

NATURAL DRUGS

ANTIOXIDANT AND ANXIOLYTIC ACTIVITIES OF
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Abstract: Hawthorn has been present for a long time in traditional medicine as antihypertensive, hypolipidemic, anti-inflammatory, gastroprotective, antimicrobial agent. Hawthorn can be used for the cure of stress, nervousness but there is no published paper about actions of *Crataegus nigra* Wald. et Kit. fruits. The present study was carried out to test free-radical-scavenging and anxiolytic activity of *C. nigra* fruits. DPPH (α,α -diphenyl- β -picrylhydrazyl) assay was used to measure antioxidant activity. BHT, BHA, PG, quercetin and rutin were used as standards. The total amount of phenolic compounds, procyanidins, and flavonoids in the extracts, was determined spectrophotometrically. Results were expressed as equivalents of gallic acid, cyanidin chloride and quercetin equivalents, respectively. LC-MS/MS was used for identification and quantification of phenolic composition. The anxiety effect, expressed as the difference in time spent in the open and closed arms, was measured and compared between groups. Phenolic compound content of *Crataegus nigra* fruits was 72.7 mg/g. Yield of total flavonoid aglycones was 0.115 mg/g. Procyanidins were 5.6 mg/g. DPPH radical-scavenging capacity of the extracts showed linear concentration dependency, IC₅₀ value were 27.33 μ g/mL. Anxiolytic effect was observed. Species *Crataegus nigra* fruits hydroalcoholic extract showed antioxidant and anxiolytic activity.

Keywords: *Crataegus nigra*, anxiolytic effect, DPPH, total phenols, flavonoids

In traditional medicine leaves, flowers, and berries of species of genus *Crataegus* have been used as astringent, antispasmodic and diuretic agents. Hawthorn products are introduced as an alternative treatment for hypertension, angina, arrhythmia. Furthermore, they can be used to treat indigestion, diarrhea, abdominal pain, hyperlipidemia (1). Although the main application is for cardiovascular disease, hawthorn fruits have also been used as a medicament against stress, nervousness, sleep disorders in traditional medicine (2, 3). It was shown that hawthorn preparations are safe and well tolerated by patients (4). The most common adverse side-effects are vertigo, gastrointestinal pains, headache and migraine. There were no reports of drug interactions. Hawthorn fruits, leaves, and flowers contain: amines, flavonoids (vitexin, vitexin-2''-*O*-rhamnoside, chlorogenic acid, hyperoside, quercetin, isoquercitrin, rutin, etc.), procyanidins, organic acids, tannins, and triterpene derivatives (1,

5, 6). Some flavonoids, procyanidins, tannins, chlorogenic acid in various plants containing these constituents have anxiolytic and sedative activities (7–10). There are no published studies examining CNS activities or anxiolytic actions of *C. nigra* fruits, although the evidence of usage in the traditional medicine of hawthorn fruits do exist for the cure of stress, nervousness and sleep disorders. The presented study evaluated possible effects of *C. nigra* fruits extract on the CNS. There is a number of medicinally active phytochemicals that have been isolated from hawthorn, with most of the data generated in studies of those species that are native to Europe and Asia.

EXPERIMENTAL

Extract preparation

Crataegus nigra Wald. et Kit. was collected from autochthonous sources. *C. nigra* is located on

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flooded plains, alluvial terrain near major rivers. In Serbia, it is prevalent near the Danube and Sava, where it is common and abundant (11). Pannonian hawthorn (*Crataegus nigra* Wald. et Kit.) is a shrub or low tree. Mature fruit is black, almost round in shape, glossy and soft. The voucher specimens (the number BEOU 16406) were deposited in the Department of Biology and Ecology, Faculty of Sciences, University of Kragujevac and botanical garden of Department of Biology, Faculty of Sciences, University of Belgrade.

Species *C. nigra* was collected and separated. The fruit was collected in the fall, September of 2010. It was taken in the vicinity of Beocin, Serbia. The collected material was dried under the shade.

