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Phytochemical, Free Radical Scavenging and Antifungal Profile of *Cuscuta campestris* Yunck. Seeds

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Abstract

This work was conceptualized with the goal to investigate the phytochemical, free radical scavenging and antifungal profile of *Cuscuta campestris* Yunck. seeds. Total phenolics, amino acid and carbohydrate contents were evaluated in ethanolic, acetone and chloroform extract. Effective antioxidant activity was evaluated throughout seven antioxidant methods. The antifungal activity was assessed against eight fungal strains and *Candida albicans*. The results showed total phenol, flavonoid, flavonols and phenolic acids contents in amount of 1.51-6.35 mg GAE/mL, 78-425 µg RU/mL, 1.04-2.98 mg QU/g and 12.01-30.58 µg CAE/mL respectively. The total amino acids and carbohydrates content ranged from 8.29-185.45 µg Gly/mL and 0.05-0.12 µg Glu/mL. The ethanolic extract showed the best antioxidant activity in phosphomolybdenum, DPPH free radical scavenging, ferric reducing power, and lipid peroxidation assays. The best activity in ferrous ion chelating and H_2O_2 assays had acetone, whereas the best hydroxyl radical scavenging activity had chloroform extract. Ethanolic extract at a concentration of 6 mg/mL proved to be the most effective antimycotic since it inhibited the growth of all tested fungi except *Penicillium verrucosum*. The obtained results indicate that *C. campestris* seeds could be attributed to a potential source of natural antioxidants in food and pharmaceutical products.

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Introduction

Cuscuta campestris YUNCK. (preferred common name: field dodder, golden dodder, family Convolvulaceae) is nonspecific parasite of wide range of commercial host species such as alfalfa, sugar beet, onions, potato, tomato, clover, carrots, legumes, *etc*. Therefore, this parasite has become one of the major causative agents that limit productivity of crops in different parts of the world [1]. Since the plant is parasitic in nature, it absorbs organic and inorganic solutes from host plant, containing number of active phytopharmaceuticals [2]. The phytochemical compounds present in the traditional medicinal plants can be used in the treatment of different types of diseases [3]. In Indian, Bangladesh, Nepal and Chinese folk/tribal medical practice various parts of this plant were used to treat many diseases. In Chinese medicine dodder is used to strengthen the musculoskeletal system, retard ageing and improve vision, and it has been used for impotence, premature ejaculation, urinary frequency and incontinence, diarrhea, low back pain, pre-senile dementia, prostate problem, threatened abortion, tinnitus, vertigo and blurred vision. European dodder has been used by Western herbalists as a laxative and for renal and hepatic disease [4]. Until now, the high antioxidant, anticancer, pharmaceutical and biological activities of the extracts from whole or different part of *Cuscuta* sp. (flower, steam, seeds) have been reported [5]. *Cuscuta* seeds have an immunostimulating activity [6], prevent liver against damage [7], improve defective kidneys [8] and alleviate inflammation/pain [9]. The beneficial activities of the plant have been attributed to the high level of phenolic compounds. Phytochemical screening of genus *Cuscuta* (*C. chinensis*, *C. reflexa*, *C. epithymum*, *C. europea*) revealed a considerable amount of phenolics, including flavonoids, flavonols, phenolics acids, alkaloids, steroids, volatile oils. Compared to abovementioned species that are to large-scale investigated, *C. campestris* is still insufficiently studied. More precisely, one research study of *C. campestris* whole plant revealed its analgesic, anti-inflammatory and CNS-depressant activities [10]. Recent study of *C. campestris* whole plant extracts confirmed the presence of isorhamnetin, kaempferol, and quercetin and their linkage with antioxidant and anticancer activity [11]. Phytochemical study of *C. campestris* seed extracts identified hyperoside, quercetin and flavonoid glycosides as the most abundant phenolics. However, the details analysis about its antioxidant, free radical scavenging and biological activities are still missing. Apart from phenolic constituents, interest in the study of antioxidant substances other than phenolics, such as amino acids and carbohydrates, is intensified. They are precursor compounds for flavonoid secondary metabolites [12]. Polysaccharides and amino acids of plants are considered as effective free radical scavenger, reducing agent, and ferrous chelator in most of the reports. Polysaccharides have been recognized as main bioactive substances which played important antitumor activity against HeL tumor cell line [13] and presented about 45% cytotoxicity against the human breast adenocarcinoma cell [14]. The antioxidant activity of free amino acids has also been revealed [15], and number of them (*Trp*, *Tyr*, *Met*, *Cys*, *His*, *Phe* and *Pro*) have been proposed to contribute positively to the antioxidant activity [16]. According to our

knowledge, polysaccharides and amino acid content and their contribution in free radical activity of *C. campestris* is still uninvestigated.

Until now, there have been no detailed studies of the phytochemical, antioxidant and antifungal activity of *C. campestris* seeds, as well as their potential use as antioxidants in food and pharmaceutical products. In accordance with the aforementioned facts, this study was aimed as much as possible to accurately examine and determine antioxidant and antifungal activities of the ethanolic, acetone and chloroform extracts of *C. campestris* seeds, using combined antioxidant methods. The total content of phenolics and non-phenolic compounds (amino acids and carbohydrates) were determined and correlated with antioxidant activity. The ultimate goal of this comprehensive study is the promotion of *C. campestris* as a source of new natural antioxidants with beneficial effects on preventing oxidative stress and their potential antioxidant-related therapeutic applications.

