Bulgarian Chemical Communications, Volume 56, Special Issue D1 (pp. 19-25) 2024 DOI: 10.34049/bcc.56.D.S2L6

The synergistic effect between Golden root and *Cannabis Sativa* L. Determination of the antioxidant activity of the extracts

N.B. Vetskov[1], I.S. Hinkov[1], K.V. Petkova- Parlapanska[2*], E.D. Georgieva[2,3], G.D. Nikolova[2], Y. D. Karamalakova[2]

[1] *University of Chemical Technology and Metallurgy, 8 Kliment Ohridski Blvd., Sofia 1756, Bulgaria*
[2] *Chemistry and Biochemistry Department, Medical Faculty, Trakia University, 11 Armeiska Str., 6000 Stara Zagora, Bulgaria*
[3]*Department of General and Clinical Pathology, Forensic Medicine, Deontology and Dermatovenerology, Medical Faculty, Trakia University, 11 Armeiska Str., 6000 Stara Zagora, Bulgaria*

Received: November 3, 2023; Revised: April 11, 2024

The study aimed to compare the chemical composition and *in vitro* antioxidant activity of an extract obtained from *Cannabis sativa* L and golden root (*Rhodiola rosea* L), which are both used as natural anxiolytics. The phytochemical profile (HPLC - DAD analysis), total polyphenol content (TPC), and antioxidant capacity (AOC) of the combination of *Rhodiola rosea* extract and *Cannabis sativa* oil were investigated to study their effects. To determine the DPPH radical-scavenging activity, an electron paramagnetic radical spectrometer (EPR-X-band) was used as a promising technique. The results indicated that the new extract derived from the combination of *Rhodiola rosea* and *Cannabis sativa* was richer in phenolic compounds and exhibited higher antioxidant activity. These findings provide valuable insights for potential *in vivo* studies that involve the simultaneous use of the two plants.

Keywords: *Cannabis sativa* L*, Rhodiola rosea* L*,* antioxidant activity, phenolic composition

INTRODUCTION

Synergy refers to the phenomenon where certain substances work together to produce a greater overall effect than the sum of their individual effects. This can often be observed when different herbal products interact with each other. Identifying and utilizing these interactions is crucial, as they can be harnessed to effectively treat a wide variety of diseases [1].

Cannabis sativa L (*C. sativa*) and *Rhodiola rosea* L (*R. rosea*) are popularly used as natural anxiolytics. The occurrence of *Rhodiola rosea* is not common in some regions and is mostly cultivated due to the accumulation of active compounds in it over several years. The golden root is found in Bulgaria, mostly on rocky places and mountain meadows up to 2600 m above sea level in Rila, Pirin, Western Rhodopes, Western and Central Stara Planina mountains. Traditionally, it has been used as a central nervous system stimulant. The pharmacological effects of *R. Rosea* are believed to be due to the presence of the glucosides rosavin, rosin and rosarin. However, there is a lack of sufficient data in the literature on the presence and amount of individual phenolic compounds in *Rhodiola rosea* L extracts, and their influence on the biological action of the extracts [4]. Over the years, the medicinal properties of another anxiolytic C *sativa*, and the action of cannabidiol (CBD) in neurodegenerative diseases have been increasingly discussed [5]. There is evidence that the therapeutic benefits of the plant are based not only on cannabinoids, but also on other secondary metabolites such as terpenes and flavonoids. Different parts of the plant, such as leaves, flowers, and pollen, contain at least 26 flavonoids [6, 7]. However, to understand the contributions of each natural product to this "entourage effect", further research is needed [8].

Electron paramagnetic resonance (EPR), also known as electron spin resonance (ESR), spectroscopy has been utilized in natural product research since the 1960s. EPR experiments are designed to demonstrate specific reactivity with various radicals, providing information on intrinsic antioxidant potential with minimal environmental impact. Spectrophotometric assays using DPPH are routinely used for this purpose [9]. Although this method has its advantages, it also has a main drawback which is spectral interference. The solvent used and the presence of unwanted colored compounds in the sample can result in interference that affects the accuracy of results. However, the EPR method can avoid this interference and ensure that these factors do not influence the outcome of the analysis [10].

