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Flavonoids of the Heartwood of *Cotinus coggygria* Scop. Showing Protective Effect on Human Lymphocyte DNA

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Abstract

In continuation of our study on *Cotinus coggygria* from Serbia, 10 known flavonoids (**1-10**) were isolated from the methylene chloride/methanol extract of the heartwood. They were tested for in vitro protective effect against chromosome aberrations in peripheral human lymphocytes, using the cytokinesis-block micronucleus assay. All tested compounds (in minimal doses of 1 μg/mL) exerted a beneficial effect by decreasing DNA damage of human lymphocytes in the range of 24.2% to 54.5%, better than the radio protectant control, amifostine. Functional groups, such as 3′,4′-dihydroxyphenyl (catechol), 5-OH, 3-OH, and 4-keto in flavonoids (3-keto in aurones), which play a key role in antioxidant activity, are proposed to be responsible for the DNA protective activity of the tested compounds.

Keywords

flavonoids, *Cotinus coggygria* Scop., DNA protective activity, cytokinesis-block micronucleus assay

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Introduction

The genus *Cotinus* (family Anacardiaceae) comprises 8 species, namely *Cotinus coggygria* Scop. (syn. *Rhus cotinus* L.), *Cotinus obovatus* Raf., *Cotinus americana* Nutt., *Cotinus carranzae* Rzed. and Calderon, *Cotinus chiangii* (D. A. Young) Rzed. and Calderón, *Cotinus kanaka* (R. N. De) D. Chandra, *Cotinus nanus* W. W. Sm., and *Cotinus szechuanensis* Pénzes.[1] *C. coggygria*, a shrubby tree, also known as the European smoketree, is native to a large area from southern Europe to northern China, inhabiting mainly dry slopes, rocky and gravely areas in forest clearings, and south-facing limestone soils. In Serbia, *C. coggygria* is widely distributed, especially on limestone and sedimentary rocks and in the forests of black hornbeam and black pine. In folk medicine of various countries, as well as in Serbia, this plant is used for the treatment of different health problems. Extracts of the various parts (leaves, flowers, heartwood, and bark) and essential oils obtained from leaves of *C. coggygria* showed a broad spectrum of pharmacological activities, including antioxidant, anticancer, antigenotoxic, antimicrobial, antiviral, hepatoprotective, and antiinflammatory.[2] Flavonoids are the most important and abundant group of biologically active constituents of *Cotinus* species, followed by phenolic acids, sterols, and other secondary metabolites. Previously reported compounds isolated from the wood of *C coggygria* are sulfuretin, fustin, 3-*O*-methyl-2,3-*trans*-fustin, 3-*O*-galloyl-2,3-*trans*-fustin, fisetin, garbanzol, eriodyctiol, butein, butin, taxifolin, quercetin, catechin, liquiritigenin, isoliquiritigenin, disulfuretin, sulfurein, gallic acid, methyl gallate, pentagalloyl glucose, *β*-resorcylic acid, gallic acid, gallic acid methyl ester, 3-*O*-*β*-sitosterol glucoside, fisetinidol-(4*α*→8)-(+)-catechin, and epifisetinidol-(4*β*→8)-(+)-catechin, a dimer of butin.[5] In continuation of our examination of *C. coggygria* from Serbia[4,9] and searching for compounds with a possible antigenotoxic activity,[10] we now report the evaluation of the DNA protective

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activity of the flavonoids **1-10**, isolated from the heartwood of *C. coggygria*, based on their ability to reduce the formation of micronuclei (MN) in the cytokinesis-block micronucleus (CBMN) assay.

