https://doi.org/10.31925/farmacia.2025.1.19 **ORIGINAL ARTICLE**

COMBINATION OF SIX COMPOUNDS SYNERGISTICALLY BOOSTS ANTIOXIDANT EFFECTS *EX VIVO*

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Abstract

In this *ex vivo* study, we aimed to evaluate the antioxidant properties of the N-acetylcysteine, zinc, manganese, selenium, vitamin C and vitamin D in human serum, as well as the effect of their mixture present in the product BiVits® ACTIVA Recovery (Abela Pharm d.o.o. Belgrade, Serbia). The serum pool was formed by collecting samples from individuals with healthy profiles. To assess the redox status, total oxidant status (TOS), prooxidant-antioxidant balance (PAB), total antioxidant status (TAS), superoxide dismutase (SOD) activity and total sulfhydryl groups (SHG) concentration were determined. Subsequently, antioxidant score (AS), prooxidant score (PS), and general oxy score (OS), were calculated. These scores served as valuable indicators of the overall redox balance. The results of this study showed that although all components exhibited antioxidant properties individually, the mixture of these components contained in the product BiVits® ACTIVA Recovery had a much more pronounced antioxidant potential both after short- and long-term incubation in conditions that mimic physiological environment and in an oxidative stress milieu. The AS of BiVits® ACTIVA Recovery was much greater than the sum of AS of the individual components, indicating a synergistic effect of the components.

Rezumat

În acest studiu *ex vivo*, ne-am propus să evaluăm proprietățile antioxidante ale N-acetilcisteinei, zincului, manganului, seleniului, vitaminei C și vitaminei D în serul uman, precum și efectul amestecului acestora prezent în produsul BiVits® ACTIVA Recovery. Probele de ser au fost colectate de la indivizi cu profiluri sănătoase pentru a forma un pool de ser. Pentru a evalua statusul redox, au fost determinate nivelurile de stres oxidativ total (TOS), balanța prooxidant-antioxidantă (PAB), statusul antioxidant total (TAS), activitatea superoxid dismutazei (SOD) și concentrația totală a grupărilor sulfhidril (SHG). Ulterior, au fost calculați scorul antioxidant (AS), scorul prooxidant (PS) și scorul general de oxido-reducere (OS), acești parametri servind drept indicatori ai echilibrului redox global. Rezultatele acestui studiu au demonstrat că, deși fiecare component a manifestat proprietăți antioxidante individuale, amestecul acestor compuși, așa cum este prezent în produsul BiVits® ACTIVA Recovery, a prezentat un potențial antioxidant semnificativ mai pronunțat, atât după incubare pe termen scurt, cât și pe termen lung, în condiții ce imită mediul fiziologic și în prezența unui stres oxidativ. Mai mult, scorul AS al produsului BiVits® ACTIVA Recovery a fost considerabil mai mare decât suma scorurilor AS ale componentelor individuale, sugerând un efect sinergic al acestora.

Keywords: oxidative stress, antioxidants, supplement, synergism

Introduction

An increased level of oxidative stress occurs when the amount of free radicals (reactive oxygen species, ROS, chemically reactive and toxic compounds) exceeds the capacity of the antioxidant protective system [1]. Oxidative stress is a pathophysiological phenomenon underlying many diseases and is considered to be one of the major risk factors for various non-infectious diseases, such as cardiovascular, neurodegenerative, renal diseases, diabetes, cancer, and even infectious diseases such as COVID-19 [1-3]. The endogenous antioxidant defence system aims to neutralise the harmful effects of prooxidants and includes enzymatic and non-enzymatic antioxidants, which may be endogenous biomolecules or obtained through diet and supplements [4]. In addition to pathophysiological conditions, oxidative stress components play a homeostatic role in many physiological processes, so it is desirable to determine the redox status parameters of the organism in different physiological conditions, such as pregnancy, doing sports, special physical and psychophysiological efforts, and everyday stress. To maintain these processes, there should be a physiological, low level of free radicals in cells, called oxidative eustress, without neutralising or suppressing the basal level of reactive oxygen species [5]. However, to prevent oxidative eustress from progressing into oxidative distress, it is critical

to continuously maintain sufficient levels of reductive (antioxidative) substances.

According to the manufacturer, BiVits® ACTIVA Recovery product contains 6 active ingredients. This product combines vitamin C, N-acetylcysteine (NAC), zinc, manganese, selenium, and vitamin D3 to provide powerful antioxidant and antiviral effects. Therefore, BiVits® ACTIVA Recovery product is recommended for people with weakened immune systems, immune disorders, common colds, respiratory tract infections, and for those who are engaged in intense physical activity, such as active athletes, based on the effects of each component.

Vitamin C is known for its antioxidant activity, anti-inflammatory properties, and immunomodulatory effects [6, 7]. Vitamin C deficiency has been associated with several diseases, including cancer, atherosclerosis, diabetes, infections, anaemia, and neurodegenerative diseases [8]. Vitamin D3 has pleiotropic functions, including antioxidant activity in addition to its confirmed role in bone metabolism [9]. It also exhibits immunomodulatory effects, as evidenced by the presence of vitamin D receptors and the enzyme 1-α-hydroxylase, which is responsible for its metabolism in certain immune system cells. While its role in adaptive immunity is still unclear, its properties are particularly important for the innate immune system [9]. Zinc and manganese have immunoregulatory and antiviral effects and contribute to antioxidant protection as part of the enzyme superoxide dismutase (SOD), which plays an indirect role in the superoxide anions dismutation [10]. In addition, these microelements are involved in the synthesis and activation of several enzymes. A deficiency of zinc and manganese is associated with conditions such as diabetes, obesity, fatty liver, atherosclerosis, and hyperlipidaemia [11]. Although its function in the immune system is not fully understood, manganese sequestration during bacterial infections results in the starvation of bacteria and increased host resistance to infection [12].