Results and Discussion

Phytochemical screening of seed extracts

Phytochemical screening of *Cuscuta* seeds was included determination of the total phenols content (flavonoids, flavonols, phenolic acids and gallotannins) and some non-phenolic compounds (amino acids and carbohydrates) in EtOH, ChOH and AcOH. The results are presented in **Table 1**. The results showed that the highest amount of TPC and TFC measured in EtOH, followed by in ChOH, and the least in AcOH. The amount of TPC was in the range of 1.51-6.35 mg GAE/mL whereas the amount of TFC varied from 78-425 µg RU/mL. In contrast, the highest amount of flavonols and TFA was observed in AcOH followed by in EtOH and ChOH. The amount of flavonols and TFA was in the range of 1.04-2.98 mg QU/g extract and 12.01-30.58 µg CAE/mL respectively, in order of activity: ChOH < EtOH < AcOH. The current study revealed a minor amount of gallotannins in EtOH (0.03 mg GA/g) and AcOH (0.015 mg GA/g), however these compounds were not found in ChOH. The TPC and TFC of different *Cuscuta* sp. were partially studied previously by various investigators. Ganapaty *et al.* [17] were found about 4.12 g GAE/100 g dw and 3.76 g CE/100 g dw in MtOH of *Cuscuta epithymum* whole plant. The quantity of TPC found in *Cuscuta reflexa* and *Cuscuta europea* from different hosts was in range of 112.95-146.32 mg GAE/100 g and 97.68-189.68 mg GAE/100 g, respectively [18]. The TFC of *C. reflexa* was in the range of 38.51-68.13 mg CE/100 g whereas their content in the *C. europea* was 38.51-68.13 mg CE/100 g. Akhtara *et al.* [19] revealed TPC and TFC of *C. reflexa* herb in amount of 35 mg GAE/g dw and 9.1 mg QE/g dw, respectively. Anjum *et al*. [20] detected TPC amount of 33.17-52.29 mg GAE/g in EtOH extract of *Cuscuta* steam from different hosts. The obtained results are in agreement with results of other authors who revealed that more polar solvent was most effective in extracting phenolic components [21]. Kinalioğlu *et al.*

[22] found that ChOH of *Xanthoparmelia stenophylla* exhibited higher amount of TPC than AcOH. In general, the solubility of phenolic compounds is not only dependent on the type of solvent used but also on the degree of polymerization and interaction of phenolics with other phytochemical.

An investigation of total amino acids and carbohydrates contents showed that amount of them were in ranged 8.29-185.45 µg Gly/mL and 0.05-0.12 µg Glu/mL respectively. The amount of amino acids was decreasing as polarity of solvents decreased; therefore their highest amount was measured in EtOH and at least in ChOH. However, the amount of carbohydrates in EtOH and ChOH was almost identical and double higher compared to AcOH. Literature data provided the evidence about positive or negative contribution of some individual or groups of amino acids residues on antioxidative processes, depends on the oxidative assay system [23]. However, the current results cannot be compared with literature data since the relevant researches about amino acids and carbohydrate contents of *Cuscuta* sp. are still missing.

Table 1.

In vitro antioxidant activity

A detailed analysis of antioxidative activity of *C. campestris* seeds was performed using seven different antioxidant methods. The results for antioxidant activity of tested extracts and some natural and synthetic compounds are summarized in **Tables 1** and **2**.

The phosphomolybdenum assay, used for the determination of total antioxidant activity, showed double higher antioxidant activity in EtOH compared to AcOH and ChOH extracts of *C. campestris* seeds. The amount of AA found in EtOH was 6.64 µg/mL, whereas its quantity in AcOH and ChOH was 3.81 and 3.46 µg/mL, respectively (**Table 1**). Obviously, the highest amount of TPC is in a positive correlation with the best antioxidant activity of EtOH. In the case AcOH and ChOH, their antioxidant activity is in negative correlation with the TPC. Despite the significant lower amount of TPC, the antioxidant activity of AcOH was negligibly better compared to antioxidant activity of ChOH. However, the better antioxidant activity of AcOH than ChOH could be connected with significantly higher amount of TFC and especially TFA. Among literature data only Akhtar *et al*. [19] revealed the antioxidant activity of *C. reflexa* Roxb. by this method. According to mentioned authors, TOA of *C. reflexa* was found to be 34.7 µg AA/mL. Taking into consideration these results it is evidently that *Cuscuta* seeds could be considered as good source of natural antioxidants.

In the DPPH free radical scavenging assay (**Table 2**), EtOH showed significant better antioxidant activity with IC_{50} = 25.99 µg/mL compared to AcOH (IC_{50} = 54.47µg/mL) and ChOH (IC_{50} = 63.30 µg/mL). In relation to standards, EtOH extract showed antioxidant activity similar to rutin (IC_{50} = 23.13 µg/mL). Therefore, it revealed that the EtOH extract of *Cuscuta* seeds was a high potential natural antioxidant. The obtained results are in agreement with results of *C. reflexa* [19, 24] but much better compared to results of *C. epithymum, C. europea* and *C. chinensis* [17, 18, 25]. Anjum *et al.* [20] found significant differences among extracts of *Cuscuta* steam collected from different hosts. Raza *et al*. [24] found that DPPH activity of *C. reflaxa* extracts ranged from 9.0-62.0 µg/mL. Akhtar *et al.* [19] revealed that *C. reflexa* exhibited DPPH activity of 32.1 µg/mL. Ganapaty *et al.* [17] reported DPPH activity of *C. epithymum* at a concentration of 244.88 µg/mL. Perveen *et al.* [18] described the DPPH activity of *C. reflexa* as much potent (88.85-289.47 µg/mL) than *C. europea* (273.72-669.37 µg/mL). As in the case of TOA, scavenger activity of EtOH was in a positive correlation with TPC, whereas a negative correlation found between TPC and scavenger activity of AcOH and ChOH. These statistical data point to the difference in level of potency of various compounds in extracts.

