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Comparative assessment of antioxidant and membrane-protective properties of medicinal plant extracts

Abstract: Medicinal plants are a valuable raw material for obtaining phytodrugs with a wide range of pharmacological and therapeutic effects, which are acting fast, do not possess cumulative properties and are less accompanied by undesirable side effects. Modern assessment of the species diversity of the medicinal flora and its resource potential is especially important in designing an effective system that fights diseases caused by oxidative stress. Thus, the purpose of scientific research is to study and establish a list of medicinal plant extracts with strongest antioxidant and membrane-protective effect on liver cells exposed to oxidative stress. Determination of medicinal plant extracts that exhibit highest antioxidant and membrane-protective activities will allow us to use it in creating of an effective phytocomposition that would inhibit the action of lipid peroxidation (LPO) and osmotic stress, which lead to excessive damage of cell constituents and development of oxidative stress. For this purpose, LPO levels were detected by measuring malondialdehyde concentration in the liver microsomes whereas the osmotic hemolysis was measured in a hypotonic solution of 0.4% NaCl followed by optical density measurement at 540 nm. It was established that LPO levels in liver microsomes as well as osmotic fragility of erythrocyte membranes are plant extract dose-dependent. As a result, 3 out of 9 plant extracts have significant membrane-protective properties. Plants belong to the families: *Urtica dioica, Viola tricolor, Vacciniumvitis.* In addition*, Valeriana officinalis* (leaves), *Viola tricolor, Limonium gmelini, Vaccinium vitis, Hypericumper foratum and Capsella bursa-pastoris* showed the strongest antioxidant property. All plant extracts contained such biologically active compounds as polyphenols and flavonoids. Our results suggest further study of the antioxidant and membrane-protective effects of medicinal plant extracts tested here may guide phytotherapy to development of new dosage forms, and advance the development of unconventional therapeutic and preventive approaches against oxidative stress related diseases.

Key words: antioxidant, free radicals, lipid peroxidation, osmotic hemolysis, erythrocyte membranes, liver microsomes, plant extracts.

Introduction

Oxidative stress is the result of a disturbed balance in oxidant and antioxidant system, which appears from incessant rise of reactive oxygen species production [1]. That is the well-established balance between antioxidants and the pro-oxidants, reactive oxygen species, could be disturbed by exposure to physical, chemical or microbial toxic agents. Continued oxidative stress can result in many age-related diseases and altered lipid peroxidation may also be a producer of pigments and lipofuscins (oxidized insoluble parts of proteins, lipids and carbohydrates) [2]. Thus, accumulation in a large number of amphiphiles is accompanied by a massive introduction of them into membranes, which, like an excess of lipid hydroperoxides, leads to the formation of clusters and micro-ruptures in them. Damage to membranes and cell enzymes is one of the main causes of a significant disorder in the vital activity of cells and often leads to their death [3]. Biological membranes perform many functions, a violation of any of which can lead to a change in the vital activity of the cell as a whole and even to its death. For some unfavorable factors (stress, radiation: radiation, UV, X-ray, etc., food contamination and the environment, the effects of certain medicines and medicines procedures, severe overheating or cooling, excessive physical load, etc.) the system of antioxidant protection does not cope, and the reactionary oxygen and nitrogen com-

