethanol 70% and kept refrigerated (4 ºC) until slide preparation for counting and evaluation of the material.

Two slides per bulb were prepared for each treatment, using 2 rootlets per bulb, thus giving 1 rootlet per slide. The rootlets were hydrolyzed in HCl 1 N for 5 min, washed with distilled water, and stained with acetic orcein 2% (Guerra, Souza, 2002); the meristematic region was squashed using a small glass rod over the material placed under the coverslip.

Five hundred of cells per bulb were analyzed, 250 per slide, thus giving 2500 total cells per treatment and 17,500 total cells altogether. The slides were scored blind using a LEICA light microscope at 400X, observing the phases of the cell cycle: interphase, prophase, metaphase, anaphase, and telophase. The mitotic index (MI) was determined from these results.

Chromatographic analysis by High Performance Liquid Chromatography (HPLC-DAD)

Chemistry, equipment and general procedures

All the chemical reagents were of analytical grade, where methanol, acetic acid, gallic acid, chlorogenic acid, and caffeic acid were acquired from Merck® (Darmstadt, Germany) and catechin, quercetin, rutin, and kaempferol from Sigma-Aldrich® (St Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was carried out with a HPLC system (Shimadzu, Kyoto, Japan) and auto-injector Shimadzu (SIL-20A), equipped with alternative pumps (Shimadzu LC-20AT) linked to a degasifier (20A5 DGU) with an integrator (CBM 20A), diode array detector (SPD-M20A) and software (LC solution SP1 1.22).

Quantification of compounds by HPLC

Chromatographic analyses were undertaken in reverse phase under gradient conditions using a C18 column (4.6 mm x 150 mm) filled with 5 µm-wide particles. The mobile phase used was water containing 2% acetic acid (A) and methanol (B), and the gradient composition was: 5% (B) for 2 min, 25% (B) up to 10 min, 40, 50, 60, 70, and 80% (B) every 1 0min thereafter, following the method described by Laghari *et al*. (2011) with some modifications. The decoctions of *P. granatum* were analyzed at 5 and 10 $g.L^{-1}$. The flow used was 0.6 mL min^{-1}, injection volume of 50 µL, and the wavelength 271 nm for gallic acid, 280 nm for catechin, 327 nm for caffeic and chlorogenic acid, and 365 nm for quercetin, rutin, and kaempferol. The samples and mobile phase were filtered through a 0.45 µm (Millipore) membrane filter and subsequently degasified by an ultra-sound bath before use. Reference solutions were prepared in the mobile phase for HPLC at concentrations of 0.05-250 mg mL^{-1} for catechin, quercetin, rutin, and kaempferol; and at 0.2-200 mg mL^{-1} for gallic, chlorogenic, and caffeic acids. The chromatographic peaks were confirmed by comparing their retention times with the reference standards and by DAD spectra (200 to 600 nm). The calibration curve for gallic acid was: $Y = 13569x + 134.9$ ($r = 0.9995$), catechin: $Y = 10932x + 1258.0$ ($r = 0.9987$), chlorogenic acid: $Y = 12573x + 1206.5$ ($r = 0.9997$), caffeic acid: $Y = 11872x + 1570.3$ ($r = 0.9996$), quercetin: $Y = 13620x + 1337$ ($r = 0.9996$), rutin: $Y = 15983x + 1321.5$ ($r = 0.9998$), and kaempferol: $Y = 16423x + 1853.2$ ($r = 0.9998$). All the chromatographic operations were carried out at room temperature and in triplicate.

Statistical analysis

Comparisons between the mitotic indices as well as the chromosomal alterations were carried out by the Chi-square (χ^2) test using the BIOESTAT 5.3 program (Ayres, 2007). The HPLC-DAD chromatographic analysis was tested using the Tukey test with $p < 0.05$.

RESULTS AND DISCUSSION

Allium cepa test

Plant test systems, such as *A. cepa*, have been

previously used for studying the effect of plant extracts and for detecting genotoxicity (Teixeira *et al*., 2003; Fachinetto *et al.*, 2007). In the present study, this system was used to evaluate the genotoxic potential of the aqueous extracts of *P. granatum*.

The mitotic index (MI) varied across the treatments: from 7.32%, for the negative control in water, to 0.84% for the aqueous extract of *P. granatum* at 10 $g.L^{-1}$ (Table I). Between controls (negative and positive), the decrease in MI was significant (χ^2=95.980), where distilled water and glyphosate presented MI=7.32% and MI=1.16%, respectively (Table I). Similarly, the control in water also differed significantly from the aqueous extracts of *P. granatum*, both at 5 $g.L^{-1}$ (χ^2=116.821) and at 10 $g.L^{-1}$ (χ^2=134.119). These results indicate a decrease in the mitotic index, which gradually increases with concentration of pomegranate peels, demonstrating their antiproliferative activity.

