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ANTIOXIDANT CAPACITY OF EXTRACTS FROM BULGARIAN MEDICINAL PLANTS

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ABSTRACT

The aim of this research is to establish the redox–modulating capacity of extracts from 5 traditional Bulgarian medicinal plants: Geranium sanguineum, Artemisia annua, Tribulus terrestris, Cichorium intybus and Cotinus coggygria, comparing their antioxidant capacity at the same concentrations subsequently adding them to the composition of a sperm extender for rams. The methods used include ABTS•+ free radical reduction ability, DPPH• radical reduction, ferric iron reduction by the FRAP method, as well as reduction of cupric ions (Cu2+) to cupric ions (Cu1+) by the CUPRAC method. As a result, all tested extracts had a high potential for neutralizing free radicals. In conclusion, we can hypothesize that their use also as protective agents in ram sperm diluents will protect gametes from oxidative stress that lowers sperm quality and fertilizing potential.

Key words: : DPPH, ABTS•+, FRAP, CUPRAC, antioxidant.

Introduction

Obtaining ejaculate by artificial vagina, dilution it with sperm extenders, *in vitro* storage of ejaculate at different temperature regimes releases high levels of free radicals, respectively induces high levels of oxidative stress. Depending on activity of the free radicals and their quantity, they can have an extremely adverse effect on the cell (Andreeva *et al.*, 2021). Plasma membrane of ram sperm contains a high percentage of unsaturated fatty acids and a relatively low percentage of cholesterol (Holt, 2000). Examining the level of by–products of sperm membrane lipid oxidation is one of the most widely used methods to assess oxidative stress on gametes (Mojica–Villegas *et al.*, 2014). Numerous studies have shown that the addition of antioxidants to sperm diluents can neutralize free radicals and improve sperm function (Zhang *et al.*, 2012; Agarwal *et al.*, 2014; Gerzilov *et al.*, 2022; Gerzilov *et al.*, 2023). The use of completely synthetic drugs, antibiotics and antimycotics, artificial immunostimulants and chemical growth supplements remains further and further behind (Namkung *et al* . 2004; Oetting *et al.* 2006). The addition of extracts from several Bulgarian herbs will support and even strengthen the action of antioxidants – both enzymatic and non–enzymatic.

The aim of the study is to examine and compare the antioxidant capacity of 5 dry extracts of Bulgarian herbs, dissolved in destilated water at the same initial concentration. According to the results, their antioxidant capacity as protectors of the sperm membrane can be assessed.

Materials and methods

1. Design of the experiment

We examined 5 herbal extracts provided by the company Vemo 99, with a certificate for each of them, describing the extraction method and the exact quantitative and qualitative composition of plant metabolites. All herbal extracts are dissolved in distilled water in the same concentration – 1mg/1 ml. For each tested herbal extract, 6 replicates were made. The extracts studied are:

Tribulus terrestris L. (Granny's teeth) – an annual plant of the Zigophyllaceae family. The presence of steroid saponins of the furostanol and spirostanol type and sapogenins has been proven. It also contains flavonol glycosides astragalin, as well as tanning substances and fatty oils.

Artemisia annua (Wild Wormwood/Sweet Wormwood) – all its active ingredients have antioxidant and antiviral properties. Artemisinin is the major component and is found in the glandular trichomes of leaves, stems and inflorescences. It is mainly concentrated in the upper parts of the plant, where the new growth also appears. Artemisia annua also contains micro– and macroelements such as lead, cadmium and selenium.

Cotinus coggygria Scop. is a tall shrub of the Anacardiaceae family. Contains tannic compounds, determined as tannin, flavonoids, polyphenols determined as catechin, gallotannins, organic acids. Contains also micro– and macroelements – selenium, lead, cadmium.

Cichorium intybus L. (Chicory) belongs to the Asteraceae family and is common in the lowlands of the whole country. The plant is rich in polyphenols, organic acids, mineral acids, inulin and intibin. Coumarins and flavonoids were isolated from the aerial part of the plant, and lactones from the leaves and stems.