Extraction was performed in the Soxhlet extractor. Ninety grams of dried and chopped herbs and 500 mL of 80% ethanol has been used for extraction. The powder fruits have been extracted for 12 h at 80°C. Dry extracts were obtained by rotating vacuum evaporators (RV05 basic IKA, Germany) at 40°C, under reduced pressure (12).

Determination of phenols

Determination of the total phenolics content was carried out according to the standard method of Singleton et al. (13), customized for 96-well microplates (14). In this study, Folin-Ciocalteu reagent (FC) (Fisher Scientific, UK), anhydrous Na₂CO₃ (Analytika, Czech Republic), and gallic acid (Sigma Aldrich, Germany) as standard have been used. We examined the following extract concentrations: 0.5, 0.25, 0.125 and 0.063 mg/mL. Gallic acid (100–0.063 µg/mL), was used as a standard for plotting a calibration curve. Thirty microliters of each extract or standard solution, was added to 150 µL of 0.1 mol/L FC reagent and mixed with 120 µL of sodium carbonate (7.5%) after 6 min. Absorbance at 760 nm was measured after 120 min. The content of total phenol compounds was expressed as mg of gallic acid equivalents (GAE) per gram of dry extract weight.

Prepared extract was diluted in mobile phase (0.05% HCOOH : MeOH, 1:1, v/v) to final concentrations of 20 mg/mL as well as 2 mg/mL, and analyzed by high-performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). For the purpose of quantification, a serial dilutions of 45 reference standard mixtures were prepared in 1.5 ng/mL to 25 µg/mL range.

Separation was achieved using Agilent Technologies 1200 Series liquid chromatograph coupled with Agilent Technologies 6410A Triple Quad mass selective detector with electrospray ion source (ESI). Five microliters of extract/standard

was injected, and compounds were resolved on a Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 µm) column, set at 50°C. Mobile phase, consisting of 0.05 % HCOOH and MeOH, was delivered in gradient mode (0 min 30% B, 6 min 70%, 9–12 min 100%, post time 3 min), at 1 mL/min flow rate. Eluted compounds were detected in dynamic SRM mode.

Obtained results were analyzed using MassHunter Workstation Software – Qualitative Analysis (B.03.01). For each compound, a calibration curve (MRM peak area vs. concentration) was plotted.

Content of procyanidins

The content of procyanidins was calculated by standard method described in European Pharmacopoeia 6.0 and expressed as equivalent of cyanidin chloride (15). Butanol (BuOH) (POCh, Poland) and cyanidin-chloride (Carl Roth, Germany) were used. The investigated extract was hydrolyzed under reflux by an EtOH/HCl mixture. Procyanidins were separated with BuOH from the aqueous layer. The absorbance was measured at 550 nm by spectrophotometer (Cecil CE 2021) (16).

Content of flavonoids

The content of flavonoids was calculated by aluminum chloride colorimetric method (17) adapted for 96-well microplates. AlCl₃ × 6H₂O, CH₃COONa × 3H₂O (Centrohem, Serbia), quercetin (Sigma-Aldrich Germany) and methanol (MeOH) (J.T. Baker, USA) were used. Investigated extract was prepared in concentrations of 10.0, 5.0, 2.5, 1.25 and 0.625 mg/mL, quercetin was used as a standard. Thirty microliters of extract or standard was diluted by 90 µL of methanol and 6 µL of 10% aluminum chloride, 6 µL of 1 mol/L sodium acetate, and 170 µL of distilled water were added. Absorbance at 415 nm was measured after 30 min. All samples were made in triplicate, and the mean values of flavonoid content were expressed as milligrams of quercetin equivalents per gram of dry extract weight calculated according to the standard calibration curve.

