Isolation and characterization of antimicrobial compounds from *Cotinus coggygria* Scop. ethyl acetate extract

Ali Şen¹, Ayşe Seher Birteksöz Tan², Şükran Kültür³, Leyla Bitiş¹

¹Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, İstanbul, Turkey
²İstanbul Univesity, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, İstanbul, Turkey
³İstanbul Univesity, Faculty of Pharmacy, Department of Pharmaceutical Botany, İstanbul, Turkey

ORCID IDs of the authors: A.Ş. 0000-0002-2144-5741; A.S.B.T. 0000-0003-2123-1468; Ş.K. 0000-0001-9413-5210; L.B. 0000-0003-1167-6666

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ABSTRACT

**Background and Aims:** *Cotinus coggygria* leaves are traditionally used in the treatment of various diseases, including their use for antifungal purposes. The aim of this study was to evaluate the antimicrobial activity of the extracts of *C. coggygria* and to discover compounds that may be responsible for the activity of the most active extract.

**Methods:** The antimicrobial activities of extracts and compounds were assessed by the microbroth dilution technique. Major compounds of active extract were isolated using chromatographic methods and identified by spectroscopic methods.

**Results:** Diethylether (CCD), ethyl acetate (CCEA), methanol (CCM), ethanol (CCE) and water (CCW) extracts of *C. coggygria* exhibited noticeable antifungal activities against *Candida albicans* with MIC values of 39, 4.9, 4.9, 4.9 and 9.8 μg/mL, respectively. Also, CCEA extract showed good antibacterial activity against *Proteus mirabilis* with an MIC value of 156 μg/mL. Two major compounds, gallic acid and methyl gallate, were isolated from CCEA, the most active extract. Gallic acid was found to be highly active against *C. albicans* and *C. tropicalis* with an MIC value of 9.8 μg/mL (for both fungi). In addition, gallic acid showed moderate antimicrobial activity against *Staphylococcus aureus*, *S. epidermidis* and *C. parapsilosis* with MIC values of 78, 156 and 156 μg/mL, respectively.

**Conclusion:** This study is the first study to reveal the compounds responsible for the activity of CCEA extract with antifungal activity. These results suggest that gallic acid, along with other phenolic compounds, is responsible for the antifungal activity of CCEA. Also, it confirms the ethnobotanical use of *C. coggygria* for antifungal purposes.

**Keywords:** Antimicrobial activity, *Candida* species, *Cotinus coggygria*, ethyl acetate extract, gallic acid, methyl gallate

INTRODUCTION

Infectious diseases, especially in tropical and developing countries, are responsible for more than 50% of deaths worldwide, according to World Health Organization (WHO) reports (Zhang et al., 2013). At the same time, microbial contamination is one of the major concerns of the food and pharmaceutical industries, and the increasing tendency of microorganisms to develop resistance to existing antibiotics has led researchers to find new antimicrobial agents (Singh, Pandey, Agnihotri, Singh, & Pandey, 2017). Recent scientific studies have shown that there is a significant agreement between the traditional use of plants by the people in the treatment of specific symptoms and the experimental studies carried out in the laboratory such as anti-bacterial, anti-fungal, antitumor, and antiviral activities (Zhang et al., 2013). At the same time, the fact that *Cotinus coggygria*, which is used for wound-he-
aling purposes, has shown an important wound-healing effect on normal and diabetic wounds in the previous pharmacological studies conducted by our team supports this idea (Aksoy et al., 2016a; Aksoy et al., 2016b). Various compounds with antimicrobial activity have been isolated from higher plants, and many of these compounds have shown promising potential to treat infectious diseases (Madzikizela, Aderogba, Finnie, & Van Staden, 2014). Therefore, it is important to conduct research on plants to discover promising new and natural antimicrobial compounds or products.