The CBMN assay used in this study is a technique that is widely recognized as one of the most reliable and successful assays for genotoxic studies. The recording of the frequency of MN, commonly used as a cytogenetic biomarker (and MN expression in peripheral blood lymphocytes) is well established as a method for monitoring chromosome damage in human populations. The method originally proposed by Countryman and Heddle[14] was subsequently improved with the development of CBMN assay, which has been extensively used to evaluate chromosome damage in human populations.[15] This technique, a couple of years ago endorsed by the Organisation for Economic Co-operation and Development, is considered one of the most robust methods for assessing cytotoxicity and genotoxicity.[18] The application of CBMN assay for antioxidant evaluation of heterocyclic compounds, including flavonoids (until 2011), is reviewed elsewhere.[13]

An important effect of flavonoids is the scavenging of oxygen-derived free radicals. This has been the subject of several studies in the past few years, and important structure–activity relationships of the antioxidant activity have been established.[19] Antioxidants protect against molecular damage in living organisms from reactive oxygen species (ROS), such as DNA damage altering gene expression, cell growth, and differentiation.[20,21] As oxidative DNA damage is considered to be a pathogenic event in the induction of many illnesses (eg, Alzheimer's and Parkinson's diseases, cancer, and diabetes),[22] decreasing the degree of such damage by plant polyphenols may reduce the risks.

Results and Discussion

Compounds **1-10** (comprising 4 flavonoid subclasses), that is, chalcone butein (**1**), aurone sulfuretin (**2**), auronolignan cotinignan A (**3**), and 7 flavanones, eriodictyol (**4**), taxifolin (**5**), butin (**6**), fustin (**7**), *cis*-methylfustin (**8**), fustin-3-*O*-gallate (**9**), and garbanzol (**10**) (Figures 1 and 2) were isolated from the heartwood of *C. coggygria* using silica gel column chromatography (CC) followed by preparative thin-layer chromatography (TLC) and semipreparative reversed-phase high-performance liquid chromatography (HPLC). These compounds, except garbanzol, have already been isolated from Serbian *C. coggygria* in our previous work and the most abundant compounds by far were fustin and sulfuretin, isolated in amounts over 100 mg, while other compounds were isolated in the range of 2 to 10 mg (see the "Experimental" section).[9] Considering that oxidative stress causes DNA damage with the formation of MN, and with 10 isolated flavonoids—well-known antioxidants,[19] our idea was to examine these compounds by CBMN assay and determine their possible protective effect against chromosome aberrations in peripheral human lymphocytes.

The majority of the studied compounds, occurring in many plant species, belong to the group of pharmacologically active metabolites with a wide spectrum of biological activities such as anticancer, antioxidant, antiinflammatory, and antiviral.[25] All tested flavonoids, except **3** and **10**, contain a 3′,4′-dihydroxyphenyl group (catechol structure) as the B ring (Figure 1), possessing electron-donating properties and being a radical target, which is beneficial for the antioxidant activity of flavonoids.[19]

The CBMN assay of **1-10** was carried out according to the standard procedure applied previously in our laboratory,[10] using the radio protectant amifostine WR-272 (1.0 μg/mL) as a positive control, and the alkylating agent mitomycin C (MMC) (0.2 mg/mL) as a negative control.[39] The results of the CBMN assay are summarized in Table 1 and Figure 2. The treatment with alkylating agent MMC at a concentration of 0.2 μg/mL gave a significant ($P < .01$) increase in the MN frequency of 32.6% compared to the control cell cultures. The cell cultures treated with amifostine WR-2721 at a concentration of 1.0 μg/mL effected a significant ($P < .01$) decrease in the MN frequency of 18.3% compared to the control cell cultures (Table 1). The highest activity for all compounds was obtained at a concentration of 4.0 μg/mL.

