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Antioxidant activity and nontoxicity of extracts from *Valeriana officinalis, Melissa officinalis, Crataegus monogyna, Hypericum perforatum, Serratula coronata* and combinations Antistress 1 and Antistress 2

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In recent years, large number of preclinical and clinical studies support the hypothesis of a link between oxidative stress, anxiety and depression. In search of novel sources of antioxidants in the last years, medicinal plants traditionally used in folk medicine have been extensively studied for their antioxidant activity (AOA). The purpose of this study was to determine the antioxidant activity of the extracts of medicinal plants *Valeriana officinalis, Melissa officinalis, Crataegus monogyna, Hypericum perforatum, Serratula coronata* and their combinations Antistress 1 and Antistress 2, which used as food supplements are recommended for chronic fatigue, anxiety and stress. This was done though measuring the Oxygen Radical Absorbance Capacity (ORAC), Hyrdoxyl Radical Averting Capacity (HORAC) and via electrochemical method (EM). The most pronounced is AOA of the extracts from *M. officinalis* (ORAC, 6751.0±214.3 μmol TE/g, HORAC, 1887.8±51.0 μmol GAE/g and EM, 24.901±1.445 μmol/l.min) and *H. perforatum* (ORAC, 5950.5±328.4 μmol TE/g, HORAC, 2128.3±200.1 μmol GAE/g and EM, 23.605±1.334 μmol/l.min), which could be a result of the high concentration of rosemary acid in the first extract and of flavonoids in the second. They contribute to the greatest extent of the activity of Antistress 1 and Antistress 2. The conducted study for acute toxicity *in vivo* reported 100 percent survival of experimental animals, indicating that both individual and combined extracts are non toxic to the tested animals.

Key words*: herb extracts, toxicity, antioxidant activity, ORAC, HORAC, voltammetry*

INTRODUCTION

Oxidative stress represents a violation of pro- and antioxidant balance of the body, which is a result of either increased formation of reactive oxigen species (ROS) in the cell, which may damage lipids, proteins and DNA; or of a reduced activity of natural antioxidant system. There is plenty of evidence about the involvement of pro-oxidant agents such as peroxide radicals (ROO•), hydroxide radical (HO•), superoxide anion (O_2^-) and singlet oxygen ($^1O_2$•) in the pathophysiology of aging, mutagenesis and many chronic degenerative diseases such as cancer, cardiovascular disease, Alzheimer's disease, Parkinson's disease and others [1]. The definition, which is given to antioxidant is generally "a compound which is opposed to oxidation or inhibits reactions caused by oxygen and peroxides" [2]. In biochemistry and medicine antioxidants are enzymes or nonenzymatic substances that have the ability to dispose of ROS, or prevent their formation [3]. Phenolic compounds are a group of secondary metabolites, which include flavonoids (anthocyanins, flavones, catechins, etc), phenolic acids, stilbenes, tannins and others with antioxidant and chelating properties, and could act as reducing agents, hydrogen donors or singlet oxygen scavengers [4]. Epidemiological studies have shown that long-term intake of foods and herbal medicines rich in plant polyphenols, provides certain protection against neurological, cardiovascular disease, diabetes, osteoporosis, neurodegenerative diseases [5, 6, 7]. In the recent years, the depression is largely considered to be associated with the oxidative stress in the organism [8], which in turn leads to a demand for natural antioxidants to combat it. It is well known that the

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extracts from *Valeriana officinalis*, *Melissa officinalis* and *Crataegus monogyna*, *Hypericum perforatum* have anxiolitic and sedative properties [9], *Serratula coronata* has anabolic and neuroprotective effects [10] and are successfully used in the traditional medicine for centuries. A better effect is sought by preparing combinations of these elements. One of the most famous figures of herbalism in Bulgaria in the last century is Peter Dimkov who has published three volumes of traditional herbal medicine he has collected over decades [11]. The dietary supplements Antistress 1 and Antistress 2 which are the objects of our research are based on one of these recipes. Therefore the purpose of this study is to determine the antioxidant activity of the extracts of medicinal plants *Valeriana officinalis, Melissa officinalis, Crataegus monogyna, Hypericum perforatum, Serratula coronata* and the combinations from them Antistress 1 and Antistress 2, as well as establishing their nontoxicity on experimental animals.