The present study aims to evaluate the combined effects of *Cannabis sativa* oil and *Rhodiola rosea*

* To whom all correspondence should be sent:
E-mail: kamelia.parlapanska@trakia-uni.bg

extract and compare the chemical composition of their mixture in terms of polyphenol composition and antioxidant activity. The total plyphenol content was determined using the Folin-Ciocalteu method, while individual phenolic compounds were determined using HPLC-DAD. The antioxidant activity was evaluated through DPPH, PBN, and TEMPOL assays using an Electron paramagnetic radical spectrometer (EPR-X-band).

EXPERIMENTAL

Plant material and extraction procedure

The extract of golden root (*Rhodiola rosea* L) was obtained by extracting 1 gram of golden root with 10 mL of 50 vol. % ethanol solution (solid to liquid ratio 1:10). The weight of the samples was measured by Sartorius analytic balance (precision 0.1 mg). The extraction process was performed once in a stirred round-bottom two-neck flask, thermostated in a silicon oil bath (operational temperature was controlled *via* immersed thermometer and a reflux condenser was mounted to avoid evaporation of the solvent, at stirring frequency of 800 min^{-1}). The flask was centered and immersed to the level of the solvent without contact with the bottom of the bath. For separating the solid from the liquid after the extraction Büchner funnel vacuum filtration was used.

Cannabis sativa oil was purchased from the pharmacy - DragonflyCBD™. *Cannabis* oil has a low THC percentage (0.3 %), so it is non-psychoactive. *Cannabis sativa* oil is usually obtained by steam distillation. Still, the only effective method to extract all valuable substances is supercritical extraction, using not only CO_2 but propane or butane, dimethyl ether, ethanol, or mixtures of these extractants. [11].

Sample preparation

Rhodiola rosea L extract (50 % EtOH) was mixed with *Cannabis sativa* oil in a 1:1 ratio.

Analysis of the extracts

TPC - total phenol content. The total phenol content (TPC) of the extracts was determined by the Folin - Ciocalteu method described by Singleton *et al*. with some modifications [12] using gallic acid as a standard for deriving the calibration line. Double - beam UV-VIS spectrophotometer (T 60, PG Instruments Ltd., Great Britain) was used to analyze the samples. Light absorption was measured at 765 nm. To avoid accidental errors, each analysis was repeated at least three times. The results are presented as g gallic acid equivalent (GAE) per mL. The results of the analyses performed in the present study are presented as mean ± RSD (n=2).

HPLC- DAD analysis

The method of high-performance liquid chromatography with a diode detector (HPLC-DAD) was used to determine 8 components: epicatechin gallate, catechin, rutin, quercetin, myricetin, kaempferol, gallic acid [13]. Analyses were performed with an Agilent 1100 HPLC system (Agilent 1100 HPLC, Agilent Technologies, CA, USA) equipped with a DAD detector (G1315B, Agilent Technologies, CA, USA) and operated by an HP Chemstation. The column used was Purospher star, Hiber RT 125 - 4; RP18 (Purospher star, Merck, Germany). The column temperature was 25 °C. Separation was performed using a linear gradient of 0.1 % TCA (A) and 100 % acetonitrile (B). The gradient started with 5 % B, 15 % B at 16.5 min, 33 % B at 22.5 min, 100 % B at 30.5 min, 5 % B at 35 min until the 40^{th} to re-equilibrate. The flow rate was 1.6 mL/min. The injection volume for samples and standards was 30 µL. DAD acquisition was set in the 200 - 400 nm range. Identification of the main compounds was performed by comparing the retention times and UV spectra of the peaks obtained in both the sample and standard chromatograms.

EPR spectroscopy

DPPH radical scavenging activity. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging capacity of the tested extracts was determined by the method of Bernardo Dos Santos *et al*. [14], modified by Zheleva *et al*. [15]. To measure DPPH scavenging capacity, 98 % ethanolic DPPH solution (80 mM, stock) and test natural products (5 mM) were mixed and homogenized. After 1 minute of incubation in the dark, 0.20 µL of the mixture were transferred to non-heparinized capillary tubes (Micro- 221), which were placed as sample tubes in standard EPR quartz tubes. Generation of the system (DPPH- H/R) was initiated after 1 minute and the DPPH solution was used as an internal standard. DPPH radical scavenging ability was calculated relative to the equation:

$$\text{Scavenged DPPH (\%)} = [(I_O - I)/I_O] \times 100\%$$

where: Io - the double-integrated intensity of the DPPH signal for the blank; I - the double-integrated DPPH signal intensity of the test sample.