All the tested compounds (in minimal doses of 1.0 μg/mL) decreased the DNA damage of human lymphocytes in the range of 24.2% to 54.5%, more effectively than amifostine at the same concentration. Among the tested compounds, eriodictyol (**4**) exhibited the most prominent effect decreasing significantly ($P < .01$) the frequency of MN by 54.5% when compared with the control cell cultures. Compound **4** is abundantly present in a wide range of medicinal plants, citrus fruits, and vegetables that are considered to have potential health importance. Several studies have indicated that eriodictyol exerts multiple therapeutic effects including antioxidant, antiinflammatory, anticancer, neuroprotective, cardioprotective, antidiabetic, antiobesity, hepatoprotective, and others.[25] The same concentration (1.0 μg/mL) of the related co-occurring 5-hydroxy flavanone, possessing a 3-OH substituent, taxifolin (**5**) also caused a significant ($P < .01$) decrease in the MN frequency by 47.6%. As reported previously, taxifolin was found to act as a powerful antioxidant, antiradical, and metal-chelating agent in different in vitro bioassays.[26] The smaller effect of **5**, in comparison with that of **4**, could be in accordance with the relative order of their antioxidant efficacy, that is, **4**>**5**, reported elsewhere.[27] Taxifolin is a flavonoid commonly found in onion, milk thistle, French maritime (*Pinus pinaster*) pine bark, and Douglas fir (*Pseudotsuga menziesii*) bark. Taxifolin showed promising pharmacological activities in the management of inflammation, tumors, microbial infections, oxidative stress, cardiovascular, and liver disorders. The anticancer activity was more prominent than other activities evaluated using different in vitro and in vivo models.[28] The remaining 3′,4′-dihydroxy group of flavanones lacking a 5-OH substituent (**6**-**9**) exhibited a less protective effect (25.6-41.4%) than **4** and **5** at the same concentration (1.0 μg/mL). The flavanone, butin (**6**), with the highest activity in this group (41.4%), is one of the most widely distributed flavonoids. As previously reported, this compound showed pronounced antioxidant activity.[29] The protective effect of **6**