Selenium is one of the most important trace elements in the body and is essential for redox reactions. It protects against oxidative stress and viral infections, especially as a component of the antioxidant enzyme glutathione peroxidase [13]. Selenoproteins, the active form of selenium, enhance the potency of natural killer cells and leukocytes against invading pathogens through their antioxidant activity [14]. Selenium also promotes antibody production and the production of interferon γ and T-helper cells [15].

The effects of NAC have been extensively studied and well documented, and it has been used in clinical practice for several decades. NAC has mucolytic and antioxidant properties and plays a crucial role in the synthesis of reduced glutathione, an important cellular antioxidant. Its protective effect against oxidative damage can be attributed to its ability to maintain a high intracellular glutathione concentration and scavenge free radicals through various mechanisms [16, 17].

The structure of all active components of BiVits® ACTIVA Recovery is shown in Figure 1. Zinc and manganese are present in the form of +2 oxidation state ions, while selenium is organically bound as selenomethionine. Manganese is available in various supplement forms, but manganese gluconate, with its chelated structure, provides excellent bioavailability compared to inorganic forms such as oxide or chloride [18]. Similarly, chelated zinc compounds are the preferred choice for supplementation [19]. In BiVits® ACTIVA Recovery, zinc is present as zinc citrate. Although the body can utilise selenium from both organic and inorganic forms, organic forms are more beneficial due to their better absorption. Selenomethionine represents the optimal selenium source with a very high absorption rate. Despite NAC being a direct precursor of L-cysteine, it is a superior supplement option compared to native cysteine, as it exhibits better bioavailability and is less susceptible to oxidation [20-22]. The composition and masses of specific antioxidants, microelements, and vitamins *per* 1 tablet of BiVits® ACTIVA Recovery are provided in Figure 1.

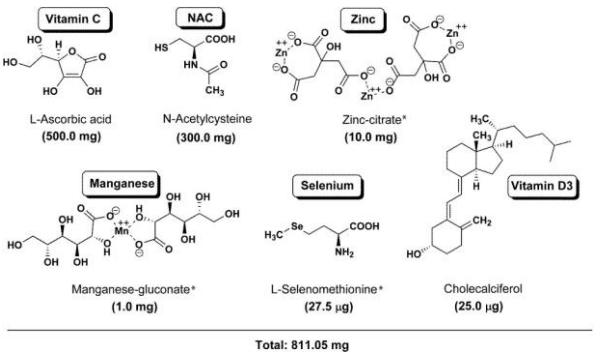

Figure 1.

Structures of active components and their mass *per* 1 tablet of BiVits Recovery®

*****Stated masses are related to the metal content in the product

Based on the aforementioned information and the unique composition of BiVits® ACTIVA Recovery, our study aims to evaluate ex vivo the antioxidative potential of this combination of vitamins, minerals, immunomodulators, and antioxidants using biological material, consisting of healthy individuals' human serum pool. We analysed various redox status parameters in the serum, both before and after the addition of individual components, as well as the BiVits® ACTIVA Recovery product (as a combination of all components).

Materials and Methods

The serum pool was formed by collecting samples from a cohort of fifty healthy subjects, who were not taking any medications or supplements, and whose baseline biochemical parameters such as glucose and lipid parameters (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides), liver enzymes and renal biomarkers (urea, creatinine, uric acid) were within the reference range. The subjects whose serum residues were used for the serum pool collection were at their regular medical check-up at the health centres. Prior to sample collection, these individuals had provided consent for any excess serum remaining after routine biochemical analyses to be used for the purposes of this study. BiVits® ACTIVA Recovery product was sourced from manufacturer AbelaPharm d.o.o., while individual components were obtained from various vendors: L-Ascorbic acid (Lambda Int. Trading, USA), N-acetylcysteine (TNJ Chemical Industry, China), Zinc citrate (Jost Chemical Co., USA), Manganese gluconate (Jost Chemical Co., USA), L-Selenomethionine (Axo Industry Int., Belgique), and Cholecalciferol (DSM Nutritional Prod., Switzerland). The vendors for the individual components were the same suppliers used by the manufacturer for sourcing ingredients for the BiVits® ACTIVA Recovery product. The antioxidant activity of the BiVits® ACTIVA Recovery product was evaluated by *ex vivo* analysis, separately for each individual component and the original formulation containing a combination of all 6 components. The antioxidative activity was evaluated in natural conditions and in the conditions of induced oxidative stress.

Two incubation times were used: one for 2 h and one for 24 h, both at 37°C. Following incubation, selected parameters of the redox status were determined by spectrophotometric methods, and prooxidant, antioxidant, and total oxy scores were calculated. Dilutions were prepared in duplicate for each individual component, representing concentrations of 100%, 50%, 25%, and 10% of its original concentration in 1 tablet, which hypothetically covers the range of possible systemic circulation levels after gastrointestinal absorption. Additionally, an exogenous oxidant was added to a set of samples to determine the effects of incubated samples in the conditions of oxidative stress. Since the absorption rate is unknown, BiVits® ACTIVA Recovery samples were prepared in duplicate at various dilutions to cover a range from 10% to 100% of a single dose's absorption. The concentration of the product needed to be achieved in the serum was calculated as follows: The total content of all 6 components in 1 BiVits® ACTIVA Recovery tablet is 811.05 mg. Assuming complete absorption of the tablet (100%), dispersing its content in 5 L of blood (approximated total blood volume in circulation), considering that approximately 2/3 of blood is serum, and assuming that the components do not enter blood cells but remain in the serum (used for analysis), the concentration was calculated as 270 mg of active components *per* 1 L of serum (0.27 g/L). Concentrations for individual components were calculated according to their content in a BiVits® ACTIVA Recovery product. All components were dissolved in dimethyl sulfoxide (DMSO) in a concentration 10 times higher than calculated above in order to achieve proper dilutions with serum (50 μL of substances DMSO solution added in 450 μL serum for 100% concentration). Several blood samples were treated with trolox (Acros Organics Geel, Belgium; 2 mmol/L in DMSO, 50 μL was added in 450 μL serum), a substance with a known and potent antioxidant effect. The obtained results of redox status for individual components and the product were compared with results from samples incubated with trolox. Blood samples were prepared as described previously, and *tert*-butylhydroperoxide (TBH, Acros Organics, Geel, Belgium) was added to each sample (in equal volume quantities) as a strong exogenous oxidant (5 μL/10 mL DMSO). The analyses were also compared with an empty serum pool serving as a sample blank.

