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Biological properties of the *Cotinus coggygria* methanol extract

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Abstract

Background and Purpose: *The purpose of this study was to determine the possible antimicrobial and genotoxic effect of the methanol extract obtained from the stem of the plant Cotinus coggygria Scop. (Anacardiaceae).*

Subjects and Method: *The in vitro antimicrobial activity of the methanol extract of C. coggygria and gallic acid was examined on six different bacterial species and Candida albicans, using the cylinder plate and macro broth dilution method. The genotoxicity of the 5% methanol extract from the stem of C. coggygria and synthetic gallic acid in a concentration of 5% was tested on the eukaryotic model system Drosophila melanogaster using the sex-linked recessive lethal (SLRL) test.*

Results and Conclusions: *The results suggest that the methanol extract from C. coggygria showed antimicrobial activity against all test microorganisms, on the other hand, synthetic gallic acid exhibited less antimicrobial activity than methanol extract. Under our experimental conditions, the synthetic gallic acid and methanol extract of the plant C. coggygria showed genotoxic effects inducing increases in the frequency of mutants in both postmeiotic (spermatids and spermatozoids) and premeiotic (spermatocytes) germ cell lines of eukaryotic species Drosophila melanogaster.*

INTRODUCTION

Plants are not only important to the millions of people to whom traditional medicine serves as the only opportunity for health care and to those who use plants for various purposes in their daily lives, but also as a source of new pharmaceuticals *(1)*. So far the extracts of many plant species have been examined for a number of biological activities, and their antimicrobial, anti-inflammatory, antioxidant, antimutagenic and cancer preventive effect have been partially described *(2, 3, 4, 5)*.

Cotinus is a small genus of the family Anacardiaceae with two species: *C. coggygria* Scop. (syn.: Rhus cotinus L.) and *C. obovatus* Raf., American smoketree. *C. coggygria* is usually either considered large shrubs or small trees. It has a wide distribution from southern Europe, the Mediterranean, Moldova and the Caucasus to central China and the Himalayas *(6)*. The flora of Serbia defines two varieties of *C. coggygria*: var. laevis and var. arenaria *(7)*. In folk medicine the plant is used for its antiseptic, anti-inflammatory, antimicrobial, antihaemorrhagic, anti-diarrhea and wound healing properties *(8)*.

It has been suggested that gallic acid (3,4,5-trihydroxybenzoic acid) and its derivatives are biologically active compounds which are present in several plants. According to Westenburg *et al*. *(9)*, Stathopoulou *et al*.

(10) and Antal *et al*. *(11)* gallic acid had been previously isolated from this species. 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 *(12, 13)*.

The objective of this study was to compare selected biological activities of *C. coggygria* methanol extract with natural polyphenols as dominant constituents, versus the biological activities of gallic acid.

MATERIAL AND METHODS

Plant methanol extraction

The plant material was collected from Rujište on Rogozna mountain in the North of Kosovo, in May-June 2007. The species was identified and the voucher specimen deposited (16178, BEOU) in the Department of Botany, Faculty of Biology, University of Belgrade.

The air-dried *C. coggygria* stem (170 g) was broken into small pieces 2–6 mm by using a cylindrical crusher and extracted with methanol (500 mL) using Soxhlet apparatus. The extract was filtered through a paper filter (Whatman, No. 1) and solvent was evaporated. Dry extract (4.7 g) was stored in a dark glass bottle for further processing.

Identification of methanol extracts

Total soluble phenolics compounds in the methanol extract of *C. coggygria* stem were determined with Folin-Ciocalteu reagent *(14)* 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 phenolics compounds in the *C. coggygria* stem was determined as mg of pyrocatechol equivalent g dry weight of extract, by using an equation that was obtained from the standard pyrocatechol graph (*15, 16*). All samples were analyzed in triplicate.

Flavonoids fraction was precipitated according to Alberto *et al. (17)* by mixing 10 mL of the extract dissolved in methanol (0.02 g/mL) with 10 mL HCl (1:3) and 5 mL of HCHO (8 mg/mL). After 24 h the mixture was filtered through a filter paper (Whatman No.5). Nonflavonoid components were determined from the filtrate with Folin-Ciocalteu reagent, by using the same spectrophotometric method as for determining total phenolics concentration, absorbance was measured at 765 nm on the spectrophotometer. Nonflavonoid content was expressed as mg of pyrocatechol per g of dry weight through the calibration curve with pyrocatechol. All samples were analyzed in triplicate.