Geranium sanguineum (Blood Geranium) is a perennial herbaceous plant of the Geraniaceae family. The composition of the plant includes some flavonoids, catechin tannins, polyphenolic acids, condensed tannins, lactones, anthocyanidis.

2. Methods used to determine the antioxidant activity of plant extracts:

Reduction of DPPH• radical

DPPH is the powdered organic chemical compound 2,2–diphenyl–1–picrylhydrazyl. It is a collection of stable free radical molecules. It is used for antioxidant analysis as well as a standard for the position and intensity of paramagnetic resonance signals. DPPH is a "trap" for other radicals. The decrease in the rate of the chemical reaction upon the addition of DPPH is used as an indicator of the radical nature of the substance under investigation. The reaction is colorimetric and the color change of the reagent is monitored. Measurement of DPPH radical scavenging activity was performed according to the method of Brand–Williams (1995). Reduction of DPPH• to DPPHH results in color changes (from deep violet to light yellow) that are read at 517 nm after 30 min of incubation at room temperature. The percent scavenging activity (AA%) is detected as follows: AA% = 100 − [(Abs (sample) − Abs (blank) × 100)/Abs (control).

ABTS•+ free radical reduction

The chemical compound ABTS is used to measure the kinetics of specific enzymes. It can be used to indirectly monitor the kinetics of an enzymatic reaction or to quantify the level of hydrogen peroxide. The method is widely used in the food industry. By adding sodium persulfate, ABTS is converted to its radical cation, which is blue in color and absorbs light. The reaction is colorimetric and from the dark blue ABTS radical cation solution, the solution is decolorized. The reaction can be monitored spectrophotometrically. According to the method of Re *et al.* 1999, Trolox is used as a standard and the results are expressed as TEAC % (Trolox equivalent antioxidant capacity vit. E).

Radical reduction of ferric iron by the FRAP method;

Ferric reduction antioxidant power (FRAP) assay, by the method of Benzie and Strain, 1996 with some modifications. Absorbance was measured at a wavelength of 593 nm. FeSO4 is used as a standard and the result is presented as μM (micromolar) FeSO4 equivalent/1g extract. The essence of the reaction is the transition of the iron cation from the third valence to the second valence, and during the reaction, the release of an electron from the reagent and the capture of a free radical leads to a change in the saturation intensity of the solution, it is even possible to discolor it. This method is simple, the result is read colorimetrically and gives sufficient clarity about the antioxidant activity of the investigated substance.

Reduction of cupric ions (Cu^{2+}) to cupric ions (Cu^{1+}) by the CUPRAC method

Test for reducing antioxidant capacity CUPRAC (CUPric Reducing Antioxidant Capacity) according to the method of Apak *et al.* (2004) with some modifications. The method is based on the reaction of a Cu(II)–neocuproine complex (CUPRAC reagent, Cu(II)–Nc) with an antioxidant, resulting in a yellow–orange product, a Cu(I)–neocuproine chelate complex and an absorption maximum at 450 nm. For the standard curve, Trolox was used at different concentrations ranging from 0.1 mM to 1.0 mM and the results were expressed as CUPRAC [μM Trolox)/g extract].

Results

All herbal extracts were dissolved in destilated water in concentration 1mg/1 ml and tested by the four methods with the same starting concentration.

When determining the activity by reduction of DPPH• radical method, close to 50% inhibition with the studied concentration was found in Cichorium intybus L. (56.87±0.73%), Geranium sanguineum (42.56±1.07%) and Artemisia annua (58.85±0.75). (Figure 1).

