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Short Communication

Genotoxic potential of *Cotinus coggygria* Scop. (Anacardiaceae) stem extract *in vivo*

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Abstract

The intention was to evaluate the possible *in vivo* genotoxic potential in different cell-types, of a methanol extract obtained from the plant stem of *Cotinus coggygria* Scop., using the sex-linked recessive lethal (or SLRL) test and alkaline comet assay. The SLRL test, revealed the genotoxic effect of this extract in postmeiotic and premeiotic germ-cell lines. The comet assay was carried out on rat liver and bone marrow at 24 and 72 h after intraperitoneal administration. For genotoxic evaluation, three concentrations of the extract were tested, viz., 500, 1000 and 2000 mg/kg body weight (bw), based on the solubility limit of the extract in saline. Comet tail moment and total scores in the group treated with 500 mg/kg bw, 24 and 72 h after treatment, were not significantly different from the control group, whereas in the groups of animals, under the same conditions, but with 1000 and 2000 mg/kg bw of the extract, scores were statistically so. A slight decrease in the comet score and tail moment observed in all the doses in the 72 h treatment, gave to understand that DNA damage induced by *Cotinus coggygria* extract decreased with time. The results of both tests revealed the genotoxic effect of *Cotinus coggygria* under our experimental conditions.

Key words: comet assay, *Cotinus coggygria*, extract, genotoxic effect, SLRL.

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Many plant extracts have been used as sources of medicinal agents, in the cure of urinary tract infections, cervicitis vaginitis, gastrointestinal disorders, respiratory diseases, cutaneous affections, helminitic infections and inflammatory processes (Brantner and Grein, 1994; Meyer *et al.*, 1996). Nevertheless, of late, the safeness of their use has been questioned in view of the reports of illness and fatalities (Stewart *et al.*, 1999; Ernst, 2002; Veiga-Junior *et al.*, 2005). Considering the complexity of herbs in general and their inherent biological variation, it is now necessary to evaluate their safety, efficacy and quality (WHO, 2002).

Cotinus coggygria is one of two species constituting a minor genus of the family Anacardiaceae, viz., *Cotinus coggygria* Scop. (syn.: *Rhus cotinus* L.) itself and *Cotinus obovatus* Raf., the American smoketree. Its wide distribution extends from southern Europe, the Mediterranean, Moldova and the Caucasus, to central China and the Himalayas (Novakovic *et al.*, 2007). Plants of the family Anacardiaceae have a long history of use by various peoples for medicinal and other purposes. *Rhus glabra* is traditionally used in the treatment of bacterial diseases such as syphilis, gonorrhea, dysentery and gangrene, while *R. coriaria*, besides its common use as a spice consisting of ground dried fruits with salt, is also widely used as a medicinal herb, particularly for wound healing (Rayne and Mazza, 2007). In folk medicine, *Cotinus coggygria* is routinely used as an antiseptic, anti-inflamatory, antimicrobial and antihaemorragic agent in wound-healing (Demirci *et al.*, 2003), as well as for countering diarrhea, paradontosis, and gastric and duodenal ulcers (Ivanova *et al.*, 2005).

This study was undertaken with the aim of determining, by way of SLRL testing and comet assay, the *in vivo* genotoxic effects of acute administration of a *Cotinus coggygria* stem extract on different models of eukaryotic organisms.

Cotinus coggygria plants were collected at a place called Rujiste, in the Rogozna mountain, in northern Kosovo, during the period May to June 2007. The species was identified and a voucher specimen (16178, BEOU) deposited at the Department of Botany, the Faculty of Biology, University of Belgrade. The extract was obtained, first by breaking air-dried stem (1.157 g) into small pieces (2-6 mm) with a cylindrical crusher, and then by applying the Soxhlet procedure using methanol (500 mL) as solvent. The extract, firs filtered through a paper filter (Whatman,

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No. 1), was then evaporated so as to remove the solvent. The residue (32 g) was stored in a dark glass bottle for further processing.

The sex-linked recessive lethal test for mutagenicity (SLRL test) was carried out with laboratory stocks of *Drosophila melanogaster* (obtained from the Umea Stock Centre, Sweden). The stocks were maintained and all experiments performed under optimal conditions (25 °C, 60% relative humidity and a 12/12 h light/dark regime) on a standard nutritive medium for *Drosophila*, (corn flour, yeast, agar, sugar and nipagin to prevent the occurrence of mould and infections).