Table 2.

The ferric reducing antioxidant power assay, characterized by the reduction of Fe^{3+} to Fe^{2+}, is frequently employed to assess the efficiency of antioxidants for their electron transfer capacity. Among tested extracts, EtOH of *C. campestris* seeds possessed the highest reducing capacity with IC_{50} value of 761 µg/mL, which is better than values obtained for AcOH (IC_{50} = 620 µg/mL) and ChOH (IC_{50} = 540 µg/mL). Again, antioxidant activity of extracts in terms of their electron transfer capacity was decreasing along with decreasing polarity of solvent. Comparison of current results could only be possible with standard compounds because of the lack of studies on the *Cuscuta* species in the literature. With regard to natural and synthetic compounds, the tested extracts were less effective (TBHQ > GA > BHT > α-tocopherol > BHA > AA) (**Table 2**).

One more assay used to evaluate the antioxidant activity of *C. campestris* seeds extracts was ferrous ion chelating activities. The transition metal ion Fe^{2+} possesses the ability to perpetuate the formation of free radicals by gain or loss of electrons. Therefore, the reduction of the formation of ROS can be achieved by the chelation of metal ions with chelating agents. Literature data do not provide the evidence about chelating ability of *C. campestris* nor any other *Cuscuta* sp. Therefore, the obtained results are compared only with the standard compounds (**Table 2**). The current results have shown that presence of *C. campestris* seeds extracts in reaction mixture led to decline in formation of Fe^{2+}-ferrozine complex suggesting chelation of iron by phytochemicals present in the seeds. Other reports [26, 27] on chelation of iron by plant extracts also substantiate these findings. From the data presented in **Table 2**, it can be seen that extracts demonstrated 50% iron chelating capacity at

concentrations of 173.11, 173.64 and 215.44 μg/mL in AcOH, ChOH and EtOH, respectively. The best chelating ability of AcOH was in correlation with the highest amount of TFA and flavonols in the extract. This observation is in line with finding that some phenolics acids such as chlorogenic acid contribute highly to the metal chelating capacity as a result of the ortho-dihydroxy substitution in the aromatic ring (catechol) which is effective at chelating transition metals such as Iron(II) [28]. However, the tested extracts showed week ability to chelate ferrous ions compared to tested standards in following order: AA > GA > guercetin > α-tocopherol > BHT.

H_2O_2 is a non radical compound, and is of potential biological significance because of its ability to penetrate biological membranes. H_2O_2 itself is not very reactive, but it sometimes could be toxic to the cell because it may give rise to hydroxyl radical in the cells (singlet oxygen and $HO^{\bullet}$ radicals) that causes DNA damage [29]. Thus, removal of H_2O_2 is very important for protection the biological systems and food components. The antioxidant compounds which donate the electrons to H_2O_2, neutralize it into H_2O molecule. As shown in **Table 2**, reduction potential of *C. campestris* seeds in H_2O_2 assay showed IC_{50} values at concentrations of extracts 160, 320 and 250 µg/mL in order of its activity: AcOH > ChOH > EtOH. Nevertheless, the extracts apparently possessed week reduction potential in comparison with tested standards (AA > GA >TBHQ > α-tocopherol > BHT > BHA).

Hydroxyl radical scavenging activity assay was used to understand the scavenger potential of extract against short-lived radicals, *viz.*, $HO^{\bullet}$ radicals. $HO^{\bullet}$ radicals were reported to abstract H-atoms from lipid membranes, and thus bring about peroxide reactions of lipids. As the **Table 2** shows, the IC_{50} values of the extracts in order to their scavenging activity were: ChOH (16.2 µg/mL) > AcOH (62.0 µg/mL) > EtOH (250 µg/mL). The activity of seeds was increasing with polarity extract decreased. Statistical data showed that IC_{50} values of extracts were significantly different (p<0.05). Ganapaty *et al*. [17] found IC_{50} value of hydroxyl radical of MtOH of *C. epitimum* whole plant at a concentration of 213.69 µg/mL. This result is very similar to the current result obtained in EtOH. Considering the results of all tested extracts and standards, it can be concluded that ChOH showed high scavenging activity compared to standards such as AA, QU, BHA but low compared to GA and TBHQ.

Lipid peroxidation contains a series of free radical-mediated chain reaction processes and is also associated with several types of biological damage. It can be inhibited by using standard antioxidants, compounds isolated from the plants and fungi, or their extracts which possess antioxidant activity. Polyunsaturated fatty acids, such as linoleic acid, are easily oxidized by oxygen in the air. This auto-oxidation leads to the formation of linoleic acid peroxides. The ability of *C. campestris* extracts and referent standards to reduce the formation of hydroperoxide after 72 h obtained IC_{50} values of EtOH, AcOH and ChOH were 758, 910 and 1062 µg/mL respectively. Sharma *et al.* [30] found IC_{50} = 37 µg/mL in EtOH of

Cuscuta reflexa. Compared to tested standards, all extracts had significant lower ability (p<0.01) to reduce the formation of hydroperoxide, as follows: α-tocopherol > BHT> QU>AA > GA > EtOH > AcOH > ChOH.