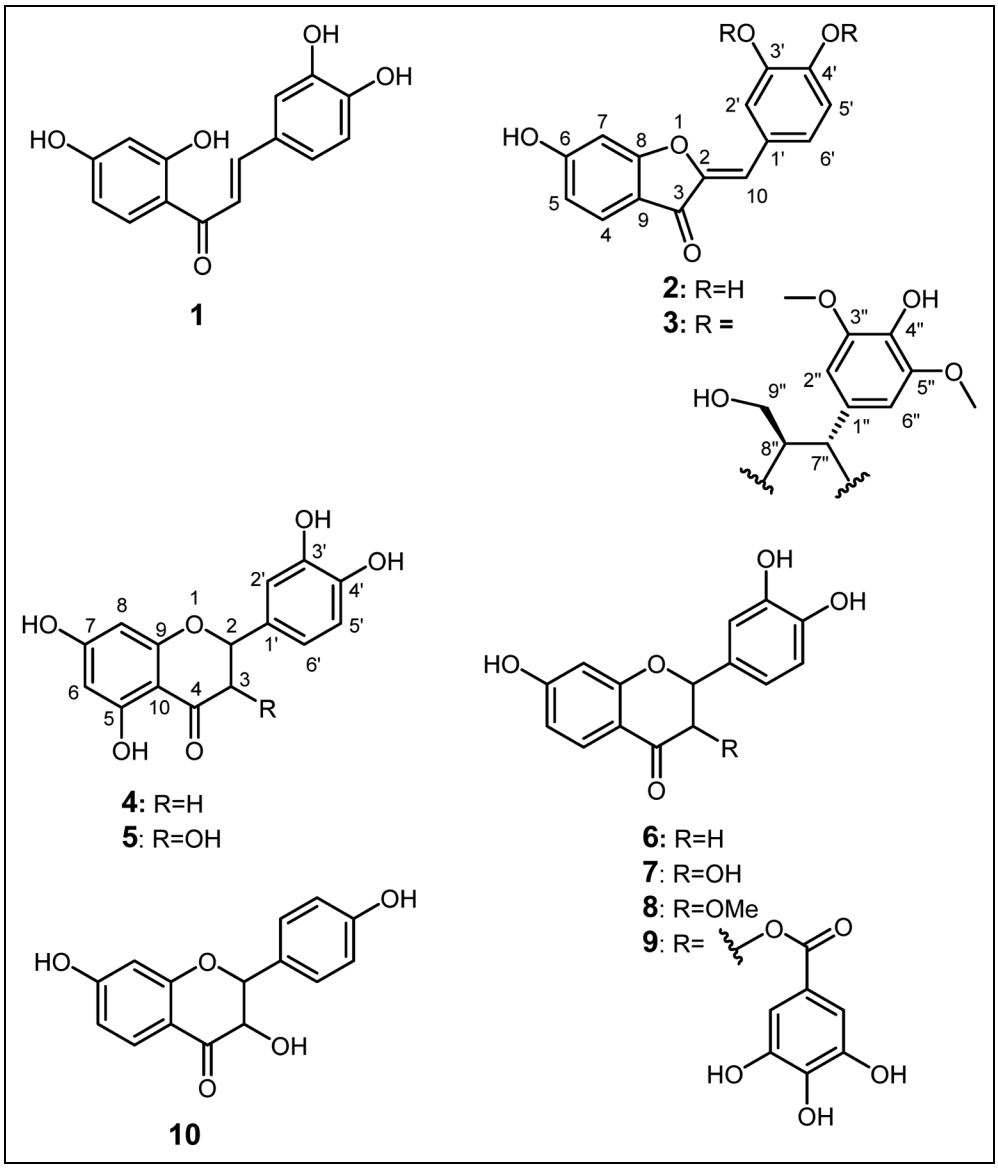

Figure 1. Flavonoids from *Cotinus coggygria* that were tested for DNA protective activity.

against hydrogen peroxide-induced apoptosis by scavenging ROS and activating antioxidant enzymes has also been reported previously.[30] A rather potent antioxidant, fustin (**7**),[31] having the ability to act against disorders caused by oxidative stress,[32] exhibited a lower (30.2%), but still substantial activity in comparison with that of **5**. Methylation of the 3-OH group, comparing compounds **7** and **8**, slightly enhanced the activity of **8** (34.9%). It is interesting to note that the protective activity (25.6%) of the 3-*O*-galloyl-derivative of fustin (**9**) was lower than that of fustin (30.2%), despite the fact that gallic acid itself, as well as the related 3-*O*-galloyl flavonoid derivatives, lacking a carbonyl function at C-4, such as gallocatechins, are among the strongest antioxidants.[39] As expected, the lowest activity (24.2%) was observed for garbanzol (**10**), the only flavanone among the tested compounds lacking a 3′,4′-dihydroxyphenyl group in the B ring, which is the key structural feature known to be responsible for the radical scavenging, antioxidant, antiinflammatory, and antiprotein aggregation activities.[40]

Butein (**1**) and sulfuretin (**2**), belonging to the flavonoid subclasses with emerging therapeutic potential, that is, chalcones and aurones, respectively, also exhibited significant DNA protective activities at a concentration of 1.0 μg/mL (35.3% and 40.5%, respectively), which is due to their radical scavenging ability.[35,41] Hitherto, butein (**1**) has been found in different parts of several plant species belonging to diverse families: for example, Anacardiaceae, Asteraceae, and Fabaceae.[36] This compound is one of the major biologically active components of the bark and stems of *Rhus verniciflua* Stokes.[42] In far eastern countries, such as Korea, Japan, and China, butein (**1**) has been