Total oxidant potential (TOS) and prooxidant-antioxidant balance (PAB) were determined as indicators of oxidative stress, while the total antioxidant capacity (TAS), total content of sulfhydryl groups (SHG), and activity of the enzyme superoxide dismutase (SOD) were determined as markers of antioxidant protection.

Measurement of total oxidative status

The TOS values were determined spectrophotometrically using a modification of the automated colorimetric method for measuring total oxidant status [23]. Oxidants present in the sample oxidised ferrous ion-o-dianisidine complex to ferric ion. The amount of oxidants was directly proportional to the colour intensity of the reaction mixture. The assay was calibrated with aqueous hydrogen peroxide solution. The results were expressed as μmoL H_2O_2 equivalent/L [24].

Measurement of pro-oxidative-antioxidative balance

The used colorimetric method is based on the reaction of 0.6% 3,3'-5,5'-tetrametylbenzidine (TMB, Sigma-Aldrich, Germany) with hydrogen peroxide and antioxidants simultaneously. Reaction with hydrogen peroxide is catalysed by peroxidase, while the reaction of antioxidants in serum and TMB is a non-catalysed reaction. The enzymatic reaction causes TMB oxidation to blue product, while the non-catalysed reaction is colourless. The standard solution was prepared by mixing different proportions of 1 mmol/L of H_2O_2 with 6 mmol/L uric acid. The values of PAB are expressed in arbitrary units, corresponding to the percentage of H_2O_2 in the standard solution [25].

Measurement of total antioxidative status

TAS was measured according to Erel's method [26] in which oxidation of 2,2-azino-bis-3-ethyl-benzo-thiazoline-6-sulfonic acid (ABTS, Sigma-Aldrich, Germany) by hydrogen peroxide present in an acidic medium, gives to the reaction mixture emerald colour. The antioxidants present in the sample cause the reagent discoloration, which is proportional to their concentration. The reaction was calibrated with trolox (analog of Vitamin E, Sigma-Aldrich, Germany). TAS value in the results is given as μmoL trolox equivalent/L [24].

Measurement of total concentration of sulfhydryl groups

The total concentration of sulfhydryl groups in the serum was determined by modification Ellman's method [27]. This spectrophotometric method is based on the reaction of 2,2'-dinitro-5,5'-dithio-benzoic acid (DTNB) with aliphatic thiol compounds in an alkaline medium (pH 9.0). This reaction generates 1 mol of p-nitrophenol anion *per* mol of thiol. It requires rapid read-out of the generated anion. The total concentration of SH groups can be determined *via* molar extinction coefficient of p-nitrophenol at 412 nm [24].

Measurement of superoxide dismutase

Serum SOD activity was measured using a slightly modified method of Misra and Fridovich, which relays on the ability of the SOD enzyme to inhibit the autooxidation of epinephrine in an alkaline medium (0.05 mmol/L bicarbonate buffer, pH 10.2). The absorbance of the obtained pink oxidised product is measured at 480 nm. SOD activity is calculated as a percent of the inhibition of epinephrine autooxidation [28].

Statistical analysis

Prooxidant, antioxidant, and total oxy scores were also calculated using Z-score statistics with the formula: $(xi - \mu)/\sigma$, where xi represents the mean value in a sample, μ represents the population mean value (obtained from a serum pool of healthy subjects), and σ represents the population standard deviation [29]. The prooxidant score was calculated as the average of the Z-scores of the oxidative stress parameters (TOS and PAB), while the antioxidant score was calculated as the average of the z-scores of the antioxidant parameters (TAS, SHG and SOD). The summary oxy score was calculated as the difference between the prooxidative and antioxidative scores.

Statistical analysis of the results involved the Shapiro-Wilk test to assess the normality of distribution. The Wilcoxon paired test was used to compare redox parameters between serum samples treated with the BiVits® ACTIVA Recovery product, samples with individual compounds and trolox, under eustress and distress conditions.

All the parameters have been implemented and validated in our laboratory and have previously been published [24, 29].

Results and Discussion

Numerous studies have consistently demonstrated the protective effects of antioxidants derived from natural products in combatting non-communicable diseases characterised by redox imbalances, such as cardiovascular disease and cancer. As a result, it is reasonable to anticipate a significant focus on the development of an optimal combination of antioxidants. The objective is to create an ideal antioxidant mixture that can effectively address the underlying redox disturbances associated with many diseases [30]. An ideal antioxidant combination should be readily absorbable, stable in the internal environment, in sufficient doses to reach adequate biological activity and demonstrate strong synergistic effects. The utilisation of such a combination is recommended for patients with an urgent need for redox balance preservation during infections, in increased requirements or after the acute illnesses and post-recovery states [31-33].

In this article, the antioxidant properties of 1 complex product, containing antioxidants, vitamins and minerals, were studied and compared to the antioxidant properties of the product's individual components. The serum samples and tested compounds were incubated for 2 and 24 h in native serum and with exogenously induced oxidative stress by TBH.