Evaluation of antioxidative activity. DPPH assay

DPPH scavenging effect of plant extract was carried out according to the method of Soler-Rivas et al. (18). It was adapted for 96-well microplates (14, 18). The following materials were used: DPPH (α,α-diphenyl-β-picrylhydrazyl) (Fluka, Switzerland), BHT (butylated hydroxytoluene) (Alfa Aesar, USA), BHA (butylated hydroxyanisole) (Merck,

Germany), quercetin (Sigma-Aldrich, Germany), rutin (Fluka, Switzerland), PG (propyl gallate) (Alfa Aesar, USA), DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Germany). Ten microliters of investigated extract solutions, in series of seven concentrations of double dilution in DMSO, (5.0–0.078 mg/mL), was added to 100 μ L of 90 μ mol/L DPPH solution in methanol, and the mixture was diluted with 190 μ L of methanol. As a control, the exact amount of extract was substituted with DMSO. Absorption at 515 nm was measured by the microplate reader (Multiskan Spectrum, Thermo Corporation) after 60 min. As a positive control, synthetic antioxidant BHT, BHA, PG, quercetin and rutin were used. The radical-scavenging capacity (RSC) was calculated by the equation:

$$\text{RSC} = 100 - (A_{\text{average}} - A_{\text{corr}}) / A_{\text{control}} \times 100$$

where A_{average} = average absorbance of the probes for a given concentration sample level; A_{corr} = correction of extract absorbance (with no reagents); A_{control} = absorbance of the DPPH radical (with no extract). The extract concentration inducing 50% of DPPH radicals inhibition (IC_{50}), was calculated from the RSC concentration curve.

Experimental anxiety effect model

Anxiety effect of investigated extract was evaluated by experimental anxiety on elevated plus-maze (EPM) test. The source of anxiety was EPM arms height. Treated animals received researched extract, while control animals received distilled water or diazepam, 30 min prior to their setting on the EPM. The anxiety effect, expressed as the difference in time spent in the open and closed arms (19, 20), was measured and compared between groups.

We used diazepam (Bensedin[®] Galenika, Serbia; IUPAC name: 7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3H)-one) (1 mg/kg), normal saline (Hemofarm, Serbia). Female and male BALB/c mice 5–6 weeks old, 20–22 g, (purchased from the Military Medical Academy Belgrade, Serbia) were used in this study. They were kept in environmentally controlled conditions (22°C, 12h light-dark cycle), with free access to standard pellet diet and water. Animals were kept in metal cages. Prior to the experiment, they fasted for 15 h and acclimatization to the test environment lasted 2 h before the experiment. All of the animal procedures were approved by the Ethics Committee of Medical Faculty, Kragujevac, which complies with the National Institutes of Health guidelines for treatment of laboratory animals.

Animals were divided into 6 groups of 8 animals – 4 groups were treated with different concen-

tration of examined ethanolic extracts of *C. nigra* (10, 100, 300, 600 mg/kg). Two groups served as control, receiving vehicle (normal saline) and diazepam (1 mg/kg), in order to control and measure anxiety effect. Treated groups received extracts intraperitoneally. Anxiety was induced by arm height. The apparatus consisted of two open and two closed arms. The each arm was wooden, two of them were closed and black and other were open and white. The whole maze was lift to a height of 60 cm above floor level (21). Testing was carried out in a quiet room and a stifled light. To start experiment, mice were settled on the open arm in the center of the maze. The time spent in the open and closed arms was noted through 5-min test period.

Experimental ketamine-induced sleeping time (hypnotic effect)

We used diazepam (Hemofarm, Serbia) as a standard drug, saline (Hemofarm, Serbia), ketamine (Laboratorio Sanderson, Chile; IUPAC name: (RS)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone) (100 mg/kg). Animals were divided into 4 groups of 8 animals – two groups were treated with different concentration of researched ethanolic extracts of *C. nigra* (300 and 600 mg/kg). Two groups served as control, receiving vehicle and diazepam (1 mg/kg), in order to control and measure hypnotic effect, which was induced by ketamine (100 mg/kg). The time from the loss to regaining the righting reflex was taken as the duration of sleep (22). Mice were considered awake in the moment they could stand on all four paws. The hypnotic effect, expressed as the difference in duration of sleep, was measured and compared between groups (23).