In vitro indirect EPR study of alpha-phenyl-N-tetrabutylnitrone (PBN)

To measure the *in vitro* inhibitory ability, a PBN solution (5 mM, stock) dissolved in DMSO and 5 mM of the tested natural product was mixed and homogenized. After 30 minutes of incubation in the dark, 0.20 µL of the mixture were transferred to non heparinized capillary tubes. The PBN-generated adduct was a sextet. The amount of spin adducts formed between the PBN and the lipid radicals present was calculated after integrating the area under the EPR spectra recorded in the control sample and the samples containing the test product. The double - integrated PBN signal intensity of the test sample was calculated by:

$$\text{Scavenged PBN (\%)} = [(I_O - I)/I_O] \times 100\%$$

Generation of the system was initiated after 30 minutes and PBN/DMSO solution was used as an internal standard at field center 3513 G, microwave power 2.05 mW, modulation amplitude 10.00 G, field width 200.00 G, 1 number of scans performed. The PBN inhibitory capacity of the test samples was calculated according to the given equation, where I_O is the double integrated intensity of the PBN/DMSO signal for the blank.

In vitro EPR estimation of TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) radical levels

Nitroxide radical solution with a starting concentration of 2 mM was added to the studied samples in a ratio of 1:5 and stirred on a vortex for 5 s at room temperature. An aliquot of the sample was taken in a microcapillary (volume 10 µL) and placed in the EPR cavity, after which the measurement started. For each measurement, a new amount of the reaction mixture (nitroxide/ sample) was taken, and the analysis was performed at different incubation times (1, 10, 30, 60, 90, and 120 min). The EPR data were calculated as a percentage of the control, 0.2 mM TEMPOL/DMSO [16].

Statistical analysis

EPR spectral processing was performed using Bruker Win-EPR and Simfonia software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one - way ANOVA, and Student - t - test to determine significant differences between data sets. Results were expressed as mean ± standard error (SE). A value of $p < 0.05$ was considered statistically. Kinetic data were expressed as the average of two independent measurements, which were processed using the computer programs Origin 6.1 and Microsoft Excel 2010.

RESULTS AND DISCUSSION

TPC - total phenol content

The results obtained for TPC are presented in Fig. 1. The analysis of the presented results shows that TPC (43.2 ± 1.38 mg GAE/mL) in the combined extract increased by about 30 %.

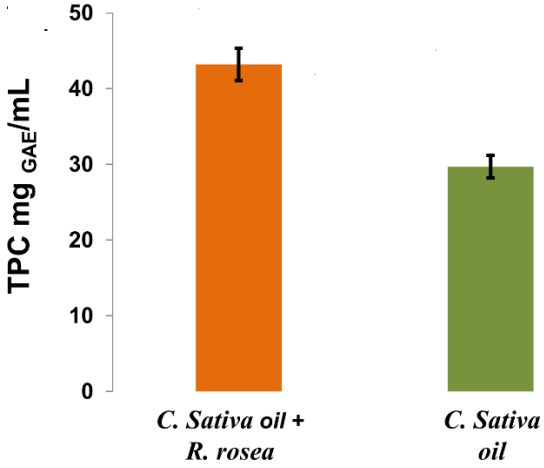

Fig. 1. Total phenol content (TPC) of combined extract (*C. sativa* and *R. rosea*) and *C. sativa* oil.

The *C. Sativa* and *R. rosea* combined extract contained a significantly higher amount of TPC than the extract of *C. Sativa* oil ($p < 0.05$). This finding highlights the potential benefits of using a combination of these two extracts in various applications. Although there is limited literature data on total phenolic compounds in *R. rosea* extracts, the results indicated that further investigation of the use of *R. rosea* in combination with other natural extracts is needed [17].