Examination of the short-term and long-term BiVits® ACTIVA Recovery product effects and comparison with the individual components and trolox in eustress conditions

To investigate the immediate effect of the tested product, we evaluated the differences in serum redox status parameters between a serum sample treated with diluted BiVits® ACTIVA Recovery product and with its individual components (Zn, Se, Mn, NAC, vitamins C, D) and trolox after 2h incubation. The antioxidant values (TAS and SHG) were significantly higher in the serum samples treated

with the product than in those treated with individual components and trolox. A similar discrepancy was observed in the enzymatic activity of SOD, whereas there was a certain similarity between the effects of vitamins D, C and BiVits® ACTIVA Recovery product. As for oxidants, the PAB values were 2 to 3 times lower after treatment with BiVits® ACTIVA Recovery compared to the other treatments, and these differences were statistically significant. In the samples incubated for 2h, low concentrations of TOS were detected. The highest TOS concentration was found in the sample with trolox, followed by the samples treated with Zn, while the lowest concentration was observed with the BiVits® ACTIVA Recovery product. However, the statistical significance of the differences in TOS values was not established.

To investigate the long-term impact of the tested product, we analysed the differences in redox status parameters after 24h incubation at 37°C. Notably, the serum pool aliquots treated with BiVits Recovery® product exhibited significantly higher values of antioxidants (TAS and SOD) compared to the samples treated with individual components of the product and trolox. PAB values were significantly lower in samples treated with BiVits® ACTIVA Recovery than in samples with individual components. In the samples incubated for 24 h, low concentrations of TOS were determined, with the lowest concentration after treatment with the BiVits® ACTIVA Recovery product. However, this difference in TOS concentration was not statistically significant. Detailed results are presented in Table I.

Table I

Redox status parameters in samples with individual components and the original BiVits® ACTIVA Recovery after 2h and 24h incubation

Incubation time	**2h**					**24h**				
Sample	TAS	SOD	SHG	TOS	PAB	TAS	SOD	SHG	TOS	PAB
BiVits® ACTIVA Recovery (1)	1728 (1651 - 1728)	150 (149 - 169)	3.618 (2.139 - 4.923)	< 1.0	26 (25 - 28)	1713 (1642 - 1733)	148 (147 - 186)	0.301 (0.132 - 0.954)	3.4 (2.0 - 4.4)	26 (25 - 27)
Zn (2)	828*** (804 - 854)	121*** (119 - 123)	0.304*** (0.294 - 0.310)	4.5 (3.4 - 4.8)	77*** (71 - 81)	771*** (758 - 783)	90*** (88 - 94)	0.260 (0.226 - 0.302)	3.6 (3.3 - 5.4)	96*** (87 - 102)
Se (3)	844*** (826 - 861)	123*** (122 - 125)	0.336*** (0.325 - 0.339)	3.7 (2.3 - 4.1)	58*** (57 - 59)	778*** (759 - 792)	81*** (80 - 82)	0.236 (0.226 - 0.241)	3.5 (2.8 - 3.5)	75*** (74 - 76)
Mn (4)	846*** (837 - 873)	106*** (102 - 112)	0.306*** (0.278 - 0.332)	3.7 (3.3 - 3.8)	61*** (60 - 62)	818*** (796 - 823)	58*** (55 - 58)	0.130 (0.059 - 0.225)	7.7 (6.4 - 18.1)	80*** (79 - 82)
NAC (5)	858*** (846 - 866)	123*** (121 - 124)	0.382*** (0.365 - 0.393)	3.8 (3.7 - 3.9)	51*** (46 - 57)	786*** (748 - 805)	72*** (71 - 75)	0.243 (0.229 - 0271)	3.7 (3.6 - 3.8)	74*** (73 - 75)
Vitamin C (6)	832*** (807 - 843)	124 (122 - 126)	0.340*** (0.316 - 0.367)	3.9 (3.8 - 4.3)	59*** (57 - 60)	782*** (776 - 835)	90*** (85 - 98)	0.205 (0.193 - 0.213)	2.1 (1.7 - 3.1)	75*** (74 - 76)
Vitamin D (7)	901*** (870 - 950)	145 (144 - 146)	0.312*** (0.305 - 0.318)	3.5 (3.2 - 3.7)	54*** (49 - 56)	780*** (768 - 792)	84*** (83 - 106)	0.239 (0.216 - 0.283)	3.4 (3.1 - 3.6)	75*** (74 - 76)
Serum (8)	934*** (888 - 980)	124 (120 - 127)	0.384*** (0.373 - 0.396)	6.5 (6.4 - 6.6)	53*** (51 - 56)	964 (937 - 991)***	80 (73 - 88)	0.326 (0.323 - 0.328)	4.8* (4.0 - 5.0)	115*** (111 - 119)
Trolox	948*** (936 - 970) *#1, 2, 3, 4, 5, 6*	91*** (85 - 105) *#1, 7*	0.232*** (0.218 - 0.245) *#1, 5, 6, 8*	2.4 (1.8 - 3.5) *#1, 2, 4, 5, 6, 8*	50*** (27 - 60) *#2, 4*	868*** (836 - 915) *#1, 2, 3, 4, 5, 6, 7, 8*	91*** (88 - 95) *#1, 3, 4, 5*	0.242 (0.239 - 0.245) *#6, 8*	2.2 (1.9 - 3.3) *#2, 4, 5, 7, 8*	54*** (30 - 64) *#1, 2, 3, 4, 5, 6, 7, 8*

All samples: *p < 0.05, *** p < 0.001 *vs.* BiVits ACTIVA Recovery, Wilcoxon's paired test

For better evaluation of the antioxidant effect of the tested compounds, the prooxidant, antioxidant, and oxy score values were calculated and a comparative analysis in relation to the treatment administered was conducted (refer to Table II). The antioxidant score values exhibited a remarkable increase, ranging from 6 to 20 times, while the oxy score values were significantly lower in the samples treated with BiVits® ACTIVA Recovery product, compared to the other samples. Notably, the prooxidant score reached its lowest point 2 hours after the treatment with the product, and this value was significantly lower compared to the other treatments, except in the case of samples treated with vitamin D. It is important to note that the prooxidant score values were approximately half as low in samples incubated with the product compared to samples with vitamin D.