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pounds and free radicals begin to damage vital DNA molecules, proteins and lipids [4]. Biological membranes contain a large number of unsaturated fatty acids, metalloproteins, activating molecular oxygen. Therefore, it is not surprising that lipid peroxidation processes can develop in them [5]. One of the products of this reaction is lipid hydroperoxide - a relatively stable compound. In addition, a peroxide radical can form a lipid endoperoxide radical, the decomposition of which leads to the formation of a number of products, including malonic dialdehyde [5]. Polyunsaturated fatty acids, both in the free form and in the lipids, can undergo spontaneous peroxidation, which proceeds at a rather high speed in lipid films and solutions, homogeneous systems, as well as in aqueous media where lipids form liposomes and films, systems with different phases [6]. Free radicals change the permeability (and hence the barrier function) of cytoplasmic membranes in connection with the formation of channels of increased permeability, which leads to disruption of the water-ion homeostasis of the cell [7]. These radicals are particularly active in interacting with membrane lipids, containing unsaturated bonds, which leads to violation of many exchange processes. Peroxide oxidation of membrane phospholipids is one of the most common mechanisms of destruction of membrane structures, it is recorded during the development of a number of pathological conditions [8]. Nevertheless, the processes of lipid peroxidation take place in a normal cell. They are regulated by a number of enzymes: NADP(H) - dependent microsomal oxygenases, cyclooxygenases and lipoxygenases. Phospholipids in native membrane systems are effectively protected from non-enzymatic peroxidation by the presence of antioxidants in biomembranes, by the structural organization of membranes, and by special enzymatic systems regulating the concentrations in the membrane of reactive oxygen species inhibiting lipo-oxidation development [9]. Products of lipid peroxidation are precursors of the synthesis of prostaglandins, thromboxanes, prostacyclin, leukotrienes and lipoxins [10]. The most active free radicals break bonds in molecules DNA, thereby damaging the genetic apparatus of cells regulating their growth, that can lead to oncological diseases [11]. Low-density lipoproteins after oxidation by free radicals easily deposited on the walls of the vessels, leading to the accelerated development of atherosclerosis and cardiovascular diseases. At present, it is shown that hundreds of diseases are preceded by an oxidative stress [12].Therefore, the modern assessment of the species diversity of the medicinal flora and its resource potential is especially important in designing an effective system that fights diseases caused by oxidative stress.

Medicinal plants are a valuable raw material for obtaining phytodrugs with a wide range of pharmacological and therapeutic effects, which are acting fast, do not possess cumulative properties and are less accompanied by undesirable side effects. Also, they are the source of a variety of medicines. Currently, about 40% of all medicines are obtained from plants [13]. Medicinal plants are valuable because of they are source of bioactive compounds possessing potential biological and physiological effects [14; 15]. One group of substances is herbal polyphenols. These compounds are secondary plant metabolites, which done functions of defense in plant cells. However, it was proved various benefit influence of them on human organism cells in vitro and in vivo studies. Multisided effects of plant polyphenols make them perspective object in medicinal researches and elaborations. There was observed expressed polyphenolic compounds' antioxidative properties out of the dependence in structure and group, which cause their positive effect at disorders characterized by developing oxidative stress [16-18].

Actively developing research in the fields of biochemistry of natural compounds and phytotherapy constantly increases the number of discovered medicinal species that have high antioxidant effect on mammalian cells. However, many species as a result of haphazard collection have become rare, such as Rhodiola rosea, peony Maryin root, Ural licorice, marjoram, etc. The state of populations of many medicinal plants is alarming, therefore strict control over their collection is necessary [19].

For the formation of a stable raw material base of the domestic pharmaceutical industry and the creation of new phytoextracts, the study of pharmacopoeial and perspective medicinal plant species found in regions of Kazakhstan is one of the goals of this research. However, the main objective of this work is to study and establish a list of medicinal plant extracts with strongest antioxidant and membrane-protective effect on male white rats liver cells exposed to oxidative stress. The purpose of this scientific research is to determine medicinal plant extracts with strongest antioxidant and membrane-protective effect on liver microsomes and erythrocyte membranes respectively.

Materials and methods

Medicinal plant extracts collected in the Almaty region, Kazakhstan. Each plant was dried and weigh-

ing 1 g was milled and powdered and then placed in 10 ml of 50% ethanol solution. The mixture was left for 20 hours in the dark place. After, the mixture had undergone centrifugation at a speed of 20,000×g for 10 min. The supernatant then was left in rotary evaporator until it is completely dry. It should be noted that all solutions with dissolved dried extracts in them (100 mg/ml) were freshly prepared in 50% ethanol prior to experiments.

For experiments was chosen healthy male white rats weight 300-350 g. Animals were kept under standard conditions that include normal light and dark cycle and free access to food and water. The blood and liver tissue were collected according to the experimental protocols approved by the Committee for the Ethical Care and Use of Animals in Experiments.