The genus *Cotinus*, a member of the Anacardiaceae family, is represented by a single species in Turkey (Davis, 1967). This species is *C. coggygria* and the leaves of this species are used directly fresh, or in the form of an infusion or decoction. Leaves of *C. coggygria* have been used in the treatment of stomach pain, gastritis, ulcer, cuts, burns, wounds, eczema, fractures, diabetes, urinary diseases, cardiac diseases, kidney stones, nephritis, cancer, cough, abdominal pain, arm numbness, asthma, hemorrhoids, enteritis and anthrax in Turkey. It is also used as an antifungal, antihypertensive and a vasodilator (Kültür, 2007). Previous studies on phytochemical analysis of *C. coggygria* indicated the presence of flavonoids (fisetin, fusitin, sulfuretin, myricetin, quercetin, myricetin-3-O-arhamnoside, myricetin-3-O-β-galactoside, 2,10-oxo-10-methoxy sulfuretin, cotinignan A, sulfuretin, 2,3-trans-fusitin, fisetin, butin, butein, taxofolin, eriodictyol, 3',5',7'-tetrahydroxyflavone, 3',4,7-trihydroxyflavone, 3-O-methyl-2,3-trans-fusitin, 3-O-galloyl-2,3-trans-fusitin); anthocyanins (leucodelphinidin, leucocyanidin, delphinidin 3-galactoside, cyanidin 3-galactoside, petunidin 3-glucoside, delphinidin 7-glucoside, cyanidin 3-glucoside-7-rhamnoside); essential oil (limonene, (Z)-β-oxime, (E)-β-oxime); gallic tannin (gallic acid), methyl gallate, gallocatechin, pentagalloyl glucose, biaroun, β-resorcylic acid and 3-O-β-sitosterol glucoside (Hegnauer, 1964; Tanev & Timberlake, 1969; Weist-Munburg et al., 2000; Demirci, Demirci, & Baser, 2003; Kultur & Biler, 2004; Novakovic et al., 2007; Dulger, Hacioglu, & Bilen, 2009; Özbek et al., 2019; Novakovic et al., 2019).

There are studies in the literature showing that different extracts obtained from the leaves, stem and aerial parts of *C. coggygria* have antimicrobial and antifungal activity (Tunc, Hoş, & Guneş, 2013; Marcetić et al., 2013; Matic, Stanic, Soljic, Milosevic, & Niciforovic, 2011). However, a report on compounds that may be responsible for the antimicrobial activity of this plant has not been published so far. To the best of our knowledge, this study is the first to find compounds that may be responsible for the activity of the ethyl acetate extract, which has been found to have potent antifungal activity. Therefore, the aim of our current study is to evaluate antimicrobial activities of the various extracts obtained by Soxhlet and maceration from *C. coggygria* and to isolate the compounds that may be responsible for the activity of the extract that is showing the best activity.

**MATERIALS AND METHODS**

**Plant material**

*C. coggygria* leaves were collected in the flowering periods from the Kirklareli province of Turkey and identified by Dr. Sukran Kultur, a botanist of the Faculty of Pharmacy, University of Istanbul. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE No: 80926).

**Extraction**

*C. coggygria* leaves were dried in the shade and powdered by a mechanical grinder. A total of 20 grams of powdered material was extracted with solvents of increased polarity such as petroleum ether, chloroform, diethyl ether, ethyl acetate, methanol using Soxhlet apparatus and extraction was continued until the solution became colorless. A total of 20 grams of powdered material was separately extracted with 96% ethanol and water using maceration method. Also, about 300 g of the plant was weighed for isolation and similar extraction procedures described above were carried out.

**Antimicrobial activity**

In this study, the *in vitro* antimicrobial activities of the extracts and isolated compounds was determined using the microbroth dilution technique described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2006; CLSI, 2008). The minimum inhibitory concentrations (MICs) of the extracts were investigated against *Staphylococcus aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *C. parapsilosis*, ATCC 22019, *C. tropicalis* ATCC 750. Serial twofold dilutions ranging from 5000 to 4.8 µg/mL were prepared in Mueller–Hinton broth (MHB) (DIFco, Detroit, MI, USA) for the bacteria and RPMI-1640 medium buffered to pH 7.0 with MOPS for yeast strain were used as the test medium. (Sigma, St. Louis, MO, USA) medium for the yeast. DMSO was used as a solvent for the extracts. Each well was inoculated with 50 µL of a 4–6 h broth culture to give a final concentration of 5×10^5 cfu/mL for the bacteria and 0.5×10^3 to 2.5×10^3 cfu/mL for the yeast in the test trays. The trays were covered and placed in plastic bags to prevent evaporation. The trays containing MHB were incubated at 37°C for 24 h, while those containing the RPMI-1640 medium were incubated at 30°C for 48 h. The MIC of each extract and isolated compounds were defined as the lowest concentration of compound required for complete inhibition of visible growth. Ciprofloxacin and fluconazole were used as reference antimicrobials for bacteria and yeast, respectively. Also, as a control, the antimicrobial effects of the dimetil sulfoxide were investigated against test microorganisms. According to the values of the controls, the results were evaluated. The MIC values of the ciprofloxacin and fluconazole were within the accuracy range in CLSI throughout the study (CLSI, 2014).