Three-to-four-days-old wild type males of *Drosophila melanogaster* (test group 1, N = 30) were left to starve in empty bottles for 5 h and then transferred and exposed to a 1% sucrose (as negative control), according to the method of Lewis and Bacher (1968). The second group (test group 2, N = 15) was treated with 0.75 ppm ethyl-methane sulfonate (EMS) in 1% sucrose (positive control). The third group (test group 3, N = 15) was treated with a 5% plant extract dissolved in sucrose. After 24 h of treatment and a further 24 h resting on fresh medium, males were individually mated to two-to-five-days-old virgin *Basc* females (brood I). The males were then remated in fresh vials with three other virgin *Basc* females at two-to-three-days intervals (brood II), so as to test all germ-cell stages for the presence of mutations. Once again, the males were then transferred to fresh vials containing three *Basc* virgins (brood III). These males remained with the females for three days, to then be removed. The females were left alone for five days to lay eggs, and then removed. When the F_1 flies emerged, brother-sister mating was allowed, whereupon ten pairs from each progeny were individually placed together, from the same number. The F_2 generation was examined for the presence or absence of wild-type males. It was noted that, when this was so, these all contained the same treated X-chromosome in hemizygous condition. Any recessive lethal therein would be expressed before the adult stage, whereat males would not emerge. Cells exposed in successive spermatogenesis stages were tested for induced mutations, to thus check the effects on postmeiotic (spermatozoa, spermatids), meiotic (spermatocytes) and premeiotic (spermatogonia) cells (Würgler and Graf, 1985).

Two to two-and-half-months-old male albino rats of the Wistar strain (*Rattus norvegicus*), each weighing 220-250 g, were used for the comet assay. The rats were kept in an experimental room under controlled conditions of temperature and humidity, with food and water available *ad libitum*. Lighting was controlled to provide 12 h artificial light followed by 12 h darkness. All animal procedures had been previously approved by the Ethical Animal Care and Use Committee of the Institute for Biological Research, Belgrade, which acts in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85/23, revised 1986).

In order to evaluate the genotoxicity of the *Cotinus coggygria* stem extract, rats were divided into four groups of five animals each. For three of the groups, each received (i.p.) a single dose of different concentrations of extract disolved in saline solution: 500, 1000 and 2000 mg/kg bw. These concentrations were based on the solubility limit of the methanol extract of *Cotinus coggygria* in saline solution. The fourth group (control), received a saline solution. Four separate experiments were carried out. The animals were sacrificed by decapitation 24 and 72 h after treatment. Liver and femur bone marrow, obtained from each group of animals, was quickly removed and separately processed, so as to obtain cell suspensions.

The alkaline version of the comet assay was carried out by the standard procedure originally described by Singh *et al.* (1988). Immediately prior to analysis, the slides were stained with 90 μL of SYBR GREEN I (Sigma-Aldrich, S 9430). Comets were visualized and captured with the 40x objective lens of a Leica DMLB fluorescence microscope, attached to a CCD camera. One hundred comet images per slide were randomly captured and analyzed. The extent of DNA damage was measured by means of two complementary methods, the tail moment quantitative method and a qualitative method of damage distribution. Comets, first analyzed by the visual scoring method, as described by Collins (2004), were then classified into five categories, defined as types 0, 1, 2, 3 and 4, where 0 indicates no or very low damage, and 1, 2 and 3 low, medium and long DNA migration, respectively, with 4 as the highest level of degradation, viz., comets with very small heads and long tails. The total score was calculated by the following equation modified from Manoharan and Banerjee (1985): (% cells in class 0 x 0) + (% cells in class 1 x 1) + (% cells in class 2 x 2) + (% cells in class 3 x 3) + (% cells in class 4 x 4), to finally appear in a 0 (all undamaged) to 400 (all maximally damaged) range. Images were analyzed with software TriTekCometScore Freeware v1.5 available at web page AutoComet.com.