Statistics

The results are expressed as the mean \pm standard error of measurement (SEM). The data were normally distributed. The one-way analysis of variance (one-way ANOVA) followed by Bonferroni *post hoc* test were used for statistical analysis. The probability of null hypothesis lower than 0.05 ($p < 0.05$) was considered to be an indicator of statistically significant difference among experimental groups. All calculations were made by statistical software SPSS version 18.

RESULTS

Quantitative phytochemical analysis of major compounds found in the berries of *C. nigra* is presented in Table 1.

Table 1. Quantitative phytochemical analysis of ethanolic extract of *C. nigra* berries (Cn-B) (the mean value \pm SD of three measurements).

Active constituents	Cn-B
Total phenolics (mg of GA ^a /g)	72.7 \pm 16
Flavonoid content (mg of K ^b /g)	1.88 \pm 1
Flavonoid aglycones content (mg/g)	0.115 \pm 5.5
Procyanidins content (mg of C ^c /g)	5.6 \pm 0.76

^aGA – gallic acid, ^bK – quercetin, ^cC – cyanidin chloride

Quantitative and qualitative analyses of individual compounds found in berries of *C. nigra* are presented in Table 2 and Figure 1.

Examined extracts of *C. nigra* have shown DPPH free-radical-scavenging activity. When the extract of *C. nigra* berries was applied in the concentration range of 166.7–2.6 μ g/mL, its DPPH free-radical-scavenging activity varied from 95.3 to 3.0% (IC₅₀ value 27.33 \pm 0.369 μ g/mL). Results of DPPH assay performed on *C. nigra* extract are presented in Table 3.

Elevated plus maze test

The elevated plus-maze is one of the most frequently used models for testing anxiolytic activities (19). Hydroalcoholic extract of *C. nigra* significantly increased the total time spent in the open arms, according to the control at doses of 300 and 600 mg/kg. The most expressed effect of *C. nigra* was generated at the dose of 300 mg/kg. In this case, the total time spent in the open arms was 3.25 \pm 0.15 min (195 \pm 8.83 s) (65 %) compared with the control 1.05 \pm 0.15 min (63.12 \pm 9.15 s) (21%). Diazepam was also proven to show significantly higher effect in comparison with the control. Extract of *C. nigra* has not shown significantly greater effect than diazepam control, although it does have the greatest effect of all the doses tested (Table 4).

Ketamine sleeping time test

The mice in control group, who were treated with saline, showed total sleep time 1542 \pm 78 s. Diazepam group significantly increased total sleeping time by 146% compared with the control. Also, *C. nigra* extract at doses of 300 and 600 mg/kg significantly increased total sleeping time, by 138% and 135%, respectively, compared with the saline control, but not with the diazepam control (Table 5).

DISCUSSION AND CONCLUSION

Hawthorn has been present for a long time in traditional medicine of many nations. Accordingly,

in many pharmacopoeias in Germany, France, China, some species of hawthorn are officially listed (24). Numerous standardized hawthorn preparations (tablets, drops), which are sold in pharmacies, are being used for heart disease treatment. In Serbia, any herbalist dealing with collecting and selling medicinal herbs can offer teas of leaves, flowers and fruits of different hawthorn species. Due to the great popularity of the plant in traditional medicine of all nations, numerous studies have been derived and done on different species of hawthorn in order to prove pharmacological effectiveness. It was proven that different hawthorn species have antihypertensive (25), hypolipidemic (26), anti-inflammatory, gastro-protective, antimicrobial (27) activity. *C. nigra* is a widely spread plant in a part of Serbia and Hungary (11). In traditional Serbian medicine it was intensively used for the same purpose as other hawthorn species, however, for this particular species no pharmacological research has been conducted or published so far. This study has dealt with chemical characteristics of fruits and has shown their antioxidant and anxiolytic effects.