Other studies have also revealed this type of activity in aqueous extracts of plant species used in folk medicine. According to Fachinetto *et al*. (2007), the mitotic indices found for the infusions of *Achyrocline satureioides* Lam. decreased compared to the control. The same was observed in *Artemisia verlotorum* Lamotte by Souza *et al*. (2010). Bagatini *et al*. (2009), while studying *Solidago microglossa* D.C., found antiproliferative activity in the extract with the highest concentration compared to the negative control.

The *A. cepa* test can be considered a bioindicator for monitoring the genotoxic potential of several chemical compounds. Chauan *et al*. (1999) used meristematic cells of *A. cepa* to study the clastogenic potential of the pesticides cypermethrim and fenvalerate and obtained a good correlation with the test in mammals. Besides this, other studies have been carried out using both plant and animal test systems in conjunction, showing correlations of 75-91.5% (Grant, 1978; Grant, 1982; Grover *et al*., 1990).

In the roots treated with aqueous extracts of *P. granatum,* chromosomal alterations were observed (Table II), such as chromosomal breakages (Figure 1) at 5 $g.L^{-1}$ and micronuclei (Figure 2) at 10 $g.L^{-1}$. The number of alterations at 5 and 10 $g.L^{-1}$, respectively, was not significant compared to the control in water:

TABLE I – Treatments with number of cells observed in different *Allium cepa* cell cycles

Treatments	Number of Total Cells	Interphase	Cells in Division	Prophase	Metaphase	Anaphase	Telophase	MI%
Water (- ctrl)	2500	2317	183	86	34	14	49	7.32^{a}
Tea 5 g/L	2500	2471	29	23	3	1	2	1.16^{c}
Tea 10 g/L	2500	2479	21	15	2	0	4	0.84^{c}
Glypho (+ ctrl)	2500	2460	40	8	26	0	6	1.16^{bc}
Glypho + water	2500	2435	65	28	13	0	24	2.6^{bde}
Glypho + tea 5 g/L	2500	2459	41	20	11	5	5	1.64^{ce}
Glypho + tea 10 g/L	2500	2477	23	15	1	0	7	0.92^{c}

Means followed by the same letter do not differ significantly at the 5% level, according to the χ^2 test. Critical χ^2= 11,0705.

TABLE II – Chromosomal alterations found for the different treatments on *Allium cepa* test

Treatments	Chromosomal alterations in cell cycle			Total cells with alterations
	Micronuclei in interphase	Chromosomal breakages	Telophasic breakages	
Water (cont. -)	0	0	0	0^{c}
Tea 5 g/L	0	3	0	3^{c}
Tea 10 g/L	5	0	0	5^{c}
Glypho (cont. +)	1	24	0	25^{a}
Glypho + Water	0	4	7	11^{ac}
Glypho + tea 5 g/L	0	6	1	7^{ac}
Glypho + tea 10 g/L	0	1	0	1^{bc}

Means followed by the same letter do not differ significantly at the 5% level, according to the χ^2 test. Critical χ^2= 11,0705.

χ^2=2.998 and χ^2=4.995. When compared to glyphosate, the aqueous extract at 5g.L^{-1} demonstrated significantly fewer chromosomal alterations (χ^2=17.190), with the same occurring at the higher concentration of the extract (χ^2=13.254).

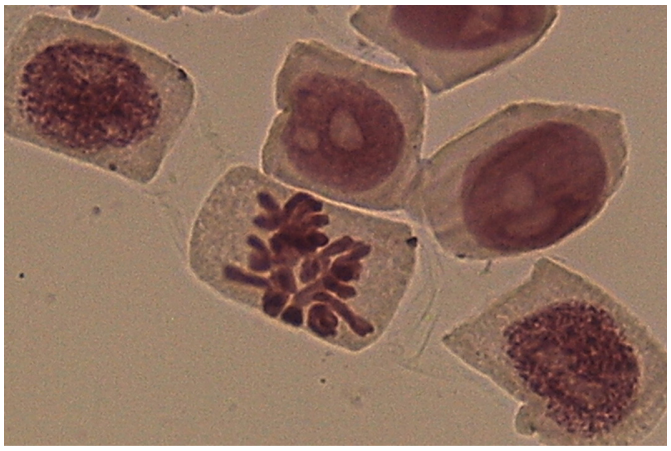

FIGURE 1 - Metaphase with chromosomal breakages.