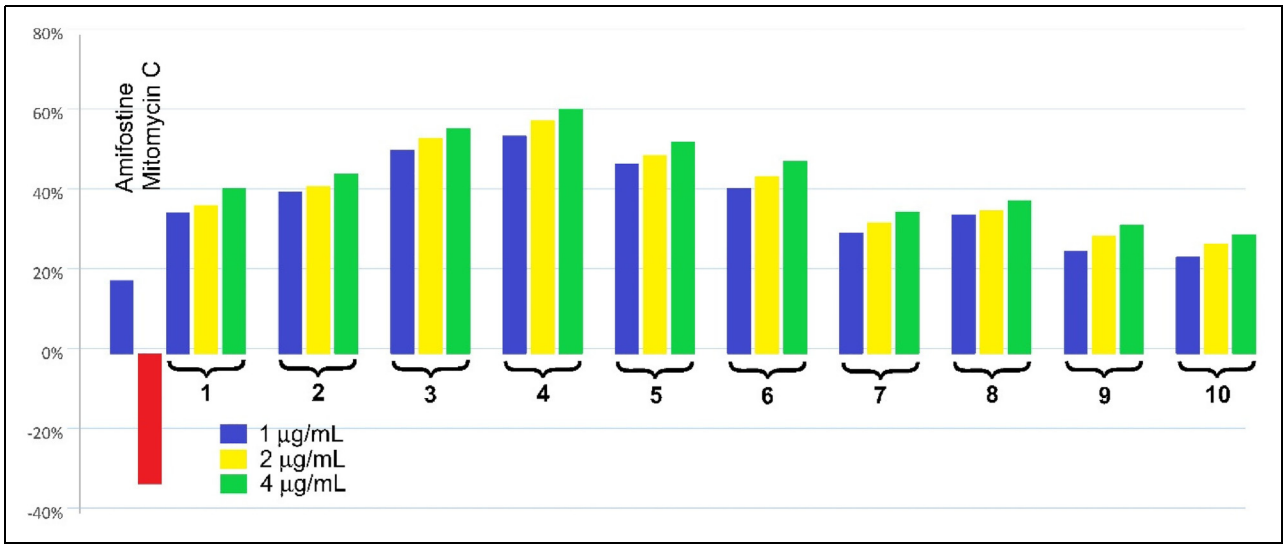

Figure 2. Decrease in frequency of MN (%) in comparison with the control (100%) (see Table 1) effected by flavonoids **1**-**10**; amifostine (positive control) and mitomycin C (negative control).

traditionally used for the treatment of pain, thrombotic disease, gastritis, stomach cancer, and parasitic infections.[42] In Korea, it has also long been used as a food additive.[43] Its significant chemotherapeutic potential has been reviewed elsewhere.[35,41,44] The antioxidant activities of **1** are primarily attributed to its free radical scavenging actions and metal ion chelation.[44]

Sulfuretin (**2**), one of the major biologically active constituents of the heartwood of *R. verniciflua* [33] and *C. coggygria*[9] is known to have various biological activities, including antiinflammatory, antimutagenic, anticancer, antioxidative stress, antiplatelet, and antiarthritis effects. In a previously reported activity-guided isolation of constituents of *C. coggygria*[6], as well as in the identification of *R. verniciflua* compounds exhibiting free radical scavenging and antiapoptotic properties, sulfuretin was identified as one of the significant antioxidative component.[45]

Cotinignan A (**3**), recently isolated from *C. coggygria*[9] and representing a new subclass of secondary metabolites—auronolignans, exhibited a protective activity of 50.9% (at the concentration of 1.0 µg/mL), higher than that of sulfuretin (Figure 2 and Table 1). Such a result could possibly be explained by the presence of a free 4″-OH group, which is important for the radical scavenging activity of flavonolignans, as well as the enhanced lipophilicity caused by blocking of reactive 3′,4′,3″,5″-OH groups.[46]

Conclusion

In this study, the DNA protective activity is presented of 10 known flavonoid compounds from *C. coggygria*. All of the investigated compounds exhibited better activity than the radio protectant amifostine, while eriodyctiol was the best protector, decreasing the MN frequency by 54.5%. Functional groups responsible for the antioxidant activity of flavonoids, 3′,4′-dihydroxyphenyl (catechol), 5-OH, 3-OH, and 4-keto (3-keto in aurones) are supposed to influence DNA protective activity in CBMN assay as well. Further investigation, including DNA protective activity against irradiation damage, will give a wider picture of the protective activity of *C. coggygria* flavonoids.

Experimental

General Experimental Procedures

For CC, Merck silica gel (Si gel) (particle size 0.063-0.200 mm), methanol, methylene chloride, and chloroform were used. Preparative TLC (Prep TLC) was performed on glass plates precoated with silica gel 60 (particle size <0.063 mm) with a layer thickness of 0.75 mm. Analytical TLC was performed on aluminum plates precoated with Merck silica gel 60 F_{254} (0.25 mm thickness). The nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III 500 (500 MHz for ^{1}H; 125 MHz for ^{13}C), in CD_3OD as a solvent. Chemical shifts (δ) were expressed in ppm and coupling constants (*J*) in hertz (Hz). Semipreparative reversed phased HPLC was performed on an Agilent Technologies 1100 Series HPLC-DAD, and a Zorbax Eclipse XDB C18 column (150 × 9.4 mm, 5 µm) was used.

