

Anxiolytic Effect and Acute Toxicity of Two Novel Combinations of Plant Extracts - Antistress I and Antistress II on Rats

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ABSTRACT

Anxiety is a significant social problem that could deteriorate the quality of life of individuals. It disturbs the normal circadian rhythms, leading to an increase of cardio-vascular diseases and distortions in the functions of the immune system. Antistress I and Antistress II are herbal combinations containing in different proportions dry extracts obtained from *Serratula coronata*, *Hypericum perforatum*, *Valeriana officinalis*, *Crataegus monogyna* and *Melissa officinalis*. The present study evaluates the acute toxicity and the anxiolytic effect of these combinations and the individual extracts which they contain after their oral administration to male Wistar rats. Doses of 5g/kg b.w. and 10g/kg b.w. are used for the evaluation of acute toxicity. Assessment of the anxiolytic effect is carried out at three doses – 100, 250 and 500 mg/kg b.w. by using the test - Elevated plus maze. The results about the acute toxicity show a survival rate of 100% for all extracts at a dose up to 10g/kg b.w. The evaluation of the anxiolytic effect on an acute stress model in rats demonstrates that both combinations Antistress I and Antistress II possess anxiolytic properties which are significant only at the highest dose. The results also give us the reason to conclude that the effect of Antistress II on anxiety is better compared to Antistress I.

Keywords: Elevated plus maze, rats, combinations of plant extracts, *Serratula coronata*, *Hypericum perforatum*, *Valeriana officinalis*, *Crataegus monogyna*, *Melissa officinalis*.

INTRODUCTION

Half of the global pharmaceutical market is occupied by plant-based therapy and drugs¹. Plant products used for therapeutic and prophylactic purposes, have an easy and convenient method of use that provides better compliance of patients in the prevention and treatment of various diseases. Natural products possess unique chemical formulas that are the base for the creation of new drugs. 25% of all medicines originate from plant source and they could be included in new combinations². Combining plant extracts leads to different mechanisms of action and influences various aspects in the pathogenesis of stress, which is risk factor for socially significant diseases such as anxiety³, depression^{4,5}, hypertension³, diabetes⁶, allergic reactions⁷, autoimmune diseases⁸ and infections⁹. The purpose of the present experimental study is to assess the anxiolytic effect of two combined plant extracts Antistress I and Antistress II after the application of acute stress model to rats. The combinations contain in different proportions individual plant extracts obtained from *Serratula coronata*, *Hypericum perforatum*, *Valeriana officinalis*, *Crataegus monogyna* and *Melissa officinalis*.

MATERIALS AND METHODS

"Antistress I" is a combination containing dry extracts from *Valeriana officinalis*, *Melissa officinalis*, *Crataegus*

monogyna and *Serratula coronata* in a ratio of 4: 3: 3: 1. "Antistress II" contains *Valeriana officinalis*, *Hypericum perforatum* and *Serratula coronata* in a ratio of 4,5: 4,5: 1. The chemical identification of the extracts is carried out at the Department of Chemistry and Biochemistry, Faculty of Pharmacy, Medical university of Plovdiv by using High-performance liquid chromatography (HPLC). Each extract is standardized on the base of the following substances:

Valeriana officinalis – valerenic acid and bornyl acetate; *Melissa officinalis* – rosmarinic acid and caffeic acid; *Crataegus monogyna* – rutin, quercetin and hyperoside; *Serratula coronata* – 20-hydroxyecdysone and quercetin, *Hypericum perforatum* – hypericin.

The experiments with laboratory animals are conducted at the Department of Pharmacology and clinical pharmacology, Faculty of Medicine, Medical university of Plovdiv in order to evaluate the acute toxicity and the anxiolytic effect of the two combinations and the individual dry extracts which they contain. 250 male Wistar rats weighting 180-200g are used in these experiments. (160 animals for studying the anxiolytic effect and 90 animals for studying the acute toxicity). The use of animals is authorized by the Bulgarian food safety agency (Permit № 127 from 09.12.2015) and approved by the ethical commission at the Medical university of

Plovdiv (protocol №3 from 21.04.2016). This experiment is part of university project HO-10/2015.

A. Design of the experiment used for evaluation of acute toxicity.

Experimental animals (90 in total) are divided into 15 groups of 6 animals in each group. Table 1 shows the design of the experiment used to evaluate the acute toxicity of combinations Antistress I and II and the individual plant extracts included in these combinations.

