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Original scientific paper

GENOTOXIC EFFECT OF GALLIC AND ELLAGIC ACIDS IN SOMATIC AND GERM CELLS OF *DROSOPHILA MELANOGASTER*

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ABSTRACT. Phenolic acids are a large class of compounds occur naturally in a variety of plants and exhibit a wide range of biological activities, but toxic effects have also been observed. This study was designed to assess genotoxic effect of two selected phenolic acids, gallic and ellagic, in somatic and germ cells of *Drosophila melanogaster* using the sex-linked recessive lethal (SLRL) test and comet assay *in vivo*. The obtained results revealed that tested phenolic acids did not induce genotoxic effect and therefore have a safety margin for therapeutic use.

Key words: ellagic acid, gallic acid, *Drosophila melanogaster*, genotoxicity

INTRODUCTION

The therapeutic effects of many plants have been assigned to the presence of phenolic acids such as cichoric acid, echinacoside, chlorogenic acid (LETCHAMO *et al*., 1999). The naturally occurring phenolic acids have been investigated in the prevention and treatment a wide range of ailments. Various reports have shown beneficial effects of phenolic acids such as cytoprotective (VIEIRA *et al*., 1998), neuroprotective (MANSOURI *et al*., 2013; IBRAHIM *et al*., 2015), anticancer (TANAKA *et al*., 1998; BAEZA *et al*., 2014), antimicrobial (ANTONIO *et al*., 2011; ALMEIDA *et al*., 2012), and antidiabetic (GHASEMZADEH and GHASEMZADEH, 2011; PADMA *et al*., 2011).

In addition to known activity, phenolic acids have been tested in order to assess genotoxic and potential antigenotoxic activities. Phenolic acids, as ferulic, caffeic and gentisic, are known to exhibit protective effect against the genotoxicity of acridin orange and ofloxacin in *Salmonella typhimurium* and *Euglena gracilis* (KRIŽKOVÁ *et al*., 2000; BELICOVÁ *et al*., 2001; BIROŠOVÁ *et al*., 2005). YAMADA and TOMITA (1996) described that caffeic and chlorogenic acid possesses inhibitory effect on the mutagenicity of 3-amino-l,4-dimethyl-5*H*-pyrido[4,3-b]indole (Trp-P-1), and 2-aminodipyrido[l,2-a:3',2'-*d*]imidazole (Glu-P-2). These phenolic acids may inhibit the formation of N-nitroso compounds because they are inhibitors of the N-nitrosation reaction *in vitro* (KONO *et al*., 1995). VATTEM *et al*. (2006) demonstrated that rosmarinic acid inhibited the mutagenic potential of sodium azide and N-methyl-N'-nitro-N-nitrosoguanidine in the *Salmonella typhimurium* test system. Also, rosmarinic acid reduced

the frequency of micronuclei in human lymphocytes (SÁNCHEZ-CAMPILLO *et al*., 2009) and in V79 cells (FURTADO *et al*., 2010). Furthermore, rosmarinic acid showed antigenotoxic effects against ethanol in mice peripheral blood and brain cells (DE OLIVEIRA *et al*., 2012) and in Wistar rat brain tissue or peripheral blood using the comet assay (PEREIRA *et al*., 2005).

The results concerning the genotoxic effect of phenolic acids are contradictory. Not all phenolic acids are necessarily beneficial, some of them have genotoxic effect. According to MAISTRO *et al*. (2011) caffeic, cinnamic and ferulic acids exhibited the genotoxic potential in rat hepatoma tissue cells (HTCs) using the *in vitro* micronucleus assays. Cichoric acid showed mutagenic activity in the Ames test with *Salmonella typhimurium* TA98 and TA100 strains (MIKULÁŠOVÁ *et al*., 2005) while caffeic acid and chlorogenic acid induced mutations in TA102 strain.

The *in vivo* study by EL HAJJOUJI *et al*. (2007) demonstrated the genotoxic effect for gallic acid using *Vicia faba* micronucleus test. Also, genotoxic effect has been reported for gallic acid by sex-linked recessive lethal test in *Drosophila melanogaster* (STANIĆ *et al*., 2009). LABIENIEC and GABRYELA (2003) previously reported that tannic, ellagic and gallic acids have genotoxic and cytotoxic properties in the Chinese hamster cells.

Gallic acid, a naturally occurring compound, and ellagic acid, a dimeric derivative of gallic acid, have been commonly found in gallnuts, tea, tree barks, herbs, flowers, fruits and vegetables including cranberry, blackberry, blackcurrant, strawberry, and raspberry (BREWER, 2011; NAKAMURA *et al*., 2012; SINGH *et al*., 2013). Various studies have shown a positive effect for ellagic and gallic acids such as antioxidant, antimutagenic and anticancer properties (SALDANHA *et al*., 2018). LOARCA-PIÑA *et al*. (1996) tested the antimutagenicity of ellagic acid against the aflatoxin B1 used the Salmonella microsuspension assay. Also, AYRTON *et al*. (1992) evaluated ability of ellagic acid to inhibit the mutagenicity of the food mutagen 2-amino-3-methylimadazo[4,5-f]-quinoline (IQ) using Ames test.