MATERIALS AND METHODS

Plant materials.

Individual herbs (*V. officinalis, M. officinalis, C. monogyna, H. perforatum* and *S. coronata*) were collected in the Rhodope Mountains, Bulgaria in July 2014. Dry extracts were prepared with extraction of individual herbs with 40% ethanol (v:v) according to the industrial technology of "Extractpharma" Ltd. Sofia, Bulgaria. Antistress1 is a combination of *V. officinalis, M. officinalis, C. monogyna* and *S. coronata* in proportion 4:3:3:1, and Antistress2 is compound of *V. officinalis, H. perforatum* and *S. coronata* in proportion 4.5:4.5:1. These combinations are registered as food supplements by the company "Avicena Herb" Ltd. Plovdiv, Bulgaria.

Animals.

In acute toxicity tests 90 male Wistar rats weighing 180-200 grams were used. The animals was housed under standard laboratory conditions: 12:12 dark-light cycle, 45% relative humidity, temperature 26.5 ± 1ºC and free access to food and water. The experiments were approved by the Committee on Animal Ethics of the Bulgarian Agency for Food Safety permit №127 and decision of the ethical committee at MU Plovdiv protocol №3/21.04.2016.

Determination of Total Polyphenols.

The determination is performed by the method of Singleton and Rossi [12]. Determinations are performed on spectrophotometer Spekol 10 (Carl Zeiss, Germany).

Determination of Antioxidant Activity.

Oxygen Radical Absorbance Capacity (ORAC) method - The method developped by Ou et al., was used with some modifications [13]. This method measures the ability of an antioxidant to neutralize peroxid radicals. The method is based on the inhibition of the decline of fluorescence of fluorescein during its oxidation in the presence of an antioxidant. The thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is used as a peroxid radical generator. The results are expressed in μmol Trolox equivalents per gram of extract. Measurements are performed on FLUOstar OPTIMA fluorometer (BMG LABTECH, Offenburg, Germany). The excitation wavelength of 485 nm and emission wavelength of 520 nm were used.

Hydroxyl Radical Averting Capacity (HORAC) method - The method was developed by Ou *et al.* [14], and measures the ability of an antioxidant to form complexes in conditions of Fenton reaction, caused by the interaction between Co (II) and H_2O_2. The results are expressed in μmol gallic acid equivalents per gram of extract. Measurements are performed on FLUOstar OPTIMA fluorometer (BMG LABTECH, Offenburg, Germany). The excitation wavelength of 485 nm and emission wavelength of 520 nm were used.

Electrochemical Method for Determination of Antioxidant Activity. The electrochemical method was used to determine the antioxidant activity [15]. The experiment's methodology consists in taking voltamperogram of cathodic electroreduction of oxygen using the "Analyst AOA" (RU.C.31.113.A N28715), connected to a PC. The antioxidant activity of the tested samples was calculated according to kinetic criterion K (in micromoles per litre·minute) indicating the quantity of the reactive oxygen species in time.

Design of Experiments to Study the Acute Toxicity of Plant Extracts. The experimental animals (90 in total) were divided into 15 groups of 6 animals. The animals were treated orally with the extracts once with a dose of 10g / kg b. w. by using a probe. The survival of the animals 24 hours after administration of the extracts is recorded.

RESULTS AND DISCUSSION

The results of the AOA of the medicinal plants we tested, determined by ORAC method are specified in Table 1 (column 2).