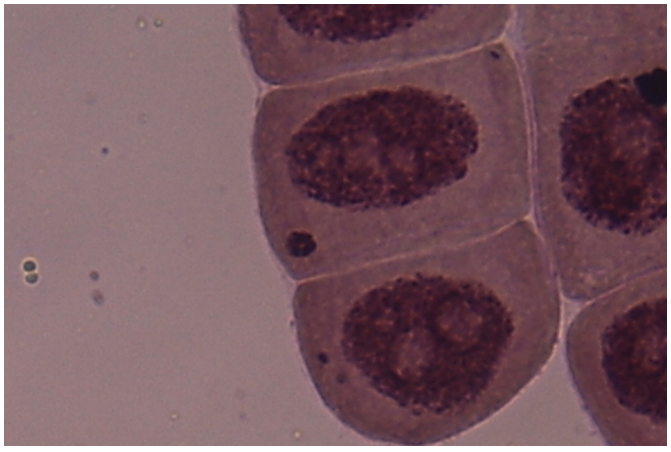

FIGURE 2 - Micronucleus in interphase.

Compared with the negative control, these results did not indicate potential genotoxicity in the extracts of *P. granatum* fruit peels. Likewise, Frescura *et al*. (2012) using *Luehea divaricata* Martius extracts, also found that the extracts studied showed no genotoxic potential; however, antiproliferative capacity was evident, akin to the pomegranate extracts studied.

There was a significant difference (χ^2=59.075) in the mitotic indices of the roots treated using glyphosate with posterior recovery in water relative to those treated with water alone (negative control), where the respective mitotic indices were 2.6 and 7.32. When recovery of rootlets after treatment with glyphosate was tested, alterations such as breakages in metaphase and bridge in anaphase were found (Figure 3) although did not reach significance (χ^2=10.976). A decrease in MI was observed for the other two groups with recovery compared to the control in water. For the treatment with recovery in tea at 5 g.L^{-1}, MI was 1.63, differing significantly to water (χ^2=94.240). The same occurred when the roots were recovered in tea at 10 g.L^{-1}, with MI of 0.92, also showing a significant decrease (χ^2=129.612). For these last two treatments, metaphases with breaks and bridges in telophase were also found, albeit not significant compared to the negative control (χ^2=6.990 and χ^2=1.000, respectively). Akin to the recovery in *P. granatum* extract, the recovery detected in extracts of *Psychotria brachypoda* (Müll. Arg.) Briton and *Psychotria birotula* by Smith & Downs also showed no antimutagenic activity (Frescura, 2012).

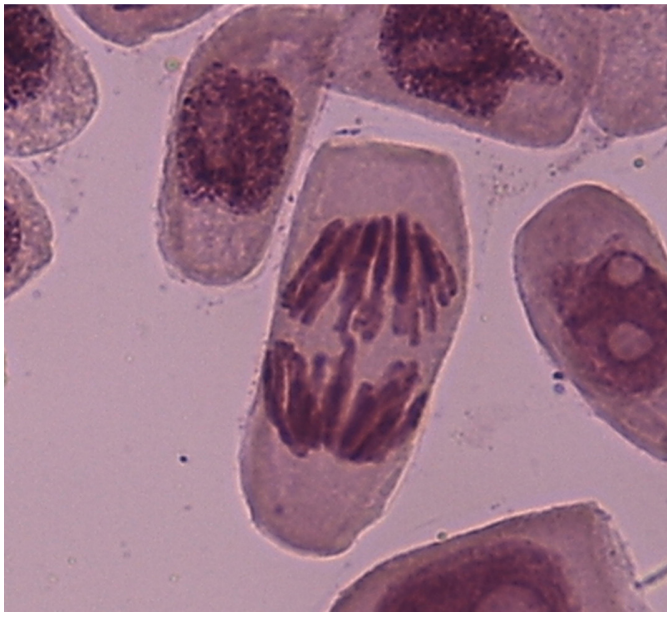

FIGURE 3 - Bridge in anaphase.

Relatively to the positive control (glyphosate), the recoveries in water and in the extract with a greater concentration presented no significant variation (χ^2=6.080 and χ^2=4.646, respectively), where the same was observed for recovery at 5 g.L^{-1} (χ^2=0.013). With regard to the number of chromosomal alterations of these treatments in relation to the positive control, the results observed were not significant.

