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GENOTOXICITY TESTING OF THE METHANOL EXTRACT OF THE PLANT *COTINUS COGGYGRIA* AND GALLIC ACID ON *DROSOPHILA MELANOGASTER*

SNEŽANA STANIĆ[1], SANJA MATIĆ[1], SLAVICA SOLUJIĆ[2], and TANJA MILOŠEVIĆ[2]

[1]*Institute of Biology and Ecology, Faculty of Science, University of Kragujevac,* 34000 Kragujevac, Serbia
[2]*Institute of Chemistry, Faculty of Science, University of Kragujevac,* 34000 Kragujevac, Serbia

Abstract — The genotoxic activity of methanol extract obtained from the stem of *Cotinus coggygria* Scop. and synthetic gallic acid were investigated using the *Drosophila* sex-linked recessive lethal test (or SLRL test). In the tested methanol extract of *C. coggygria* (1 g), 62.50 mg of pyrocatechol equivalent of phenols was detected. Also, 46.76 mg of flavonoids and 15.75 mg of nonflavonoids were observed in 1 g of dry weight of extract. Methanol extract of *C. coggygria* in a concentration of 5% and 5% synthetic gallic acid were shown to be clearly genotoxic, inducing sex-linked recessive lethal mutations on the X-chromosome of *Drosophila melanogaster* males in all three broods.

Key words: *Cotinus coggygria*, genotoxic activity, methanol extract, gallic acid, *Drosophila melanogaster*

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INTRODUCTION

Cotinus is a small genus of the family Anacardiaceae with two species: *Cotinus coggygria* Scop. (syn.: *Rhus cotinus* L.) and *Cotinus obovatus* Raf., American smoketree. *Cotinus coggygria* is a deciduous, polygamous shrub or little tree up to 7 m tall. It has a wide distribution from Southern Europe, the Mediterranean, Moldova, and the Caucasus to Central China and the Himalayas (Novaković et al., 2007). *Flora of Serbia* defines two varieties of *Cotinus coggygria*: var. *laevis* with form *atropurpurea* and var. *arenaria* (Josifović et al., 1973).

Leaves and young branches are utilized for the production of essential oil with a terpenic scent for use in perfumery (Tsankova et al., 1993). Yellow/orange color can be obtained from the root and stem of *Cotinus cogygria* and can be used for fabric coloring. Leaves and bark are a good source of tannins (Grieve, 1971).

In folk medicine, the plant is used for its antiseptic, anti-inflamatory, antimicrobial, antihemorrhagic, and wound-healing effects and against diarrrhoea (Demirci et al., 2003). The dried leaf and twig of *C. coggygria* are used in Chinese traditional medicine to eliminate «dampness» and «heat», and as an antipyretic (Huang, 1999).

Extracts of aromatic plants obtained using organic solvents or fluidized gasses, essential oils, fractions and isolates of extracts, and essential oils are utilized in flavor and fragnance, food, perfumery, cosmetics and toiletries, fine chemicals, and the pharmaceutical industry and in therapy. They are used as such or in diluted forms in the budding aromatherapy sector.

The objectives of this study were to identify chemical components and investigate the genotoxicity of methanol extract of the plant *Cotinus coggygria* and to investigate the genetic effects of synthetic gallic acid using the SLRL test.

MATERIAL AND METHODS

Plant Material

Cotinus coggygria plants were collected from the Rujište locality on Mt. Rogozna in Northern Kosovo during May-June 2007. The species was identified

and the voucher specimen was deposited (16178, BEOU) at the Department of Botany, Faculty of Biology, University of Belgrade.

Chemicals

Total soluble phenolic compounds in methanol extract of *C. coggygria* stem were determined with Folin-Ciocalteu reagent (FC) using pyrocatechol as a standard. Methanol extract was soluted to a concentration of 0.02 g/mL. Of the soluted extract, 0.5 mL was mixed with 2.5 mL of FC reagent (previously diluted 10-fold with distilled water) and 2 mL of $NaHCO_3$ (7.5%). After 15 min of stirring at 45°C, the absorbance was measured at 765 nm on a spectrophotometer (ISKRA, MA9523-SPEKOL 211).