For SLRL testing, the frequency of sex-linked recessive lethal cultures was calculated according to the ratio between the numbers of lethal cultures and the total number of treated X-chromosomes. The total number of treated X-chromosomes is equal to the sum of lethal and non-lethal cultures. The significance of percentual differences in lethal cultures was arrived at by testing for large independent samples, and then testing the difference between proportions (Petz, 1985). For comet analysis, average tail moment and standard deviation per treatment were obtained. Variance analysis was performed by using one-way analysis (ANOVA). Variance homogeneity and data distribution were determined with the Levene and Kolmogorov-Smirnov tests, respectively. Post-hoc comparison between control and treated groups was performed with a T3 Dunnett or

Bonferroni test when variance was not homogeneous. Statistical analysis was performed using the SPSS statistical software package, version 10.0 for Windows. The results were considered to be statistically significant at $p < 0.05$.

From a phytomedicinal point of view, the evaluation of genotoxicity is of particular importance, since the genotoxic effects of chemicals or complex mixtures may be crucially important at the population level.

In the present study, the genotoxic effect of a *Cotinus coggygria* extract was observed via SLRL test and comet assay using *Drosophila melanogaster* as an insect and Wistar rats as a mammal model.

Various mechanisms are involved in this form of genotoxicity testing. Essentially, the SLRL test is used for detecting the occurrence of mutations, both point mutations and small deletions, in the germ line. Mutations in the X-chromosome of *Drosophila melanogaster* are phenotypically expressed (presence or absence of white-eyed) in males carrying the mutant gene. The sex-linked recessive lethal test in *Drosophila melanogaster* has been proved to be an excellent screening test for the detection of natural plant mutagens (Stanic *et al.*, 2008). Over several decades, *Drosophila* has been widely used as an insect model, due to its well-elucidated genetics and developmental biology. In the present study, the genotoxicity of a methanol extract of *Cotinus coggygria* was examined, using a short test for the detection of mutagenicity under *in vivo* conditions. The frequency of germinative mutations induced by the *Cotinus coggygria* extract in SLRL test is significantly higher than that induced by sucrose as negative control (Table 1), this being a clear indication of its mutagenic effect. Ethylmethane sulfonate in a concentration of 0.75 ppm was shown to be clearly genotoxic, by inducing significant increases in the frequency of mutants in all the three broods. On the other hand, and in comparison, the *Cotinus coggygria* extract induced recessive lethal X-linked mutations in premeiotic germinative cell lines, *i.e.* spermatozoids and others of this line, as well as spermatocytes, while spermatids proved to be more resistant to the genotoxic effects of the extract.

Over the last decade, *in vivo* alkaline comet assay, besides gaining widespread use in various areas, has emerged as a standard tool in the pharmaceutical industry for assessing the safety of new drugs and, increasingly, as a means of evaluating genotoxicity testing (Tice *et al.*, 2000; Hartmann *et al.*, 2001; Brendler-Schwaab *et al.*, 2005). In the present study we evaluated the extent of DNA damage by determining average tail moment and by analyzing qualitative tail length distribution in comet images. In the liver sample from animals treated with 500 mg/kg bw of the extract, no statistically significant difference in total score between treated animals and control was observed at 24 and 72 h after treatment (Table 2). Most of the comets remained undamaged, with only a few cells denoting minor damage (class 1), and less still medium (class 2). However, this was not the case with animals treated with 1000 and 2000 mg/kg, where significant differences were noted (Table 2). In the case of rats exposed to 1000 mg/kg bw, this was apparent in comet tails being longer (classes 3 and 4) when compared to the control group. The highest level of DNA damage was observed in the group treated with 2000 mg/kg bw of the extract after 24 and 72 h.

The percentage of comets assigned to damage categories in bone marrow, at 24 and 72 h after *in vivo* exposure to 500 mg/kg bw of the extract, are presented in Table 3. With a predominance of comet class 0 and almost no presence of classes 1 and 2, no statistically significant difference between treated and untreated animals could be observed. Under the same circumstances, this was not the case in the groups treated with 1000 and 2000 mg/kg bw of the extract,

Table 1 - Frequencies of SLRL mutations after treatment of *Drosophila melanogaster* males with a methanol extract from *Cotinus coggygria* plants

