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# THE QUALITATIVE COMPOSITION AND CONTENT OF PHENOLIC COMPOUNDS IN SHOOTS OF *Casuarina equisetifolia* L.

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Abstract

The species *Casuarina equisetifolia* L. is widely used in forestry in many countries with a tropical climate. Extracts from the shoots of *C. equisetifolia* are known to be rich in phenolic compounds which play an important role in plant growth and development, as well as in adaptation to abiotic and biotic environmental factors. Additionally, they exhibit antiviral, antibacterial, anti-inflammatory, anti-tumor, neuroprotective, and other activities. In this study, the composition of phenolic compounds primarily consisting of monomeric ellagitannins was comprehensively investigated for the first time in the shoots of *C. equisetifolia*. The aim of this study was to investigate the composition and content of phenolic compounds in *C. equisetifolia* shoots using ultra performance liquid chromatography coupled with photodiode and mass spectrometric detectors (UHPLC-PDA-MS). The study focused on the green one-year-old photosynthetic shoots of the *C. equisetifolia* tree grown in the greenhouse of the All-Russian Institute of Medicinal and Aromatic Plants (VILAR, Moscow). Samples were collected in the first decade of July 2019. The shoots were frozen, lyophilized, and ground. A 15 mg specimens were extracted with 1 ml of 80 % acetone for 60 min at room temperature with constant stirring. The extract was centrifuged for 20 min at 14000 rpm and evaporated to dryness at 45 °C. The extraction was repeated two more times. The resulting dry extract was dissolved in 1 ml of deionized water for 60 min, centrifuged for 20 min at 14000 rpm, diluted five times with deionized water, and filtered. An ultra-high performance liquid chromatographic system (UHPLC, Acquity UPLC® 2.9.0, Waters Corporation, USA) with a photodiode array detector (190-500 nm) and triple quadrupole mass spectrometer (Xevo TQ, Waters Corporation, USA) was used for the analysis of phenolic compounds. Separation was carried out in an Acquity UPLC® BEH Phenyl column (2.1×100 mm, 1.7 μm, Waters Corporation, Ireland). Data analysis was performed using the DataAnalysis 4.0 software. Phenolic compounds were identified based on mass spectrometry data by determining the m/z value of the [M-H] ion and its m/z fragments. The content of different classes of phenolic compounds such as gallolyl-glucoses, ellagitannins, condensed tannins, flavonoids (quercetin and kaempferol derivatives) was determined using multiple reaction monitoring. The extract was found to contain 16 phenolic compounds, with 14 belonging to the class of hydrolyzable tannins and 2 to the class of flavan-3-ols. It was discovered that *C. equisetifolia* shoots accumulate monomeric ellagitannins with molecular masses ranging from 784 to 1068 Da, containing glucose as a polyol in either cyclic or linear form. Among the ellagitannins of *C. equisetifolia*, casuarinin, two isomers of pedunculagin, stachyurin, chebulic acid, casuarininin, and casuarictin were identified for the first time. Two compounds with a molecular mass of 1068 Da were preliminarily identified as isomers of pterocarinin A. Ellagic acid and its derivatives, ellagic arabinoside and ellagic rhamnoside, were also identified in shoots. The total content of phenolic compounds was 55 mg/g dry weight, with ellagitannins being the main phenolic compounds. Their content reached 42 mg/g, or 76 % of the total amount of all phenolic compounds. Galloyl-glucose and condensed tannins each accounted for 10 % of the total amount of all phenolic compounds. These findings suggest the potential use of *C. equisetifolia* shoots as a raw material for obtaining individual ellagitannins and studying their antiviral, anti-inflammatory, and anti-tumor activities.

Keywords: *Casuarina equisetifolia* L., *Casuarinaceae*, liquid chromatography, mass spectrometry,

phenolic compounds, hydrolysable tannins, ellagitannins

*Casuarina equisetifolia* L. is a fast-growing evergreen woody plant of the family *Casuarinaceae* [1] with highly reduced scale-like leaves in whorls on long thin shoots, reminiscent of the needles of *Pinaceae* plants [2]. Unlike other species of the genus, *C. equisetifolia* has the largest natural area [3]. In many countries with tropical climates, the plant is used to restore degraded ecosystems, prevent erosion and stabilize sand, when planting coastal windbreaks, and in forestry in dry areas [4, 5].