Antifungal activity of seeds extracts

Antifungal activity of *C. campestris* seeds extracts is shown in **Table 3**. The best antifungal activity was shown EtOH since their concentration of 6 mg/mL inhibited the growth of all tested fungi with exception of *P. verrucosum*. The AcOH extract had a selective effect on the growth regardless of the types of fungi. The most sensitive species were *T. viride, P. cyclopium* and *A. niger*. The growth of these fungi was inhibited by the extract of a concentration at 6 mg/mL. As most resistant species, the *C. albicans* was identified. For 50% inhibition of yeast growth, it was necessary concentration of the extract at 25 mg/mL. Among all extracts, the ChOH showed the lowest antifungal activity. At a concentration of 6 mg/mL of ChOH, only the growth of *P. cyclopium* was inhibited (50%) whereas the concentration of ChOH at 12 mg/mL inhibited the most tested fungi. The fungi species such as *P. chrysogenum* and *C. cladosporioides* were the most resistant to the activity of this extract. For the inhibition of their growth, it was required a concentration of extract at 25 mg/mL. By increasing the solvent polarity, antifungal activity of *P. chrysogenum* and *C. cladosporioides* was decreased. However, the polarity of the solvent had no effect on antifungal activity of *P. verrucosum*. The MIC values obtained with extracts against tested fungi and yeast were 10 to 20 fold greater than that of reference antimycotic on the corresponding microorganisms. Especially, the *A. niger* exhibited almost the same susceptibility for EtOH and AcOH extracts when compared to reference antimycotic. Antifungal activity of some *Cuscuta* sp. was described in literature. According to Inamdar *et al*. [31], EtOH extract of *C. reflexa* stem at a concentration of 200 µg/mL was effective against fungi in following order: *A. niger* > *P. citrinum* > *P. aeruginosa*. Perveen *et al.* [18] found antifungal activity of EtOH extract of *C. europea* and *C. reflexa* from different hosts against *A. niger* and *A. flavus*. Conversely, Ferraz *et al.* [32] did not found antifungal activity of *C. racemosa* EtOH extract against *A. niger* and *C. albicans*. Obviously, antifungal activity of *Cuscuta* species depends on different factors: the plant species, the part of plant, their hosts, macro- and microclimate, type of solvent, extraction conditions, type of fungi, *etc*.

Table 3.

Statistical analysis

Relationships among different antioxidant variables and total phenolics and non-phenolic compounds are presented in **Table 4**. A strong positive correlation between TPC and TFC (r=0.889**) and negative correlation between TPC and gallotannins (r= -0.751*) were obtained at p<0.01 and p<0.05 levels respectively, in a two-tailed Pearson correlation. Likewise, the strong correlation between flavonols and TFA (r=0.875**), as well as between

flavonols and gallotannins (r=0.810**) was found. The strongest linear correlation was found between TFA and gallotannins (r=0.993**). The correlation between phenolic and non-phenolic compounds was also detected. In fact, TCHC were strongly positive correlated with TPC (r=0.943**), TFC (r=0.711**) or negative correlated with TFA (r= -0.843**) and gallotannins (r= -0.988**). With regards to the TAAC, a very strong correlation was only verified with flavonols (r=0.921**). These correlation data indicate that *C. campestris* seeds contain polyphenols in a large extent and simple phenols in minor extent. A strong correlation found among different phenolics could be brought into connection with their common metabolic pathways, i.e. competition for substrate. Based on correlation coefficient obtained between TCHC and TFC it could be conclude that conjugated flavonoids (glycosides) are the most abundant form of flavonoids in the extracts. High correlation found between TAAC and flavonols indicates that these compounds are highly associated at the biosynthetic level (phenolpropane pathway).

The linear correlation between phenolics and non-phenolic compounds and free radical scavenging activities was evaluated in the next step. As the **Table** shows, a very strong correlation between TPC and hydrogen peroxide assay (r=0.906**) and moderate to weak correlation between TPC and phosphomolybdenum (r=0.749*), hydroxyl radical scavenging activity (r=0.690**) and chelating activity (r=0.605**) assays were found. However, no significant correlation was observed between TPC and other antioxidant assays. Unlike TPC, TFC correlated strong with all free radical scavenging assays, apart from lipid peroxidation assay. The correlation coefficients found between TFC and phosphomolybdenum, DPPH, hydrogen peroxide, hydroxyl radical, reducing power, chelating ability and lipid peroxidation assays were: r=0.969**, r= -0.930**, r=0.969**, r=0.999**, r=0.945**, r=0.864**, r=0.987** and r= -0.767**, respectively. Flavonols was in strong correlation with lipid peroxidation assay (r= -0.811**) and in moderate to weak correlation with reducing potential assay (r=0.698*). Like TFA, TAA well correlated with all antioxidant assays with exception of hydrogen peroxide assay. A very strong correlation was noted between TAA, reducing ability (r=0.914**) and inhibition of lipid peroxidation (r= -0.974**); strong correlation was found between TAA, DPPH (r= -0.857**) and hydroxyl radical scavenging activity (r=0.834**) whereas moderate correlation was observed between TAA, phosphomolybdenum (r=0.785*) and chelating activity (r=0.725**) assays. The THCH correlated only with hydrogen peroxide (r=0.738**) and lipid peroxidation (r= -0.901**) assays. These statistical data indicates that TFC had dominant role in free radical scavenging activity whereas non-flavonoid polyphenols had a minor role in this activity. Indeed, the different phenolic compounds are responsible for quenching free radicals. The distinctive structure of each phenols compound (number of OH groups, side chain on benzoic acids) explains their special ability for scavenges different free radicals [33].

Finally, relationship between different antioxidant assays was evaluated. It is important to note that a very strong correlation found between phosphomolybdenum assay and all tested antioxidant assay (DPPH, hydrogen peroxide, hydroxyl radical scavenging, lipid peroxidation, reducing capacity and chelating ability). From the methodological point of view, these results indicate strong relationship between applied antioxidant methods.

Table 4.