Flavonoids content was determined from residuum of the total phenolics and nonflavonoid content. Flavonoids content was expressed as mg of pyrocatechol per g of extract. All samples were analyzed in triplicate.

For the purpose of comparative analysis synthetic gallic acid (Sigma-Aldrich) was used.

Microorganisms

Bacterial strains and yeast used in these experiments were: *Staphylococcus aureus* (IPH), *Bacillus subtilis* (IPH), *Klebsiella pneumoniae* (B26), *Escherichia coli* (ATCC 25923), *Staphylocossus aureus* (ATCC 25923), *Micrococcus lysodeikticus* (ATCC 4698) and yeast *Candida albicans* (ATCC 10259).

All of the tested bacteria cultures were obtained from the Institute for Health Protection (IPH) in Kragujevac and the Faculty of Science, University of Belgrade, Serbia. The identity of the bacterial strains and yeast was confirmed in the Laboratory for Microbiology at the Department of Biology (B), Faculty of Science, University of Kragujevac and University of Belgrade, Serbia.

Antimicrobial activity determined by the cylinder plate method

Petri dishes containing 10 mL Muller Hinton Agar (for bacteria) and Sabouraud dextrose agar (for yeast) with 1 mL volume of microbial suspension. For bacteria, 24 h old culture and for yeast, 72 h old, were adjusted with sterile water to 6.5×10^6 CFU/mL for bacteria and 3×10^4 CFU/mL for yeast. The plates incubated at 37°C for approximely 20 min until microbial overlay had dried on the surface. Then sterile vertical cylinders were placed alternatively on the Petri plates and samples of methanol extract and gallic acid, respectively (150, 300 and 500 μg) were aseptically poured into the vertical cylinder using micropipettes *(18)*. The plates were subsequently incubated for 24 h at 37°C for bacteria and 48 h at 28°C for yeast. Negative controls were prepared using the same solvents (5% DMSO) employed to dissolve the extract. Amracin (100 μg for bacteria) and Nistatin (100 μg for yeast) were used as positive controls. The diameter of zones of inhibition was measured in mm. All experiments were performed in duplicate.

Antimicrobial activity determined by the macro broth dilution method

The minimal inhibitory concentration (MIC) *(19, 20)* of the methanol extract was determined by the macro broth two-fold serial technique. A series of two-fold dilutions of the gallic acid and extract, ranging from 7.8 μg/mL to 500 μg/mL (in 5% solution of DMSO) was prepared in Mueller-Hinton broth with the addition 0.1 mL of a suspension of the microbial spores (5.4×10^6 CFU/mL for bacteria and 3×10^4 CFU/mL for yeast). The MIC values were determined after 24 h as the lowest concentration of the extract, which inhibited visible growth of each organism. Amracin and Nystatin were chosen as the positive control drugs for bacteria and C. albicans, respectively. Negative control contained only 5% solution of DMSO.

Genotoxicity determination by sex-linked recessive lethal (SLRL) test

The sex-linked recessive lethal test for mutagenicity was carried out by the standard procedure *(21)* with laboratory stocks of *Drosophila melanogaster* (obtained from the Umea Stock Centre, Sweden).

The stocks were maintained and all experiments were 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 mould and infections).

Three to four day old wild type males of *Drosophila melanogaster* (test group 1, N = 30) were starved in empty bottles for 5 h and then transferred and exposed to the 1% sucrose by methods of Lewis and Bacher *(22)* and served as the negative control group. The other group of individuals (test group 2, N = 30) was treated with 0.75 ppm ethyl-methane sulfonate (EMS) dissolved in 1% sucrose and served as the positive control group. The third group of individuals (test group 3, N = 30 males) was exposed to the methanol extract dissolved in 1% sucrose, while the fourth group of individuals (test group 2, N = 15 males) was treated with 5% synthetic gallic acid dissolved in 1% sucrose.

