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Gadetskaya A.V., *Zhussupova A.I., Shalakhmetova T.M.,
Murzakhmetova M.K., Zhussupova G.E.

Al-Farabi Kazakh National University, Almaty, Kazakhstan
*E-mail: aizhan.zhusupova@gmail.com

Natural antioxidants of plant origin

In pharmacotherapy and prevention of "free radical diseases", including diseases of the cardiovascular system, gastrointestinal tract, malignancies, herbal medicinal products become important, the effect of which is based on synergistic action of the major groups of natural compounds, such as polyphenols (mainly condensed tannins and flavonoids), amino, phenolic, polyene carboxylic acids, vitamins and minerals. Herbal substances of antioxidant action obtained on the basis of Kazakhstani plants are presented and described. The allocated herbal substances are notable by their bioavailability, low toxicity, absence of allergic reactions and cumulative effects, possibility of their long-term use for treatment and prevention of diseases.

Key words: antioxidant action, Limonium Mill, polyphenols.

Introduction

According to the World Health Organization the Republic of Kazakhstan takes the leading position among the countries of Central Asia on the prevalence of "free radical diseases". The pathogenesis of these diseases reclines on common fundamental mechanisms of damage to biological membranes of body tissues associated with the increased formation of free radicals and peroxide compounds of organic and inorganic nature [1-2]. Main substrates of free radical oxidation are unsaturated lipids. A necessary condition for cells functioning is to maintain normal levels of free radical oxidation. Marked and prolonged exposure to lipid peroxidation leads to decrease in the content of biological membranes of more easily oxidized polyunsaturated fatty acids with a simultaneous increase of the fatty acid radicals and secondary products of lipid peroxidation. The damaging effect of lipid peroxidation products on cell is caused by forming hydrophilic channels in a lipid layer of membranes dramatically violating their permeability, inactivation of energy generating thiol enzymes, uncoupling of oxidative phosphorylation, which, in turn, results in the cleavage of membrane lipids, alteration of lipid-protein interactions and other irreversible consequences.

Speed and regulation of lipid peroxidation is performed by multicomponent antioxidant system [3], which provides binding and modification of free radicals, preventing the formation and destruction of peroxides, shielding the functional groups of proteins and other biomolecules. The ratio of the intensity of free radical oxidation and antioxidant activity defines the so-called status of cells, tissues and body as a whole. Aging, cardiovascular diseases, cancer, disorders of the central and peripheral nervous systems, acquired immune deficiency syndrome, diabetes, arthritis, cataracts, asthma, diseases of the gastrointestinal tract as well as other inflammatory diseases are caused or accompanied by oxidative stress, failure or defect in the physiological antioxidant defense system in a state of indivisible unity with other human defence systems – hemoprotective and immune [4-6].

To correct the imbalances of the body caused by hyperoxidation and leading to such pathological conditions, use of drugs with antioxidant action is recommended. In this regard, search of compounds with high antioxidant activity or estimation of the biological activity of this type for traditionally used drugs has great medical and general biological significance. Antioxidants, which are substances present in the systems at significantly lower concentrations compared to the oxidized substrate and inhibiting its oxidation, are used for prevention and complex therapy of peroxide pathologies [7-9].

Among the substances with antioxidant, immunostimulating and other adaptogenic properties are substances with high biological activity, derived from herbal raw materials occupy an important place.

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Compatibility of herbal medicines with physiological antioxidant system by virtue of their similarity capable of specifically inducing protective and mobilizing resources that practice the principle of "treat the body, not a disease". The advantage of herbal medicines lays in their gentle effect on the body and complexity of their therapeutic action with low toxicity, absence of cumulative effects, addiction, inducing rare allergic reactions, which is especially important in case of diseases requiring long-term treatment.

Among natural antioxidants best known are the plant polyphenols because of their potential to reduce the risk of disease and in the treatment of many diseases such as cancer, diabetes, disorders of the cardiovascular system, atherosclerosis, neurodegenerative diseases and other inflammatory processes [10-13]. First of all, it is condensed tannins and flavonoids. Condensed tannins include dimeric, oligomeric and polymeric forms of flavan-3-ols, the latter are among the most reduced form of flavonoids.

Highly efficient substance "Limonidin" is produced on the basis of commercially important plant *Limonium gmelinii* by a simple, economically and environmentally feasible technological scheme with a high yield, using aqueous solution of ethyl alcohol generated in the process as a suitable extracting agent or excipient. It is rich in polyphenolic compounds and their variety, with dominance of condensed tannins [14]. Proanthocyanidins are usually represented by linear molecules separate monomers of which are capable of limited rotation around correspondent connecting bonds, whereby the molecule may acquire a relatively stable helical conformation with phenolic groups located at the periphery.