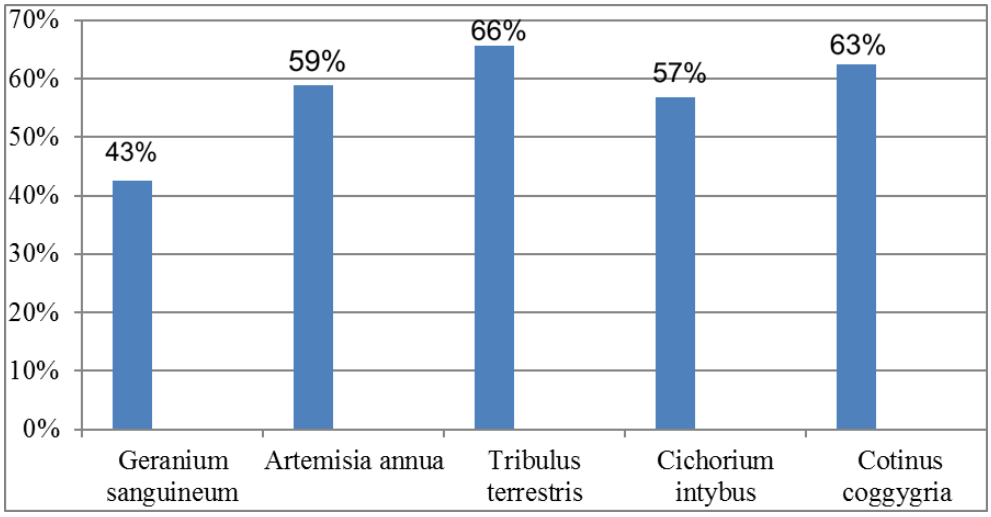

Figure 1: DPPH–capture activity

The antioxidant activity of the five herbs according to ABTS•+ free radical reduction method. The results are expressed relative to TEAC % (Trolox equivalent antioxidant capacity vit. E). In first place is *Cotinus coggygria* with 6.845%, in second place *Geranium sanguineum* with activity

3.108%, in third place *Cichorium intybus* with activity 0.975 %, in fourth place is *Artemisia annua* – 0.667%, last place is taken by *Tribuls terrestris* – 0.549 % (Figure 2).

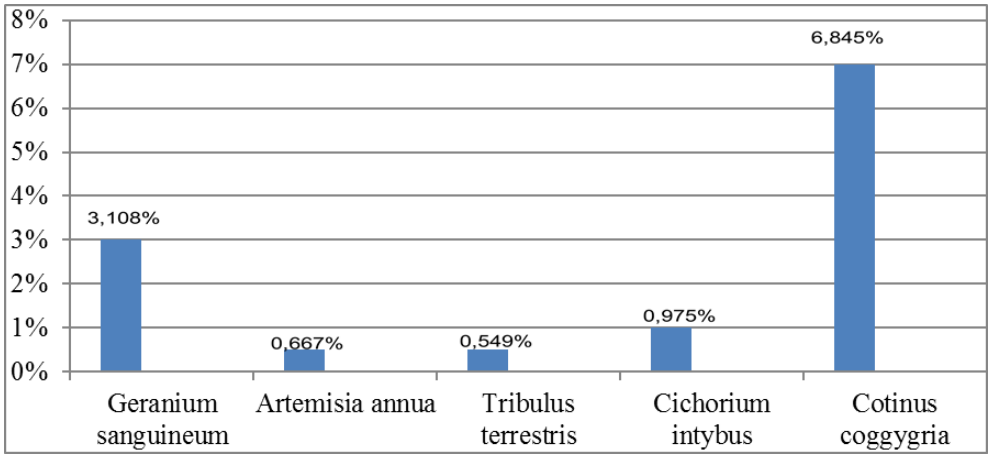

Figure 2: ABTS•+ free radical reduction method; TEAC% (Trolox equivalent antioxidant capacity)

The antioxidant activity of the five herbs according to radical reduction of ferric iron by the FRAP method. The results are expressed relative to µM $FeSO_4$ equivalent/1g extract. In first place is *Cotinus coggygria* with 51.58 ± 0.0057 µM $FeSO_4$, in second place *Geranium sanguineum* with activity 44.96 ± 0.037 µM $FeSO_4$, in third place *Tribulus terrestris* with activity 37.08 ± 0.015 µM $FeSO_4$, in fourth place is *Cichorium intybus* – 34.37 ± 0.028 µM $FeSO_4$, last place is taken by *Artemisia annus* – 31.78 ± 0.018 µM $FeSO_4$ (Figure 3).

