Biological properties of the *Cotinus coggygria* methanol extract

**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-diarrhoea 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 Rujiste 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 NaHCO3 (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 extract. All samples were analyzed in triplicate.

Flavonoids content was determined from residual 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 (IPU), Bacillus subtilis (IPU), Klebsiella pneumoniae (B26), Escherichia coli (ATCC 25923), Staphylococcus 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 (IPU) 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<sup>5</sup> CFU/mL for bacteria and 3 × 10<sup>5</sup> CFU/mL for yeast. The plates incubated at 37°C for approximately 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 Nystatin (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<sup>6</sup> CFU/mL for bacteria and 3 × 10<sup>6</sup> 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 F2 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>
<thead>
<tr>
<th>Microorganism</th>
<th>Zones of inhibition (mm)</th>
<th>Gallic acid</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. coggygria extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>150 µg</td>
<td>300 µg</td>
<td>500 µg</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. aureus (IHP)</td>
<td>14 ± 1.0</td>
<td>6 ± 0.5</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>0</td>
<td>8 ± 1.0</td>
<td>9 ± 0.5</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>0</td>
<td>0</td>
<td>10 ± 0.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>29 ± 1.0</td>
<td>15 ± 0.5</td>
<td>17 ± 0.5</td>
</tr>
<tr>
<td>S. aureus</td>
<td>15 ± 0.5</td>
<td>19 ± 0.5</td>
<td>10 ± 0.5</td>
</tr>
<tr>
<td>M. lysodeikticus</td>
<td>20 ± 0.5</td>
<td>8 ± 0.5</td>
<td>18 ± 0.5</td>
</tr>
<tr>
<td>Yeast</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values are mean ± S.E based on two replicates, zone of inhibition in mm

**“O” absence of antimicrobial activity

Negative control (DMSO) was negative

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.

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 stages 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 therapeutic 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 3**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Minimal inhibitory concentration (µg/mL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. coggygria</em></td>
<td>Synthetic gallic acid</td>
<td>Standard*</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> (IHP)</td>
<td>250</td>
<td>0</td>
<td>2.500</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>125</td>
<td>0</td>
<td>1.250</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>250</td>
<td>500</td>
<td>0.625</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>250</td>
<td>0</td>
<td>0.625</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>250</td>
<td>500</td>
<td>1.250</td>
</tr>
<tr>
<td><em>M. lysodeikticus</em></td>
<td>250</td>
<td>0</td>
<td>1.250</td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>125</td>
<td>500</td>
<td>5</td>
</tr>
</tbody>
</table>

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

**TABLE 4**

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>I brood Σ</th>
<th>II brood Σ</th>
<th>III broods Σ</th>
<th>1+II+III Σ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of lethal</td>
<td>% of lethal</td>
<td>% of lethal</td>
<td>% of lethal</td>
</tr>
<tr>
<td>Test group 1</td>
<td>300</td>
<td>269</td>
<td>252</td>
<td>821</td>
</tr>
<tr>
<td>1% Sucrose</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>negative control</td>
<td>1.67</td>
<td>1.86</td>
<td>2.38</td>
<td>1.95</td>
</tr>
<tr>
<td>Test group 2</td>
<td>265</td>
<td>193</td>
<td>140</td>
<td>598</td>
</tr>
<tr>
<td>0.75 ppm EMS</td>
<td>88</td>
<td>65</td>
<td>36</td>
<td>189</td>
</tr>
<tr>
<td>positive control</td>
<td>32.21</td>
<td>33.68</td>
<td>25.71</td>
<td>31.61</td>
</tr>
<tr>
<td>Test group 3</td>
<td>269</td>
<td>284</td>
<td>252</td>
<td>805</td>
</tr>
<tr>
<td>5% <em>C. coggygria</em> extract</td>
<td>34</td>
<td>17</td>
<td>43</td>
<td>94</td>
</tr>
<tr>
<td>12.64</td>
<td>5.99</td>
<td>17.06</td>
<td>11.67</td>
<td></td>
</tr>
<tr>
<td>Test group 4</td>
<td>134</td>
<td>130</td>
<td>96</td>
<td>360</td>
</tr>
<tr>
<td>5% Synthetic gallic acid</td>
<td>13</td>
<td>12</td>
<td>16</td>
<td>41</td>
</tr>
<tr>
<td>9.7</td>
<td>9.2</td>
<td>16.6</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>% sucrose/extract</td>
<td>5.45***</td>
<td>2.57*</td>
<td>5.72***</td>
<td>8.15***</td>
</tr>
<tr>
<td>% sucrose/gallic acid</td>
<td>3.02**</td>
<td>2.76**</td>
<td>3.65***</td>
<td>5.42***</td>
</tr>
<tr>
<td>% EMS/extract</td>
<td>5.71***</td>
<td>7.57***</td>
<td>2.25*</td>
<td>9.09***</td>
</tr>
<tr>
<td>% EMS/gallic acid</td>
<td>6.13***</td>
<td>6.25***</td>
<td>1.70*</td>
<td>8.4***</td>
</tr>
</tbody>
</table>

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 pheno-
colic 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 anti-
carcinogenic effects and potential to prevent cardiovascular and cerebrovascular diseases (29). Our results dem-
strate (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 anti-
microbial compounds from various sources such as ani-
imals, microorganisms and plants. Plants possess antimicro-
natural products to protect themselves (30, 31). Antimicrobial activities of various herbs and spices in plant leaves, flowers, stems, roots or fruits have been re-
ported (32, 33, 34, 35).

The results obtained regarding the antimicrobial ac-
tivity 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 ac-
tive against all examined pathogenic and phytopatho-
genic bacteria with the inhibition zones ranging from 9 to 18 mm (Table 2). Very sensitive bacteria toward meth-
anol 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 phytopa-
thogenic bacteria were sensitive in the presence of the ex-
tract 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 anti-
bacterial 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 Amrachine, 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 produ-
t 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 antibac-
terial activity.

In general, pure gallic acid showed lower antimicro-
bial activities than the methanol extract. From six investi-
gated 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 respect-
ively. 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 me-
lanogaster* 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 genotoxicity of the methanol extract of plant *C. coggygria* and synthetic gal-
ic acid using a short test for the detection of mutageni-
city in vivo conditions. According to the results, metha-
nol 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 (spermatozoids and spermato-
oids) and premeiotic (spermatocytes) germ cell lines. For the purpose of comparative analysis we used syn-
thetic 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 sper-
matocytes represent a sensitive stage of spermatogenesis.

The antimicrobial studies revealed that methanol extract of *C. coggygria* is more effective against all tested microor-
ganisms 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 num-
ber of articles showing adverse effects of the drug. For example, two antibacterial compounds, metronidazole and furazolidone, were tested for their genotoxic effects in so-
matic and male germ line cells of *Drosophila melanogas-
ter*. The results show that metronidazole is only genoto-
xic at the highest concentration (100 mM) both in the so-
matic and germ line cells, whereas furazolidone is geno-
toxic 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 chro-
matid 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. Fur-
ther work is required to establish if any other compo-
nents of this plant have any role in the activity of the *C. coggygria* extracts. Also, further in vitro and in vivo stud-
ies are needed before definitive conclusions about the mutagenic potential of *C. coggygria* can be drawn.

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