Preparation of rat liver microsomes. In the experiment rats were initially sedated by administering isoflurane anesthesia and then physically euthanized by using cervical dislocation technique. The rat livers were isolated and collected, washed, and sprayed with cold saline solution. Liver tissue was minced and homogenized (1:10 w/v) in 10mM solution of potassium phosphate buffer with pH 7.4 and containing 1mM EDTA placing a mixture on ice. After the homogenate was centrifuged at a speed of 10,000×g for 20 min under a temperature of 4 °C. The obtained supernatant was further centrifuged at a speed of 100,000×g, for 60 min, after which we obtained the microsomal part of the liver. Microsomes were carefully isolated and suspended in a buffer containing 10mM histidine with pH 7.2, 25% (v/v) glycerol, 0.1mM EDTA and 0.2mM $CaCl_2$, and were stored under a temperature of 20°C. Moreover, the protein content was determined by the Lowry assay using bovine serum albumin as a standard.

Isolation of erythrocytes. The rats were put under isoflurane anesthesia, then were euthanized and the blood through cardiac puncture was collected. Then blood was centrifuged at a speed of 1000×g, for 10 min, after which white blood cells were removed. Erythrocyte pellets were washed twice with 5mM Na_2HPO_4 (pH 7.4) and 150mM NaCl buffer, and immediately undergone osmotic resistance tests.

Assessment of microsomal lipid peroxidation. An assessment of LPO was held by measuring the presence of malondialdehyde in the form of thiobarbituric acid-reacting substances (TBARS) [20]. Cincisely, liver microsomes were preincubated together with test agents in 50mM KH_2PO_4 (pH 7.2) and 145mM NaCl buffer at a temperature of 37 °C, for 10 min constantly stirred. 0.02mM Fe^{2+} and 0.5mM ascorbic acid-induced microsomal LPO along with basal was then established in a reaction mixture with added 0.4% SDS, 20mM thiobarbituric acid and 0.9M sodium acetate buffer with pH 3.5, followed by incubation at a temperature of 95 °C for full 60 min [21]. After a mixture was cooled down at room temperature, the mixture was washed with n-butanol: pyridine solution with ratio of 15:1, v/v and centrifuged at a speed of 3,000×g, for full 5 min. After collecting organic layer of the analyzing mixture, it is absorbance was measured at 532 nm by PD-303UV spectrophotometer. The MDA concentration is represented in nmol of TBARS per mg protein.

Assessment of osmotic resistance of erythrocytes. Osmotic resistance of erythrocytes was measured as it is done in the in vitro studies. Preincubated at 37°C for 10 min isolated erythrocytes with vehicle/test agents were placed into a hypotonic 0.4% NaCl solution at 37°C, for 20 min, after which the mixture had undergone centrifugation. An absorbance of hemoglobin in the supernatant was determined by spectrophotometer at 540 nm. In the experiment with rats, a level of hemolysis of erythrocytes induced by 0.4% NaCl solution was determined and measured directly not performing preincubation. The level of hemolysis was expressed in percentage of overall hemolysis caused by 0.1% Na_2CO_3 solution.

Assessment of total phenolic and flavonoid content. The amount of total phenolics in the extracts was measured using the Folin-Ciocalteu reagent method [22]. The 0.5ml of each extract (l.0 mg/ml) was added into test tubes containing 2.5 ml of 10% Folin-Ciocalteu reagent and 2.0 ml of 2% sodium carbonate solution and the tubes were shaken thoroughly. The mixture was incubated at 45°C for 15 min with intermittent shaking. Absorbance was measured at 765 nm using a PD 303 UV-Vis spectrophotometer (Shimadzu, Japan). Gallic acid was used as a standard to obtain a calibration curve (ranging from 0 to 1 mg/ml). The results were expressed in percent per mg dry extract.

The total flavonoid contents were determined according to colorimetric assay [23], with rutin as standard. The 0.5 ml of each extract (l.0 mg/ml) were mixed with 2 ml of distilled water and 150 µl of 5% sodium nitrate. After 6 min, 150 µl of 10 % aluminum chloride and 2 ml of 1 M sodium hydroxide were added and left at room temperature for 15 min. Absorbance of the mixtures was measured at 510 nm. The calibration curve was prepared in the same manner using 0-1.0 mg/ml of rutoside solutions in methanol. The results were expressed in percent per mg dry extract.

Statistical data analysis. Results are statistically processed using the program Microsoft Excel. Given the Fisher-Student performance recorded changes were considered significant at $p \leq 0.05$.

Results and discussion

Herbal extracts were able to decrease erythrocyte hemolysis, however, their anti-hemolytic effect is observed on different levels (Table 1).