Chromatographic analysis by HPLC-DAD

The chromatograms of *P. granatum* revealed gallic acid (t_R = 8.71 min; peak 1), catechin (t_R = 14.65 min; peak 2), chlorogenic acid (t_R = 20.19 min; peak 3), caffeic acid (t_R = 23.46; peak 4), quercetin (t_R = 36.59 min; peak 5), rutin (t_R = 45.92 min; peak 6), and kaempferol

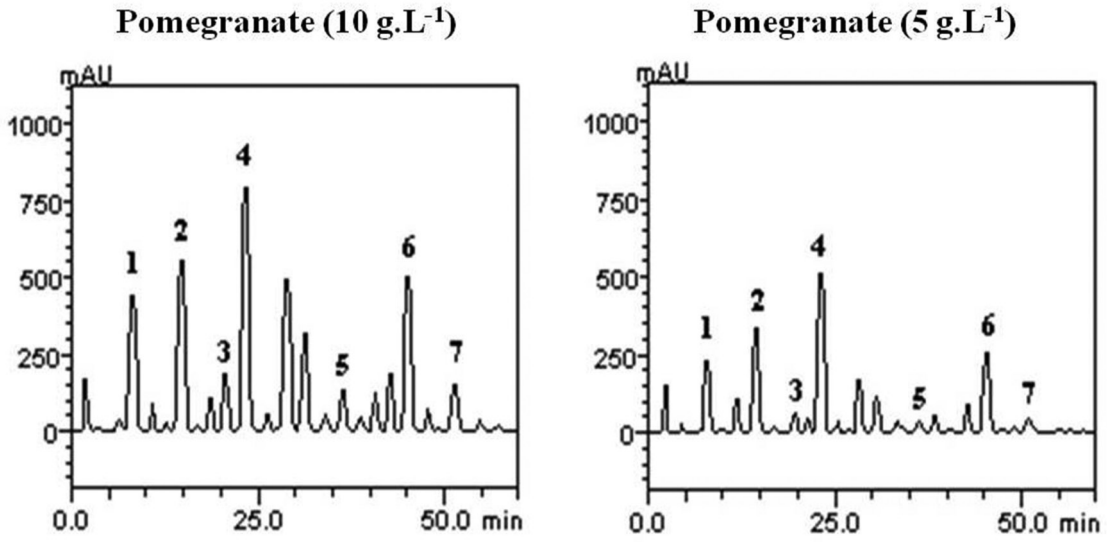

FIGURE 4 - Chromatographic profile of *Punica granatum*, UV detection at 327 nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), quercetin (peak 5), rutin (peak 6), and kaempferol (peak 7). Chromatographic conditions are described in methods.

TABLE III – Composition of phenolic and flavonoid compounds of *Punica granatum*

Compounds	*Punica granatum*			
	10 g.L^{-1}		5 g.L^{-1}	
	mg.L^{-1}	%	mg.L^{-1}	%
Gallic acid	27.15 ± 0.01 ^{c}	2.71	13.62 ± 0.03 ^{c}	1.36
Catechin	35.94 ± 0.02 ^{b}	3.59	17.45 ± 0.01 ^{b}	1.74
Chlorogenic acid	9.07 ± 0.01 ^{d}	0.90	4.87 ± 0.02 ^{d}	0.48
Caffeic acid	43.28 ± 0.03 ^{a}	4.32	24.19 ± 0.03 ^{a}	2.41
Quercetin	7.52 ± 0.01 ^{d}	0.75	2.39 ± 0.01 ^{e}	0.23
Rutin	28.63 ± 0.04 ^{c}	2.86	13.73 ± 0.04 ^{c}	1.37
Kaempferol	8.45 ± 0.02 ^{d}	0.84	3.42 ± 0.01 ^{de}	0.34

Results are expressed as mean ± standard deviation (DS) of three determinations. Columns followed by different letters differ on the Tukey test ($p < 0.05$).

(t_R = 52.03 min; peak 7) (Figure 4 and Table III). HPLC analysis revealed that flavanoids (quercetin, rutin, and kaempferol), phenolic acids (gallic acid, caffeic acid, and chlorogenic acid), and tannins (catechin) were present in the *P. granatum* extract.

Gallic acid, catechin, caffeic acid, and rutin are found at higher concentrations in *P. granatum* fruit peels. These compounds have properties that can explain the antiproliferative capacity of the species studied. In addition, caffeic acid, the compound found at the highest concentration in pomegranate extracts, has antioxidant activity as observed in plants of *Rosmarinus officinalis* L. (rosemary) (Ramalho, Jorge, 2006).

CONCLUSION

Extracts from fruits of *P. granatum* were found to have antiproliferative capacity, since an increase in the concentration of the extracts of fruit peels decreased mitotic indices. Additionally, the extract exhibited no antimutagenic activity or significant genotoxic activity. These effects might be related with the phenolic compounds found in the extracts of *P. granatum*.

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