We also examined the changes in prooxidant, antioxidant, and Oxy scores in the treated samples. The antioxidant score values exhibited a substantial increase, ranging from 6 to 20 times higher, while the oxy score values (refer to Figure 2.) were lower in the sample treated with the BiVits® ACTIVA Recovery product compared to the other samples. Furthermore, the prooxidant score reached its lowest point 24 hours after treatment with the product, and this value was significantly lower compared to the other treatments, except in the case of incubation of the serum pool with trolox.

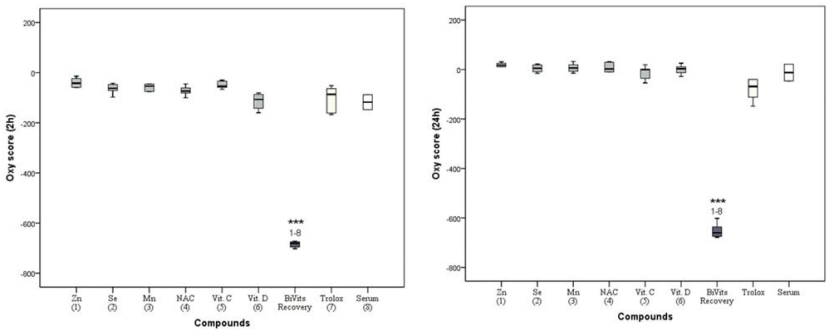

Figure 2.
Oxy scores in samples incubated at 37°C with distinct compounds for 2 h and 24 h
Zn (1): Zinc-citrate, Se (2): Selenomethionine, Mn (3): Manganese-gluconate, NAC (4): N-Acetylcysteine, Vit. C (5): L-ascorbic acid, Vit. D (6): Cholecalciferol.
***p < 0.001 *vs.* components' number in parenthesis.

Examination of the short-term and long-term effect of the BiVits ACTIVA Recovery product compared to individual components after exposure to exogenous oxidants

Aliquots of the serum pool were incubated with a combination of TBH and BiVits® ACTIVA Recovery product or TBH and product`s individual components, to evaluate the antioxidant effects under conditions of induced oxidative stress. After the incubation, TAS values were significantly elevated in the samples treated with the combination of TBH and BiVits® ACTIVA Recovery product, compared to the results of serum exposed to the combination of each individual component of the product and TBH. However, no significant changes were observed in the SOD and SHG values in samples treated with the product and TBH compared to the samples treated with TBH and individual components, except for the samples with Mn and NAC. These samples exhibited a decrease in SOD and SHG values compared to the samples without TBH, but also to the samples treated with the product and TBH combination. PAB values were several times lower in the sample simultaneously treated with the product and TBH in contrast to the other treatments. The increase in the number of SH groups indicates a successful synergistic effect of the recovery ingredients. Although NAC is known to act as a radical scavenger, a reductant of disulfide bonds, and a precursor for glutathione biosynthesis, our results demonstrate that its effectiveness is significantly enhanced in the presence of other recovery components. Investigating the detailed mechanisms behind this synergistic effect could present an exciting challenge for future research. Additionally, it would be valuable to test the antioxidant activity of various combinations of the recovery ingredients and compare their effectiveness.

The serum pool aliquots were incubated for 24 h with a combination of TBH and the product and with TBH and product`s individual components. This experimental setup aimed to assess the antioxidant effects under the conditions of increased and prolonged exogenous oxidative stress. Values of antioxidants TAS and SOD were significantly higher in the sample incubated with the product. SHG values were decreased both in samples incubated with the product and with individual components upon the TBH exposure, as compared to the native serum samples. On the other hand, SHG values in samples with BiVits® ACTIVA Recovery product were significantly higher when compared to the samples treated with Mn. Moreover, samples incubated with Mn for 24h showed significant increase in TOS, compared to all other samples, including ones treated with the BiVits® ACTIVA Recovery product. It is worth mentioning that even in the TBH sample, a decrease in TOS was observed after the 24 h incubation at 37°C. This could be explained by spontaneous balance reestablishment, which may occur in biological material like serum. On the contrary, PAB value remained increased after the 24 h incubation at 37°C in all samples, except for the sample with BiVits® ACTIVA Recovery product. This product successfully managed to lower prooxidants' concentration measured by PAB test (Table III).

Table II

Prooxidant, antioxidant and oxy score in samples incubated with individual components and the samples with original BiVits Recovery® product (2 h and 24 h incubation)

Incubation time	**2h**			**24h**		
Sample	Prooxidant score	Antioxidant Score	Oxy score	Prooxidant score	Antioxidant score	Oxy score
BiVits Recovery®**(1)**	-33.1 (-33.2 - -32.3)	648 (642 - 662)	-681 (-695 - -675)	-28.0 (-31.0 - - 27.1)	632 (605 - 645)	-660 (-647 - -636)
Zn (2)	-7.3*** (-8.6 - -5.5)	33*** (18 - 50)	-42*** (-59 - -23)	-0.9*** (-4.1 - 5.7)	-14*** (-24 - -6)	17*** (11 - 25)
Se (3)	-15.1* (-18.3 - -14.4)	46*** (33 - 56)	-62*** (-71 - -47)	-9.1** (-10.4 - - 8.6)	-14*** (-27 - - 3)	5*** (-9 - 18)
Mn (4)	-14.1* (-15.1 - -13.6)	40*** (33 - 61)	-54*** (-75 - -46)	1.4*** (-1.5 - 22.2)	2*** (-10 - 5)	6*** (-7 - 19)
NAC (5)	-18.5 (-19.8 - - 16.0)	55*** (44 - 61)	-73*** (-81 - -61)	-8.6** (-9.3 - -8.4)	-11*** (-37 - 1)	2*** (-9 - 29)
Vitamin C (6)	-14.2* (-15.0 - -13.6)	38*** (19 - 44)	-54*** (-59 - - 34)	-11.2** (-12.5 - -9.4)	-10*** (-12 - 29)	-1*** (-35 - 1)
Vitamin D3 (7)	-17.4 (-19.1 - -16.2)	90*** (70 - 123)	-107*** (-142 - -87)	-9.1** (-9.4 - - 8.4)	-12*** (-20 - 2)	3*** (-12 - 11)
Serum	15.9*** (9.3 - 27.3)	102*** (91 - 133)	-86*** (-161 - -64)	-18.8 (-30.8 - - 15.7)	-7.0*** (-41 - 27)	-12*** (-46 - 21)
Trolox	-11.6*** (-12.9 - -10.5) *#1-7*	106*** (75 - 137) *#1 - 6*	-117*** (-147 - -88) *#1*	-19.2 (-19.6 - - 18.9) *#2, 4*	50*** (27 - 81) *#1 - 7*	-68*** (-112 - -40) *#1*