*C. equisetifolia* can accumulate large amounts of phenolic compounds. For example, in the bark extract their total content varies from 43 to 76 mg/g [6, 7], and in the shoots it reaches 100 mg/g [9]. Among the phenolic compounds of *C. equisetifolia*, there are flavonoids, condensed and hydrolyzed tannins [9]. The main flavonoids are rutin, hesperetin, and the aglycones quercetin, narenginin, and kaempferol [10].

Condensed tannins are polymers of procyanidin, prodelphinidin and propelargonidin with a degree of polymerization of up to 30 [11]. In addition, monomeric precursors of condensed tannins, epicatechin and catechin, have been identified [10]. The composition of hydrolyzable tannins in *C. equisetifoli*a has not been studied in detail, but a study of the related species *C. stricta* shows that ellagitannins are the main phenolic compounds [12]. Pedunculagin, casuarinine, stachyurin, tellimagrandin I, strictinin, casuariin, casuarictin, 2,3-hexahydroxydiphenoyl-glucose and 4,6-hexahydroxydiphenoyl-glucose have been isolated and identified [12].

Phenolic compounds in plants perform a variety of physiological and environmental functions. They play an important role in growth, development, and adaptation to abiotic and biotic environmental factors, such as UV radiation, low temperatures, plant pathogens, and phytophagous insects [13, 14]. Currently, the mechanism of *C. equisetifolia* resistance to salinity and drought is being actively studied at the transcriptome and metabolome levels [15, 16]. Drought tolerance in *C. equisetifolia* is associated with changes in phenylpropanoid biosynthesis and an increase in condensed tannin content [15, 17]. In addition, many phenolic compounds have pharmacological activity, antioxidant, antimicrobial, anti-inflammatory, antitumor, and other beneficial properties [18-21]. Therefore, *C. equisetifolia* is of significant interest with regard to the efficient isolation of individual phenolic compounds and the study of their pharmacological activity.

In this work, the composition of phenolic compounds, which were mainly represented by monomeric ellagitannins, was studied for the first time in detail in the shoots of *C. equisetifolia*.

Our goal was to assay the composition and content of phenolic compounds in the shoots of *Casuarina equisetifolia* using ultra-performance liquid chromatography combined with photodiode and mass spectrometric detectors (UPLC-DAD-MS).

*Materials and methods.* Green annual photosynthetic shoots of the tree *C. equisetifolia* grown in the greenhouse of the Botanical Garden of the All-Russian Institute of Medicinal and Aromatic Plants (VILAR, Moscow) were collected in the first ten days of July in 2019. The shoots are articulated, about 1 mm thick, with reduced, fused scale-like leaves, collected in whorls of 6-8. Samples.

The shoots were frozen, freeze-dried (FreeZone 2.5 L, Labconco Corporation, USA) and ground (MM 400, Retsch GmbH, Germany). A sample of dry crushed shoot weighing 15 mg (CPA 225D, Sartorius AG, Germany) was extracted with 1 ml of 80% acetone for chromatography (Component-Reaktiv, Russia) for 60 min at room temperature and constant stirring (VORTEX Genie 2 , Scientific

Industries, Inc., USA). The extract was centrifuged for 20 min at 14,000 rpm (5430R, Eppendorf AG, Germany) and evaporated to dryness at 45°C (CentriVap concentrator, Labconco Corporation, USA). Sample extraction was repeated 2 more times. The resulting dry extract was dissolved in 1 ml of deionized water (Direct-Q3, Merck KGaA, Germany) for 60 min, centrifuged for 20 min at 14,000 rpm, diluted 1:5 with deionized water and filtered (PTFE filter Clean 2 , 0.45 rm, Thermo Fisher Scientific, Inc., USA).

To analyze phenolic compounds, an ultra-performance liquid chromatography system (UPLC, Acquity UPLC® 2.9.0, Waters Corporation, USA) witha photodiode detector (190-500 nm) and a triple quadrupole mass spectrometer (Xevo TQ, Waters Corporation, USA). Separation was carried out on an Acquity UPLC® BEH Phenyl column (2.1×100 mm, 1.7 μm, Waters Corporation, Ireland) in a gradient of 0.1% formic acid (A) and acetonitrile (B) according to the program: 0-0.5 min, 0.1% B in A; 0.5-5.0 min, 0.1-30.0% B in A (linear gradient); 5.0-6.0 min, 30-35% B in A (linear gradient). The eluent flow rate was 0.5 ml/min, and the injected sample volume was 5 μl [22]. To register phenolic compounds, the mass spectrometer operated in negative ionization mode. The obtained data were analyzed using the DataAnalysis 4.0 program.