Plant Material, Extraction, Isolation, and Identification of Compounds

The *C. coggygria* heartwood was collected at Deliblatska Peščara (Deliblato Sand), Vojvodina province, Serbia, in June 2018. Plant material was identified by Professor Milan Veljić, Faculty of Biology, University of Belgrade, and voucher specimen BEOU 17422 was deposited at the Herbarium of the

Table 1. Incidence of MN, Cytokinesis-Block Proliferation index, Distribution of MN per Cells, and Frequency of MN Measured in Cell Cultures of Human Lymphocytes Treated with Different Concentrations of the Isolated Compounds From *Cotinus coggygria*.

Compound	conc. (μg/mL)	MN/1000 BN cells	% BN cells with MN	MN/BN cells	CBPI	Frequency of MN
Control		27.83 ± 0.40	2.36 ± 0.06	1.19 ± 0.03	1.68 ± 0.05	100%
Amifostine	1.0	22.74 ± 0.51[a]	1.88 ± 0.04	1.21 ± 0.03	1.64 ± 0.02	81.7%
MMC	0.2	36.90 ± 0.85[a,b]	3.37 ± 0.13	1.09 ± 0.03	1.68 ± 0.08	132.6%
1	1.0	18.00 ± 0.61[a,b,c]	1.53 ± 0.07	1.18 ± 0.03	1.71 ± 0.08	64.7%
	2.0	17.50 ± 0.38[a,b,c]	1.47 ± 0.07	1.19 ± 0.05	1.63 ± 0.01	62.9%
	4.0	16.31 ± 0.60[a,b,c]	1.39 ± 0.03	1.17 ± 0.05	1.60 ± 0.02	58.6%
2	1.0	16.57 ± 0.84[a,b,c]	1.51 ± 0.10	1.10 ± 0.04	1.62 ± 0.04	59.5%
	2.0	16.18 ± 0.56[a,b,c]	1.51 ± 0.09	1.08 ± 0.03	1.58 ± 0.03	58.1%
	4.0	15.32 ± 1.14[a,b,c]	1.32 ± 0.07	1.15 ± 0.04	1.70 ± 0.11	55.0%
3	1.0	13.66 ± 0.30[a,b,c]	1.18 ± 0.05	1.17 ± 0.04	1.65 ± 0.04	49.1%
	2.0	12.81 ± 1.26[a,b,c]	1.05 ± 0.09	1.21 ± 0.05	1.62 ± 0.02	46.0%
	4.0	12.13 ± 1.51[a,b,c]	1.09 ± 0.11	1.10 ± 0.04	1.53 ± 0.01	43.6%
4	1.0	12.67 ± 0.44[a,b,c]	1.07 ± 0.11	1.21 ± 0.10	1.66 ± 0.08	45.5%
	2.0	11.59 ± 0.47[a,b,c]	0.95 ± 0.06	1.19 ± 0.01	1.66 ± 0.08	41.6%
	4.0	10.76 ± 0.42[a,b,c]	0.86 ± 0.05	1.22 ± 0.06	1.70 ± 0.07	38.7%
5	1.0	14.58 ± 0.83[a,b,c]	1.32 ± 0.08	1.10 ± 0.02	1.70 ± 0.08	52.4%
	2.0	14.00 ± 1.28[a,b,c]	1.28 ± 1.06	1.12 ± 0.07	1.61 ± 0.03	50.3%
	4.0	13.07 ± 0.47[a,b,c]	1.09 ± 0.01	1.21 ± 0.05	1.61 ± 0.03	47.0%
6	1.0	16.31 ± 0.80[a,b,c]	1.44 ± 0.08	1.13 ± 0.03	1.67 ± 0.03	58.6%
	2.0	15.49 ± 0.94[a,b,c]	1.32 ± 0.04	1.16 ± 0.07	1.66 ± 0.03	55.6%
	4.0	14.43 ± 0.81[a,b,c]	1.22 ± 0.10	1.18 ± 0.06	1.63 ± 0.04	51.8%
7	1.0	19.43 ± 0.76[a,b,c]	1.67 ± 0.14	1.18 ± 0.11	1.64 ± 0.01	69.8%
	2.0	18.70 ± 0.53[a,b,c]	1.60 ± 0.08	1.12 ± 0.05	1.72 ± 0.08	67.2%
	4.0	17.93 ± 0.70[a,b,c]	1.57 ± 0.07	1.14 ± 0.04	1.77 ± 0.08	64.5%
8	1.0	18.11 ± 0.50[a,b,c]	1.61 ± 0.12	1.13 ± 0.08	1.61 ± 0.02	65.1%
	2.0	17.88 ± 0.41[a,b,c]	1.47 ± 0.08	1.21 ± 0.05	1.66 ± 0.03	64.2%
	4.0	17.18 ± 0.55[a,b,c]	1.37 ± 0.03	1.24 ± 0.06	1.61 ± 0.02	61.7%
9	1.0	20.71 ± 0.70[a,c]	1.75 ± 0.10	1.19 ± 0.08	1.66 ± 0.06	74.4%
	2.0	19.61 ± 0.69[a,b*,c]	1.67 ± 0.05	1.18 ± 0.06	1.63 ± 0.03	70.5%
	4.0	18.87 ± 0.61[a,b,c]	1.64 ± 0.05	1.17 ± 0.02	1.64 ± 0.02	67.8%
10	1.0	21.11 ± 0.62[a,c]	1.75 ± 0.10	1.16 ± 0.06	1.66 ± 0.01	75.8%
	2.0	20.17 ± 0.43[a,b,c]	1.65 ± 0.12	1.24 ± 0.09	1.67 ± 0.01	72.5%
	4.0	19.54 ± 0.70[a,b,c]	1.64 ± 0.07	1.20 ± 0.09	1.62 ± 0.03	70.2%