	Sucrose negative control	EMS positive control	*Cotinus coggygria* extract	$t_{sucrose/EMS}$	$t_{sucrose/extract}$	$t_{EMS/extract}$
I broods S	300	221	269	9.51	5.45	5.26
No of lethals	5	73	34	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
% of lethals	1.67	33.03	12.64			
II brood S	269	161	284	8.38	2.57	7.04
No of lethals	5	54	17	$p < 0.001$***	$p < 0.05$*	$p < 0.001$***
% of lethals	1.86	33.54	5.99			
III broods S	252	117	252	5.85	5.72	1.92
No of lethals	6	30	43	$p < 0.001$***	$p < 0.001$***	$p < 0.1$
% of lethals	2.38	25.64	17.06			
I+II+III S	821	499	805	13.81	8.15	7.92
No of lethals	16	157	94	$p < 0.001$***	$p < 0.001$***	$p < 0.001$***
% of lethal	1.95	31.46	11.67			

Statistically significant difference: $p < 0.05$*; $p < 0.01$**; $p < 0.001$***

Table 2 - DNA migration in the comet assay for the assessment of genotoxicity of *Cotinus coggygria* extract in the livers of albino wistar rats, 24 and 72 h after treatment.

Treatments	Levels of damage					Total score (mean ± SD)
	0	1	2	3	4	
Negative control	84.1 ± 1.32	10.6 ± 0.40	6.30 ± 0.3	0.00 ± 0.00	0.00 ± 0.00	23.20 ± 1.10
C. coggygria 24 h						
500 mg/kg	78.8 ± 0.42	15.6 ± 0.44	5.60 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	26.80 ± 2.30
1000 mg/kg	43.8 ± 0.33	32.8 ± 0.20	17.7 ± 0.40	4.70 ± 0.40	1.00 ± 0.21	86.30 ± 0.83*
2000 mg/kg	27.2 ± 0.26	38.8 ± 0.34	25.7 ± 0.39	6.10 ± 0.19	2.01 ± 0.21	116.50 ± 1.14*
C. coggygria 72 h						
500 mg/kg	81.0 ± 0.84	13.4 ± 0.16	5.70 ± 0.84	0.00 ± 0.00	0.00 ± 0.00	24.80 ± 0.45
1000 mg/kg	41.6 ± 0.26	45.0 ± 0.42	10.1 ± 0.18	3.10 ± 0.24	0.00 ± 0.00	74.50 ± 1.14*
2000 mg/kg	30.4 ± 0.32	47.2 ± 0.35	16.6 ± 0.37	2.90 ± 0.24	1.03 ± 0.25	92.10 ± 1.22*

*Significantly different from the negative control $p < 0.05$.

Table 3 - DNA migration in the comet assay for the assessment of genotoxicity of *Cotinus coggygria* extract in bone marrow of albino wistar rats, 24 and 72 h after treatment.

Treatments	Levels of damage					Total score (mean ± SD)
	0	1	2	3	4	
Negative control	79.8 ± 0.41	20.2 ± 0.35	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	21.0 ± 1.60
C. coggygria 24 h						
500 mg/kg	76.4 ± 0.48	20.4 ± 0.43	3.20 ± 1.51	0.00 ± 0.00	0.00 ± 0.00	26.8 ± 0.24
1000 mg/kg	31.1 ± 0.41	40.2 ± 0.28	19.8 ± 0.38	7.40 ± 0.36	1.50 ± 0.47	108.0 ± 0.65*
2000 mg/kg	27.4 ± 0.48	32.1 ± 0.27	30.2 ± 0.33	6.60 ± 0.20	3.80 ± 0.42	127.5 ± 0.43*
C. coggygria 72 h						
500 mg/kg	72.2 ± 0.58	27.7 ± 0.47	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	25.2 ± 1.90
1000 mg/kg	42.3 ± 0.42	47.3 ± 0.43	8.70 ± 0.40	1.90 ± 0.42	0.00 ± 0.00	70.4 ± 1.50*
2000 mg/kg	27.2 ± 0.40	49.5 ± 0.45	19.10 ± 0.2	3.20 ± 0.23	1.01 ± 0.26	101.3 ± 3.60*

*Significantly different from the negative control $p < 0.05$.

where there was a significant increase in damages, when compared to the control group.

The average values of comet tail moment in liver and bone marrow are shown in Table 4. Comet tail moment in the group treated with 500 mg/kg bw of the extract was not significantly different from the control group. Nevertheless, when compared to the control, there was a significant increase in tail moment at both 24 and 72 h after treatments with 1000 and 2000 mg/kg bw. It should be pointed out, however, that after 72 h, a slight decrease in tail moment, both in the liver and bone marrow, was detected when compared to the 24 h time point.