According to this method, the most pronounced is AOA of the extracts from *M. officinalis* and *H. perforatum*, which could be attributed to the high content of rosemary acid (55.6 ± 0.30 mg/g) in the

first extract and flavonoids respectively (62.36 ± 0.71 mg/g) in the second [16]. They contribute to the fullest extent to the activity of Antistress 1 and Antistress 2 given their share in them. The explanation for the low activity of extracts from *V. officinalis* and *S. coronata* is probably the lesser content of polyphenolic compounds on account of their characteristic terpenes and ecdysteroids.

The ORAC method is widely used by a number of teams to analyze the AOA in dry plants [17] or foods and spices [18]. Wojcikowski et al. used the ORAC method to investigate the antioxidant activity of 55 medicinal plants after sequential extraction with three solvent as a result of which they reported higher activity of the herbal extracts [19]. Kratchanova et al. performed a similar study of 25 medicinal plants as compared AOA of water and acetone extract of the dry palnts [20]. They recorded higher activity of acetone extracts of *C. monogyna, M. officinalis* and *H. perforatum* (2163, 1121 and 1141 μmol TE/g) compared to water extracts (364, 996 and 629 μmol TE/g). The activities we recorded for the same plants are much higher, as they are calculated per gram of dry extract.

The flavonoids' ability to form complexes, which allows them to manifest themselves as AO was demonstrated by HORAC method, but in the scientific literature there is scarcely any information on its use. Only in recent years it was established as a criterion for determining the AOA together with ORAC method. Denev *et al.* used it for analysis of AOA of six herbal extracts [21] and Wasek *et al.* used it for twenty-seven food supplements [22]. The teams used both methods to more fully characterize the antioxidant properties of the respective objects while determine the polyphenol content. Like them, our team used HORAC method of determining the AOA of extracts from *V.officinalis, C. Monogyna, S. Coronata, M. Officinalis, H. perforatum* and the combinations Antistress 1 and Antistress 2. The results are presented in table 1 (column 3).

The most pronounced AOA is the one of the extract of *H. Perforatum*, which is largely due to four major flavonoid - rutin, hyperozide, quercetin and apigenin with a total amount of 62.36 ± 0.71 mg/g [16]. The high activity of the Antistress 2 respectively is due to the fact that *H. Perforatum* is 4.5/10 thereof. *M. officinalis* extract also has high activity, though flavonoids were 0.9 mg/g, but dominated phenolic acids (57.1 mg/g), represented primarily by rosemary acid, then by caffeic, ferulic and p-coumaric ones [16].

In the literature there is data about the relationship between AOA and content of polyphenol compounds in herbs. Some authors report good linear relationship between these two parameters [20, 23] while others do not observe such [24]. In our experiments, good correlations were found between the total amount of polyphenols in the extracts (table1, column 4) and their ORAC (r^2 = 0.9042) and HORAC (r^2 = 0.9293) values, represented on Figure 1.

Our research is the first to make comparative assessment of ORAC and HORAC AOA of the abovementioned plants, and combined extracts Antistress 1 and Antistress 2. Determination of the antioxidant activity of each of the extracts gives better idea about their ability to act independently as antioxidants, as well as their contribution to the activity of the combined extracts.

Table 1. ORAC and HORAC antioxidant activity and polyphenol content of extracts from *V.officinalis, C. Monogyna, S. Coronata, M. Officinalis, H. perforatum* and their combinations Antistress 1 and Antistress 2

Extracts	ORAC, μmol TE/g	HORAC, μmol GAE/g	Polyphenols, mg/g
M. officinalis	6751.0±214.3	1887.8±51.0	238.96±4.8
H. perforatum	5950.5±328.4	2128.3±200.1	222.29±6.4
C. monogyna	3917.3±227.8	1052.1±32.5	113.04±1.9
Antistress 2	3774.7±99.3	1132.4±44.8	132.36±2.4
Antistress 1	3746.2±180.1	861.7±25.2	128.57±2.4
S. coronata	1142.7±25.5	575.2±18.8	95.24±0.3
V. officinalis	820.5±21.9	381.6±14.0	43.36±1.3

Results are presented as mean ± S.D.