Location of phenolic groups on "surface" of proanthocyanidins is important from the point of view of possibility of the formation of multiple hydrogen bonds with the natural substrates, such as proteins, which underlies their pharmacological action. Blocking specific regions of the surface of enzymes might lead to modification of their activity. Screening of activity of many flavans in relation to enzymes suggests that one possible mechanism of action is chelating of metal ions in the active site of enzymes, as well as the formation of insoluble complexes [15]. Many microbial enzymes (cellulase, glycosyltransferase, pectinase, etc.) inhibit their activity in presence of tannins. The authors suggest that proanthocyanidins act on the outer shell of bacteria consisting of polysaccharides and proteins, fixing it at very low concentrations. Furthermore, due to the presence of ordinary phenolic hydroxyl groups they can chelate metal ions, thereby blocking access of metal ions to microbes. It was shown that an increase in activity of hydrolyzable tannins depends on the amount of galloyl hexahydroxydiphenyl groups, condensed tannins, degree of their polymerization and the amount of phenolic groups [16]. It was found that interaction of proanthocyanidins with phospholipids of cell membranes and lysosomes restricts oxidants access to the membranes, preventing their destruction, at that more active are hexamers [17].

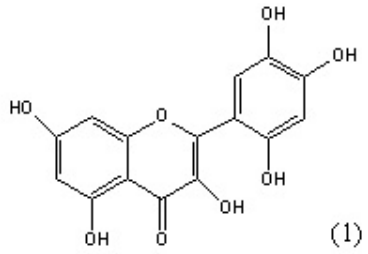
It has been experimentally shown that substance "Limonidin" enhances the flow of anabolic processes in the body, directly reducing accumulation of lactic acid (LA) in tumor and tissues of the body, displacing the redox process towards formation of pyruvic acid (PA). Use of substance "Limonidin" caused a sharp decline of lactate in almost all of the analyzed organs. The greatest decrease (from 17.04 to 0.3 mmol/g) was detected in kidneys; almost on 1.5 orders lower was its content in liver, on order – in tumor and spleen, three and two fold decrease was observed in lungs, heart and skeletal muscles. When analyzing marked deviations it should be noted that substance "Limonidin" facilitated restoring the normal LA content in most part of the internal organs; the greatest effect was detected in parenchymal organs – kidneys, liver and spleen. On the other hand, substance "Limonidin" facilitates increase of PA content in liver, spleen and muscles by 30-40%, in tumor – 70% in lungs, heart and kidneys – by 2-4.2 times.

The results of a comparative study of antiviral activity of substance "Limonidin" with commercial agents – amphotericin B, Oxolinic ointment and Ribavirin on models of myxovirus A/FPV/Rostock/34 and NDV (of La Sota) have shown that substance "Limonidin" has high level of suppression of reproduction of influenza and parainfluenza viruses more than commercial formulations. It is assumed that the antiviral activity is provided by attaching of tannin molecules to the viral protein envelope or to the host cell membrane and thus the virus penetration stops. This marked the activity pattern between tannins and their structure; condensed tannin activity increases with the degree of condensation [18, 14].

Substance at different doses was tested on sufficient number of laboratory animals (white mongrel rats) in different models of acute and chronic liver damage (poisoning by carbon tetrachloride, heavy metals, propellants and other xenobiotics) [19]. Silibor – an effective herbal hepatoprotective agent, widely used in medicinal practice, was used as reference drug in the experiments. It has been found that hepatoprotective activities of substance "Limonidin" and Silibor are comparable; however, single thera-

peutic dose of "Limonidin" is 2 times less than that of Silibor. Substance "Limonidin" is registered by the Ministry of Health Care and Social Development of the Republic of Kazakhstan (PhA RK 42-1259-08, RK-MP-5No.008963 from 22.09.2008) and is approved for use in medicine.

During the chemical study of *L. gmelinii* roots and substance "Limonidin" flavonols (myricetin, quercetin, isorhamnetin, myricetin monomethyl ether and new flavonol previously undescribed in literature 3,5,7,3',4',6' hexahydroxyflavon (1)), their glycosides (miritsitrin, rutin, 3-β-galactosylquercetin and 3-β-galactomyricetin, other mono- and biozides, as well as described for the first time in literature 3-α-galactopyranoside myricetin and 3-O-α-L-(2"-galloyl)-arabinopyranoside myricetin), pyrogallol, gallic and ellagic acids were identified. Main monomeric flavan is (-)-epigallocatechin gallate; various forms of flavan-3-ols: 3,5,7,3',4',6'-hexahydroxyflavan (2) (-)-epigallocatechin-(4β→8)-(-)-3,5,7,3',4',6' – hexahydroxyflavan (3) (+)-gallocatechin-(4α→8)-[(-)-epigallocatechin]5-(4β→8)-(-) – epigallocatechin gallate (4) are identified as new, previously undescribed in literature [20-21]:



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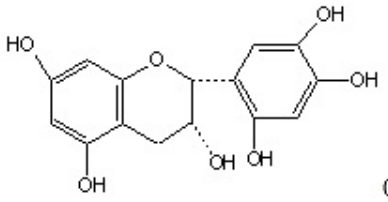

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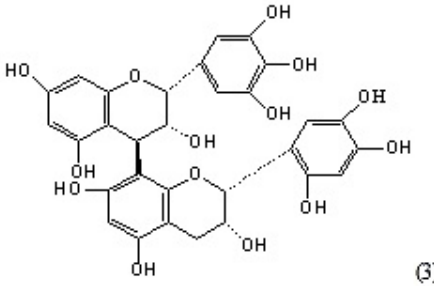

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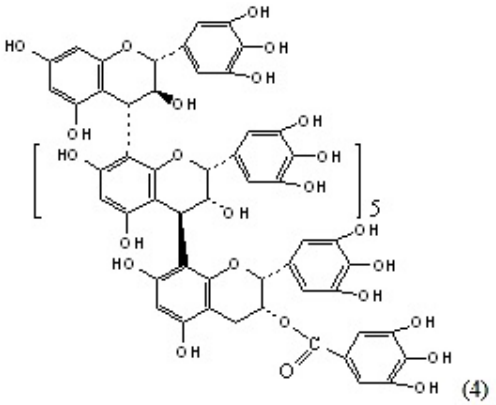

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Previously unknown for this species amino and fatty acid, carbohydrate and microelement contents, vitamins and xantofills were identified. From sterols along with the known 3-O-β-D-glucopyranoside campesterol was described for the first time. Vitamins E and C, along with polyphenols are powerful antioxidants that are used to treat and prevent many diseases, pathogenesis of which is connected with increase of lipid peroxidation associated with changes in functional activity of membranes.

The amount of heavy metals does not exceed the permissible norms for herbal substance and the vital macro- and microelements are contained in required quantities, which appears as a result of synergies with other important components and causes a wide range of physiological actions of resulting preparation – substance "Limonidin".

Active antiinflammatory effect of *Cistus incanus* plant extracts, used against skin diseases, is caused by the presence of condensed tannins and proanthocyanidins [22]. *Cuscuta campestris* Juncker has characteristics, which can be used against various human pathologies, what has previously been demonstrated by the data of traditional medicine for other types of dodder, in particular, *Cuscuta chinensis* Lam. and *Cuscuta europaea* L. [23].

Materials and methods

In the experiment with CCl_4 Silibor was used as reference for Limonidin. Introduction of certain doses of aqueous Limonidin and Silibor solutions was conducted once by intragastric gavage 1 hour prior to use of hepatotoxin – carbon tetrachloride (CCl_4) for 14 days. Animal euthanasia was conducted under Nembutal anesthesia between 9 and 10 am. The content of malondialdehyde (MDA) and lipid hydroperoxide (LPO) and enzyme activity was measured in rat liver homogenate. Preclinical tests were conducted on white outbred male rats weighing 220-250 g, divided into 4 groups (of 10 animals each):

I – intact animals (control),

II – animals received 5 mg/kg of CCl_4 (50% solution in olive oil), single, intragastric;

III – animals received 100 mg/kg of Limonidin and 5 mg/kg CCl_4 once, intragastric;

IV – animals received 200 mg/kg of Silibor and 5 mg/kg CCl_4 under the same conditions.

Dried samples of field dodder were grounded to the final size of 1-3 mm. Under extraction with 50% ethanol and dimethyl sulfoxide (DMSO) two substances were obtained in the form of liquid extracts. Lipid peroxidation was induced for 60 minutes by Fe^{2+}/ascorbate system. The obtained field dodder substances were preincubated with LPA for 15 minutes at 37°C.

Results and their discussion

Animal intoxication with CCl_4 induces activation of lipid peroxidation processes, i.e., increase of MDA and LPO and inhibition of antioxidant processes by reducing the activity of enzymes – superoxide dismutase (SOD) and catalase. Table 1 shows the results of biochemical studies of rat control and experimental groups at their intoxication with CCl_4 and the combined use of CCl_4 and herbal preparations.

According to the results shown in Table 1 hepatotoxin CCl_4 when administered to animals activates LPO, content of MDA and LPO increases by 2.2 and 1.6 times and suppression of antioxidant processes by reduction of SOD and catalase enzymes by 7.0 and 1.8 times, respectively. The processes of lipid peroxidation, induced by CCl_4, are largely reduced when receiving Limonidin (IIIrd group). This is evidenced by a decrease in the content of primary and secondary products of lipid peroxidation (LPO and MDA content decrease by 1.4-1.7 and 1.3-1.4 times), as well as increased activity of antioxidant enzymes in these animals (SOD and catalase activities increase by 5.5-5.7 and 1.2 times, respectively, compared with rats fed only with CCl_4).