The animals are treated with the extracts orally by using a gavage. The survival rate is reported 24 hours after the administration of the extracts.

B. Design of the experiment used for evaluation of anxiolytic effect of combinations and individual plant extracts in different doses after applying an acute stress model to rats.

160 male Wistar rats are divided into 23 groups. For 14 days, the animals from different groups are treated daily by using a gavage at doses of 100, 250 and 500 mg / kg b.w. with two combinations and five individual plant extracts, contained in them. Table 2 shows the design of the experiment used for the evaluation of the anxiolytic effect in model of acute stress of the two combinations - Antistress I and Antistress II and the individual extracts included in them.

On the 15th day of the experiment one hour after the oral treatment, all animals (excluding these without stress – group C₀) are put in refrigerator at 5°C for one hour (model of acute cold stress). The anxiolytic effect is evaluated by using the test – Elevated plus maze. The following indicators are measured: 1. Time spent in opened arms (Totk) (s), 2. Number of entries in opened arms (Notk), 3. Total number of entries in both arms (Ntot), 4. Ratio of opened/total arm entries (Notn). Criteria for anxiolytic effect are: increase of Totk, Notk and Notn. Criterion for sedative effect is decrease of Ntot.

Statistics

Statistical assessment of the results is made with IBM SPSS 17.0. Normality of distribution is determined by using Shapiro-Wilk test. Arithmetic mean and SEM are identified for each indicator. Comparison of the results between groups is done by using the Independent Sample T test for normal distribution or Mann-Whitney U test when the distribution is not normal. P value < 0,05 is considered statistically significant.

RESULTS

In the study of acute toxicity, all animals survive 24 hours after the oral administration of the combinations and the individual extracts at dose up to 10g/kg b.w thus the survival rate is 100%. Moreover the usage of such high doses in this experiment leads us to the conclusion that the tested extracts are practically non-toxic. Referring to the available literature data, the usual doses of the individual extracts range from 100 mg/kg b.w. to 500mg/kg b.w. The following results are observed by studying the anxiolytic effect of the extracts at doses of 100, 250 and 500 mg/kg b.w. using a model of acute stress:

Results of the evaluated combinations and individual extracts at doses of 100, 250 and 500 mg/kg b.w. compared to the results of the control group after applying a model of acute stress Table 3 compares the time spent in the opened arms of Elevated plus maze measured in seconds - Totk (s) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

The comparison between the two control groups shows that in group C₀ there is a significant increase of Totk compared to group C. This proves that the test - Elevated plus maze is appropriate for evaluation of behavioral changes in rats exposed to acute stress model. Except in C₀ there is an increase of Totk in groups AS1₂₅₀, AS1₅₀₀, AS2₁₀₀, AS2₅₀₀, SER₁₀₀, SER₅₀₀, VAL₁₀₀, VAL₅₀₀, CRA₁₀₀, CRA₅₀₀, MEL₂₅₀, MEL₅₀₀ compared to group C. The increase is statistically significant for animals from groups AS1₅₀₀ and AS2₅₀₀. As for the individual extracts a significant growth is observed for groups, SER₅₀₀, VAL₅₀₀, CRA₅₀₀, MEL₂₅₀, MEL₅₀₀. All the other groups demonstrate decrease of this indicator compared to group C.

Table 4 compares the number of entries in the opened arms of Elevated plus maze Notk after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

When compared to the control group – C, Notk increases in all groups, with the exception of HYP₂₅₀ in which the indicator decreases. In groups C₀, AS1₅₀₀, AS2₅₀₀, VAL₅₀₀, CRA₁₀₀, CRA₅₀₀, MEL₂₅₀ and MEL₅₀₀ the increase is statistically significant.

Table 5 compares the total number of entries in both arms of Elevated plus maze (Ntot) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

Ntot is increased significantly for the groups treated with the combinations – AS1₅₀₀ and AS2₅₀₀ compared to the control group – C. The growth is more pronounced in AS2₅₀₀. As for the individual extracts only groups CRA₁₀₀, CRA₅₀₀ and MEL₂₅₀ increase significantly Ntot compared to C. For all other groups the observed increase in this indicator is statistically insignificant and for groups SER₂₅₀, HYP₂₅₀, MEL₁₀₀, CRA₂₅₀ there is a decrease in Ntot compared to C.