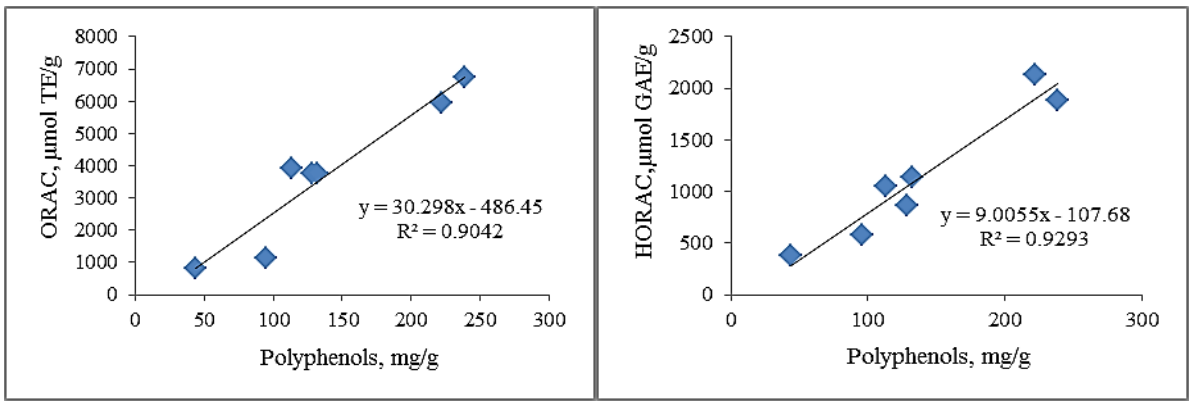


Figure 1. Correlation between the amount of polyphenols and AOA of the surveyed extracts determined by ORAC and HORAC methods

Table 2. Antioxidant activity of the extract from *V.officinalis, C. Monogyna, S. Coronata, M. Officinalis, H. perforatum* and the combinations Antistress 1 and Antistress 2, as measured by electrochemical method

Extracts	K, µmol/l.min±SD	AOA
Antistress 2	28.795±1.125	3.551
Antistress 1	27.136±1.874	3.346
M. officinalis	24.901±1.445	3.071
H. perforatum	23.605±1.334	2.911
V. officinalis	20.751±1.521	2.559
C.monogyna	17.878±0.832	2.204
S. coronata	15.643±0.761	1.929
Trolox	8.109±0.010	1.000

To more complete characterization of the tested extracts' AOA, an electrochemical method was used as well. The kinetic criterion values for each of the samples and the AOA calculated relative to that of Trolox are given in Table 2.

While AOA determined by ORAC and HORAC correlates directly proportional to the content of polyphenols, the activity determined by electrochemical method does not follow this correlation. From the literature it is known that the AO may act by three mechanisms: by attachment of the radical to a conjugated system of double bonds, giving hydrogen and participating in reactions with a transmission electron [25]. The used electrochemical method is applicable to AO which exhibit antioxidant activity by any of the three mechanisms. Therefore AOA of not only phenol type compounds is measured by this method. The combined extracts Antistress 1 and Antistress 2 show the most prominent AOA. This is probably due to the synergistic effect among their components - compounds of phenolic type, terpene derivatives (valerenic acid, bornyl acetate), phytosteroids such as 20-hydroxyecdysone, anthracene derivatives (hypericin) [16].

The extract from *V. officinalis* has the lowest activity based ORAC and HORAC methods but according electrochemical method it ranked fifth. This method determines the overall antioxidant potential of all types compounds in the sample. Yashin *et al.* create a database of AOA for different groups of foods and beverages using the described method [26]. According to the results obtained by Tewari *et al.*, mixed extracts combine in optimal proportion the components of different herbs, which voids the possibility of overdose in their individual administration [27].