The concentration of total phenolic compounds in the *C. coggygria* stem was determined as mg of pyrocatechol equivalent/g of dry weight of extract using an equation obtained from the standard pyrocatechol graph. All samples were analyzed in three replications.

The flavonoid fraction was precipitated by mixing 10 mL of the extract dissolved in methanol (0.02 g/mL) with 10 mL of HCl (1: 3) and 5 mL of HCHO (8 mg/mL). After 24 h, the mixture was filtered through filter paper (Whatman No. 5). Nonflavonoid components were determined from the filtrate with Folin-Ciocalteu reagent using the same spectrophotometric method as for determining total phenolic concentration; absorbance was measured at 765 nm on a spectrophotometer. Nonflavonoid content was expressed as milligrams of pyrocatechol per gram of dry weight through the calibration curve with pyrocatechol. All samples were analyzed in three replications.

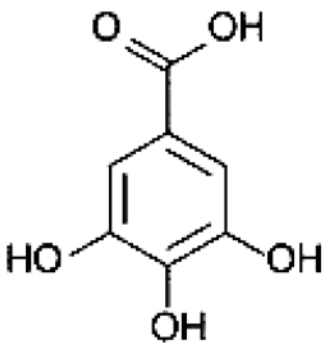

Fig 1. $C_6H_2(OH)_3COOH$; MW 170.12; 3,4,5-trihydroxybenzoic acid (gallic acid).

Flavonoid content was determined from the residium of total phenolic and nonflavonoid content. Flavonoid content was expressed as milligrams of pyrocatechol per mg of extract. All samples were analyzed in three replications.

Synthetic gallic acid (SIGMA ALDRICH) was used for comparative analysis (Fig. 1).

Genotoxicity

The sex-linked recessive lethal test for mutagenicity (SLRL test) was performed with laboratory stocks of *Drosophila melanogaster* (obtained from the Umea Stock Center, Sweden). *Canton-S* line flies had a normal phenotype (*wild* type), while *Basc* line flies were characterized by individuals homozygous for an X-chromosome balancer carrying three genetic markers: *Bar* (*B*), which produces a narrow eye shape in homo- and hemizygous conditions and a kidney-shaped eye when heterozygous in females (the character can be regarded as partially dominant); *white-apricot* (w^a), which alters the red eye color to light-orange and is expressed only in homozygous females and hemizygous males; and *scute* (*sc*), which is a recessive mutation that reduces the number of thoracic bristles [the given mutation is linked with a long inversion on the X-chromosome, necessary for suppression of crossover that could potentially change the existing gene combinations on the treated chromosome (Lee et al., 1983)].

The stocks were maintained and all experiments performed under optimal conditions (t = 25°C, relative humidity = 60%, 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 mold and infections).

Test procedure

Three-day-old *Canton-S* males (test group 1, N = 30) were starved in empty bottles for 5 h prior to the treatment, then transferred and fed in bottles containing filter paper soaked with 5% methanol

extract for 24 h. After another 24 h of recovery on a standard medium, each male was mated individually to three *Basc* females in bottles, which yielded brood I. Two days later, males were transferred to new set of vials containing three virgins of the *Basc* line (thus creating brood II). After three days, males were transferred again to fresh vials containing three *Basc* virgins (brood III). These males stayed with females for three days and were removed afterwards. Females were left alone for five days to lay eggs, and then removed.

Another group of individuals of the same age (test group 2, N = 15 males) was treated with 5% synthetic gallic acid, the solvent 1% sucrose (test group 3, N = 30 males) serving as a negative control (Lewis and Bacher, 1968).