Despite the potential of the gallic and ellagic acids, there have very little studies on the possible genotoxic effects of these natural compounds. Therefore, the aim of this study was to evaluate the genotoxic activity of these two phenolic compounds in *Drosophila melanogaster*, using the comet and SLRL assays.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used for analyses of genotoxic effect were of analytical grade. Phosphate-buffered saline (PBS) without calcium and magnesium, agarose for DNA electrophoresis, low-melting point agarose (LMA), and collagenase were obtained from Alfatrade Enterprise D.O.O.; methyl 4-hydroxybenzoate, ethyl methanesulphonate (EMS) and gallic acid (CAS 149-91-7, technical grade > 98.0%) were purchased from Sigma-Aldrich, St. Louis, MO, USA and ellagic acid (CAS 133039-73-3, technical grade > 98.0%) was obtained TCI, Tokyo Chemical Industry CO., LTD.

Fly strain

Flies and larvae of wild type strain of *Drosophila melanogaster* (*Canton S*, available from Bloomington Stock Center, Indiana, USA) were cultured at 25°C, 60% humidity and a 12:12 h light/dark regime on standard corn medium containing agar, sugar and yeast.

SLRL test

In the negative control group 30 males were exposed to 1% sucrose. The positive control group was treated with 0.75 ppm ethylmethane sulfonate (EMS). The third and fourth

groups were treated with a 100 ppm of gallic or ellagic acids dissolved in sucrose. The standard procedure for the detection of sex linked recessive lethal mutations on *D. melanogaster* was applied (WÜRGLER and GRAF, 1985) as previously described in detail in MLADENOVIĆ *et al*. (2013).

Comet test

Third instars larvae of *D. melanogaster* (20 larvae per group) were divided into four groups. A negative control group, without treatment, received standard Drosophila diet while a positive control group treated only with ethyl methanesulphonate (EMS, 5 mM in PBS). In order to determine the potential for gallic and ellagic acids to damage DNA in somatic cells, the larvae of *D. melanogaster* were fed with the medium containing 1 mM gallic or ellagic acid for 24 h. The comet assay was performed according to SINGH *et al*. (1988) with minor modifications as described by MUKHOPADHYAY *et al*. (2004). Immediately before use slides were stained with 80 mL of ethidium bromide (20 mg/mL). The images were visualized and captured with 40x objective lens of fluorescence microscope Nikon (Ti-Eclipse) attached to CCD camera. One hundred randomly selected cells (50 cells per two replicate slides) were analyzed per treatment. The DNA damage was quantified as percentage of DNA in the comet tail.

Statistical analysis

A statistically significant difference in lethal cultures in the SLRL test was found for large independent samples by testing the difference between proportions (PETZ, 1985). The results in the Comet test were expressed as mean ± SEM and a statistical evaluation of the data was carried out by means of one-way analysis (ANOVA) using the SPSS statistical software package, version 13.0 for Windows. The significance level was set at $p < 0.05$.

RESULTS AND DISCUSSION

The genotoxicity of gallic and ellagic acids was evaluated *in vivo* in germ and somatic cells of *D. melanogaster* by the SLRL and Comet tests. As shown in Tab. 1 EMS significantly increased the frequency of germinative mutations compared with the untreated control in all the three broods. There were no statistically significant differences in the frequency of germinative mutations between the males treated with gallic acid (Tab. 1) and the males in the control group (1% sucrose). Compared to the males in the positive control group, the males treated with gallic acid showed a statistically significant difference.

The results of the SLRL test performed in *D. melanogaster* males showed no genotoxic activity of ellagic acid (Tab. 2). The increase in number of lethal was observed in the EMS treated group and no increase in the number of lethal in the ellagic acid treated group.

Contrary to our results, EL HAJJOUJI *et al*. (2007) reported the genotoxic activity of gallic acid in *Vicia faba* micronucleus test. Furthermore, LABIENIEC and GABRYELA (2003) reported that ellagic and gallic acids has genotoxic effect in the Chinese hamster cells and in human carcinoma cells (WENG *et al*., 2015)

Also, we have previously described the genotoxic activity of the gallic acid in concentration of 5% using SLRL test (STANIĆ *et al*., 2009) in *D. melanogaster*. Our results indicate that the genotoxic effect of gallic acid occurs in a concentration-dependent manner. There are cases where low concentrations of polyphenols cause DNA protection whereas high concentrations of the same compounds cause DNA damage (WÄTJEN *et al*., 2005).