Taking into account the presented results, it is clear that *C. camperstris* seeds can be regarded as promising candidates for natural plant source of antioxidants (phenolics and non-phenolics) with significant antioxidant properties. This plant can be explored as a viable source for the isolation of natural antioxidants and high-valued bioactives which may serve as leads for the isolation of new antioxidants and bioactives for development of functional foods/nutraceutical and pharmaceutics.

Experimental part

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ascorbic acid, quercetin, butylated hydroxytoluene, ammonium molybdate, sodium phosphate, sulphuric acid, ferrous sulfate heptahydrate, Folin–Ciocalteu reagent, aluminum chloride, potassium ferricyanide, trichloroacetic acid, ferric chloride, linoleic acid, sodium acetate, Tween-20, ammonium thiocyanate, hydrochloric acid, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*′-disulfonic acid monosodium salt hydrate, sodium carbonate, potassium sodium tartrate tetrahydrate, rutine, were obtained from Sigma-Aldrich St. Louis, MO, USA; ethanol, methanol, acetone, chloroform, were obtained from Fluka Chemie AG Buchs, St. Louis, USA.

Plant material

The fresh aerial parts of plant *C. campestris* growing on different plants (host) were collected from field around Belgrade (Serbia) in June, 2014. *C. campestris* was separated from the host and authenticated by Dr Sava Vrbničanin, Faculty of Agriculture, University of Belgrade, Serbia. Voucher specimen of *C. campestris* (CCFA 03) was deposited in the Herbarium of Faculty of Agriculture, University of Belgrade, Serbia.

Preparation of seed extracts

The air dried seeds of *C. campestris* (30 g) was powdered and extracted with ethanol, acetone and chloroform (100 mL). The samples were placed in water bath at 40 °C over the night. The each extract was filtered through a filter paper (Whatman No. 1) and residues were re-extracted by same solvent for 3 times and obtained fractions were collected. All extracts were allowed to evaporate at room temperature until the dry weight mass were obtained. The crude extracts were stored in a dark glass bottle at 4 °C to prevent oxidative damage until the further analysis.

Phytochemical studies

Total Phenolic Content

The total phenolic content in the extracts was determined according to the Folin-Ciocalteu method by Singleton and Rossi [34] with slight modifications. To 1 mL of each extract dissolved in methanol, 2 mL of 7.5% (w/v) sodium carbonate solution was added and vortexed vigorously. After 5 min, 1 mL of 1:10 diluted Folin-Ciocalteu's phenol reagent was added and vortexed again. Same procedure was repeated for the standard solution of gallic acid. All the tubes were incubated at room temperature for 30 min and then the absorbance was measured at 765 nm. The total phenolic content in the extracts was calculated from the standard curve and values are expressed as gallic acid equivalent (GAE) in mg/g of dry weight (DW) extract.

Total Flavonoid Content

The aluminum chloride method was used for the determination of the total flavonoids content of the sample extracts [35]. Aliquots of extract solutions were taken and made up to volume 3 mL with methanol. Then 0.1 mL $AlCl_3$ (10%), 0.1 mL potassium sodium tartrate and 2.8 mL distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was measured after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using known concentrations of rutine. The concentration of flavonoids in the test samples was calculated from the standard curve and values are expressed as rutine (RU) equivalent in mg/g of DW extract.

Total Flavonol Content

Total flavonols in the plant extracts were estimated using the method of Kumaran and Mbaebie [36] with slight modification. Briefly, 1 mL of extract or fractions (1 mg/mL) was added to a centrifuge tube with 2 mL of $AlCl_3$ prepared in ethanol and 3 mL of 50 g/L sodium acetate solution. This was then mixed thoroughly with a vortex mixer and incubated for 1 hour. Absorbance was then measured at 440 nm with a spectrophotometer. Total

flavonol content was calculated as quercetin equivalent (mg/g) using the following equation based on the calibration curve Y = 0.0297x + 0.1288, R^2= 0.9729, where X is the absorbance and Y is the quercetin equivalent. X = (A ×m_0×10)/(A_0× m).Where: X = flavonoids content, mg quercetin/g; A = the absorbance of sample; A_0 = the absorbance of standard quercetin.

Gallotannins content

Gallotannins can be detected quantitatively by the potassium iodate assay. This assay is based on the reaction of potassium iodate (KIO_3) with galloyl esters [37], which will form a red intermediate and ultimately a yellow compound. The concentration of the red intermediate can be measured spectrophotometrically at 550 nm. The reaction was performed by adding 1.5 mL of a saturated potassium iodate solution to 3.5 mL of extract at a temperature of over 40 °C until maximum absorbance was reached (regardless of time). Gallotannin content was determined using gallic acid as standard.

Total phenolics acids

Total phenolic (hydroxycinnamic) acids were determined by the Polish Pharmacopoeia method from the monograph of dandelion leaves [38]. Briefly, 1 mL of extract (1 mg/mL) was added to 5 mL water, followed by 1 mL of 0.1 M HCl, 1 mL of Arnow reagent (10% w/v of sodium molybdate and 10% w/v sodium nitrite), 1 mL 1 M NaOH, filled up to 10 mL in a volumetric flask and the absorbance read immediately at 490 nm. The results were expressed as caffeic acid equivalents (mg CAE/g extract).

Nonphenolic content

Quantitative determination of amino acids content. The amino acids content of extracts were determined spectrophotometrically according to procedure described by Swamy [39]. One mL of test sample (1 mg/mL) was transferred in test tube and evaporated to dryness. After evaporation, 2 mL 0.2 M citrate buffer (pH 5.0) and 1 mL KCN-Acetone-Ninhydrin reagent were added in each tubes, and they vortexed again. Reaction mixture was incubated in boiling water bath for 20 min and after cooling the absorbance was measured at 570 nm. Citrate buffer (pH 5.0) was used as blank. Glycine (0-400 µg/mL) was used for the preparation of standard curve.