All samples: *** p<0.001 *vs.* BiVits Recovery® # p<0.05 Trolox *vs.* distinct components (signed with number in parenthesis), Wilcoxon's paired test

Prooxidant, antioxidant and oxy scores (Table IV) for obtained data set were calculated. Prooxidant scores were significantly decreased in the samples treated the BiVits® ACTIVA Recovery product compared to the samples treated with individual components. Prooxidant score in the samples treated with individual components were slightly increased (not statistically significant) compared to the serum samples treated with TBH. The Oxy score values in the samples with BiVits® ACTIVA Recovery product were more than ten times lower compared to the samples treated with individual components (Figure 3). The antioxidant score was several times higher in the samples with the product in comparison to the samples treated with individual components and TBH. Prooxidant, antioxidant and oxy scores (Table IV) were investigated after 24h incubation with product and its individual components in conditions of exogenously induced oxidative stress. After incubation with the BiVits® ACTIVA Recovery product, a significant decrease in the oxy score was observed in comparison to the samples treated with individual components or with TBH (Figure 3).

A similar change was found in the case of the prooxidant score, in vitamin C samples affecting this parameter to the same extent as the BiVits® ACTIVA Recovery product. Interestingly, Zn and Mn samples had positive prooxidant score, which was even significantly higher in the case of Mn compared to TBH sample. The antioxidant score was several times higher in the sample treated with the product, compared to all the other samples. The sum of median values of antioxidant scores of individual components (2 h incubation) was 331 while for the BiVits® ACTIVA Recovery product was 612. The same findings were noticed for 24 h incubation: the sum of median antioxidant score values for individual components reached 456, while the antioxidant score value in BiVits® ACTIVA Recovery product samples was 641. These results imply the presence of synergistic effects rather than simple additive effects among the 6 compounds.

The oxy score was the lowest in the samples treated with BiVits® ACTIVA Recovery after 2h. The OS was ranked in the following order: samples with trolox, vitamin D, NAC, and Se. After treatment with vitamin C and Mn, the OS was found to be equal, while the highest OS was observed after treatment with Zn (Figure 2).

Low negative OS for the samples incubated for 2h with vitamin C, NAC and trolox indicates good antioxidant activity of these components. Oxy scores for samples incubated with Zn, Se and Mn were around zero (Figure 2). Although these components do not have direct redox properties by themselves, they have a great influence on other antioxidants, such as GSH, metallothionein and SOD [7, 35]. Some metal complexes can show reactivity toward free radicals. It is known that their reactivity can be significantly changed in the presence of vitamins C and E [36]. Selenium compounds can reduce peroxides and have a great influence on the release of zinc by metallothionein [22, 34, 37]. However, the lowest OS value was calculated for the sample with BiVits® ACTIVA Recovery, which possesses the strongest antioxidant effect.

Table III

Redox status parameters in the samples with individual components and with BiVits® ACTIVA Recovery, after 2 h and 24 h incubation in the presence of TBH

Incubation time	**2h**					**24h**				
Sample	TAS	SOD	SHG	TOS	PAB	TAS	SOD	SHG	TOS	PAB
BiVits® ACTIVA Recovery +TBH (1)	1715 (1713 - 1716)	84 (80 - 102)	0.257 (0.225 - 1.936)	< 1	29 (28 - 30)	1728 (1681 - 1733)	149 (146 - 150)	0.130 (0.112 - 0.202)	4.4 (3.1 - 11.7)	25 (24 - 26)
Zn+TBH (2)	890*** (878 - 906)	88 (86 - 94)	0.172 (0.164 - 0.184)	31*** (29 - 31)	127*** (124 - 128)	936*** (892 - 967)	95*** (91 - 106)	0.147 (0.126 - 0.151)	7.6 (6.1 - 9.4)	86*** (81 - 91)
Se+TBH (3)	878*** (874 - 897)	94 (59 - 99)	0.161 (0.155 - 0.164)	28.0*** (26.6 - 29.3)	120*** (119 - 121)	918*** (898 - 955)	87*** (85 - 89)	0.108 (0.096 - 0.139)	5.5 (5.2 - 5.6)	80*** (79 - 81)
Mn+TBH (4)	893*** (866 - 925)	54** (52 - 73)	0.158 (0.141 - 0.181)	27.6*** (26.3 - 28.7)	120*** (119 - 121)	896*** (887 - 955)	79*** (78 - 81)	0.120 (0.103 - 0.149)	18.6 (9.5 - 22.7)	83*** (79 - 84)
NAC+TBH (5)	893*** (890 - 905)	64* (62 - 66)	0.172 (0.151 - 0.196)	24.6*** (21.7 - 27.1)	117*** (115 - 120)	897*** (894 - 910)	88*** (84 - 89)	0.136 (0.113 - 0.162)	4.8 (4.6 - 4.9)	79*** (78 - 80)
Vitamin C+TBH (6)	879*** (832 - 895)	112 (101 - 126)	0.180 (0.162 - 0.190)	26.1*** (25.9 - 27.5)	116*** (115 - 120)	928*** (915 - 951)	86*** (83 - 87)	0.129 (0.122 - 0.138)	3.6 (3.4 - 3.7)	78*** (77 - 79)
Vitamin D+TBH (7)	861*** (855 - 895)	98 (96 - 99)	0.180 (0.170 - 0.181)	27.0*** (23.9 - 29.3)	122*** (120 - 122)	894*** (890 - 904)	88*** (86 - 95)	0.148 (0.110 - 0.154)	4.5 (4.1 - 4.9)	76*** (75 - 80)
Serum+TBH	916*** (901 - 931) *#1, 6*	81 (76 - 87) *#4, 5, 6, 7*	0.209 (0.168 - 0.251) *#ns*	20.2*** (18.9 - 21.6) *#1, 3, 4, 6*	120*** (119 - 121) *#1, 2*	906*** (892 - 921) *#1*	97*** (87 - 107) *#1, 4, 6*	0.150 (0.106 - 0.194) *#ns*	4.1 (3.8 - 4.4) *#2, 3, 4*	82*** (80 - 84) *#1, 3, 5, 6*