HPLC- DAD analysis

HPLC-DAD analysis revealed that the combined extract of the two plants had a significantly increased quercetin content (Table 1, Fig. 2). The concentration (µg/mL) is presented as mean ±RSD (n = 2) of each identified compound. This information is valuable in understanding the potential health benefits of these plants and their extracts. Quercetin has been shown to exhibit synergistic effects with cisplatin in human cancer cell lines. The significant antioxidant properties of *C. sativa* seed oil generally depended on the phenolic compounds present in the sample, which is in agreement with the data reported by other researchers [18]. The presence of a phenolic pattern and alkyl and hydroxyl groups on the phenolic ring (as seen in apigenin, quercetin, luteolin, catechin, epicatechin, and canflavin A and B) enhances the antioxidant capacity of the oils [19]. However, there is a possibility that CBD increases the antioxidant activity of terpenes and/or polyphenols through a

synergistic effect, as an increase in antioxidant activity in *C. sativa* extracts has been reported when cannabinoids are accompanied by a large amount of polyphenols [20]. Quercetin was found to be present in the hydroalcoholic extract of the four cultivars [21, 22]. It is interesting to note that samples with different concentrations of the main cannabinoid CBD exhibited similar radical scavenging activity. Therefore, it can be concluded that the antioxidant activity is a consequence of the presence of many different compounds, i.e. flavonoids and phenolic compounds. Thus, the authors explain that the antioxidant capacity may be the result of a synergistic effect between CBD and the phenolic types of metabolites that are present in these cannabis oils [23].

Table 1. Polyphenol content of *C. sativa* and *R. rosea* combined extract and *C. sativa* oil.

	Analyte	Concentration, µg/mL
Cannabis sativa	rutin	19.95
	quercetin	4.12
Cannabis sativa + *Rhodiola rosea*	rutin	19.82
	quercetin	7.21

From the results presented in Table 1 it is clear that two flavonols - rutin and quercetin - were identified using the validated liquid chromatography method. A higher content of quercetin (7.21 ± 0.13 µg/mL) was found in the combined extract compared to that in *C. sativa* oil (4.12 ± 0.17 µg/mL).

Investigation of the formation of stable radical structures in natural antioxidants by direct EPR spectroscopy

The radical scavenging activity of medicinal extracts is linked to their capacity to create stable radical structures. Direct EPR spectroscopy, in the extract of *C. sativa* and *R. rosea*, registered a stable EPR singlet signal (Fig. 3). The values of the measured g factors were 2.0041 ± 0.0002 for the combined extract and 2.0042 ± 0.0002 for the extract obtained from *C. sativa* oil alone, respectively. The measured peak-to-peak distance was of the order of 9 G.

The extraction of these two plants together did not destroy the stable radical structure. Based on the data obtained, in the identification of the recorded stable free-radical form, we hypothesized that the stable structure is due to the high content of quercetin after our HPLC analysis [18].

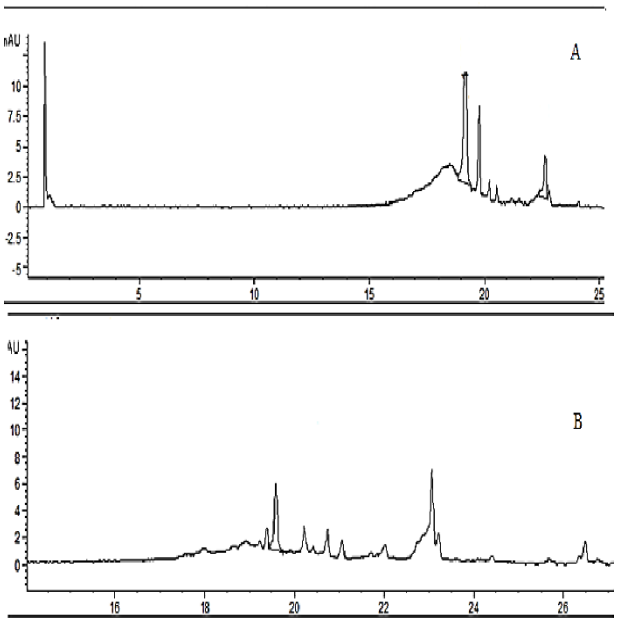

Fig. 2. HPLC chromatogram of the *Cannabis sativa* oil (A) and *C. sativa* and *R. rosea* combined extract (B)-chromatogram at 368 nm.

DPPH radical scavenging activity

The antioxidant properties were evaluated by determining the DPPH radical scavenging capacity in the extract of *R. rosea* and *C. sativa*. The results are presented in Fig. 4.