After 24 h treatment and further 24 h resting on the fresh medium, males were individually mated to two-five day old virgin *Basc* females (which made brood I). The males were then remated in new vials with three new virgins *Basc* females at two-three day intervals (thus creating brood II), to test all germ cell stages for the presence of mutations. Males were then transferred again to the fresh vials containing three *Basc* virgins (brood III).

The F_2 generation was examined for the presence or absence of *wild* type males. All *wild* type males in this generation contained the same treated X-chromosome in hemizygous condition. Any recessive lethal on it will be expressed before the adult stage and such males will not emerge. Cells exposed in successive spermatogenesis stages, were tested for induced mutations **(23)**.

Statistical analysis

Statistical evaluation of the antimicrobial data was performed by Student's t-test. The results are expressed as mean ± standard deviation. The frequency of sex-linked recessive lethal cultures was calculated according the ratio between the numbers of lethal cultures to 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 the percentage difference regarding lethal cultures was examined by testing for big independent samples – testing the difference between proportions *(24)*.

RESULTS

In the methanol extract of *C. coggygria* (1 g), 62.50 mg pyrocatechol equivalent of phenols was detected. Also, 46.76 mg flavonoids and 15.75 mg nonflavonoids were

TABLE 1

Total phenolics, flavonoids and nonflavonoid content of the *Cotinus coggygria* methanol extract determined spectrophotometrically with Folin-Ciocalteu reagent.

C. coggygria methanol extract	Total phenolics mg/g extract	Flavonoids mg/g extract	Nonflavonoids mg/g extract
	62.50 ± 2.55[a]	46.75 ± 3.05[a]	15.75 ± 1.50[a]

[a]Expressed as mg of pyrocatechol equivalent per g dry weight of extract
All measurements were repeated three times

TABLE 2

Antimicrobial activity of the methanol extract of *C. coggygria* stem and synthetic gallic acid by cylinder plate method.

Microorganism	Zones of inhibition (mm)[a,b,c]						
	C. coggygria extract			Gallic acid			Standard[d]
	150 µg	300 µg	500 µg	150 µg	300 µg	500 µg	100 µg
Bacteria							
S. aureus (IHP)	14 ± 1.0	6 ± 0.5	9 ± 0.5	0	0	0	26 ± 1.0
B. subtilis	0	8 ± 1.0	9 ± 0.5	0	0	0	30 ± 1.0
K. pneumoniae	0	0	10 ± 0.5	10 ± 1.0	13 ± 0.5	0	31 ± 0.5
E. coli	29 ± 1.0	15 ± 0.5	17 ± 0.5	29 ± 0.5	31 ± 0.2	30 ± 0.5	36 ± 0.1
S. aureus	15 ± 0.5	19 ± 0.5	10 ± 0.5	9 ± 0.5	9 ± 1.0	10 ± 0.1	32 ± 0.5
M. lysodeikticus	20 ± 0.5	8 ± 0.5	18 ± 0.5	18 ± 0.3	19 ± 0.5	0	40 ± 0.5
Yeast							
C. albicans	0	0	0	0	0	0	32 ± 0.5

[a] Values are mean ± S.E based on two replicates, zone of inhibition in mm
[b] "O" absence of antimicrobial activity
[c] Negative control (DMSO) was negative
[d] Positive control: Amracin 100 µg for the bacteria and Nistatine 100 µg for the yeast

detected in 1 g of dry weight of extract. Results of the determination of total phenolics, flavonoid and nonflavonoid contents are given in Table 1.

The antimicrobial activities by cylinder plate method of the methanol extract of *C. coggygria* stem and synthetic gallic acid against the test bacteria and *C. albicans* are presented in Table 2, while the data of antimicrobial activities by macro broth dilution method of the methanol extract of *C. coggygria* stem and synthetic gallic acid were given in Table 3. These results showed that the methanol extract has higher antimicrobial activity than synthetic gallic acid.

TABLE 3

Antimicrobial activity of the methanol extract of *C. coggygria* stem and synthetic gallic acid by macro broth dilution method.