All samples: *** $p < 0.001$ *vs*. BiVits® ACTIVA Recovery; # $p < 0.05$ TBH *vs*. distinct components (signed with number in parenthesis), ns-nonsignificant

Table IV

Prooxidant, antioxidant and oxy score in samples incubated with individual components and samples with BiVits® ACTIVA Recovery product, in the presence of TBH (2 h and 24 h incubation)

Incubation time	**2h**			**24h**		
Sample	Prooxidant score	Antioxidant score	Oxy score	Prooxidant score	Antioxidant score	Oxy score
BiVits Recovery® (1)	-31.8 (-32.0 - - 31.6)	612 (609 - 634)	-644 (-666 - -641)	-26.8 (-29.3 - - 12.9)	641 (609 - 644)	-652 (-675 - -637)
Zn (2)	63.5*** (61.4 - 65.2)	65*** (54 - 75)	-1*** (-12 - 9)	3.4*** (-1.3 - 8.8)	96 (66 - 119)	-93*** (-109 - -68)
Se (3)	55.5*** (54.1 - 59.5)	57*** (42 - 70)	0*** (-16 - 11)	-3.1** (-3.7 - -2.9)	80*** (67 - 104)	-83*** (-107 - -70)
Mn (4)	55.3*** (53.3 - 57.7)	55*** (41 - 69)	0*** (-18 - 17)	23.7*** (4.1 - 30.1)	63*** (57 - 79)	-57*** (-64 - -33)
NAC (5)	47.5*** (42.6 - 56.7)	56*** (54 - 64)	-12*** (-17 - 0)	-4.6** (-5.4 - - 4.4)	67*** (63 - 76)	-71*** (-81 - -69)
Vitamin C (6)	52.5*** (50.3 - 57.6)	63*** (31 - 74)	-8*** (-23 - 17)	-7.6 (-9.2 - -7.1)	85*** (78 - 101)	-93*** (-111 - -84)
Vitamin D3 (7)	54.5*** (49.3 - 59.9)	46*** (42 - 69)	7*** (-12 - 14)	-5.7** (-7.9 - - 5.6)	65*** (64 - 70)	-71*** (-76 - -65)
Serum	9.5*** (6.6 - 12.5)	111*** (90 - 131)	-101*** (-119 - -84)	-9.2 (-10.3 - - 8.1)	100*** (97 - 104)	-110*** (-112 - -107)
TBH	41.2*** (38.7 - 43.8) *#1*	78*** (70 - 85) *#1*	-36*** (-42 - -31) *#1*	-4.8** (-5.4 - - 4.3) *#4*	76*** (70 - 82) *#1*	-80*** (-86 - -75) *#1*

All samples: *** $p < 0.001$ *vs*. BiVits® ACTIVA Recovery # $p < 0.05$ TBH *vs*. distinct components (signed with number in parenthesis), Wilcoxon's paired test

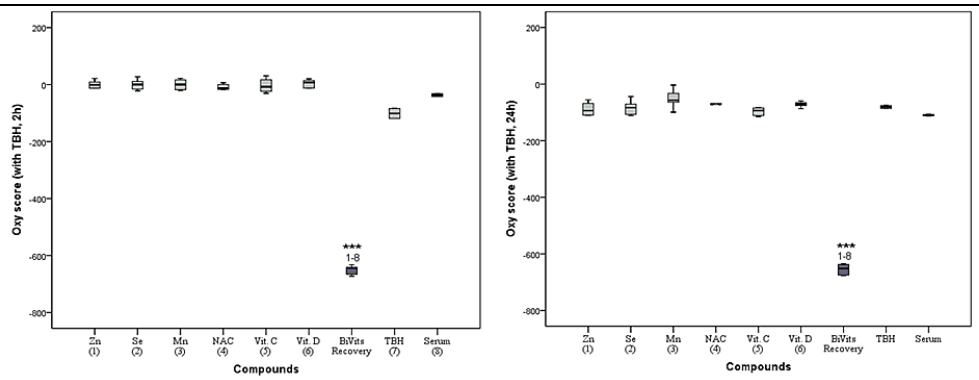

Figure 3.

Oxy scores in samples incubated at 37°C with distinct compounds for 2h, 24h, with TBH

Zn (1): Zinc-citrate, Se (2): Selenomethionine, Mn (3): Manganese-gluconate, NAC (4): N-Acetylcysteine, Vit. C (5): L-ascorbic acid, Vit. D (6): Cholecalciferol.