After F_1 emerged in all three-test groups, brother-sister mating was allowed for several days, and 10 females from each vial were put individually into new vials. Each vial would give the progeny of one treated X-chromosome. In $F_{2,}$ the phenotypes were scored according to eye color and shape. The absence of *wild* type males indicated the presence of a recessive lethal agent induced by the test substance.

The total number of treated X-chromosomes is equal to the sum of lethal and non-lethal cultures, and the frequency of sex-linked recessive lethal cultures was calculated from the ratio between the number of lethal cultures to the total number of treated X-chromosomes. Significance of the percentage difference of lethal cultures was determined through testing for large independent samples by testing the difference between proportions (Petz, 1985).

RESULTS

In methanol extract of *C. coggygria* (1 g), 62.50 mg of pyrocatechol equivalent of phenols was detected. Also, 46.76 mg of flavonoids and 15.75 mg of nonflavonoids were detected in 1 g of dry weight of extract. Results of determining total phenolic, flavonoid, and nonflavonoid content are given in Table 1.

The results of testing the genotoxic effect of methanol extract (test group 1) and synthetic gallic acid (test group 2) are shown in Table 2. In our experiment, a 5% concentration of methanol extract was shown to be clearly genotoxic, inducing significant increases in the frequency of mutants in all three broods (I, II, and III). Also, 5% synthetic gallic acid induced sex-linked recessive lethal mutations on the X-chromosome of *Drosophila melanogaster* males in all three stages of spermatogenesis.

DISCUSSION

Plant extracts and essential oils, as well as their constituents, are used in the food, cosmetics, and pharmaceutical industries (Stammati et al., 1999). Extracts of many plant species have been examined for a number of biological activities so far, and their antimicrobial, anti-inflammatory, antioxidant, antimutagenic, and cancer-preventive effects have been partially described (Baricevic and Bartol, 2000; Mitić et al., 2001; Vujošević and Blagojević, 2004; Faried et al., 2007).

Phytochemical investigation of methanol extract of the plant *Cotinus coggygria* led to the isolation of several phenolic compounds (Stathopoulou et al., 2007; Zdunić et al., 2007). Our results demonstrate that in methanol extract of *C. coggygria* (1 g), 62.50 mg of pyrocatechol equivalent of phenols are detected.

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989). Phenolic compounds may contribute directly to antioxidative action (Duh et al., 1999). It is suggested that

Table 1. Total phenolics, flavonoids, and nonflavonoid content of *C. coggygria* stem methanol extract.

Extract (MeOH)	Total phenolics mg/g of extract	Flavonoids mg/g of extract	Nonflavonoids mg/g of extract
	62.50 ± 2.55 mg	46.75± 3.05 mg	15.75 ± 1.50 mg

Table 2. Frequencies of SLRL mutations after treatment of Drosophila melanogaster males with methanol extract of the plant Cotinus coggygria and synthetic gallic acid (statistically significant differences: $p < 0.05^{*}$; $p < 0.01^{**}$; $p < 0.001^{***}$).

	Methanol extract of *Cotinus coggygria*	Synthetic gallic acid	Sucrose-negative control		
	(Test group 1)	(Test group 2)	(Test group 3)	$t_{sucrose/extract}$	$t_{sucrose/gallic\ acid}$
I brood Σ	269	134	300	5.45	3.02
No. of lethal	34	13	5	$p < 0.001^{***}$	$p < 0.01^{**}$
% of lethal	12.64	9.7	1.67		
II brood Σ	284	130	269	2.57	2.76
No. of lethal	17	12	5	$p < 0.05^{*}$	$p < 0.01^{**}$
% of lethal	5.99	9.2	1.86		
III brood Σ	252	96	252	5.72	3.65
No. of lethal	43	16	6	$p < 0.001^{***}$	$p < 0.001^{***}$
% of lethal	17.06	16.6	2.38		
I + II + III Σ	805	360	821	8.15	5.42
No. of lethal	94	41	16	$p < 0.001^{***}$	$p < 0.001^{***}$
% of lethal	11.67	11.4	1.95		

polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka et al., 1998; Yoshida et al., 2000; Tsuda et al., 2004).