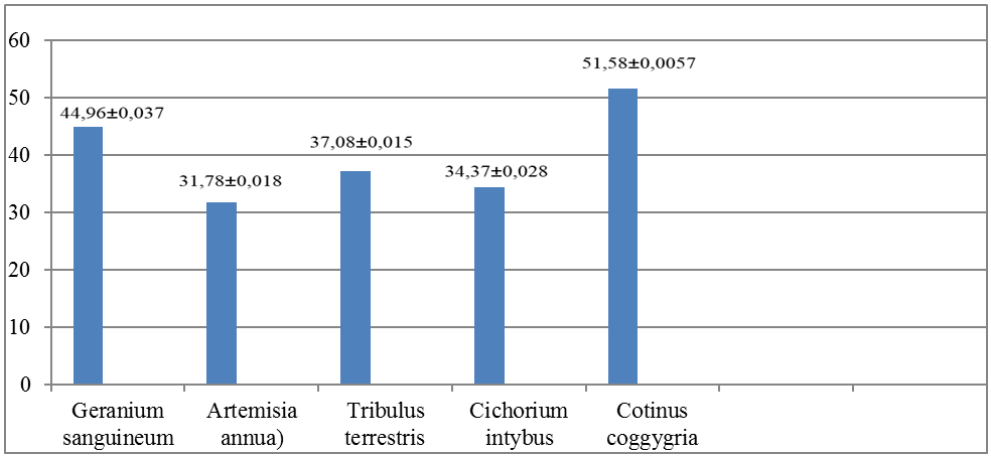

Figure 3: Radical reduction of ferric iron by the FRAP method

The antioxidant activity of the five herbs by reduction of cupric ions (Cu^{2+}) to cupric ions (Cu^{1+}) by the CUPRAC method. The results are expressed relative to TEAC % (Trolox equivalent antioxidant capacity vit. E). In first place is *Cotinus coggygria* with 1193,565 ± 0,124 µM Trolox equivalent/1mg extract, in second place *Geranium sanguineum* with activity 1088,83±0,317 µM Trolox equivalent/1mg extract, in third place *Cichorium intybus* with activity 527,217±0,111 µM

Trolox equivalent/1g extract, in fourth place is *Tribulus terrestris* 425,217±0,076 μM Trolox equivalent/1g extract, last place is taken by *Artemisia annua* 361,87±0,026 μM Trolox equivalent/1g extract 9 (Figure 4).