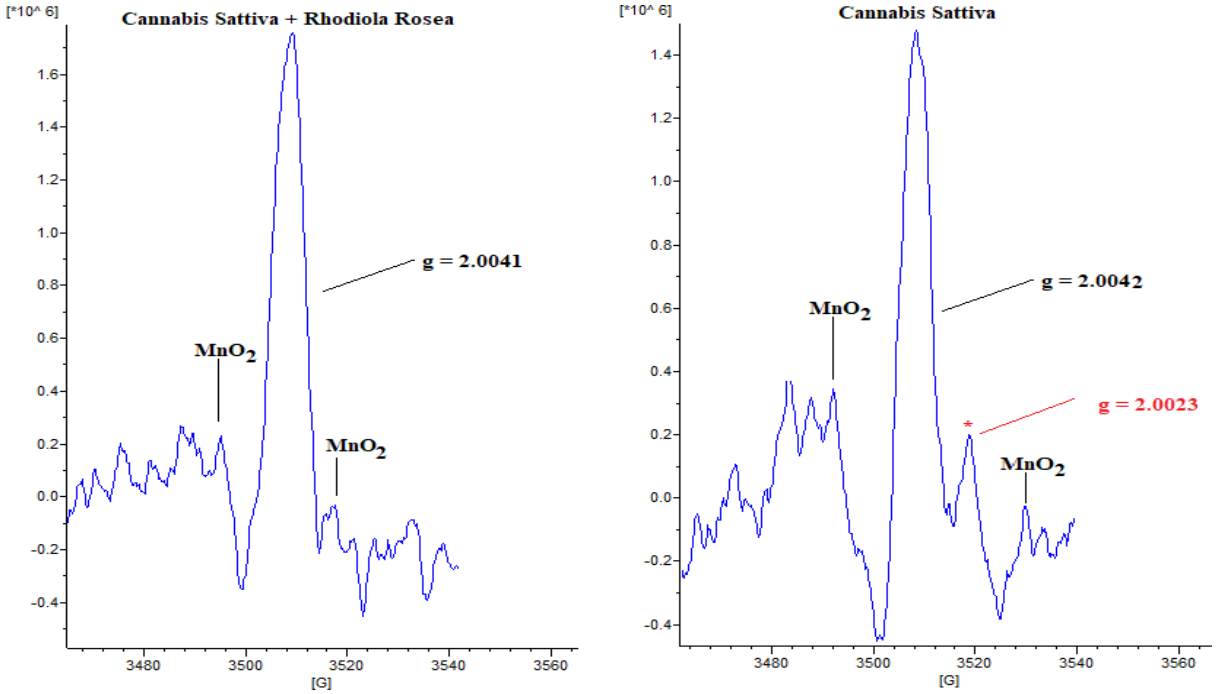

Fig. 3. Direct EPR spectra of the extract of *C. sativa* and *R. rosea* and spectra obtained from *C. sativa* oil. Values are the arithmetic mean of 3 consecutive measurements.

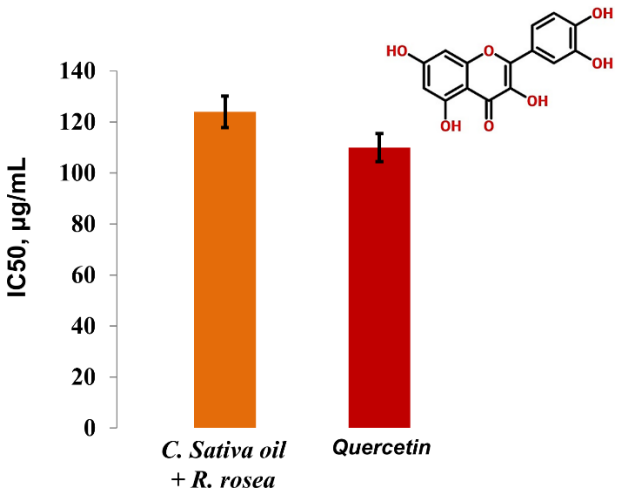

Fig. 4. DPPH radical scavenging capacity of *C. sativa* and *R. Rosea* combined extract. Pure quercetin was used as control

Based on the presented IC_{50} values in Fig. 4. (IC_{50} = 120.17 ± 1.87 µg/mL), it was discovered that *C. sativa* and *R. rosea* combined extract exhibited high antioxidant activity comparable to pure quercetin. The results of the study found 87.3 % scavenged ($p < 0.002$) DPPH radicals at the concentration of the extract (2 %), compared to the control sample.