From alcoholic extract of *C. coggygria*, gallic acid and its derivatives methyl gallate and pentagalloyl glucose were isolated (Westenburg et al., 2000). Polyphenolic gallic acid and its derivatives are biologically active compounds present in many plants (Kahkonen et al., 1999; Lee et al., 2000). They are widespread in plant foods and beverages such as tea and wine and are present in *Cotinus coggygria*, both in the free state and as part of the tannin molecule (Trpinac et al., 1983).

Many plants and herbs have potential antioxidant activity. Gallic acid is a strong natural antioxidant (Aruoma et al., 1993; Heinonen et al., 1998; Khan et al., 2000; Zheng and Wang, 2001). It was reported as a free radical scavenger and as an inducer of differentiation and apoptosis in leukemia, lung cancer, and colon adenocarcinoma cell lines, as well as in normal lymphocyte cells (Inoue et al., 1994; Kawada et al., 2001; Salucci et al., 2002; Sohi et al., 2003). Several plant species with anti-cancer activity have already been discovered, one of them being *Cotinus coggygria*. Gallic acid from this plant has been shown to display selective cytotoxicity against tumor cells and to induce apoptosis in tumor cells (Isuzugawa et al., 2001).

In the present study, we examined the genotoxicity of methanol extract of the plant *Cotinus coggygria* and synthetic gallic acid using a short test for detection of mutagenicity under *in vivo* conditions. Our results suggest, as evident from Table 2, that the components of methanol extract of *Cotinus coggygria* in a concentration of 5% induced sex-linked recessive lethal mutations on the X-chromosome of *Drosophila melanogaster* (test group 1) in all three broods (I, II, and III). We used synthetic gallic acid for comparative analysis (test group 2). This polyphenolic acid was shown to be clearly genotoxic, inducing significant increases in the frequency of mutants in both post-meiotic (spermatids and spermatozoids) and pre-meiotic (spermatocytes) germ cell lines of the eukaryotic species *Drosophila melanogaster.*

Employing *in vivo* experimental methods, the

present study showed a significant genotoxic effect of gallic acid on *Drosophila melanogaster*. Also, methanol extract of the plant *Cotinus coggygria* induced mutations in male germinative cells of this eukaryotic species, while certain chemical components (except gallic acid) in methanol extract manifested a genotoxic effect. Further studies are needed to prove the genotoxicity of these chemical substances.

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ГЕНОТОКСИЧНО ТЕСТИРАЊЕ МЕТАНОЛСКОГ ЕКСТРАКТА БИЉКЕ *COTINUS COGGYGRIA* И ГАЛНЕ КИСЕЛИНЕ НА *DROSOPHILA MELANOGASTER*

СНЕЖАНА СТАНИЋ[1], САЊА МАТИЋ[1], СЛАВИЦА СОЛУЈИЋ[2], и ТАЊА МИЛОШЕВИЋ[2]

[1]*Институт за биологију и екологију, Природно-математички факултет, Универзитет у Крагујевцу,* 34000 Крагујевац, Србија

[2]*Институт за хемију, Природно-математички факултет, Универзитет у Крагујевцу,* 34000 Крагујевац, Србија

Испитиван је генотоксични ефекат метанолског екстракта биљке *Cotinus coggygria* и галне киселине вештачког порекла коришћењем SLRL теста. Повећање фреквенције полно везаних рецесивних летала код тестираних група мужјака еукариотске врсте *Drosophila melanogaster*, у односу на негативну контролу представља позитиван резултат. Статистички значајне разлике установљене за I, II и III легло указују на подједнаку осетљивост ћелија премејотичког и постмејотичког ступња сперматогенезе на компоненте метанолског екстракта и галну киселину вештачког порекла.