Quantitative determination of carbohydrates content. To determine the carbohydrates content in plant extracts modified anthrone reagent was used [40]. Sulfuric acid and water were mixed in a 2.3:1.0 (v/v) ration and allowed to cool. An anthrone reagent was freshly prepared by dissolving 0.2% (w/v) anthrone in the diluted sulfuric acid. The extracts samples and standard were diluted in methanol in final concentration of 1 g/mL. An 25 µL aliquots of

each extract as well as glucose (standard) were mixed with 3 mL of anthron reagent and heated in screw-cut test tubes in boiling water bath for 10 min. Tubes were then removed from water bath, cooled to room temperature and absorbance at 630 nm was recorded. Methanol was used as blank.

In vitro antioxidant activity

Total antioxidant capacity. The total antioxidant capacity was determined by phosphomolybdenum method according to Prieto *et al.* [41]. To 1 mL of samples or standard at different concentration performed from stock solutions (1 mg/mL), 2 mL reagent solution (ammonium molybdate 4 mM, sodium phosphate 28 mM, and sulphuric acid 0.6 M) was mixed vigorously. All the reaction tubes were incubated at 95 ºC for 90 min. The absorbance was measured at 695 nm against blank (methanol) after cooling at room temperature. Ascorbic acid (AA) was used as standard and total antioxidant capacity extracts is expressed as mg AA/g of DW extract.

DPPH assay. For the estimation of anti-radical potential, DPPH free radical scavenging activity of all the extracts was conducted using DPPH method [42]. Working solution of extracts was carried out by dilution stock solution (2 mg/mL) of extracts. DPPH was dissolved in methanol to obtain a concentration at 8 µg/mL. To 1 mL of DPPH solution, 1 mL of various concentrations of the extracts or the standard solution was added separately. The reaction mixtures were incubated at 37 ºC for 30 min, following by absorbance measured at 517 nm using methanol as blank reference. The DPPH scavenging activity (%) of extracts and standards AA, gallic acid, butylated hydroxytoluene (BHT), α-tocopherol, quercetin was determined using the following equation (1):

$$\% \text{ radical scavenging activity} = [(Ac - As) / Ac] \times 100 \qquad \text{Eq (1)}$$

Where Ac was absorbance of control reaction and As the absorbance in presence of the sample.

Reducing power assay. The reducing power assay was determined according to method described by Oyaizu [35]. Serial dilutions were carried out with the stock solution (1 mg/mL) of each extract. To 1 mL sample extract at different concentrations, 2.5 mL 0.2 M phosphate buffer pH 6.6, and 2.5 mL 1% potassium ferricyanide were added followed by mixed vigorously. After incubation at 50 ºC for 20 min, 2.5 mL 10% trichloroacetic acid was added to mixture followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 mL of upper layer of mixture was added to 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride, and absorbance of resulting solution was read at 700 nm against a blank. AA was used as standard. The reducing capacity of extracts was calculated using Eq. (1), and results are expressed as IC_{50} value.

Ferrous ion chelating ability. The ferrous ion chelating activity extracts was measured by the decrease in absorbance at 562 nm of the iron (II)-ferrozine complex according to Carter [43] and Yan *et al.* [44]. One mL of 0.125 mM $FeSO_4$ was added to 1 mL sample extract at different concentrations (from 2 mg/mL to 3.91 μg/mL), followed by 1 mL 0.3125 mM ferrozine. The test tubes were allowed to equilibrate at room temperature for 10 min. The absorbance was measured at 562 nm against blank. AA, BHT, α-tocopherol and quercetin were used as positive control. The ability of the extract to chelate ferrous ion was calculated using Eq. (1), and results are expressed as IC_{50} value.

Inhibitory activity against lipid peroxidation. The antioxidant activity of different extracts on inhibition of lipid peroxidation was determined according to the ferric thiocyanate method as reported by Kuo *et al.* [45]. A solution of 0.02 mM linoleic acid emulsions was prepared by dissolving 0.28 g linoleic acid and 0.28 g Tween 20 in 50 mL of 0.02 M phosphate buffer (pH 7.0). Aliquots of this emulsion (2.5 mL) and 2 mL of 0.2 M phosphate buffer (pH 7.0) were transferred into different test tubes containing 4 mL of various concentrations (5 - 50 mg/mL) of the sample extract in 80% methanol. After incubated at 55 °C in darkness for 72 h, 0.1 mL of the mixture was sampled and combined with 4.7 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 20 mM ferrous chloride (in 3.5% hydrochloric acid). For control, 80% methanol was used in the reaction instead of the sample extracts. After addition ferrous chloride exactly 3 min, the absorbance of the reaction mixture was measured at 500 nm. The inhibitory effect was calculated according to the following equation:

% inhibitory effect = [1- (Absorbance of sample/ Absorbance of control)] x 100 Eq (2)

The percentage of inhibitory effect obtained was subsequently plotted against log sample concentration. The antioxidant activity of extracts was expressed as IC_{50}, which was defined as the concentration in mg/mL.

Antifungal activity

Test organisms. The antifungal activity of each extract of *C. campestris* seed was tested against 8 fungi species such as *Aspergillus niger*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Trichoderma viride*, *Trichoderma harzianum*, *Penicillium chrysogenum*, *Penicillium verrucosum*, *Penicillium lividum* and yeast *Candida albicans*. All test organisms were obtained from Institute for Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. The fungi were cultivated on Potato dextrose agar and yeast on Sabouraund dextrose agar plates. The cultures were stored at +4 °C and sub-cultured once a month.