This difference in the response seen in the comet assay and SLRL test is not surprising, since the comet assay is a rapid, simple and highly sensitive method for detecting single and double DNA strand breaks and alkali-labile sites. In contrast, the comet assay is not used to detect mutations, but rather to detect genomic lesions that could lead to a mutation (Gontijo and Tice, 2003).

The fact that certain plants may have genotoxic effects depends on the various compounds present in their extracts. The partial chemical analysis of the methanol extract of *Cotinus coggygria*, showed flavonoids, tannins and phenolic compounds to be the main compounds (Stanic *et al.*, 2009). In a previous study, total soluble phenolic compounds in the methanol extract of *Cotinus coggygria* stems were determined with the Folin-Ciocalteu reagent, using pyrocatechol as a standard. In the methanol extract of *C. coggygria* (1 g), 62.50 mg pyrocatechol equivalent of phenols was detected, while 46.76 mg of flavonoids and 15.75 mg of nonflavonoids were detected in 1 g of dry weight (Stanic *et al.*, 2009).

Fractionation of the methanol extract from *Cotinus coggygria* performed by Stathopoulou *et al.* (2007), led to the isolation of sulfuretin, fisetin, 7,3',4'-trihydroxy-fla-

Table 4 - Average comet tail moment and standard deviation, 24 and 72 h after *in vivo* exposure of albino wistar rats to *Cotinus coggygria* methanol extract.

Treatments	Tail moment - liver		Tail moment - bone marrow	
	24 h	72 h	24 h	72 h
Negative control	2.7 ± 0.2		5.4 ± 0.25	
C. coggygria				
500 mg/kg	3.1 ± 0.11	2.8 ± 0.16	5.80 ± 0.17	5.6 ± 0.12
1000 mg/kg	11.7 ± 0.13[a]	6.7 ± 0.15[a]	16.55 ± 0.43[a]	13.8 ± 0.40[a]
2000 mg/kg	17.1 ± 0.38[a]	9.6 ± 0.32[a]	20.80 ± 0.63[a]	17.5 ± 0.36[a]

[a]Significantly different from the negative control $p < 0.05$ (multiple comparisons T3 Dunnett test), n = 5 rats per group.

vanone, 5,7,4'-trihydroxy-flavanone, 4,2',4'-trihydroxy-chalcone, 2,3-dihydro-fisetin, 2,3-dihydro-quercetin, methyl gallate, 3,4,2',4'-tetrahydroxy-chalcone, quercetin, 4',7-dihydroxy-flavanone and 4',7-dihydroxy-2,3-dihydroflavonol. Dominant compounds in the ethyl acetate partition of *Cotinus coggygria* were disulfuretin, sulfuretin, sulfurein, gallic acid, methyl gallate and pentagalloyl glucose (Westenburg *et al.*, 2000).

It has been suggested that polyphenolic compounds, besides having been shown to exert anticarcinogenic effects, are potential preventives against cardiovascular and cerebrovascular diseases. Gallic acid and its derivatives are biologically active compounds which are present in several plants. This polyhydroxyphenolic acid has been reported to be a free radical scavenger, as well as an inducer of differentiation and apoptosis in leukemia, lung cancer, colon adenocarcinoma cell lines, and normal lymphocyte cells (Kawada *et al.*, 2001; Sohi *et al.*, 2003).

Flavonoids, such as fisetin, are naturally occurring molecules with antioxidant, cytoprotective and anti-inflammatory actions. Tannins, as one such class of compounds, are suspected of possessing protective properties. Fedeli *et al.* (2004) showed that they are capable of protecting against DNA breakage at low concentrations, although at high levels they could be genotoxic.

The results in this study imply that the methanol extract from *Cotinus coggygria* plant stems is capable of giving rise to genotoxic effects *in vivo* under our experimental conditions, thereby indicating caution in its use. The fact that 500 mg/kg body weight of the *Cotinus* extract was not genotoxic in the alkaline comet assay, and that the manifest antigenotoxic activity might be attributed to the presence of polyphenolic constituents, indicates that further studies are required, in order to evaluate antigenotoxic activity *in vivo*, and to isolate these constituents and decipher their mode of action.

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