Table 6 compares the ratio of opened/total arm entries in Elevated plus maze (Notn) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

The evaluation of the indicator Notn demonstrates significant increase in two of the groups treated with the combinations - AS1₅₀₀ and AS2₅₀₀ compared to group C. As for the individual extracts, it could be observed statistically significant growth of the indicator for groups VAL₅₀₀, CRA₁₀₀, CRA₅₀₀, MEL₂₅₀ and MEL₅₀₀ compared to group C. Moreover, Notn increases significantly for group C₀ compared to C.

Comparison of the results regarding the anxiolytic effect of the two combinations, administered at doses of 100, 250 and 500 mg/kg b.w.

Table 1: Design of the experiment in the study of acute toxicity in male Wistar rats.

Groups	Per os treatment with
1. Control	Distilled water 10ml/kg b.w.
2. Combination	Antistress I 5g/kg b.w.
3. Combination	Antistress I 10 g/kg b.w.
4. Combination	Antistress II 5 g/kg b.w.
5. Combination	Antistress II 10 g/kg b.w.
6. Individual extract	Serratula 5g/kg b.w.
7. Individual extract	Serratula 10 g/kg b.w.
8. Individual extract	Hypericum 5 g/kg b.w.
9. Individual extract	Hypericum 10 g/kg b.w.
10. Individual extract	Valeriana 5 g/kg b.w.
11. Individual extract	Valeriana 10 g/kg b.w.
12. Individual extract	Crataegus 5 g/kg b.w.
13. Individual extract	Crataegus 10 g/kg b.w.
14. Individual extract	Melissa 5g/kg b.w.
15. Individual extract	Melissa 10 g/kg b.w.

The comparison between Antistress I and Antistress II at doses of 100 and 250 mg/kg b.w. shows no significant difference in the values of the indicators for anxiolytic effect. However at a dose of 500 mg/kg b.w. there is a statistically significant increase of Totk, Notk and Ntot for group AS₂₅₀₀ compared to group AS₁₅₀₀

Comparison of the results regarding the anxiolytic effect of Antistress I at doses of 100, 250 and 500 mg/kg b.w. and the individual extracts contained in this combination at the same doses.

The comparison of AS₁₀₀ with SER₁₀₀, MEL₁₀₀, VAL₁₀₀

and CRA₁₀₀ doesn't demonstrate statistically significant difference between the indicators of anxiolytic effect. Similar results could be reported when comparing AS₁₂₅₀ with SER₂₅₀, MEL₂₅₀, VAL₂₅₀ and CRA₂₅₀. At a dose of 500 mg/kg b.w. there is a significant increase of Totk for groups CRA₅₀₀ and VAL₅₀₀ compared to group AS₁₅₀₀.

Comparison of the results regarding the anxiolytic effect of Antistress II at doses of 100, 250 and 500 mg/kg b.w. and the individual extracts contained in this combination at the same doses.

There is no statistically significant difference in the indicators of anxiolytic effect when comparing group AS₂₁₀₀ with groups SER₁₀₀, HYP₁₀₀ and VAL₁₀₀. The comparison of AS₂₂₅₀ with SER₂₅₀, HYP₂₅₀ and VAL₂₅₀ shows similar results. Totk, Notk, Ntot and Notn decrease significantly for group SER₅₀₀ compared to group AS₂₅₀₀. The indicators -Totk, Notk and Ntot decrease for group HYP₅₀₀ compared to group AS₂₅₀₀. Only Notk and Ntot decrease significantly for group VAL₅₀₀ compared to group AS₂₅₀₀.

Comparison of the results regarding the anxiolytic effect between the different doses (100, 250 and 500) of the combination Antistress I and comparison of the results regarding the anxiolytic effect between the different doses (100, 250 and 500) of the combination Antistress II.

The comparison of AS₁₁₀₀ and AS₁₂₅₀ doesn't show statistically significant change of the indicators for anxiolytic effect. Similar results could be reported after comparing AS₁₂₅₀ and AS₁₅₀₀. However a significant increase of Totk and Notk could be observed for AS₁₅₀₀ after comparing this group with AS₁₁₀₀.

Table 2: Design of the experiment used for the evaluation of the anxiolytic effect in model of acute stress of the two combinations - Antistress I and Antistress II and the individual extracts included in them.