Microorganism	Minimal inhibitory concentration (μg/mL)		
	C. coggygria extract	Synthetic gallic acid	Standard[a]
Bacteria			
S. aureus (IHP)	250	0	2.500
B. subtilis	125	0	1.250
K. pneumoniae	250	500	0.625
E. coli	250	0	0.625
S. aureus	250	500	1.250
M. lysodeikticus	250	0	1.250
Yeast			
C. albicans	125	500	5

[a] Standard: Amracin 5 μg/mL for the bacteria and Nistatine 5 μg/mL for the yeast
[b] "O" absence of antimicrobial activity

The genotoxic effect of *C. coggygria* methanol extract (test group 3) and synthetic gallic acid (test group 4) are shown in Table 4. Ethyl-methane sulfonate in a concentration of 0.75 ppm (test group 2) was shown to be clearly genotoxic, inducing significant increases in the frequency of mutations in all the three broods. The frequency of germinative mutations induced by the *C. coggygria* extract in SLRL test is significantly higher than that induced by sucrose as negative control (Table 4). Compared to the EMS as a positive control group, the extract induced recessive lethal X-linked mutations in all three stage of spermatogenesis. On the other hand, the synthetic gallic acid in the concentration of 5% induced significant increases in the frequency of mutations in III brood compared to the EMS, based on which we may conclude that spermatocytes fall into and represent a sensitive stage of spermatogenesis.

DISCUSSION

The use of a natural product with therapeutical properties has a long history. Plants are invaluable sources of pharmaceutical products *(25)*. Many plant extracts have been used as a source of medicinal agents to cure urinary

TABLE 4

Frequencies of SLRL mutations after treatment of *Drosophila melanogaster* males with methanol extract of plant *C. coggygria* and synthetic gallic acid.

Treatment	I brood Σ No of lethal % of lethal	II brood Σ No of lethal % of lethal	III broods Σ No of lethal % of lethal	I+II+III Σ No of lethal % of lethal
Test group 1	300	269	252	821
1% Sucrose	5	5	6	16
negative control	1.67	1.86	2.38	1.95
Test group 2	265	193	140	598
0.75 ppm EMS	88	65	36	189
positive control	32.21	33.68	25.71	31.61
Test group 3	269	284	252	805
5% *C. coggygria*	34	17	43	94
extract	12.64	5.99	17.06	11.67
Test group 4	134	130	96	360
5% Synthetic	13	12	16	41
gallic acid	9.7	9.2	16.6	11.4
$t_{sucrose/extract}$	5.45***	2.57*	5.72***	8.15***
$t_{sucrose/gallic\ acid}$	3.02**	2.76**	3.65***	5.42***
$t_{EMS/extract}$	5.71***	7.57***	2.25*	9.09***
$t_{EMS/gallic\ acid}$	6.13***	6.25***	$1.70^{\#}$	8.4***

Statistically significant difference: $p < 0.05$*; $p < 0.01$**; $p < 0.001$***
Frequencies that are not significantly different: $p > 0.05^{\#}$

tract infections, cervicitis vaginitis, gastrointestinal disorders, respiratory diseases, cutaneous affections, helminitic infections and inflammatory process *(26, 27)*.

Phytochemical investigation of the methanol extract of plant *C. coggygria* led to the isolation of several phenolic compounds *(10, 28)*. Polyphenolic compounds are known to have antioxidant activity and it is likely that the activity of the extracts is due to these compounds. It is suggested that polyphenolic compounds have shown anticarcinogenic effects and potential to prevent cardiovascular and cerebrovascular diseases *(29)*. Our results demonstrate (Table 1) that in the methanol extract of *C. coggygria* (1 g), 62.50 mg of pyrocatechol equivalent of phenols are detected.

Many efforts have been made to discover new antimicrobial compounds from various sources such as animals, microorganisms and plants. Plants possess antimicrobial natural products to protect themselves *(30, 31)*. Antimicrobial activities of various herbs and spices in plant leaves, flowers, stems, roots or fruits have been reported *(32, 33, 34, 35)*.