Hydroalcoholic extract of *C. nigra* in doses of 300 and 600 mg/kg has led to the significant increase of time that the mice spent in the open arms and to the decrease of time spent in closed arms of elevated plus-maze. For most of the plants that have anxiolytic effect, a hypnotic one has been proven as well (28–31). In this study, *C. nigra* in doses of 300 and 600 mg/kg has shown a significant prolongation of sleeping time compared to placebo. Thus, this hawthorn species presumably also has both hypnotic and anxiolytic effect.

Chemically analyzing *C. nigra* extract, rutin, kaempferol, quercetin, hyperoside, epicatechin were identified as the main compounds. In the previously published studies, it had been confirmed that flavonoids, flavonol quercetin, kaempferol aglycones are responsible for anxiolytic effects of some plants (32–34). In the extract of *C. nigra* the authors have examined the presence of these compounds and confirmed it in similar concentrations.

Table 2. Quantitative and qualitative analysis of individual compounds found in berries (Cn-B).

Compound name ^a	t_r^b (min)	t_r^c (min)	Quantification (mg/kg)
			Cn-B
Chinic acid	0.48	0.44	475
Protocatechuic acid	0.76	0.72	385
5-O-caffeoyloic acid	0.80	0.76	172
Epicatechin	0.97	0.92	105
Hyperoside	2.14	2.07	164
Rutoside	2.17	2.10	868
Quercetin-3-O-glucoside	2.22	2.15	408
Kaempferol-3-O-glucoside	2.80	2.72	5.10

^a The numbers refer to compounds signed on the HPLC spectrum. ^b Retention times of the standards. ^c Retention times of the compounds identified in the extract.

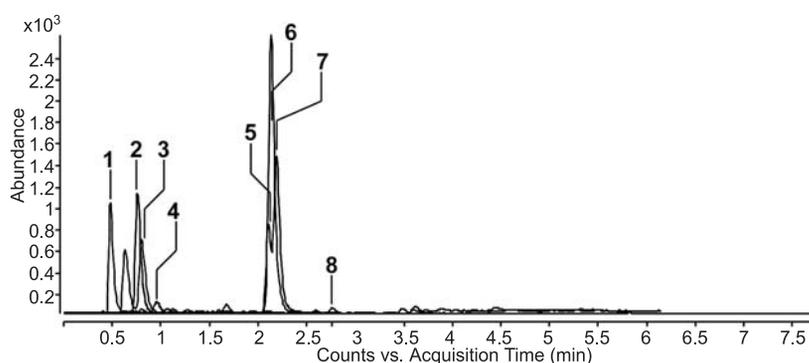


Figure 1. HPLC chromatogram of *C. nigra* berries ethanol extract: 1 – chinic acid, 2 – protocatechuic acid, 3 – 5-O-caffeoyloic acid, 4 – epicatechin, 5 – hyperoside, 6 – rutin, 7 – quercetin-3-O-glucoside, 8 – kaempferol-3-O-glucoside

Table 3. DPPH free-radical-scavenging activity of the *C. nigra* extract (Cn-B).

Sample	IC ₅₀ (µg/mL) ^a
Cn-B	27.33 ± 0.369
BHT	11.65 ± 1.144
BHA	1.57 ± 0.093
Quercetin	0.41 ± 0.169
Rutin	1.42 ± 0.173
PG	0.62 ± 0.030

^a The mean value ± SD of three measurements

However, many authors believe that the certain pharmacological activity of the plants is in fact the consequence of the present combination of chemical compounds in a plant. According to that, the anxi-

olytic effect of *C. nigra* is probably the consequence of the blend of all the compounds present in it.