Group	Label	Description	Model of acute stress	Number of rats in each group
1	C	Distilled water per os 10 ml/kg b.w.	Yes	14
2	C ₀	Distilled water per os 10 ml/kg b.w	No	6
3	AS ₁₀₀	Antistress I per os 100 mg/kg b. w	Yes	6
4	AS ₁₂₅₀	Antistress I per os 250 mg/kg b. w	Yes	6
5	AS ₁₅₀₀	Antistress I per os 500 mg/kg b. w	Yes	8
6	AS ₂₁₀₀	Antistress II per os 100 mg/kg b. w	Yes	6
7	AS ₂₂₅₀	Antistress II per os 250 mg/kg b. w	Yes	6
8	AS ₂₅₀₀	Antistress II per os 500 mg/kg b. w	Yes	8
9	SER ₁₀₀	Serratula coronata per os 100 mg/ kg b. w	Yes	6
10	SER ₂₅₀	Serratula coronata per os 250 mg/ kg b. w	Yes	6
11	SER ₅₀₀	Serratula coronata per os 500 mg/ kg b. w	Yes	8
12	HYP ₁₀₀	Hypericum perforatum per os 100 mg/ kg b. w	Yes	6
13	HYP ₂₅₀	Hypericum perforatum per os 250 mg/ kg b. w	Yes	6
14	HYP ₅₀₀	Hypericum perforatum per os 500 mg/ kg b. w	Yes	8
15	VAL ₁₀₀	Valeriana officinalis per os 100 mg/ kg b. w	Yes	6
16	VAL ₂₅₀	Valeriana officinalis per os 250 mg/ kg b. w	Yes	6
17	VAL ₅₀₀	Valeriana officinalis per os 500 mg/ kg b. w	Yes	8
18	CRA ₁₀₀	Crataegus monogyna per os 100 mg/ kg b. w	Yes	6
19	CRA ₂₅₀	Crataegus monogyna per os 250 mg/ kg b. w	Yes	6
20	CRA ₅₀₀	Crataegus monogyna per os 500 mg/ kg b. w	Yes	8
21	MEL ₁₀₀	Melissa officinalis per os 100 mg/ kg b. w	Yes	6
22	MEL ₂₅₀	Melissa officinalis per os 250 mg/ kg b. w	Yes	6
23	MEL ₅₀₀	Melissa officinalis per os 500 mg/ kg b. w	Yes	8

Table 3: Time spent in the opened arms of Elevated plus maze measured in seconds - Totk (s) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

Groups	Number	Mean \pm SEM	U	p
C	14	5,1 \pm 2,0	10,5	0,007*
C ₀	6	36,2 \pm 11,6		
C	14	5,1 \pm 2,0	38	0,724
AS1 ₁₀₀	6	2,7 \pm 1,5		
C	14	5,1 \pm 2,0	39,5	0,825
AS1 ₂₅₀	6	6,5 \pm 5,1		
C	14	5,1 \pm 2,0	19	0,010*
AS1 ₅₀₀	8	14,7 \pm 2,7		
C	14	5,1 \pm 2,0	26	0,166
AS2 ₁₀₀	6	14,7 \pm 6,8		
C	14	5,1 \pm 2,0	41	0,930
AS2 ₂₅₀	6	3,2 \pm 1,5		
C	14	5,1 \pm 2,0	1	<0,001*
AS2 ₅₀₀	8	54,9 \pm 8,4		
C	14	5,1 \pm 2,0	39,5	0,826
SER ₁₀₀	6	11,5 \pm 7,8		
C	14	5,1 \pm 2,0	41,5	0,965
SER ₂₅₀	6	3,0 \pm 1,0		
C	14	5,1 \pm 2,0	24,5	0,026*
SER ₅₀₀	8	25,2 \pm 7,6		
C	14	5,1 \pm 2,0	38,5	0,757
HYP ₁₀₀	6	3,0 \pm 1,6		
C	14	5,1 \pm 2,0	24,5	0,103
HYP ₂₅₀	6	0,5 \pm 0,5		
C	14	5,1 \pm 2,0	37	0,173
HYP ₅₀₀	8	16,4 \pm 6,8		
C	14	5,1 \pm 2,0	39	0,786
VAL ₁₀₀	6	8,8 \pm 7,2		
C	14	5,1 \pm 2,0	37	0,652
VAL ₂₅₀	6	4,7 \pm 3,6		
C	14	5,1 \pm 2,0	11,5	0,002*
VAL ₅₀₀	8	36,6 \pm 7,0		
C	14	5,1 \pm 2,0	19,5	0,055
CRA ₁₀₀	6	18,3 \pm 8,8		
C	14	5,1 \pm 2,0	37,5	0,691
CRA ₂₅₀	6	3,0 \pm 1,7		
C	14	5,1 \pm 2,0	2	<0,001*
CRA ₅₀₀	8	45,0 \pm 8,5		
C	14	5,1 \pm 2,0	24,5	0,103
MEL ₁₀₀	6	0,5 \pm 0,5		
C	14	5,1 \pm 2,0	15	0,021*
MEL ₂₅₀	6	23,0 \pm 7,0		
C	14	5,1 \pm 2,0	15,5	0,010*
MEL ₅₀₀	8	27,6 \pm 9,8		

* The results are statistically significant.