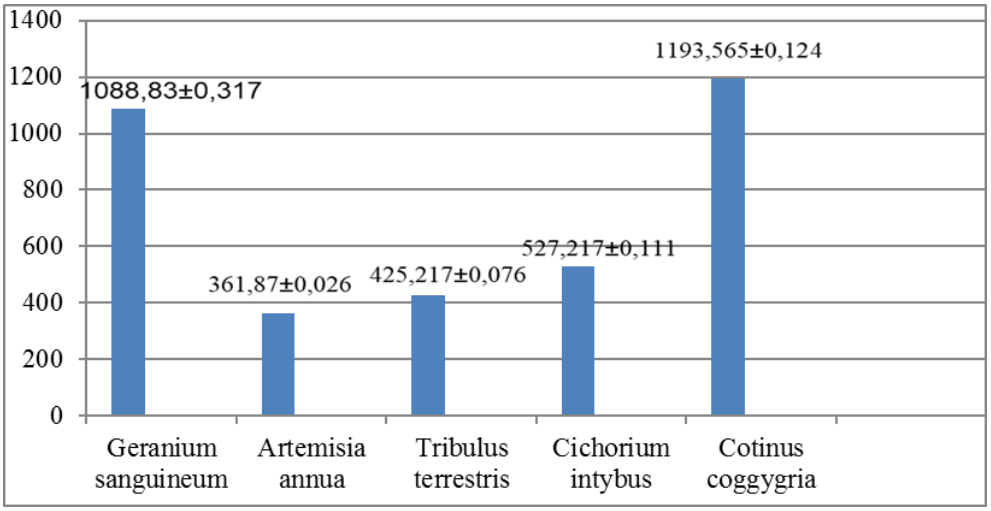

Figure 4: CUPRAC method, μM Trolox equivalent/1mg extract

Discussion

There is no common method in the literature that can be used to assess antioxidant capacity (Chochkova *et al.*, 2022).Each method ranks the plant differently, but the trend for highest activity in Cotinus coggygria remains. The values of the obtained results give us reason to categorize herbs as plant species with high antioxidant activity and the ability to protect the lipid membrane of spermatozoa from oxidative stress. Free radicals are particles with one or more unpaired electrons that are capable of independent existence (Halliwell and Gutteridge, 2007). The methodology of obtaining ejaculate through an artificial vagina, dilution, storage of sperm at different temperature regimes physical and chemical stress on the sperm membrane which successively peduces the viability of sperm and the ability to fertilization because of the oxidative stress. (Thuwanuta *et al*, 2011, Andreeva and Stefanov, 2020; Blagova *et al.*, 2021; Andreeva *et al.*, 2023). In the scientific literature, the term free radicals often overlaps and is used synonymously with the term reactive oxygen species (ROS). Of the great variety of ROS, superoxide anion radical (O2–), hydrogen peroxide (H2O2) and hydroxyl radical (–OH) are of essential importance for the cell (Andreeva, 2020). In this regard, oxidative stress is considered as the main cause of male infertility, which we can limit by the addition of various antioxidants in the storage media. Our results about the antioxidant capacity of *Cotinus coggygria Scop* coincide with those found by other authors (Georgiev *et al.*, 2014, Kashani *et al.*, 2012, Ngule *et al.*, 2013) The extract contains a high percentage of tannins, gallotannins, flavonoids, organic acids. These substances have high osmoticity and good antioxidant capacity. Furostanol–and spirostanol–type steroidal saponins, sapogenins, the furostanol saponins protodioscin and protogracilin in *Tribulus terrestris* extract are highly active antioxidants (Sun *et al.*, 2002). The leaves also contain the flavonol glycosides astragalin, as well as tannins and fatty oils (Wu *et al.*, 1996; Stefanescu *et al.*, 2020). *Tribulus terrestris* is a widely used antioxidant and stimulant as a food supplement. According to our results the plant has also high local antioxidant capacity. The phenolic

compounds of *Artemisia annua* such as coumarins, flavones, flavonoids, phenolic acids and various phytochemicals – all its active ingredients have antioxidant and antiviral properties. Our results are in agreement with those presented by other authors (Kostova et Dinchev, 2005). The composition of the *Geranium sanguineum* includes some flavonoids, catechin tannins, polyphenolic acids, condensed tannins, lactones, etc. (Leucuta, S *et al.* 2005). Our results about the antioxidant capacity of *Geranium sanguineum* are in unison with those found by other authors (Hardman, *et al.*, 2014; Abbas *et al.*, 2017; Leucuta, 2005). The extract contains a high percentage of tannins, gallotannins, flavonoids, organic acids. *Cichorium intybus*, coumarins and flavonoids are the most active, and lactones are also isolated from the leaves and stems (Al–Snafi, A., 2016). We confirm the research of Al–Snafi *et al.*on the quantitative constituents of the leaves of *Cichorium intybus* because the extract is extracted from this part of the plant.

Conclusion

All 5 herbs have high antioxidant capacity. Our research leads us to hypothesize that the use of these herbs extracts as protective agents in sperm extenders will protect sperm membrane from oxidative stress, which lowers sperm quality and fertilizing potential.

Acknowledgements

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