When identifying phenolic compounds, we used mass spectrometry data, determining the m/z value of the $[M-H]^-$ ion and its fragments, and comparing the results with those published in the literature [12, 23] and in the mass spectrometric database The Human Metabolome Database (HMDB) [24].

The content of various classes of phenolic compounds, the galloyl glucose, ellagitannins, condensed tannins, and flavonoids (quercetin and kaempferol derivatives) was measured by the multiple reaction monitoring method [22] and expressed as mg/g shoot dry weight. We used calibration graphs for standards of phenolic compounds: 1,2,3,4,6-pentagalloylglucose, ellagic acid, gallic acid, (+)-catechin, quercetin and kaempferol (Sigma-Aldrich, USA). Total content was expresses as the sum of all classes of phenolic compounds.

*Results.* UPLC-DAD-MS analysis revealed 16 phenolic compounds in the shoot extract of *C. equisetifolia*. Based on the UV spectra, 14 phenolic compounds were classified as hydrolyzable tannins or their precursors and derivatives, and 2 compounds were classified as flavan-3-ols (Fig. 1).

Compound 1 with a retention time of 1.26 min showed a UV spectrum with two absorption maxima at 218 and 274 nm, which is characteristic of galloyl glucose (Fig. 2). The deprotonated ion m/z 331 $[M-H]^-$ and its fragment m/z 169 [gallic acid-H]$^-$ were identified in the mass spectrum (Table). Based on this, compound 1 was identified as monogalloyl-glucose, a precursor for hydrolyzable tannins.

Compounds 2, 3, 4 with a retention time of 2.14; 2.55 and 2.86 min had a UV spectrum with an absorption maximum at 228-229 nm and a small shoulder in the region of 260-280 nm, which is typical for ellagitannins, the structure of which does not contain galloyl groups (see Fig. 2). These compounds had a deprotonated ion m/z 783 $[M-H]^-$ and its fragment m/z 301 [ellagic acid-H]$^-$ (see Table). As a result, compound 2 was identified as casuariin, and compounds 3 and 4 as isomers of pedunculagin (see Table). All three ellagitannins have the same mass, but differ structurally. In the casuariin molecule, glucose has a linear form, while in pedunculagin it has a cyclic form [12]. Therefore, the retention time of casuariin in reverse-phase HPLC analysis is shorter than that of pedunculagin [25].

Compound 5 was identified as (+)-catechin based on the UV spectrum characteristic of flavan-3-ols with two absorption maxima at 226 and 278 nm (see Fig. 2), the deprotonated ion m/z 289 $[M-H]^-$, fragment m/z 245 $[M-H-CO_2]^-$

and ion m/z 579 $[2M-H]^-$ (see Table).