The indicators for anxiolytic effect don't differ significantly after comparing AS2₁₀₀ and AS2₂₅₀. There is a significant increase of Totk, Notk, Ntot and Notn for AS2₅₀₀ compared to AS2₂₅₀. The comparison of AS2₅₀₀ with AS2₁₀₀ demonstrates significant increase of the indicators Totk and Notn.

DISCUSSION

For the present experiments, we have selected the Elevated plus maze as an appropriate method to evaluate the

anxiolytic effects of the combinations and the individual extracts. According to Pellow and File, in the assessment of behavioral responses of rodents, anxiety is studied most often by using the Elevated plus maze test, which is the main test for preclinical evaluation of drugs with anxiolytic effect¹⁰.

Referring to the available literature data we haven't found any information about combinations of plant extracts corresponding to the doses, the content and the ratios of the combinations included in our experiment. To explain the

Table 4: Number of entries in the opened arms of Elevated plus maze (Notk) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

Groups	Number	Mean \pm SEM	U	p
C	14	0,6 \pm 0,2	18,5	0,038*
C ₀	6	2,3 \pm 0,8		
C	14	0,6 \pm 0,2	37,5	0,684
AS1 ₁₀₀	6	0,8 \pm 0,4		
C	14	0,6 \pm 0,2	39,5	0,819
AS1 ₂₅₀	6	1,2 \pm 0,8		
C	14	0,6 \pm 0,2	15,5	0,004*
AS1 ₅₀₀	8	2,2 \pm 0,4		
C	14	0,6 \pm 0,2	26	0,156
AS2 ₁₀₀	6	1,8 \pm 0,7		
C	14	0,6 \pm 0,2	40,5	0,891
AS2 ₂₅₀	6	0,7 \pm 0,3		
C	14	0,6 \pm 0,2	2	0,001*
AS2 ₅₀₀	6	4,7 \pm 1,0		
C	14	0,6 \pm 0,2	37	0,651
SER ₁₀₀	6	1,0 \pm 0,5		
C	14	0,6 \pm 0,2	34	0,466
SER ₂₅₀	6	0,8 \pm 0,3		
C	14	0,6 \pm 0,2	31,5	0,075
SER ₅₀₀	8	1,6 \pm 0,5		
C	14	0,6 \pm 0,2	40	0,855
HYP ₁₀₀	6	0,8 \pm 0,5		
C	14	0,6 \pm 0,2	30,5	0,276
HYP ₂₅₀	6	0,3 \pm 0,3		
C	14	0,6 \pm 0,2	40,5	0,254
HYP ₅₀₀	8	1,7 \pm 0,8		
C	14	0,6 \pm 0,2	41	0,927
VAL ₁₀₀	6	1,2 \pm 0,8		
C	14	0,6 \pm 0,2	38	0,711
VAL ₂₅₀	6	0,8 \pm 0,6		
C	14	0,6 \pm 0,2	20,5	0,024*
VAL ₅₀₀	8	2,0 \pm 0,5		
C	14	0,6 \pm 0,2	16,5	0,026*
CRA ₁₀₀	6	2,3 \pm 0,8		
C	14	0,6 \pm 0,2	40,5	0,891
CRA ₂₅₀	6	0,7 \pm 0,3		
C	14	0,6 \pm 0,2	8,5	0,001*
CRA ₅₀₀	8	4,5 \pm 0,9		
C	14	0,6 \pm 0,2	37,5	0,677
MEL ₁₀₀	6	0,7 \pm 0,5		
C	14	0,6 \pm 0,2	12,5	0,011*
MEL ₂₅₀	6	3,5 \pm 1,0		
C	14	0,6 \pm 0,2	17,5	0,032*
MEL ₅₀₀	8	1,8 \pm 0,5		

* The results are statistically significant.

pharmacological effects of Antistress I and Antistress II, we have used information from the available literature related to the chemical composition and the pharmacological effects of the individual extracts which are included in these combinations.