As represented in Table 1, the nettle and violet's extracts possess the best membraneprotective qualities. Plant ethanolic extracts authentically decreased erythrocyte hemolysis in concentration range from 0.005-0.1 mg/ml. In concentration 0.1 mg/ml fragility of erythrocytes accordingly lowed up to 36.9%±1.3 and 41.0%±2.04. Antihemolytic action of plantain comes out dose-dependent.

Phytoextracts of hypericum plant, shepherd's grass and valeriana leaves occurred unsignificant influence on erythrocyte hemolysis at 0.5 mg/ml concentration – hemolysis level consists 98.6%, 95.2% and 94.1%. Neverthless in concentration upper 0.01 mg/ml there is observed strengthening of valeriana leaves and hypericum exracts protective effect on erythrocyte membranes. Shepherd's grass extracts do not change hemolysis level in concentration 0.01 mg/ml notably, but action of extract in concentrations higher than 0.05 mg/ml there was found out significant enlarging of erythrocyte membrane resistance. Comparison of valeriana roots and leaves showed that leaves' membrane protective effect is better. Thus, there are foundations to make conclusions about more effective applying of valeriana leaves to decreasing memrane fragility than using plant roots.

Table 1 – Influence of herbal extracts on erythrocyte membrane fragility

Extract name	Extract concentration (mg dry extract/ml IM)				
	0	0.005	0.01	0.05	0.1
Limonnium gmelinii	100	74.0±3.68	70.6±3.51	60.9±3.34	56.7±2.82
Plantain – Plantago major	100	73.6±3.67	66.7±3.3	66.0±3.28	61.4±3.05
Valeriana officinalis (leaves)	100	94.1±4.68	87.7±4.37	74.6±3.72	69.9±3.4
Valeriana officinalis (roots)	100	107.1±5.3	100.7±5.0	87.6±4.37	87.0±4.3
Hypericum – Hypericum perforatum	100	98.6± 4.9	78.6±3.91	61.4±3.06	51.1±2.5
Nettle – Urtica dioica	100	89.0±44.4	54.4±2.7	50.9±2.53	36.9±1.3
Lingonberry – Vaccinum vitis	100	65.4±3.25	61.8±3.08	58.8±2.92	46.8±2.3
Violet – Viola tricolor	100	59.1±2.85	58.4±2.90	49.4±2.46	41.0±2.04
Shepherd's grass – Capsella bursa-pastoris	100	95.2±47.5	92.7±4.6	81.2±4.03	72.1±3.59

Analysis of the results of the research showed that not all plant extracts possess a membrane-stabilizing property and reduce the hemolysis of red blood cells; moreover, roots of *Valeriana officinalis* had a damaging hemolytic effect on the membranes of red blood cells. Most of the extracts showed a great change in stability of erythrocyte membranes. However, only three extracts showed the lowest degree of hemolysis: *Urtica dioica, Viola tricolor, Vaccinium vitis* show the lowest intensity of hemolysis.

The results of the study of the influence of the 9 different plant extracts on the processes of LPO in liver microsomes showed that the extracts of these plants are strong antioxidants, which is confirmed by inhibition of the formation of TBA active products.

Studies of herbal extracts' influence on peroxidation processes in liver microsomes resulted that *Valeriana, Lingon berries* leaves, *Hypericum* and *Violet* herbs possess high-expressive antioxidative properties (Table 2), named extracts totally inhibited MDA generation in concentrations from 0.25 mg to 2.5 mg per 1 mg protein.

Nettle and *Limonium* extracts inhibited LPO product's generation in concentrations from 0.5 to 2.5 mg on 70% and 75%. Increasing of *Limonium* concentration do not lead to change in level of TBARS in incubation medium, as in case of nettle extract, enlarging

concentrations resulted to total suppressing of MDA generation. Total inhibition of LPO processes by shepherd's grass extract was occurs in concentrations upper than 1.25 mg. Data which were got at researches of valeriana roots and plantain extracts are differ from previous results. These herbal extracts showed prooxidative action increasing the intensity of lipoperoxidation on 23.8% and 2.3% in first researched concentrations. Neverthless, there was observed increasing of antioxidative effect of *Valeriana* root's extract in accordance with enlarging doses upper than 0.5 mg, plantain extract came out high inhibiting action on forming of lypoperoxides in 2.5 mg concentration.