**Isolation of active compounds from CCEA extract**

In the antimicrobial activity experiment, the CCEA showed the best antibacterial and antifungal activity among all extracts (Table 1). Therefore, CCEA was chosen for isolation. The CCEA (1.91 g) was fractionated by CC on polyamide, eluting with CH₃OH/H₂O mixture in increasing polarity to yield thirty fractions (Figure 1). Fractions showing similar TLC profiles were combined to give seven sub-fractions (F1-F7, F8-F10, F11-F14, F15-F17, F19-F21, F22-F23, F24-F30). When the fractions were examined on the TLC plate, isolation was continued with F11-
F14 fraction, where the major compounds appeared to be the most pure. F11–F14 was repeatedly chromatographed on a Sephadex LH-20 column, eluted with CH$_3$OH and then combined sub-fractions were re-chromatographed by preparative TLC with toluene: acetone: formic acid (5:5:1) to give gallic acid (25.7 mg) and methyl gallate (17.4 mg) (Figure 1).

RESULTS AND DISCUSSION

In this study, the antimicrobial activities of various extracts obtained by different extraction methods from C. coggygria as well as the major compounds isolated from the active extract were investigated. The antimicrobial activities of extracts were investigated against Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, and Candida albicans. Saraiva et al. (2011) suggested that plant extracts with MIC values of < 100 µg/mL were considered to be highly active antimicrobial agents; those with MICs of 100 to 500 µg/mL were considered to have low activity; and those with MICs of > 2000 µg/mL were considered to have no activity. According to this view, CCD, CCEA, CCM, CCE and CCW extracts were re-chromatographed by preparative TLC with toluene: acetone: formic acid (5:5:1) to give gallic acid (25.7 mg) and methyl gallate (17.4 mg) (Figure 1).

Table 1. Antimicrobial activity of various extracts from Cotinus coggygria leaves.

| Microorganisms          | CCP | CCC | CCD | CCEA | CCM | CCE | CCW | CPFX | FCZ |
|-------------------------|-----|-----|-----|------|-----|-----|-----|------|-----|
| **Staphylococcus aureus** |     |     |     |      |     |     |     |      |     |
| **Staphylococcus epidermidis** |     |     |     |      |     |     |     |      |     |
| **Escherichia coli**     |     |     |     |      |     |     |     |      |     |
| **Klebsiella pneumoniae**|     |     |     |      |     |     |     |      |     |
| **Pseudomonas aeruginosa**|     |     |     |      |     |     |     |      |     |
| **Proteus mirabilis**    |     |     |     |      |     |     |     | 156  | 0.31|
| **Candida albicans**     |     |     |     | 39   |     | 4.9 | 4.9 | 4.9  | 9.8 |

Abbreviations: CCP, CCC, CCD, CCEA, CCM, CCE and CCW show petroleum ether, chloroform, dichlylether, ethyl acetate, methanol, ethanol and water extracts, respectively. CPFX: Ciprofloxacin, FCZ: Fluconazole, - No activity

In a study conducted by Tunç et al. (2013), antibacterial effects of petroleum ether, chloroform, acetone, methanol, ethanol and distilled water extracts from the leaves of C. coggygria on S. epidermidis, E. coli, Salmonella typhimurium, Enterococcus faecalis, P. aeruginosa, S. aureus, and Bacillus subtilis were investigated and it was revealed that distilled water and methanol extracts showed good activity against S. aureus, S. epidermidis, and E. faecalis. In addition, it was found that distilled water extract had the highest activity with 22 mm inhibition zone against E. faecalis among these extracts (Tunç et al., 2013). In another study, Marcetic et al. (2013) tested antimicrobial activities of 70% acetone extract obtained from young shoots which included leaves and branches of C. coggygria and its chloroform, ethyl acetate and water fractions against S. aureus, S. epidermidis, Micrococcus luteus, E. faecalis, B. subtilis, P. aeruginosa, S. epidermidis, and E. faecalis. In addition, it was found that distilled water extract had the highest activity with 22 mm inhibition zone against E. faecalis among these extracts (Marcetić et al., 2013). Additionally, Matic et al. (2011) examined antibacterial and antifungal effects of the methanol extract obtained by Soxhlet from the stem of C. coggygria and found that the MIC values of extract were in the range of 3.1–200 µg/mL. When compared with our current study, it was seen in previous studies that extracts from different parts of C. coggygria have antibacterial effects against various bacterial species. In the current study, it was observed that antibacterial activities of all the extracts were quite weak except for the activity shown by CCEA extract against P. mirabilis. However, the antifungal activity values of the extracts against C. albicans were close to or higher than previous studies. This may be due to the fact that collection time, growth place, extracted part of the plant as well as
the extraction method, seasonal changes, growth stage and climate conditions are different.