The group of rats treated with the combination Antistress I at a dose of 500 mg / kg b.w. demonstrates a significant increase in the indicators of anxiety. However, the individual plant extracts contained in Antistress I - *Serratula coronata*, *Valeriana officinalis*, *Crataegus*

monogyna and *Melissa officinalis*, when administered separately at a dose of 500 mg / kg b.w. have better anxiolytic effect compared to effects on the experimental animals treated with Antistress I. This is probably due to antagonistic interactions between them which occur after they are included in the combination.

The combination Antistress II administered at a dose of 500 mg / kg b.w shows significant increase in the main indicators of anxiety - Totk, Notk and Notn and this increase is greater compared to the increase of the groups

Table 5: Total number of entries in both arms of Elevated plus maze (Ntot) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

Groups	Number	Mean \pm SEM	U	p
C	14	2,3 \pm 0,4	21,5	0,079
C ₀	6	4,7 \pm 1,2		
C	14	2,3 \pm 0,4	40,5	0,894
AS1 ₁₀₀	6	2,8 \pm 1,1		
C	14	2,3 \pm 0,4	29,5	0,276
AS1 ₂₅₀	6	3,8 \pm 1,3		
C	14	2,3 \pm 0,4	15,5	0,004*
AS1 ₅₀₀	8	5,0 \pm 0,6		
C	14	2,3 \pm 0,4	23,5	0,108
AS2 ₁₀₀	6	5,7 \pm 2,0		
C	14	2,3 \pm 0,4	29	0,258
AS2 ₂₅₀	6	3,3 \pm 0,8		
C	14	2,3 \pm 0,4	3	0,001*
AS2 ₅₀₀	6	8,5 \pm 1,6		
C	14	2,3 \pm 0,4	34	0,487
SER ₁₀₀	6	2,8 \pm 0,7		
C	14	2,3 \pm 0,4	41,5	0,965
SER ₂₅₀	6	2,0 \pm 0,4		
C	14	2,3 \pm 0,4	31	0,076
SER ₅₀₀	8	4,0 \pm 0,8		
C	14	2,3 \pm 0,4	41	0,930
HYP ₁₀₀	6	2,3 \pm 0,7		
C	14	2,3 \pm 0,4	27	0,160
HYP ₂₅₀	6	1,3 \pm 0,3		
C	14	2,3 \pm 0,4	45	0,425
HYP ₅₀₀	8	3,9 \pm 1,3		
C	14	2,3 \pm 0,4	35	0,543
VAL ₁₀₀	6	3,7 \pm 1,6		
C	14	2,3 \pm 0,4	37,5	0,683
VAL ₂₅₀	6	2,3 \pm 1,0		
C	14	2,3 \pm 0,4	25	0,062
VAL ₅₀₀	8	4,3 \pm 0,9		
C	14	2,3 \pm 0,4	14,5	0,019*
CRA ₁₀₀	6	5,7 \pm 1,5		
C	14	2,3 \pm 0,4	35	0,535
CRA ₂₅₀	6	1,7 \pm 0,3		
C	14	2,3 \pm 0,4	11,5	0,002*
CRA ₅₀₀	8	8,4 \pm 1,5		
C	14	2,3 \pm 0,4	33,5	0,441
MEL ₁₀₀	6	1,7 \pm 0,5		
C	14	2,3 \pm 0,4	13,5	0,015*
MEL ₂₅₀	6	8,8 \pm 2,5		
C	14	2,3 \pm 0,4	25	0,145
MEL ₅₀₀	8	3,5 \pm 0,8		

* The results are statistically significant.

treated with individual extracts of Valeriana officinalis, Hypericum perforatum and Serratula coronata. That could be explained with synergy between Serratula coronata and the others constituents in the combined extract. Moreover in the available literature is reported that hypericine contained in Hypericum perforatum, except its antidepressant properties, has also the ability to potentiate the anti-stress and adaptogenic effects of other plant extracts, when administered in combination with them. This explains its inclusion in the combined extract

Antistress II¹¹. Clinical study reports the beneficial effects in the treatment of anxiety-depressive disorders when Valeriana officinalis and Hypericum perforatum are applied in combination¹². A preclinical study conducted by Patidar and Associates shows anxiolytic and sedative effects of combined extract and dry crude plant drug containing Valeriana, Hypericum and Passiflora at a dose of 400 mg / kg b.w. Besides the positive effects of the combination on anxiety it is also found that the dry crude drug demonstrates stronger sedative effect compared to the

Table 6: Ratio of opened/total arm entries in Elevated plus maze (Notn) after Antistress I, Antistress II and the individual extracts contained in them are administrated on male Wistar rats for 14 days.