MN/BN cells=incidence of micronuclei in binucleated cells; CBPI=cytokinesis-block proliferation index; % BN cells with MN=% BN cells with micronuclei; MN/1000 BN cells=incidence of micronuclei in 1000 binucleated cells examined for each concentration; Frequency of MN=incidence of MN presented as % from control groups in cell cultures of human lymphocytes treated with different concentrations of the investigated compounds. The statistical significance of the difference between the data pairs was evaluated by analysis of variance (ANOVA; one-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at $P < .01$.

[a]Compared with control groups, statistically significant difference $P < .01$.

[b]Compared with amifostine WR-2721, statistically significant difference $P < .01$.

[b*]Compared with amifostine WR-2721, statistically significant difference $P < .05$.

[c]Compared with mitomycin C, statistically significant difference $P < .01$.

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The heartwood was air-dried and milled. Wood powder, 100.0 g, was extracted 3 times with 1 L of CH_2Cl_2/MeOH 2:1 for 24 h (the first hour of extraction in an ultrasonic bath) at room temperature to give 8.2 g of crude extract, which was subjected to fractionation by Si gel CC.

Isolation was performed similarly to our previous work.[9] Crude extract (8.2 g) was chromatographed on a Si gel column (750 × 45 mm), with CH_2Cl_2/MeOH (gradient elution, from 95/5 to 60/40) and afforded 230 fractions. The eluates were monitored by TLC, and the fractions with similar retention factor values were combined. Pure compounds were isolated from these fractions by additional Si gel CC and/or Prep TLC and semipreparative HPLC. All isolated compounds were identified using ^1H and ^{13}C NMR spectroscopy, and comparison to NMR data from our previous work.[9] Compounds **1** (22 mg), **3** (3 mg), **4** (10 mg), **6** (3 mg), **8** (7 mg), and **10** (2 mg) were isolated from fractions 22 to 53 from the Si gel CC (CH_2Cl_2/MeOH 95:5 ratio), while compounds **2** (>100 mg), **5** (8 mg), and **7** (>100 mg) were isolated from fractions 103 to 129 (CH_2Cl_2/MeOH 88:12 ratio). Compound **9** (2 mg)

was isolated from the combined fractions 175 to 196 (CH_2Cl_2/MeOH 75:25 ratio). The semipreparative HPLC program used for the final isolation and purification was the same as in our previous work.[9]

Subjects

Venous blood samples were obtained by heparinized sterile vacutainers (Becton Dickinson) from 6 healthy nonsmoking male volunteers who had not been exposed to chemicals, drugs, or other substances. A safety protocol concerning blood-borne pathogens/biohazards was adopted. The volunteers gave their permission for using their blood for the experiment. From each subject, 2 aliquots of blood, 5 mL each were obtained. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002). The blood samples were obtained at the Medical Unit in accordance with current Health and Ethical regulations in Serbia, Law on Health Care (2005).