Table 2 –Influence of herbal extracts on LPO level in hepatocytes

Exract name	Extract concentration (mg dry extract/mg protein)				
	0	0.25	0.5	1.25	2.5
Limonnium gmelini	100	24.4±1.2	18.3±0.91	18.0±0.9	16.5±0.82
Plantain - Plantago major	100	112.3±5.1	99.1±4.98	96.2±4.81	16.1±0.8
Valeriana officinalis (leaves)	100	10.8±0.54	5.2±0.26	5.3±0.27	5.1±0.25
Valeriana officinalis (roots)	100	123.8±6.18	80.6±4.02	59.3±2.86	22.9±1.14
Hypericum- Hypericum perforatum	100	8.5±0.42	6.2±0.31	6.0±0.3	5.7±0.28
Nettle - Urticadioica	100	31.8±1.6	5.8±0.29	4.8±0.24	2.9±0.14
Lingon berry – Vaccinum vitis	100	10.2±5.01	10.3±5.0	7.4±0.36	5.8±0.28
Violet – Viola tricolor	100	18.6±0.92	15.5±0,77	9.3±0.45	9.0±0.43
Shepherd's grass - Capsella bursa-pastoris	100	71.4±3.52	67.3±3.35	3.9±1.95	1.9±0.94

It can be seen that in the investigated concentration range, all these plant extracts almost completely suppress the formation of TBA-active products in liver microsomes except for *Plantago major* and *Valeriana officinalis* (roots). Extracts *Limonnium gmelini, Valeriana officinalis* (leaves), *Vaccinum vitis* show good antioxidant properties, dose-dependent decrease in the formation of peroxide products in liver microsomes.

There was used lower concentration range to get more detail investigations of antioxidant qualities of *Limonium, Valeriana* leaves, *Hypericum* herb, *Lingon berry* leaves and *Violet herbs'* extracts (Figure 1). Figure 1 illustrated that all investigated plants showed dose-dependent antioxidant action in low doses. In result of studies was determined that the minimal concentration for significantly inhibition of lipid peroxidation for *Limonium* extracts was 50 μg, whereas similar action of *Violet, Valeriana* leaves and *Lingon berry's* extract showed in concentration 25 μg for *Hypericum* the most minimal effective dose consisted 10 μg. In concentrations upper than 200 μg *Valeriana, Lingon berry* and *Hypericum* extracts completely retarded LPO intensity, when violet and *Limonium* extracts conceded them in similar concentration range.

Having demonstrated that different plant extract at certain concentration can affect the processes involved in lipid peroxidation and can shift the osmotic fragility of erythrocyte membranes, it is established that all nine medicinal plant extracts collected in the Almaty region, Kazakhstan, showed dose-dependent antioxidant and membrane-protective effect on mammalian cells.

There were arranged studies of having in plants such bioactive compounds as polyphenols and flavonoids to get into probable mechanisms of antioxidant potential (Figure 2).

Content of bioactive substances is different in diverse extracts. Mass fraction of flavonoids and polyphenols were higher in *Hypericum* extract in compare with other investigated plants. Also, we should say about different level of the same bioactive compounds in different parts of one plant. So plant extracts could be placed in accordance to content of total polyphenols in next row *Hypericum perforatum* > *Limonnium gmelini*> *Vaccinum vitis* >*Valeriana officinalis* (leaves) > *Capsella bursa-pastoris* > *Viola tricolor* > *Urtica dioica* > *Plantago major* > *Valeriana officinalis* (roots), whereas there are some diversities in flavonoid's content. At comparison of antioxidant potential and bioactive

substance content was brought out that there was no strong correlation between antioxidant content and antioxidative effect of extracts. Thus, content of flavonoids in violet extract is lower on 42% than so one in shepherd's grass extract, but protective effect of violet was higher.