Two major compounds, gallic acid and methyl gallate, were isolated from the CCEA extract, which showed the best antimicrobial activity. All isolated compounds were analyzed by spectroscopic methods (1H NMR, 13C NMR-APT, HMBC) and their data were compared with those reported in the literature (Xu et al., 2015; Banday et al., 2012). Also, gallic acid isolated in this study was compared on TLC with an authentic sample. The compounds were identified as follows:

**Gallic acid:** Colourless amorphous powder. 1H NMR (500 MHz, CD3OD, δ, ppm, J/Hz): 7.04 (s, 2H, H-2/H-6) (Figure 2).

**Methyl gallate:** Colourless amorphous powder. 1H NMR (500 MHz, CD3OD, δ, ppm, J/Hz): 6.95 (s, 2H, H-2/H-6), 3.71 (s, 3H, -OCH3). 13C NMR (125 MHz, CD3OD, δ, ppm): 169.05 (C-7), 146.52 (C-3/C-5), 139.74 (C-4), 121.54 (C-1), 110.11 (C-2/C-6), 52.30 (OCH3) (Figure 3).

The antimicrobial activities of major compounds were investigated against *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*. Gallic acid showed moderate antibacterial activity against *S. aureus* and *S. epidermidis* with MIC values of 78 and 156 µg/mL while it showed weak antibacterial effect against *E. coli* and *P. aeruginosa* with MIC value of 625 µg/mL (for both bacteria), respectively. Methyl gallate did not show any antibacterial effect. Gallic acid exhibited strong antifungal activity against *C. albicans* and *C. tropicalis* with MIC value of 9.8 µg/mL (for both fungi) while it possessed moderate activity 156 µg/mL against *C. parapsilosis*. Methyl gallate showed moderate antifungal effect against *C. albicans*, *C. parapsilosis* and *C. tropicalis* with MIC values of 312 and 156 µg/mL (for the last two fungi), respectively (Table 2). Especially considering the strong antifungal effect of gallic acid against different *C. species*, it suggests that this compound, with other phenolic compounds, may be highly responsible for the activity of CCEA, which exhibits strong antifungal effect against *C. albicans*. Also, previous studies showing that gallic acid has strong antifungal activity against *Candida* species support the results of our current study (Li et al., 2017; Alves et al., 2014).

**CONCLUSION**

These results indicate that *C. coggygria* have strong antifungal activity confirming its ethnobotanical use for the treatment of fungal infections. It also shows that the compound that is significantly responsible for the activity of CCEA, one of the extracts with strong antifungal effect, is gallic acid together with other phenolic compounds.

**Table 2. Antimicrobial activity of major compounds isolated from CCEA.**

| Microorganisms             | Gallic acid | Methyl gallate | CPFX | FCZ |
|----------------------------|-------------|----------------|------|-----|
| *Staphylococcus aureus*    | 78          | -              | 0.25 |     |
| *Staphylococcus epidermidis* | 156        | -              | -    |     |
| *Escherichia coli*         | 625         | -              | -    |     |
| *Klebsiella pneumoniae*    | -           | -              | -    |     |
| *Pseudomonas aeruginosa*   | 625         | -              | -    |     |
| *Proteus mirabilis*        | -           | -              | -    |     |
| *Candida albicans*         | 9.8         | 312            | 1    |     |
| *Candida parapsilosis*     | 156         | 156            |      |     |
| *Candida tropicalis*       | 9.8         | 156            |      |     |

CPFX: Ciprofloxacin, FCZ: Fluconazole, -: No activity

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