Table 1. Frequencies of sex linked recessive lethal mutations after the treatment with gallic acid.

	Treatment					
	S[a]	**EMS[b]**	**GA[c]**	**$t_{S/EMS}$**	**$t_{S/GA}$**	**$t_{EMS/GA}$**
I brood Σ	92	104	52	8.3	0.36	8.23
No of lethal	12	64	6	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	13.04	61.5	11.5			
II brood Σ	96	90	48	6.7	1.6	3.5
No of lethal	10	44	10	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	10.04	48.9	20.8			
III brood Σ	64	108	42	5.3	0.8	3.9
No of lethal	6	44	6	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	9.4	40.7	14.3			
I+II+III Σ	252	302	142	13.3	1.1	8.7
No of lethal	28	152	22	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	11.1	50.3	15.5			

Triple asterix indicates significantly higher frequency compared to EMS as positive control or to sucrose as negative control. Statistically significant differences: $p < 0.001$***
[a]S; sucrose; negative control, 1%. [b]EMS; ethyl methanesulfonate, positive control, 0.75 ppm.
[c]GA; gallic acid, 100 ppm.

Table 2. Frequencies of sex linked recessive lethal mutations after the treatment with ellagic acid

	Treatment					
	S[a]	**EMS[b]**	**EA[c]**	**$t_{S/EMS}$**	**$t_{S/EA}$**	**$t_{EMS/EA}$**
I brood Σ	92	104	54	8.3	0.95	6.14
No of lethal	12	64	10	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	13.04	61.5	18.5			
II brood Σ	96	90	44	6.7	0.7	4.4
No of lethal	10	44	6	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	10.4	48.9	13.6			
III brood Σ	64	108	30	5.3	1.4	2.7
No of lethal	6	44	6	$p < 0.001$***	$p > 0.05$	$p < 0.01$**
% of lethal	9.4	40.7	20			
I+II+III Σ	252	302	128	13.3	1.5	8.3
No of lethal	28	152	22	$p < 0.001$***	$p > 0.05$	$p < 0.001$***
% of lethal	11.1	50.3	17.2			

Triple asterix indicates significantly higher frequency compared to EMS as positive control or to sucrose as negative control. Statistically significant differences: $p < 0.01$**, $p < 0.001$***
[a]S; sucrose; negative control, 1%. [b]EMS; ethyl methanesulfonate, positive control, 0.75 ppm.
[c]EA; ellagic acid, 100 ppm.

The effects of the positive controls (EMS, 5 mM in PbS) and two phenolic acids (1 mM) on selected comet parameter and third instars larvae of *D. melanogaster* are presented in Tab. 3.

The percentages of DNA in tail were significantly higher in the group treated with 5 mM EMS than in the negative control ($p < 0.05$). The data demonstrate that tested phenolic acids did not cause an increase of the DNA damage. Ellagic acid induced only a slight increase in the % DNA in tail. The statistical analysis of these data confirms that the concentration of 1 mM of ellagic acid induced a minimum level of DNA damage after 24 h of treatment and that this DNA damage was less in comparison with those caused by the 5 mM EMS.

Table 3. Genotoxic effect of gallic and ellagic acids using comet assay

Treatment[a]	% DNA in tail
NC[b]	5.4±0.61[†]
EMS[c]	61.5±1.2[*]
GA[d]	6.2±0.5[†]
EA[e]	8.04±0.62[*†]

[a]Data are presented as the means ± SEM obtained from three independent experiments.
[b]NC; negative control. [c]EMS, ethyl methanesulfonate, 5 mM. [d]GA, gallic acid, 1 mM.
[e]EA, ellagic acid, 1 mM. [*]$p < 0.05$ when compared with the negative control group
[†]$p < 0.05$ when compared with the positive control group.

According to the data obtained in the SLRL test and Comet assay, the selected phenolic acids did not cause DNA damage suggesting that they did not present genotoxicity. These results are in accordance with earlier studies that reported the absence of genotoxic effect of ellagic and gallic acids using the *in vitro* Ames test (CHEN AND CHUNG, 2000; OKUDA, 2005; SILVA *et al*., 2014) and comet test (FERK *et al*., 2011). Similarly, study by BERNI *et al*. (2012) has reported that ellagic acid did not increase the frequency of polychromatic erythrocytes in Swiss albino mice.

In conclusion, exposure of males in SLRL test and larvae in comet test to gallic and ellagic acids did not produce genotoxic effect. The results of this research showed that further *in vivo* studies with other model organisms are needed before definitive conclusions about the absence of genotoxic potential of tested phenolic acids.

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