Groups	Number	Mean \pm SEM	U	p
C	14	0,18 \pm 0,05	16	0,025*
C ₀	6	0,41 \pm 0,09		
C	14	0,18 \pm 0,05	41	0,929
AS1 ₁₀₀	6	0,21 \pm 0,11		
C	14	0,18 \pm 0,05	42	1,000
AS1 ₂₅₀	6	0,18 \pm 0,09		
C	14	0,18 \pm 0,05	20,5	0,012*
AS1 ₅₀₀	8	0,39 \pm 0,06		
C	14	0,18 \pm 0,05	36	0,601
AS2 ₁₀₀	6	0,24 \pm 0,09		
C	14	0,18 \pm 0,05	40	0,859
AS2 ₂₅₀	6	0,15 \pm 0,07		
C	14	0,18 \pm 0,05	4	0,001*
AS2 ₅₀₀	6	0,54 \pm 0,03		
C	14	0,18 \pm 0,05	37	0,658
SER ₁₀₀	6	0,22 \pm 0,10		
C	14	0,18 \pm 0,05	25	0,135
SER ₂₅₀	6	0,33 \pm 0,10		
C	14	0,18 \pm 0,05	34	0,118
SER ₅₀₀	8	0,33 \pm 0,08		
C	14	0,18 \pm 0,05	38,5	0,757
HYP ₁₀₀	6	0,22 \pm 0,11		
C	14	0,18 \pm 0,05	31,5	0,327
HYP ₂₅₀	6	0,11 \pm 0,11		
C	14	0,18 \pm 0,05	39,5	0,233
HYP ₅₀₀	8	0,30 \pm 0,09		
C	14	0,18 \pm 0,05	38	0,717
VAL ₁₀₀	6	0,14 \pm 0,09		
C	14	0,18 \pm 0,05	38,5	0,751
VAL ₂₅₀	6	0,15 \pm 0,10		
C	14	0,18 \pm 0,05	19,5	0,023*
VAL ₅₀₀	8	0,41 \pm 0,08		
C	14	0,18 \pm 0,05	22,5	0,095
CRA ₁₀₀	6	0,34 \pm 0,08		
C	14	0,18 \pm 0,05	33,5	0,451
CRA ₂₅₀	6	0,28 \pm 0,13		
C	14	0,18 \pm 0,05	10	0,001*
CRA ₅₀₀	8	0,52 \pm 0,05		
C	14	0,18 \pm 0,05	30,5	0,282
MEL ₁₀₀	6	0,08 \pm 0,08		
C	14	0,18 \pm 0,05	19,5	0,054
MEL ₂₅₀	6	0,35 \pm 0,07		
C	14	0,18 \pm 0,05	14,5	0,018*
MEL ₅₀₀	8	0,45 \pm 0,10		

* The results are statistically significant.

extract¹³.

The doses that we have selected for the experiments - 100, 250 and 500 mg / kg b.w. are similar to those chosen by other authors¹⁴. The results of our study using a model of acute stress show that dry extract from *Serratula coronata* exhibits anxiolytic effect by increasing Totk only at a dose of 500 mg / kg b.w. There is no dose-effect relation because the increase Totk and Notk is lowest at a dose of 250 mg / kg b.w. Another study gives information about the anxiolytic and adaptogenic effect of 20-

hydroxyecdysone, which is one of the main chemical compounds found in *Serratula coronata*¹⁵. There is no evidence in the available literature explaining the in vivo effects on anxiety of total *Serratula coronata* extract. Although the individual extract of *Serratula coronata* demonstrates anxiolytic effect in our experiments, its inclusion in the combinations Antistress I and Antistress II is 10 times less than the dose causing such an effect. This leads to the conclusion that *Serratula coronata* acts

synergistically on the anxiolytic effects of the other ingredients in the combinations.