Determination of minimum inhibitory concentration (MIC). The minimum inhibitory concentrations (MICs) of *C. campestris* seed extracts against tested microorganisms were determined according to the microdilution method [46]. For determination of antifungal activity the Sabouraud dextrose broth (SDB) was used. The assay for MICs determining was performed by a serial dilution technique using 96-well microtiter plates.

The seed extracts at concentration range 10.00-0.078 mg/mL were added into the first row of the plate and in all the other rows that were filled with 50 µl of SDB, and the double dilutions were made. Thereafter, 10 µl of resazurin indicator solution (270 mg resazurin in 40 mL of sterile distilled water) and 30 µl of nutrient broth were added to the each well. A 10 µl of SDB was added in tests with fungi instead of resazurin solution. Finally, 10 µl of fungal suspension (1.0×10^6 CFU/mL) was added to the each well. For each strain, the growth conditions and the sterility of the medium were checked. The microplates were incubated for 48 h at 28 °C for fungi. Color change was then assessed visually and any color change from purple to pink or colorless was recorded as positive. The lowest concentration with no observed color change was taken as the MICs value for bacterial strains and the lowest concentrations without visible growth were defined as MICs for fungi. All tests were repeated in triplicate. Fluconazole was used as a positive control for antifungal activity.

Statistical analysis. All the results are expressed as means (MS) ± standard deviation (SD) of five independent measurements. For tested the normality of distribution, means and standard deviation, Student *t*-test at the level of significance 0.05 and 0.01 was used. Correlation coefficient was analyzed through Pearson's correlation coefficient. The IC_{50} values were calculated by nonlinear regression analysis from the sigmoidal dose-response inhibition curve. Statistical analyses of the data were analyzed using analysis of variance (ANOVA) and the group means were compared by the least significant difference test (LSD). The results were considered statistically significant if the $P < 0.05$. For statistical analysis, the SPSS 19.0 software program was used.

Conclusions

The results presented in the current study suggest that *C. campestris* seed has excellent antioxidative potential and is effective both as a primary and a secondary antioxidant. Presence of substantial amounts of phenols and flavonoids in the seed may be responsible for its effective antioxidant potency. The ethanolic extract showed the best antioxidant activity in phosphomolybdenum, DPPH free radical scavenging, ferric reducing power, and lipid peroxidation assays. Furthermore, ethanolic extract proved to be the most effective antimycotic against of all fungi species except *Penicillium verrucosum.* Based on these relevant data, it could be concluded that *C. campestris* seeds could be attributed to a potential source of natural antioxidants in food and pharmaceutical products. Further works will be focus on isolation and characterize of the functional components of these extracts,

maybe, in similar models of antioxidant and biological activities and on validate these activities *in-vivo* which may leads to the new drug formulation.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1. Phytochemical profile (phenolic and non-phenolic compounds) and total antioxidant activity of *C. campestris* seeds

Extract	Phenolic compounds					Non-phenolic compounds		Total antioxidant activity
	TPC	TFC	Flavonols	Gallotaninns	TFA	TAAC	TCHC	
	mg GAE/mL	µg RU/mL	mg QU/g	mg GA/g	mg CAE/g	µg Gly/mL	µg Glu/mL	(µg AA/g)
EtOH	6.35±0.20^{c}	425±1.63^{c}	2.72±0.10^{b}	0.03±0.00^{a}	19.60±1.40^{b}	185.45±1.55^{c}	0.12±0.01^{b}	6.64±0.18^{c}
AcOH	1.51±0.04^{a}	78±0.32^{a}	2.98±0.08^{c}	0.015±0.00^{a}	30.58±1.22^{c}	135.45±1.28^{b}	0.05±0.00^{a}	3.81±0.09^{b}
ChOH	4.35±0.15^{b}	133±0.52^{b}	1.04±0.02^{a}	0.00^{a}	12.01±0.08^{a}	8.29±0.12^{a}	0.11±0.01^{b}	3.46±0.03^{a}

TPC- Total phenolic contents, TFC- total flavonoid contents, TFA- total phenolic acids, TAAC- total amino acids content, TCHC- total carbohydrates content, EtOH- ethanolic extract, AcOH- acetone extract, ChOH- chloroform extract. Results are expressed as mean values ± SD from five independent experiments. One-Way ANOVA with Fisher's least significant difference test (LSD).

Table 2. IC_{50} values of *C. campestris* seeds extracts and some natural and synthetic antioxidants in DPPH (DPPH), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and lipid radicals (ILPO), reducing power (RP) and chelating ability (ChA) assay