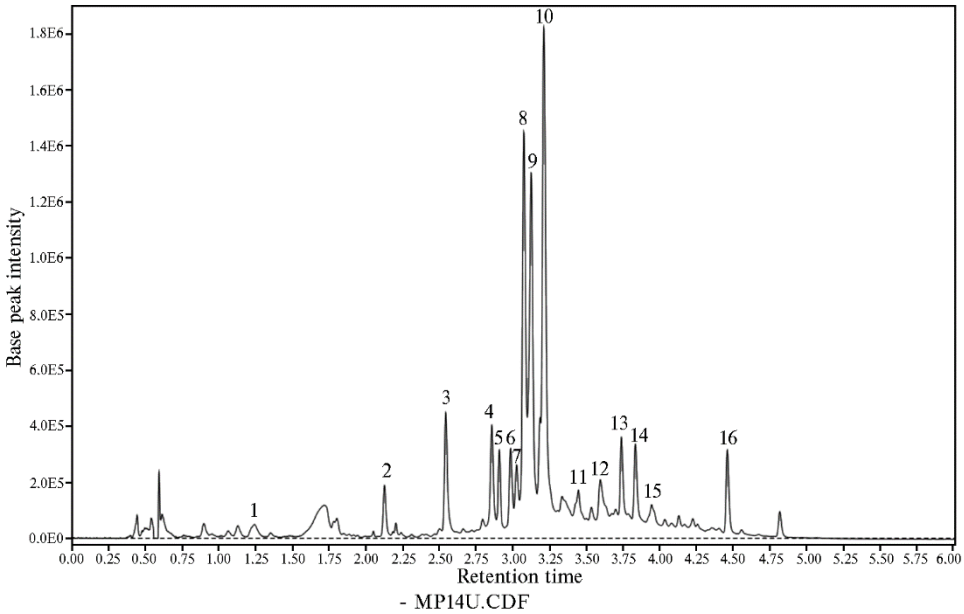


**Fig. 1. Profile of phenolic compounds from the *Casuarina equisetifolia* L. shoot extract:** 1 — monogalloyl-glucose, 2 — casuariin, 3 — pedunculagin (isomer 1), 4 — pedunculagin (isomer 2), 5 — catechin, 6 — pterocarinin A (isomer 1), 7 — pterocarinin A (isomer 2), 8 — stachyurin, 9 — chebulagic acid, 10 — casuarinine, 11 — catechin derivative, 12 — ellagitannin, 13 — ellagic acid arabinoside, 14 — casuarictin, 15 — ellagic acid, 16 — ellagic acid rhamnoside. (ultra-performance liquid chromatography with a photodiode detector (280 nm).

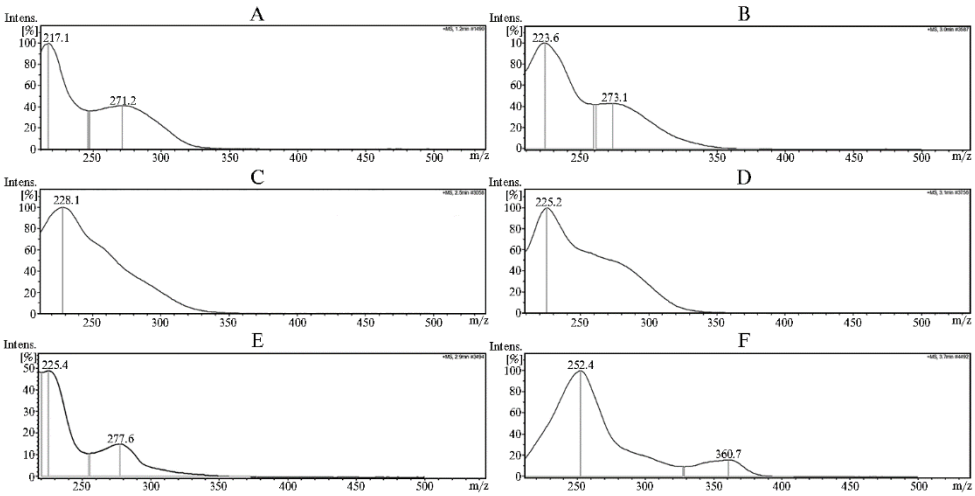


**Fig. 2. Examples of UV spectra of various phenolic compounds identified by ultraperformance liquid chromatography in the *Casuarina equisetifolia* L. shoot extracts:** A — monogalloyl-glucose, B — pterocarinin A (isomer 1), C — pedunculagin (isomer 1), D — casuarinine, E — catechin, F — ellagic acid arabinoside.

Compounds 6 and 7 had a UV spectrum with two absorption maxima at 224 and 271-273 nm (see Fig. 2), which is typical for ellagitannins containing galloyl and hexahydroxydiphenoyl groups. Examination of the mass spectrum of these compounds showed the presence of a deprotonated ion m/z 1067 $[M-H]^-$ and characteristic fragments m/z 169 [gallic acid-H]$^-$, 275 [decarboxylated hexahydroxydiphenic acid monolactone-H]$^-$ and 533 $[M-2H]^{2-}$ (see Table). As a result,

compounds 6 and 7 were tentatively identified as isomers of pterocarinin A, a monomeric ellagitannins with a C-glycosidic bond [26]. More accurate identification necessitates additional research.