The results obtained regarding the antimicrobial activity of the methanol extract and synthetic gallic acid, as evident from Table 2 and Table 3, showed that synthetic gallic acid demonstrated lower antimicrobial activity than methanol extract. In an amount of 500 μg, extract was active against all examined pathogenic and phytopathogenic bacteria with the inhibition zones ranging from 9 to 18 mm (Table 2). Very sensitive bacteria toward methanol extract are *E. coli* (in amounts of 150 μg and 300 μg inhibition zones are 29 and 17 mm, respectively) and *M. lysodeikticus* (150 and 300 μg of extracts produced inhibition zones of 20 and 18 mm, respectively). All phytopathogenic bacteria were sensitive in the presence of the extract in an amount of 300 μg to 500 μg. The highest concentration of the methanol extract of *C. coggygria* (500 μg) showed the highest inhibition zones (ranging from 9 to 18 mm). *C. albicans* was completely resistant in the presence of all examined concentration of the plant methanol extract.

Based on MIC values, the tested extract shows antibacterial activity between 125 and 250 μg/mL against all tested pathogenic bacteria (Table 3). Although the MICs obtained with the methanol extracts are high compared with those of Amracine, in general between 125–250 μg/mL, these results are of interest since they have been obtained with methanol extracts and are not a pure product and could be considered to have good potency level. Based on these results, it is possible to conclude that methanol extract of *C. coggygria* has stronger antibacterial activity.

In general, pure gallic acid showed lower antimicrobial activities than the methanol extract. From six investigated bacteria gallic acid showed activity on four bacteria species. Gallic acid (the concentrations are 150, 300, 500 μg/disc) showed strong antibacterial activity against *E. coli (36)* with inhibition zones from 29–31 mm respectively. For *S. aureus* the effect of gallic acid is 40–50% less than methanol extract. The examined concentration of gallic acid does not demonstrate inhibition effect on the growth of *S. aureus* (isolate), *B. subtilis* and *C. albicans*.

The sex-linked recessive lethal test on *Drosophila melanogaster* has been proved to be an excellent screening test for the detection of natural plant's mutagens *(37)*. In the present study, we examined the genotixicity of the methanol extract of plant *C. coggygria* and synthetic gallic acid using a short test for the detection of mutagenicity *in vivo* conditions. According to the results, methanol extract of plant *C. coggygria* in a concentration of 5% induced sex-linked recessive lethal mutations on the X--chromosome of *Drosophila melanogaster* (test group 3, Table 4) in both postmeiotic (spermatids and spermatozoids) and premeiotic (spermatocytes) germ cell lines. For the purpose of comparative analysis we used synthetic gallic acid (test group 4). Compared to the EMS as positive controle group, this polyphenolic acid induced significant increases in the frequency of mutations in III brood (Table 4), based on which we may conclude that spermatocytes represent a sensitive stage of spermatogenesis.

The antimicrobial studies revealed that methanol extract of *C. coggygria* is more effective against all tested microorganisms than gallic acid. Therefore, the extract can be used as an effective and safe source of antibacterial agent. On the other hand, the results obtained in the investigation of genotoxicity showed the genotoxic effect of the extract.

Reviewing the literature we found an increasing number of articles showing adverse effects of the drug. For example, two antibacterial compounds, metronidawle and furazolidone, were tested for their genotoxic effects in somatic and male germ line cells of *Drosophila melanogasfer*. The results show that metronidazole is only genotoxic at the highest concentration (100 mM) both in the somatic and germ line cells, whereas hrazolidone is genotoxic even at lower concentrations *(38)*. Another example is Ciprofloxacin, one of the best known drugs for the treatment of many bacterial infections and widely used in medicine. Ciprofloxacin is highly active *in vitro* against a broad spectrum of Gram-negative and Gram-positive organisms *(39)*. On the other hand, *in vitro* genotoxicity of Ciprofloxacin has been demonstrated with sister chromatid exchange and unscheduled DNA synthesis *(40)* and *in vivo* genotoxicity with the micronucleus test *(41)* and chromosomal aberrations *(42)* in lymphocytes of humans.

In conclusion, the results of this research showed that total phenolics are important components of this plant, and some of the pharmacological effects could be attributed to the presence of these valuable constituents. Further work is required to establish if any other components of this plant have any role in the activity of the *C. coggygria* extracts. Also, further *in vitro* and *in vivo* studies are needed before definitive conclusions about the mutagenic potential of *C. coggygria* can be drawn.

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