Table 1 – Content of LPO and MDA, SOD and catalase activities in rat liver

Group	Experimental conditions	LPO (conv.un./g)	MDA (мМ/g)	SOD (conv.un./g)	Catalase (conv.un./g)
I	Control	23.9±0.6	1.9±0.1	147.2±3.2	302.2±8.4
II	CCl_4	37.9±0.3	4.2±0.1	21.5±4.6	162.7±4.2
III	CCl_4+Limonidin (100 mg/kg)	29.2±4.1	2.5±0.2	119.2±2.8	175.4±1.5
IV	CCl_4+Silibor (200 mg/kg)	28.6±2.8	2.7±0.1	122.3±2.2	172.5±3.0

Despite the fact that indicators of lipid peroxidation in poisoned rats under Limonidin have completely returned to normal, the severity of structural damage in the liver of these animals was significantly smaller. Thus, the results of morphological studies have shown that a single intragastric administration of CCl_4 in rats led to the development of acute toxic hepatitis, considerable destruction centrilobular hepatocytes. Combination of CCl_4 with phytopreparation significantly reduces the degree of destructive processes caused by CCl_4. A similar trend is to reduce the content of lipid peroxidation products, and increase the activity of antioxidant enzymes was observed in group IV, where, along with CCl_4 hepatoprotector Silibor was used. It is shown that Limonidin 100 mg/kg possesses a strong antioxidant and hepatoprotective effect comparable to that of Silibor (dose – 200 mg/kg) used in medicinal practice. In addition, experiments were carried out on animals under their poisoning with heavy metals, propellants and other xenobiotics. Comprehensive studies using various techniques (histological, morphometric, quantitative cytochemical and biochemical) showed that substance "Limonidin" increases the resistance of the liver pathological effects, strengthens its neutralizing function by increasing the activity of enzymatic monooxygenase system, and helps to restore its structure under various injuries.

To isolate the microsomal fraction rat liver tissue was weighed (0.5-1.0 g) after washing with cooled saline and being placed in 10 ml of medium containing 0.85% NaCl and 50 mM KH_2PO_4 (pH 7.4 at 4°C) and homogenized in Polytron type homogenizer within 90 seconds. The homogenate was centrifuged at 10 000 g for 20 min. Microsomal fraction was obtained by centrifuging the supernatant at 30 000 g for 60 min. The supernatant was carefully decanted and the pellet, which is a fraction of heavy microsomes suspended in medium containing 25% glycerol, 0.1 mM EDTA, 0.2 mM $CaCl_2$, 10 mM histidine (pH 7.2 at 4°C) and stored at -4°C [24].

Content of malondialdehyde (MDA) and products of lipid peroxidation is determined by reaction with 2-thiobarbituric acid according to the intensity of the developing color. Content of the products reacting with thiobarbituric acid is calculated taking into account the coefficient of MDA molar extinction equal to $1.56x10^5$ $M^{-1} \cdot cm^{-1}$ [25].

Erythrocytes were obtained by centrifuging blood for 10 minutes at 1 000 g. Plasma and white blood cells are removed and erythrocytes are washed twice with medium containing 150 mM NaCl, 5 mM Na_2NPO_4 (pH 7.4). Osmotic resistance of erythrocytes is determined by the degree of hemolysis in hypotonic solutions of NaCl (0.35-0.5 g/100ml).

Studied substances of field dodder do not affect the accumulation of thiobarbituric acid reactive substances (TBARS) without induction of lipid peroxidation. Significant reduction is observed in the presence of TBARS substance isolated using DMSO as extragent versus ethanol extract at a concentration of 20 μg substance/mg of protein (3.3 and 6.3 mmol of DMSO and ethanol extracts, respectively), which is probably due to a more thorough extraction of polyphenolic compounds from the raw material with dimethyl sulfoxide.

Results of study of dodder alcoholic extract effect on the level of lipid peroxidation in rat liver microsomes revealed that the extract reduces the formation of peroxide products, depending on the dose. Extract concentration of 40 μg/mg protein or above almost completely inhibits the formation of peroxide products in rat liver microsomes.

Conclusion

The choice of research area is based on search of Kazakhstani plants, containing a complex of synergistic biologically active substances of plant origin affecting life expectancy and health by improvement of human and animal physiological features. This refers to preservation of reserve forces and adaptive mechanisms, as well as stimulation of physiological ability of organism in adaption to changing life conditions.

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