CBMN Assay

The culture lymphocytes were treated with compounds **1**-**10** isolated from *C coggygria* heartwood at 3 concentrations: 1.0, 2.0, and 4.0 μg/mL. One cell culture without added compounds served as the control. One cell culture containing amifostine WR-2721 (Marligen-Biosciences) (1.0 μg/mL) served as the positive control, and another one containing MMC (0.2 μg/mL, in phosphate buffer) as a negative control. All cultures were incubated in a thermostat at 37 °C. Treatment with compounds lasted for 19 h, after which all cultures were rinsed with pure medium, transferred into 5 mL fresh Rosewell Park Memorial Institute (RPMI) 1640 medium (RPMI-1640 medium + GlutaMAX + 25 mM HEPES; Invitrogen-Gibco-BRL), and incubated for an additional 72 h. The incidence of spontaneously occurring MN in control samples was scored.

About 2×10^6 blood lymphocytes were set up in 5 mL of RPMI-1640 medium supplemented with 15% calf serum and 2.4 μg/mL of phytohemagglutinin (Invitrogen-Gibco-BRL). One hour after the start of cell stimulation, the investigated compounds were added to the samples at a final concentration of 1.0, 2.0, or 4.0 μg/mL, respectively. The incidence of spontaneously occurring MN in control samples was scored. For MN preparation, the cytokinesis block method of Fenech was used, with some modifications.[14] Cytochalasin B (Invitrogen-Gibco-BRL), at a final concentration of 6.0 μg/mL, was added to the samples after 44 h, and the lymphocyte cultures were incubated for a further 24 h. After 72 h, the cells were washed with 0.9% NaCl (Merck, Sharp, & Dohme GMBH), collected by centrifugation, and treated with a hypotonic solution at 37 °C. The hypotonic solution consisted of 0.56% KCl + 0.9% NaCl (mixed in equal volumes). The cell suspension was prefixed in methanol/acetic acid (3:1), washed 3 times with fixative, and dropped onto a clean slide.[16] Slides were air-dried and stained with alkaline Giemsa (Sigma-Aldrich) (2%). At least 1000 binucleated (BN) cells per sample were scored, registering MN according to the criteria of Countryman and Heddle [14] and Fenech.[15,16]

Initially, we investigated the effects of isolated compounds on cell proliferation by determining the cytokinesis-block proliferation index (CBPI). Since micronucleus expression is dependent on cell proliferation, quantification of cell proliferation and cell death should be carried out to obtain a sound evaluation of cell kinetics and micronucleus frequencies.

The CBPI was calculated as suggested by Surralle et al,[17] that is

$$\mathrm{CBPI} = \frac{\text{No. mononucleated cells} + 2 \times \text{No. binucleated cells} + 3 \times \text{No. multinucleated cells}}{\text{Total number of cells}}$$

For the analysis of MN, only BN cells with well-preserved cytoplasm were scored for MN (under a light microscope with a 40 × 10 magnification). The criteria for the selection of BN cells and identification of MN given in the HUMAN project website (http://www.humn.org) were followed.

The numbers of BN cells with 1, 2, 3, or more MN were then tabulated. The data for each treatment were expressed as the frequency of MN per 1000 BN cells.

Statistical Analysis

The statistical analysis was performed using Origin software package version 7.0. The statistical significance of the difference between the data pairs was evaluated by analysis of variance (ANOVA; one-way ANOVA), followed by the Tukey test. Statistical difference was considered significant at $P < .01$. The index calculated is presented as the % of change comparing different groups.

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Ethical Approval

The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002). The blood samples were obtained at the Medical Unit in accordance with

the current health and ethical regulations in Serbia (Parliament of the Republic of Serbia. Law on Health Care. Official Gazette of the Republic of Serbia 2005; 107: 112-161)

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Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Law on Health Care, Parliament of the Republic of Serbia Official Gazette of the Republic of Serbia 2005; 107: 112-161.

Statement of Informed Consent

Verbal informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

Declaration of Conflicting Interests

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