The antioxidant activity in this study has been examined with DPPH method. The compounds that have antioxidant characteristics can reduce the DPPH radical. In fact, they are hydrogen donors and can convey DPPH radical into the neutral DPPHH form (35). This study has shown that the *C. nigra* fruit extract has a free-radical scavenging activity. This activity is lower than with BHA, BHT, quercetin and rutin, which were used like positive controls (EC₅₀ values of 27.33, 11.65, 1.57, 0.41 µg/mL, respectively). However, in the previously conducted studies, it had been established that the compounds that realize EC₅₀ with the concentration less than 50 µg/mL are active antioxidants (36). Thus, based on this, we can say that the *C. nigra* extract has the active antioxidant activity.

Table 4. Anxiolytic effect of *C. nigra* fruits.

	Time spent (s)	
	Open arm*	Closed arm*
Control	63.125 ± 9.15	236.875 ± 8.56
Diazepam 1 mg/kg	145.62 ± 14.24	54.37 ± 14.24
Extract <i>C. nigra</i> 10 mg/kg	77.25 ± 17.35	212.25 ± 22.44
Extract <i>C. nigra</i> 100 mg/kg	107 ± 11.04	193 ± 11.04
Extract <i>C. nigra</i> 300 mg/kg	95 ± 8.83	105 ± 8.83
Extract <i>C. nigra</i> 600 mg/kg	157 ± 21.68	143 ± 21.68

Numbers represent the mean ± SEM of the groups (n = 8). * p < 0.05 compared to saline (ANOVA followed by Bonferroni test).

Table 5. Sedative effect of *C. nigra* fruits on ketamine-induced sleep in mice.

	Sleeping time (s)*
Control	1542.5 ± 77.75
Diazepam 1 mg/kg	2250.62 ± 32.17
Extract <i>C. nigra</i> 300 mg/kg	2133 ± 58.38
Extract <i>C. nigra</i> 600 mg/kg	2086 ± 104.55

Numbers represent the mean ± SEM of the groups (n = 8).

* p < 0.05 compared to saline (ANOVA followed by Bonferroni test).

It has already been shown that some other hawthorn species have antioxidant activity and compared to those data *C. nigra* has shown somewhat greater effect. The blend of *C. monogina* and *C. oxyacantha* has shown EC₅₀ at the extract concentration of 52.04 µg/mL (27). *C. pinnatifida* at the concentration of 100 µg/mL shows EC₈₄ (37). According to the studies (37), some plants used like familiar antioxidants have shown less DPPH activity than *C. nigra*. However, there are many plants which are more potent antioxidants (38). Apart from hawthorn's antioxidant activity there are numerous evidence of its very significant and diverse pharmacological activity. In *C. monogyna* it has been established that phenol potency (phenolics) goes in the following order: quercetin > B2 procyanidin > epicatechin, while the two flavonol glycosides, hyperoside and rutin, are somewhat less effective (39). Generally, epicatechin and procyanidin B2 flavonoids are the most effective, and hyperoside and rutin follow them. This study has shown that the content of all the phenols and procyanidins in dried *C. nigra* fruits is higher than the same content in *C. monogina*. Also, the total content of phenol and procyanidins in *C. monogina* dried fruits is smaller than their content in *C. monogina* dried tops and flowers

(39). Thus, chemical composition of all *C. nigra* parts should be examined as well.

Since it is known that procyanidins, flavones and flavonoids are bearers of antioxidant activity, we believe that the presence of these polyphenolic compounds in the *C. nigra* extract is responsible for this extract's feature.

Benzodiazepines are mostly used in anxiety treatment today. However, they can have significant side effects, especially if they are used at the same time as depressors of CNS, leading to development of psychophysical addiction with time. This is the reason why herb preparations with anxiolytic effect are given more attention, while side effects are expected to be minimal or none. This study showed that *C. nigra* has anxiolytic and hypnotic activity. Yet it is to be shown in the future the extent to which these preparations are effective in replacing or reducing anxiolytics. Antioxidant activity of *C. nigra*, which is alongside with numerous known antioxidants, gives special significance to it.

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Conflict of interest

The authors have declared that there is no conflict of interest.

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