Regarding the individual *Valeriana officinalis* extract, it can be observed a dose-effect relation in the increase of Totk. This indicator has the smallest value at the lowest dose of 100 mg/kg b.w. and the greatest value in the highest dose of 500 mg/kg b.w. *Valeriana officinalis* contains triterpenes (valepotriates, valerenic acid, bornyl acetate, etc.). Valepotriates potentiate secretion and block reuptake of the main inhibitory CNS mediator - Gamma-aminobutyric acid (GABA)¹⁶. Valerenic acid modulates GABA_A receptors and also supports the anxiolytic action¹⁷. In our study the individual *Valeriana officinalis* extract doesn't alter significantly the locomotor activity of rats. Our results correspond with these of Hattesoehl and co-authors, who investigate *Valeriana officinalis* extract, administered orally. In their study, a dose of 1000 mg/kg b.w. doesn't exhibit sedative or miorelaxant effect¹⁸. Locomotor activity in mouse doesn't decrease in the study of Hiller and co-authors¹⁹.

The extract of *Crataegus monogyna* shows anxiolytic effect at the highest dose by increasing significantly the indicators of anxiety. The anxiolytic effect is due to the flavonoid fraction in the extract (rutin, quercetin, hiperozid etc.). The observed increase in the overall locomotor activity expressed in Ntot demonstrates a lack of sedative effect. This doesn't correspond to the results from Can and co-authors, according to who the extract of seeds and pulp from *Crataegus monogyna* at a dose of 10 to 1000 mg / kg b.w. reduces the locomotor activity²⁰. The differences in our results may be due to the fact that in our study, the extract of *Crataegus monogyna* is obtained from the leaves and flowers, but not from seeds and pulp thus has a different content of biologically active substances. In another study an ethanol extract of *Crataegus* shows anxiolytic effect in the elevated plus maze at doses of 300 and 600 mg. The mechanism of action is not fully understood, but it is probably due to influence upon GABA neurotransmission²¹. In our study, the lowest dose exhibits statistically significant effect only in Notk while increasing the locomotor activity. Furthermore, the individual extract from *Crataegus monogyna* doesn't show dose-effect relation.

In the current study it could be observed a dose-effect relation regarding Totk for the extract of *Melissa officinalis*. Our experiments also show significant increase of Totk and Notk at doses of 250 mg/kg b.w. and 500 mg/kg b.w. The anxiolytic effect of *Melissa officinalis* extract is probably due to the rosmarinic acid contained in the plant. It inhibits GABA- transaminase - the enzyme that is responsible for the degradation of GABA and thus potentiate its inhibitory effects in the CNS²². In a study of Taiwo and co-authors, *Melissa officinalis* at a doses of 100 and 300 mg / kg b.w., administered orally to rats for 15 days, exhibits a reduction of anxiety in the Elevated plus maze and the observed changes in Totk are also dose-dependents²³. Our current study shows significant increase of locomotor activity at doses of 100 and 500 mg/kg b.w., indicating a lack of sedative effect. These results regarding

the anxiolytic effect of extract of *Melissa officinalis* are similar to results of other authors²⁴.

Our current results allow us to assume direct relation between the anxiolytic effects of the combinations and the chemical composition of the individual extracts included in this study. The connection established by Leonard et al between anxiety and oxidative stress gave us reason to examine the antioxidant activity of Antistress I and Antistress II in vitro by using three methods (ORAC, HORAC and electrochemical method) and in vivo by evaluating the effect of these combinations on anxiety after applying a model of acute stress on rats²⁵. The more pronounced anxiolytic effects of the extracts of *Crataegus monogyna*, *Melissa officinalis* and *Valeriana officinalis* can be attributed to the higher percentage of polyphenolic compounds: flavonoids - rutin, quercetin, and hyperoside, vitexin; triterpenes - valepotriates, bornyl acetate, valerenic acid. The content of these compounds was determined by HPLC method developed at the Department of Chemistry and Biochemistry, Faculty of Pharmacy at the Medical University - Plovdiv. There is a correspondence between the results from the in vitro tests for antioxidant activity and the results from in vivo tests for anxiolytic activity obtained after the experiments with laboratory animals treated with Antistress I and Antistress II and the individual extracts which they contain. The results from chemical and experimental studies confirm the thesis of Halliwell for the necessity of both in vitro and in vivo pharmacodynamic interaction studies of the extracts²⁶.

Both combinations - Antistress I and Antistress II are suitable for studying anxiety on a model of acute stress but only at the highest dose used in our experiment. The results about the anxiolytic effect of Antistress II on an acute stress model in rats give us the reason to conclude that this combination could be used for anxiety disorder combined with depression. Our results are confirmed with the data for antioxidant activity by using ORAC, HORAC and electrochemical method.

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