Extracts/ standards	DPPH IC_{50} (µg/mL)	H_2O_2 IC_{50} (µg/mL)	OH IC_{50} (µg/mL)	ILPO IC_{50} (µg/mL)	RP IC_{50} (mg/mL)	ChA IC_{50} (µg/mL)
EtOH	25.99 ± 0.85^{f}	250 ± 5.75^{i}	250 ± 2.05^{f}	758 ± 2.25^{d}	0.76 ± 0.18^{b}	215.44 ± 1.75^{b}
AcOH	54.47 ± 0.78^{h}	16 ± 0.32^{e}	62 ± 1.18^{e}	910 ± 3.45^{e}	0.62 ± 0.10^{ab}	173.64 ± 1.21^{a}
ChOH	63.30 ± 0.90^{j}	62 ± 0.85^{g}	16.2 ± 0.20^{b}	1062 ± 3.38^{f}	0.54 ± 0.10^{a}	173.11 ± 1.19^{a}
AA	6.05 ± 0.36^{d}	38.40 ± 0.70^{f}	21.4 ± 0.12^{c}	250 ± 1.05^{c}	1.14 ± 0.05^{c}	352.9 ± 1.25^{c}
GA	3.97 ± 0.03^{c}	63.33 ± 2.95^{h}	1.48 ± 0.02^{a}	255 ± 1.35^{c}	21.36 ± 3.93^{g}	$>4000^{g}$
α-tocopherol	12.00 ± 0.33^{e}	11.99 ± 3.12^{bd}	nd	0.51 ± 0.03^{a}	14.96 ± 0.52^{e}	$>1000^{e}$
Quercetin	1.48 ± 0.29^{a}	nd	52 ± 3.11^{d}	2.90 ± 0.25^{b}	nd	550 ± 1.18^{d}
BHA	2.03 ± 0.28^{b}	12.09 ± 1.12^{cd}	$>1500^{g}$	nd	5.74 ± 0.23^{d}	nd
BHT	26.00 ± 0.05^{g}	12.05 ± 2.13^{bc}	nd	1.00 ± 0.23^{a}	15.54 ± 1.17^{f}	$>1500^{f}$
TBHQ	1.90 ± 0.07^{ab}	0.13 ± 0.01^{a}	0.59 ± 0.01^{a}	nd	26.81 ± 1.92^{h}	nd

AA-ascorbic acid, GA-gallic acid, BHA-butylated hydroxyanisole, BHT- butylated hydroxytoluene, TBHQ-butylated hydroquinone, nd-not determined. Results are expressed as mean values ± SD from five independent experiments. Mean values in the same column with superscript with different letters are significantly different ($p<0.05$). One-Way ANOVA with Fisher's least significant difference test (LSD).

Table 3. Minimum inhibitory concentration (MIC) of *C. campestris* seeds extracts against fungi and yeast species

Strains	Antifungal activity (MIC) in mg/mL			
	EtOH	AcOH	ChOH	Fluconazole
Alternaria alternata	6	12	12	0.6
Trichoderma viride	6	6	12	0.6
Trichoderma harzianum	6	12	12	0.6
Penicillium verrucosum	12	12	12	0.6
Penicillium lividum	6	12	12	0.6
Penicillium cyclopium	6	6	6	0.6
Aspergillus niger	6	6	12	5
Cladosporium cladosporioides	6	12	25	0.6
Penicillium chrysogenum	6	12	25	0.5
Candida albicans	6	25	12	1.25

EtOH- Etanolic extract, AcOH- acetone extract, ChOH- chloroform extract. The test was performed in triplicate, SD = 0.

Table 4. Linear correlation coefficients between phytochemical constituents and antioxidant capacities of *C. campestris* seeds

	TPC[a]	TFC[b]	Flav[c].	Gallot[d].	TFA[e]	TAAC[f]	TCHC[g]	TAO[h]	DPPH[i]	H_2O_2[j]	OH•[k]	ILPO[l]	RP[m]	ChA[n]
TPC	ns	0.889**	ns	-0.751*	ns	ns	0.943**	0.749*	ns	0.906**	0.690*	ns	ns	0.605*
TFC	0.889**	ns	ns	ns	ns	ns	0.711*	0.969**	-0.930**	0.999**	0.945**	-0.767*	0.864**	0.987**
Flavonols	ns	ns	ns	0.810**	0.875**	0.921**	ns	ns	ns	ns	ns	-0.811**	0.698*	ns
Gallotanins	-0.751*	ns	0.810**	ns	0.993**	ns	-0.899**	ns	ns	ns	ns	ns	ns	ns
TFA	ns	ns	0.875**	0.993**	ns	ns	-0.843**	ns	ns	ns	ns	ns	ns	ns
TAAC	ns	ns	0.921**	ns	ns	ns	ns	0.785*	-0.857**	ns	0.834**	-0.974**	0.914**	0.725*
TCHC	0.943**	ns	ns	-0.899**	-0.843**	ns	ns	ns	ns	0.738**	ns	ns	ns	ns
TOA	0.749*	0.969**	ns	ns	ns	0.785*	ns	ns	-0.992**	0.959**	0.996**	0.901**	0.959**	0.996**
DPPH	ns	-0.930**	ns	ns	ns	-0.857**	ns	-0.992**	ns	ns	ns	ns	ns	0.723**
H_2O_2	0.906**	0.999**	ns	ns	ns	ns	0.738*	0.959**	ns	ns	ns	ns	-0.389*	ns
OH•	0.690*	0.945**	ns	ns	ns	0.834**	ns	0.996**	ns	ns	ns	ns	ns	ns
ILPO	ns	-0.767**	-0.811**	ns	ns	-0.974**	-0.901**	-0.901**	ns	ns	ns	ns	-0.749**	-0.846**
RP	ns	0.864**	0.689*	ns	ns	0.914**	ns	0.959**	ns	-0.389*	ns	-0.749**	ns	0.965**
ChA	0.805**	0.987**	ns	ns	ns	0.725**	ns	0.996**	0.723**	ns	ns	-0.846**	0.965**	ns

[a] Total phenolic contents, [b] total flavonoid contents, [c] total flavonols, [d] total gallotannins, [e] total phenolic acids, [f] total amino acids content, [g] total carbohydrates content, [h] total antioxidant activity, [i] DPPH scavenger activity, [j] hydrogen peroxide, [k] hydroxyl radical, [l] lipid radicals, [m] reducing power, [n] chelating ability assay. Pearson Correlation Sig. (2-tailed). * Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level, ns - no significant.