Compounds 8, 10 and 14 also had a UV spectrum characteristic of ellagitannins. The mass spectrum contained a deprotonated ion m/z 935 $[M-H]^-$, an ion m/z 467 $[M-2H]^{2-}$, and a characteristic fragment m/z 301 [ellagic acid-H]$^-$ (see Table). These compounds were identified as stachyurin, casuarinine and casuarictin, respectively. Stachyurin and casuarinine are C-linked ellagitannins in which open-chain glucose forms ester bonds with two hexahydroxydiphenoyl groups, while casuarictin is a simple monomeric ellagitannin [12].

**Identification of phenolic compounds in *Casuarina equisetifolia* L. shoots (ultra-performance liquid chromatography coupled with photodiode and mass spectrometric detectors)**

| No | Time, min | $UV_{max}$, nm | m/z of adduct or fragment | | | Compounds |
|---|---|---|---|---|---|---|
| | | | $[M-H]^-$ | $[2M-H]^-$ | fragments | |
| 1 | 1.26 | 218; 274 | 331 | – | 169 | Monogalloyl glucose |
| 2 | 2.14 | 229 | 783 | – | 301; 391; 603 | Kazuriin |
| 3 | 2.55 | 228 | 783 | – | 275; 301; 391; | Pedunculagin (isomer 1) |
| 4 | 2.86 | 229 | 783 | – | 275; 301; 375; 391; 483 | Pedunculagin (isomer 2) |
| 5 | 2.91 | 226; 278 | 289 | 579 | 245 | (+)-Catechin |
| 6 | 2.99 | 224; 273 | 1067 | – | 169; 275; 533 | Pterocarinin A (isomer 1) |
| 7 | 3.03 | 224; 271 | 1067 | – | 169; 275; 533 | Pterocarinin A (isomer 2) |
| 8 | 3.08 | 227; 273 | 935 | – | 169; 275; 467 | Stachyurin |
| 9 | 3.13 | 225; 270 | 953 | – | 169; 275; 301; 476; 633 | Chebulagic acid |
| 10 | 3.21 | 232; 274 | 935 | – | 179; 275; 301; 467 | Casuarina |
| 11 | 3.45 | 226; 275 | 458 | 917 | 289 | Catechin derivative |
| 12 | 3.60 | 225; 272 | – | – | 169; 275; 301; 633; 785; 917; 1063 | Unidentified ellagitannin |
| 13 | 3.74 | 252; 300pl; 361 | 433 | 867 | 301 | Ellagic acid arabinoside |
| 14 | 3.84 | 224; 273 | 935 | – | 301; 467 | Casuarictin |
| 15 | 3.95 | 252; 300pl; 365 | 301 | – | - | Ellagic acid |
| 16 | 4.46 | 249, 300pl; 365 | 447 | 895 | 301 | Ellagic acid rhamnoside |

N o t e. Dashes indicate that the ion or fragments of the ion were not present in the mass spectrum; pl — shoulder.

Compound 9 was identified as chebulagic acid or its isomer by the presence of a deprotonated ion with m/z 953 $[M-H]^-$ and characteristic fragments with m/z 169 [gallic acid-H]$^-$, m/z 275 [decarboxylated hexahydroxydiphenic acid monolactone-H]$^-$, m/z 301 [ellagic acid-H]$^-$, m/z 476 $[M-2H]^{2-}$ and m/z 633 [corilagin-H]$^-$ (see Table).

Compound 11 had a UV spectrum with absorption maxima atof 226 and 275 nm with the presence of ions with m/z 458 and 917, which corresponded to the $[M-H]^-$ and $[2M-H]^-$ ions, and the fragment of parent ion with m/z 289 [catechol-H]$^-$. Compound 11 could not be accurately identified, but based on the data obtained, we can assume that it is a catechin derivative.

For compound 12 with a retention time of 3.60 min, it was not possible to detect the deprotonated ion in the mass spectrum and to identify it. However, the UV spectrum characteristic of ellagitannins and the presence in the mass spectrum of ions with m/z 169, 275, 633, 785, 917 indicate that this compound belongs to ellagitannins.

Compounds 13, 15 and 16 had UV spectra characteristic of ellagic acid (see Fig. 2). Analysis of the mass spectra showed the presence of a fragment with m/z 301 [ellagic acid-H]$^-$. In the mass spectrum of compound 13, ions with m/z 433 and 867 were observed, which correspond to $[M-H]^-$ and $[2M-H]^-$ (see Table). Therefore, compound 13 was identified as ellagic acid arabinoside and compound 15 as ellagic acid. Compound 16 showed the presence of ions with m/z 447 $[M-H]^-$ and 895 $[2M-H]^-$ and was identified as ellagic acid rhamnoside. Although previous studies indicate the hydrolyzable tannins in *C. equisetifolia*, their composition has not been studied in detail.