***p < 0.001 *vs.* components' number in parenthesis

After 24 hours of incubation, samples containing Zn, Mn, NAC and vitamin D exhibited positive OS values (Figure 2), indicating a transition in their redox properties from antioxidant to prooxidant over prolonged incubation. A possible explanation for this phenomenon is the binding of manganese to plasma proteins, particularly transferrin. Research has shown that Mn^{2+} must undergo oxidation to Mn^{3+} before it can bind to the transferrin. The maximum binding of manganese to transferrin is achieved after 12 hours, which could account for the increase in OS value observed after 24 h [38]. Additionally, vitamin D has been found to possess both antioxidant and prooxidant properties. Some studies have shown a relation between vitamin D and increased activity of some prooxidants [39, 40]. Halliwell proposed that certain antioxidants induce endogenous antioxidant protection through their prooxidant activities [41].

Following a 24-hour incubation period, vitamin C exhibited diminished antioxidant activity, as indicated by a calculated OS value that was negative but close to zero (Figure 2). This decrease in antioxidant properties aligns with previous findings demonstrating a significant decrease in the stability of vitamin C in serum and plasma after 24 h [42]. Conversely, trolox still displayed antioxidant properties even after 24h, albeit significantly weaker, (24 h *vs.* 2 h OS: -68 (-112 - -40) *vs.* -117, (-147 - -88), respectively). Interestingly, the antioxidant activity of BiVits® ACTIVA Recovery remained almost unchanged (24 h *vs.* 2 h OS: -660 ((-647 - -636) *vs.* -681 (-695- -675), respectively). This suggests that the synergistic effect exhibited by the multivitamin complex persists even after 24 h of incubation.

The impact of other antioxidant enzymes becomes more significant in the presence of externally applied oxidative stress, where the extended presence of essential ions (such as Mn, Se and Zn) plays a role in their functional effectiveness. Selenium, as an integral component of glutathione peroxidase, plays a crucial role in enhancing resistance against oxidative stress [43].

The antioxidative properties of all compounds were assessed in the presence of TBH, indicating their potential to withstand "circulation-born" oxidative stress. Under this condition, all compounds, except vitamin D, exhibited negative (NAC, Zn, vitamin C) or close to zero (Se, Mn) OS values after a 2 h incubation period (Figure 3). After 24 h of incubation, these OS values for all tested compounds became significantly negative (with OS median values ranging from -93 for vitamin C to -57 for Mn) (Figure 3). NAC is not a typical direct radical scavenger, as its reaction rates with most free radical species are relatively slow. However, NAC has an impact on increasing the levels of glutathione (GSH) and can help regenerate oxidised protein thiol (-SH) groups, thereby enhancing the scavenging potency of other reducing substances present in the serum. This property makes the environment more effective in combating oxidative stress, which explains, at least partially, the low OS values observed for NAC after 24 h in the presence of TBH [16, 44]. Vitamin D does not act as a direct scavenger of radicals, which explains the loss of antioxidant properties in the presence of TBH after 2 h incubation. On the other hand, it has a great influence on the reduction of lipid peroxidation and on the activity of antioxidant enzymes, which can be expressed after 24 h of incubation [45].

A similar pattern can be observed with the SOD (superoxide dismutase) activity, one of the enzymatic antioxidants that was measured in this study. Both zinc (Zn) and manganese (Mn) are essential components of the 3 isoforms of SOD. Zn is found in the active centre of cytosolic and extracellular SOD, while Mn is a part of the mitochondrial isoform [46]. Previous research, such as the study conducted by Coudray [47] already reported the enhancement of *in vitro* SOD activity in the presence of zinc salts, which

supports our findings regarding the low OS values (Figure 3).

The estimation of the exact potency of antioxidant compounds *in vivo* poses a challenge due to the lack of suitable biomarkers that can accurately correlate oxidative damage with the therapeutic effects of isolated compounds. In our *ex vivo* platform, we provide a controlled environment where the compounds come into contact with proteins, lipids, and other biomolecules that are regular constituents of the biological matrix, such as the serum pool used in our analysis. Of particular interest is the interaction between proteins and the dissolved compounds, given the abundance of proteins in serum, their high binding capacity, and the presence of reducing groups [48]. Additionally, the lipid components within lipoprotein particles present in serum should not be overlooked. Lipid biomolecules consist of various components that are susceptible to oxidation, but they also contain specific enzymes like paraoxonase-1, which is responsible for antioxidant protection across all lipid classes [49].

The sum of individual components' antioxidant scores is far smaller than antioxidant scores of BiVits Recovery® both after 2 h and 24 h of incubation which additionally supports the findings of synergistic effect of the components.

Interestingly, we did not observe a significant influence of the concentration of BiVits® ACTIVA Recovery product, indicating that even a 10% concentration is equally effective as a 100% concentration. This finding is important considering that achieving a hypothetical 100% concentration is not feasible under real conditions, taking into account factors such as tablet absorption in the gastrointestinal tract and subsequent metabolism after entering the circulation. Therefore, the use of the lowest, 10% concentration is more representative and aligns with the conditions present in the organism.

Conclusions

Both short-term (2h) and long-term (24h) incubation of BiVits® ACTIVA Recovery product in a human serum pool led to a significant increase in antioxidants and a significant decrease in oxidants, compared to the individual components of this product. The same pattern exists in conditions with the increased exogenous oxidants' load. The results of this study show that after a prolonged incubation at 37°C, the redox balance is established, favouring the antioxidants present in the sample. When exposed to prooxidants, the activation of endogenous antioxidant protection occurs, but the presence of BiVits® ACTIVA Recovery product aids in faster restoration of the redox balance by buffering or neutralising the prooxidants. The obtained *ex vivo* results are encouraging, and further *in vivo* studies are needed to prove that the observed synergistic effect of individual components can be achieved after the supplementation with BiVits® ACTIVA Recovery.

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Conflict of interest

The study was partially funded by Abela Pharm d. o. o. Belgrade, Serbia.

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