In vitro indirect EPR study of alpha-phenyl-N-tetrabutylnitrone (PBN)

Experiments involving the peroxyl (or hydroperoxyl) radical are of particular relevance to the antioxidant capacity as they predominate in lipid oxidation in biological systems [19].

The results of the *in vitro* study of superoxide anion radicals reveal that the obtained extract inhibits 94.96 % of the generation of superoxide radicals ($O2^{\bullet-}$) within the first minute. Several studies have shown that both NO and $O2^{\bullet-}$ are involved in multiple pathophysiological processes and therefore it is important to establish the activity of the product against these radicals to be used for therapeutic purposes [21].

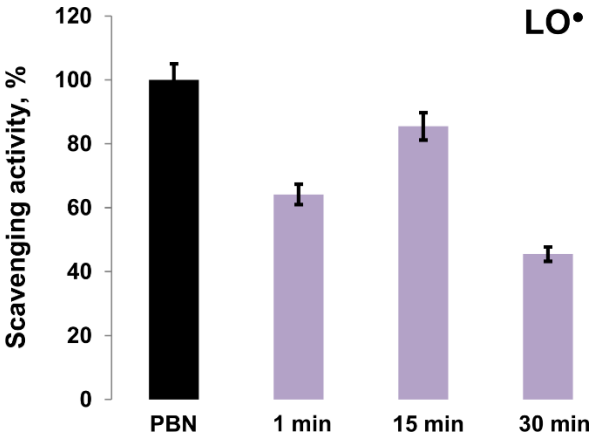

Fig. 5. Inhibition of peroxyl radical of the PBN-1- hydroxyethyl radical adducts.

Cytotoxic aldehydes produced as a result of lipid peroxidation can block macrophage action, inhibit protein synthesis, inactivate enzymes, cross-link proteins and lead to thrombin generation [24].

Therefore, lipid peroxidation plays a crucial role in inflammation, cancer and heart disease. From the obtained results, it can be concluded that the combined extract of *Cannabis sativa* oil and *Rhodiola rosea* inhibit lipid radicals ($p < 0.003$). Maximal inhibition is at 15 min, probably due to the interaction of phenolic compounds and PBN.

The antioxidant compounds in *C. sativa* and *R. rosea* combination scavenge the biggest radical amounts, 15 minutes after the start of experiment. Importantly, after this time, the antioxidant activity sharply decreases.

Importantly, after this time, the antioxidant activity sharply decreases. Quercetin is one of the most effective inhibitors of lipid peroxidation [25], and the increased amount of this flavonoid (found by HPLC-DAD analysis, Table 1) is due to lipid radical inhibition.

In vitro EPR estimation of TEMPOL radical levels

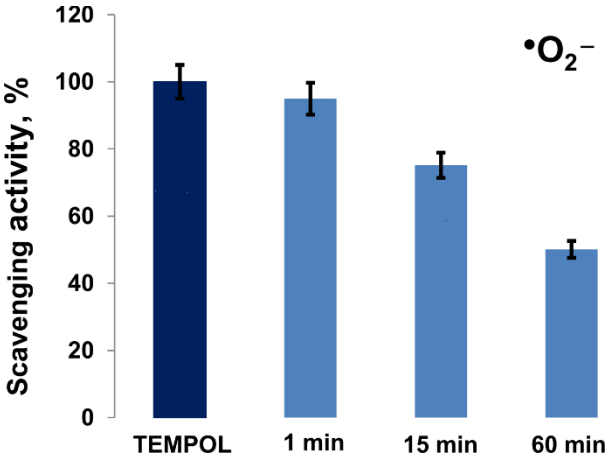

Fig. 6. Monitoring of ($\bullet O_2^-$) produced by catalase - mimic reaction.

CONCLUSIONS

The present study outlines the activity of the combined extract of *Cannabis sativa* seed oil and *Rhodiola rosea* extract in *in vitro* tests. As a byproduct of the electron transfer process, reactive oxygen species have shown their ubiquity in the cell in the form of various groups of oxygen radicals, including singlet oxygen ($^1O_2$); superoxide anion radical ($O_2\bullet^-$); hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), and their overproduction leads to the occurrence of oxidative stress. That is why it is important to determine the possibility of plant products - oils and extracts to inhibit these radicals. The phenolic composition of the secondary metabolites of *Cannabis sativa* and *Rhodiola rosea* shows a high antioxidant potential. EPR experiments - lipid peroxidation, superoxide anion ($\bullet O_2^-$) inhibition, hydroxyl radical -(•OH) and DPPH radical scavenging were designed to show specific reactivity with different radicals, providing information on antioxidant potential. As EPR studies are taken as preliminary *in vivo*, our preliminary study demonstrates the best biomedical prospects for obtaining the extract as a future drug or source of new functional products - an important protection against various diseases related to oxidative stress.