Pedunculagin, casuarinin, and casuarictin were previously isolated from plants of another species of the *Casuarinaceae* family, the *C. stricta* [12]. Chebulagic acid is found in *C. glauca* [27]. Pterocarinin A has been identified in members of the family *Juglandaceae* which belongs to the same order as *Casuarinaceae* [23]. The presence of catechin and ellagic acid in *C. equisetifolia* has also been reported previously [28].

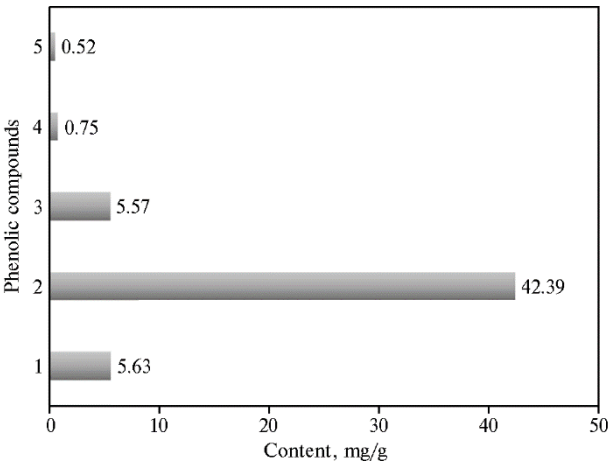


**Fig. 3. Various classes of phenolic compounds in the *Casuarina equisetifolia* L. shoots:** 1 — galloyl-glucose, 2 — ellagitannins, 3 — condensed tannins, 4 — kaempferol derivatives, 5 — quercetin derivatives.

The total content of phenolic compounds in the shoots of *C. equisetifolia* was 55 mg/g dry weight (Fig. 3). The total content of ellagitannins reached 42 mg/g dry weight of the shoot, or 76% of the sum of all classes of phenolic compounds, which basically corresponds to the data of other studies [6-8].

In the composition of ellagitannins, we revealed casuariin, two isomers of pedunculagin, two isomers of pterocarinin A, stachyurin, chebulagic acid, casuarinin, unidentified ellagitannin and casuarictin (see Table). Quantitatively, stachyurin, chebulagic acid and casuarinine predominated (see Fig. 1).

Ellagotannins play an important role in plant physiology [29], participating in growth, development and reproduction [30], and in protection from phytophagous insects and pathogens [31, 32]. The synthesis of these compounds occurs in plant cells via the shikimate pathway [33]. The composition and content of hydrolyzable tannins, including ellagitannins, depends both on the plant species and on the stage of plant development [34]. For example, at the beginning of the growing season, galloyl-glucoses predominate, which are then oxidized to ellagitannins [35].

Ellagotannins found in the shoots of *C. equisetifolia* have a variety of pharmacological activities. For example, chebulagic acid, pedunculagin and casuarinine have high antioxidant [36)] and antiviral [37, 38] activity. In addition, pedunculagin exhibits antitumor properties against cancer cell cultures [39)] and casuarinine is able to induce cell apoptosis [40]. The anti-inflammatory and anticoagulant effects of casuarinine have been established [41, 42]. Casuarictin, stachyurin and casuarinine have antibacterial activity [43].

Thus, using the method of ultra-performance liquid chromatography in combination with photodiode and mass spectrometric detectors, the composition and content of phenolic compounds in the shoots of the plant *Casuarina equisetifolia*, growing in the VILAR greenhouse complex was assayed. It has been shown that the main phenolic compounds in its shoots are ellagitannins. Casuariin, two isomers of pedunculagin, two isomers of pterocarinin A, strachiurin, chebulagic acid, casuarinin and casuarictin have been identified. The total content of ellagitannins was 42 mg/g, or 76% of the total phenolic compounds. The results obtained indicate the important role of ellagitannins in the vital activity of *C. equisetifolia*. Its shoots may be used for the preparative isolation of individual ellagitannins (stachyurin, chebulagic acid and casuarinin) in order to study their

pharmacological activity.

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