Halliwell's longtime studies on free radicals, antioxidants and their role in the body lead to the conclusion that the *in vitro* and *in vivo* experiments in this area should go hand in hand [1]. According to these studies, even if some AO exhibit very high AOA in *in vitro* models it is not certain that *in vivo* they will prove to be prooxidants. The review article by Tirzitis and Bartosz also treat this problem [28]. In line with this, our team conducted also an experiment for determining acute toxicity of the individual extracts and combinations thereof, which is the first of a series of pharmacological tests. 24 hours after oral administration of Antistress 1, Antistress 2 and the extracts included in their composition at a dose of 10 g/kg b.w. we observed 100 percent survival of experimental animals. In the open literature the doses in which the combinations and the extracts included in their composition are used are typically in the range of 100 mg/kg b.w. to 500 mg/kg b.w. [29]. Using such big dose in our experiment gives us reason to believe that the tested products are practically nontoxic.

CONCLUSIONS

High antioxidant activity of the extracts *in vitro* is quantifies by three methods – ORAC (820 - 6751 µmol TE/g), HORAC (381-2128 µmol GAE/g) and electrochemical (15.6 - 28.7 µmol/l.min). Good correlation was observed

between the total amount of polyphenols in plant extracts combinations Antistress 1 and Antistress 2 and their antioxidant activity determined by ORAC and HORAC methods. It was confirmed that by the electrochemical method antioxidant activity of the compounds not only of phenolic type is determined but also of fitosteroids, terpene and anthracene derivatives. The extracts of *Valeriana officinalis*, *Melissa officinalis*, *Crataegus monogyna*, *Hypericum perforatum* and *Serratula coronata* and combinations Antistress 1 and Antistress 2 are nontoxic, which has been shown in *in vivo* testing for acute toxicity. This is a prerequisite for the continuation of experiments to prove the anxiolytic activity of combinations Antistress 1 and Antistress 2.

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АНТИОКСИДАНТНА АКТИВНОСТ И НЕТОКСИЧНОСТ НА ЕКСТРАКТИ ОТ *Valeriana officinalis, Melissa officinalis, Crataegus monogyna, Hypericum perforatum, Serratula coronata* И КОМБИНАЦИИ АНТИСТРЕС 1 И АНТИСТРЕС 2

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(РЕЗЮМЕ)

В последните години големият брой предклинични и клинични изследвания подкрепят хипотезата за връзката между оксидативния стрес, тревожността и депресивните състояния. В търсене на нови източници на антиоксиданти напоследък все повече се изследват лечебните растения, които от векове се използват в народната медицина. Целта на настоящото проучване е да се определи антиоксидантната активност на екстракти от лечебните растения *Valeriana officinalis, Melissa officinalis, Crataegus monogyna, Hypericum perforatum, Serratula coronata* и комбинациите от тях Антистрес 1 и Антистрес 2, които са хранителни добавки, препоръчвани при хронична умора, тревожност и стрес. Това е направено чрез измерване на Oxygen Radical Absorbance Capacity (ORAC), Hyrdoxyl Radical Averting Capacity (HORAC) и по електохимичен метод. Най-изразена е антиоксидантната активност на екстрактите от *M. officinalis* (ORAC, 6751.0±214.3 μmol TE/g, HORAC, 1887.8±51.0 μmol GAE/g и 24.901±1.445 μmol/l.min) и *H. perforatum* (ORAC, 5950.5±328.4 μmol TE/g, HORAC, 2128.3±200.1 μmol GAE/g и 23.605±1.334 μmol/l.min), която вероятно е следствие от високата концентрация на розмаринова киселина в първия екстракт и съответно на флавоноиди във втория. Те допринасят в най-голяма степен за активността на Антистрес 1 и Антистрес 2. Проведено е *in vivo* изследване за остра токсичност като е отчетена 100% преживяемост на опитните животни, което доказва, че самостоятелните и комбинираните екстракти са нетоксични.

***Ключови думи**: растителни екстракти, токсичност, антиоксидантна активност, ORAC, HORAC, волтамперометрия*