Acknowledgement: *This study was funded by scientific project №14/2023 of the Medical Faculty, Trakia University, Stara Zagora, Bulgaria, and the National Research Program "Young scientists and postdoctoral students-2" of the Ministry of Education and Science, Bulgaria and the Ministry of Education and Science BG-RRP-2.004-0006" Development of research and innovation at Trakia University in service of health and sustainable well-being".*

Conflict of interest*:* The authors declare that they have no conflict of interest.

REFERENCES

1. R. Pezzani, B. Salehi, S. Vitalini, M. Iriti, F. A. Zuñiga, J. Sharifi-Rad, N. Martins, *Medicina*, **55**, 110 (2019).
2. M. Malík, P. Tlustoš, *Plants*, **12**, 1364 (2023).
3. M. N. Khanam, M. Anis, S. B. Javed, J. Mottaghipisheh, D. Csupor, *Agronomy*, **12**, 1178, (2022).
4. D. N. Olennikov, N. K. Chirikova, A. G. Vasilieva, I. A. Fedorov, *Antioxidants*, **9**, 526 (2020).
5. J. Moreira, M. Machado, M. Dias-Teixeira, R. Ferraz, C. Delerue-Matos, C. Grosso, *Acta Pharm. Sin. B*., (2023).
6. Y. Liu, H. Y. Liu, S. H. Li, W. Ma, D. Wu, T. Li, R. Y. Gan, TrAC, *Trends Anal. Chem*., **149**, 116554 (2022).
7. G. R. Gandhi, A. B. S. Vasconcelos, D. T. Wu, H. B. Li, P. J. Antony, H. Li, F. Geng, R. Q. Gurgel, N. Narain, R.Y. Gan, *Nutrients*, **12**, (2020).
8. S. Sofrás, M. F. Desimone, *Pharm. Des*., **29**, 394, (2023).
9. W. Brand-Williams, Wendy, M. E. Cuvelier, C. Berset, *LWT*, **28**, 25, (1995).
10. A. J. Fielding, *J. Nat. Prod.*, **1**, 16 (2022).
11. A. Dobreva, *Phytologia Balcanica*, **28**, (2022).
12. J. M. Peñarrieta, J. A. Alvarado, B. Bergenståhl, B. Åkesson, *Rev. Boliv. Quím*., **24**, 5 (2007).
13. V. Dimcheva, N. Kaloyanov, M. Karsheva, *Open Journal of Anal. Bioanal. Chem*., **3**, 39 (2019).
14. D. H. S. Bernardo dos Santos, V. Silva, S. Bolzani, *J. Brazilian Chem. Soc*., **20**, 1483 (2009).
15. A. Zheleva, R. Kumar, R. Sharma, Y. Karamalakova, V. Gadjeva, *Biotechnol. Biotechnol. Equip*., **39**, (2009).
16. E. Georgieva, V. Atanasov, R. Kostandieva, V. Tsoneva, M. Mitev, G. Arabadzhiev, Y. Yovchev, Y.

Karamalakova, G. Nikolova, *Int. J. Mol. Sci*., **24**, 5807 (2023).
17. M. Polumackanycz, P. Konieczynski, I. E. Orhan, N. Abaci, A. Viapiana, *Antioxidants*, **11**, 919 (2022).
18. A. Smeriglio, *Phytotherapy Research*, **30**, 1298 (2016).
19. A. Mohanan, *Food Chem*., **266**, 524 (2018).
20. A. Nuutila, *Food Chemistry*, **4**, 485 (2003).
21. A. Hacke, *Analyst*, **144**, 4952 (2019).
22. V. di Giacomo, L. Recinella, *Antioxidants,* **10**, 44 (2021).
23. C. Cantele, Bertolino, *Antioxidants*, **9**, 1131 (2020).
24. B. Halliwell, *Methods in Enzymology*, **186**, 1 (1990).
25. W. Oh, *Food Chemistry*, **364**, 130394 (2021).