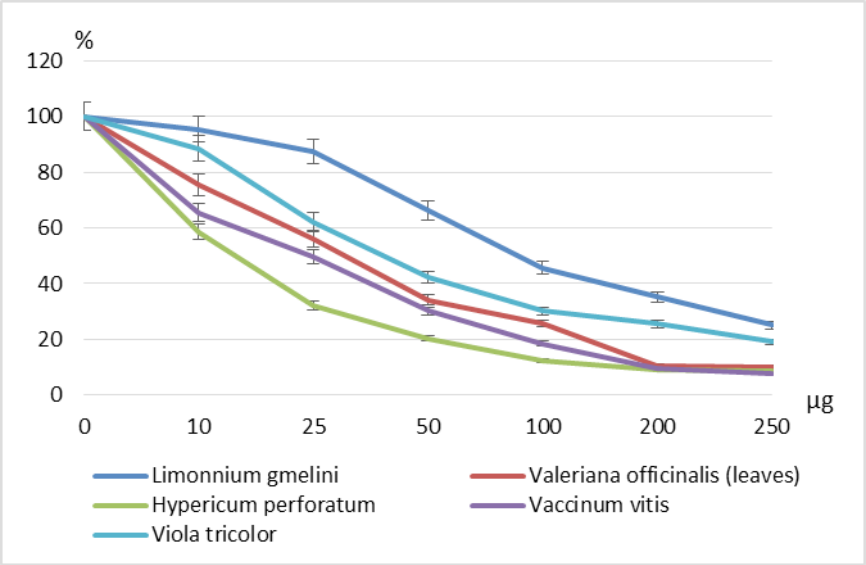

Figure 1 – Effect of selected plant extracts on the level of LPO in microsomes
Note: on the abscissa axis – plant extracts concentration µg/mg protein;
on the ordinate axis – the intensity of LPO processes, % (p≤0.05)

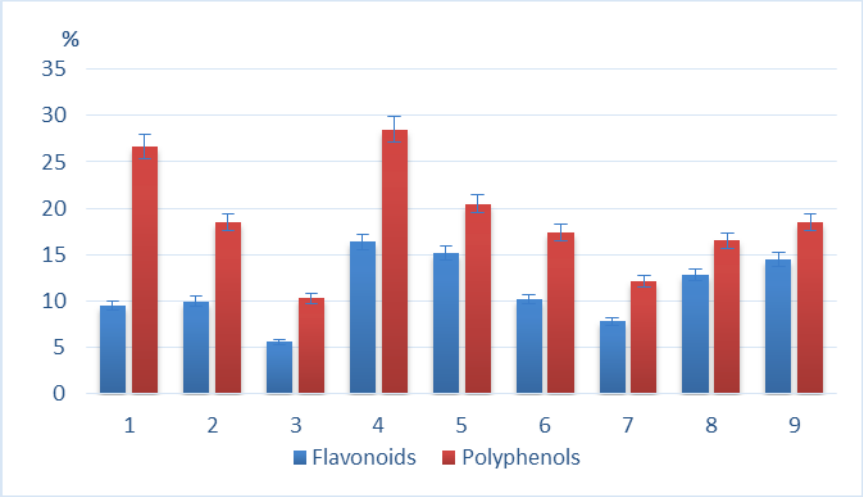

Figure 2 – Content of total polyphenols and flavonoids in extracts
Note: Mass fraction of total polyphenols and flavonoids in terms of mg of dry extract,%;
on the ordinate axis - plant extracts (1 – *Limonnium gmelini*, 2 – Valeriana officinalis (leaves),
3 – *Valeriana officinalis* (roots), 4 – *Hypericum perforatum*, 5 – *Vaccinum vitis*,
6 – *Viola tricolor*, 7 - *Plantago major*, 8 – *Urtica dioica*, 9 – *Capsella bursa-pastoris*)

Conclusion

Medicinal plant extracts, collected in the Almaty region, Kazakhstan, have antioxidant and membrane stabilizing properties, reduces the development of LPO reaction chain activation and also have a positive effect on anti-hemolytic properties of erythrocyte membranes.

Herbal plants have antioxidant and membrane-stabilizing properties that can be used to increase the resistance of the organism to the action of factors leading to excessive formation of free radicals and the development of oxidative stress.

There is observed that plant extracts' antioxidant properties based on presence of polyphenolic compounds and flavonoids. However, there was not found out clear conjunction between content of bioactive substances and protective effect. Assumingly it depends on synergetic or antagonistic interactions of different compounds in plant.

Our results suggest this research of the antioxidant and membrane-protective effects of the herbal plant extracts tested here may guide phytotherapy to development of new dosage forms, and further, to the development of unconventional therapeutic and preventive approaches against oxidative stress related diseases.

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