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PHYSICS AND CHEMISTRY OF ELEMENTARY CHEMICAL PROCESSES

Physicochemical Approaches to the Study of the Antioxidant Activity of Glycyrrhizin[1]

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Abstract—The review presents an attempt to collect and systematize the available data on the antioxidant activity of glycyrrhizin obtained by various physicochemical methods and to stimulate further discussions on the mechanisms of its activity and prospects for its use as a multifunctional drug delivery system.

Keywords: glycyrrhizin, antioxidant activity, free radicals, supramolecular complexes, micelles

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Glycyrrhizin or glycyrrhizic acid (GA) is the main active component of licorice root (*Glycyrrhiza glabra* and *G. uralensis*) [1]. In its chemical structure, GA is an amphiphilic molecule: its hydrophilic part is represented by glucuronic acid residues, and the hydrophobic part, by a glycyrrhetinic acid residue (Fig. 1).

Glycyrrhizic acid and licorice root have a very long history of use in traditional medicine. Glycyrrhizic acid has been known since ancient times in ancient China, Egypt, and Japan [2]. In recent decades, the properties of GA have been actively studied not only in Asia, but also in Europe [3–9]. Since ancient times, licorice has been used to treat diseases of the lungs, liver, stomach, various urinary tract infections, fevers, and eye diseases. Recent studies have also demonstrated a significant effect of GA and licorice root extract on coronaviruses (including SARS-CoV-2) along with other viruses (herpes, flaviviruses, hepatitis C, and influenza) [10–20]. Another promising direction for the use of GA is its antioxidant activity [21–26]. As antioxidants are involved in various processes in living systems, the antioxidant activity of GA can find wide use in the complex therapy of various diseases. It should be emphasized that, despite the abundance of examples of the antioxidant activity of GA in vivo and in vitro, there is still no consensus among scientists on the physicochemical mechanism of this activity at the molecular level, and discussions on this problem continue to this day [27–35]. It should be noted that the majority of studies devoted to GA still concern its own biological activity. In recent decades, however, a new unusual property of GA has been discovered in addition to its own therapeutic activity, namely, the ability to enhance the therapeutic activity of other drugs [3, 36]. It was demonstrated, using various physicochemical methods, that this effect is associated with the ability of GA to form inclusion complexes with various drugs [3, 8, 36–41], including antioxidants [32, 37, 39, 42]. Enhancing the solubility and membrane permeability was considered to be one of the main physicochemical mechanisms for potentiating the drug activity in complexes with GA [41].

In this context, it can be stated that GA has excellent prospects for use as delivery means in combination therapy due to its own biological activity and abil-

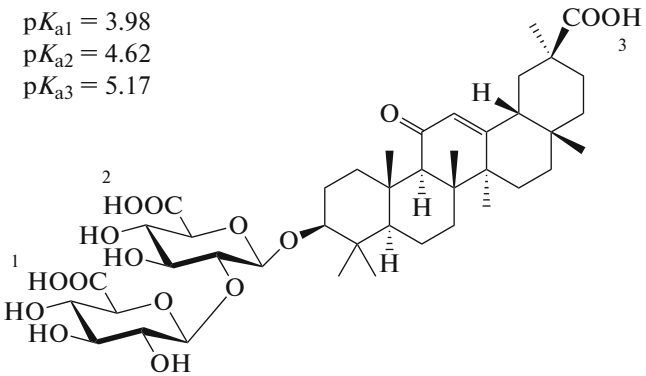

Fig. 1. Structural formula of the glycyrrhizin molecule.

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ity to potentiate other drugs. Thus, the authors of the review [8] demonstrated this possibility on the use of GA in anticancer therapy as an example. It was shown that a combination of GA with first-line drugs has the best therapeutic effect on the tumor. Complexes with GA exhibit broad-spectrum antitumor activity and enhance drug absorption [3, 8].

In this review, we attempted to collect and systematize the available physicochemical data concerning various aspects of the antioxidant activity of GA and to stimulate further discussions on the mechanisms of GA activity and prospects for its use as a multifunctional drug delivery system. Examples of reactions of GA with free radicals and solvated electron will be discussed, as well as examples of increased resistance of drug molecules to the action of oxidants due to encapsulation in micelles and gel nanoparticles of GA, and also examples of increased bioavailability and activity of other antioxidants in the presence of GA.

SELF-ASSOCIATES OF GLYCYRRHIZIN AND INCLUSION COMPLEXES WITH DRUG MOLECULES

As emphasized by many authors, it is the amphiphilicity of the GA molecule (Fig. 1) that determines its ability to self-aggregate in aqueous solutions, forming various self-associates (dimers, micelles, and gel nanoparticles) [3, 41], as well as noncovalent guest–host complexes with other molecules, among which drugs are of primary interest [3, 8, 41, 43]. Research associated with the development of innovative domestic drugs using supramolecular drug delivery systems based on GA was started at Ufa and Novosibirsk scientific centers in the 1990s under the supervision of Academician G.A. Tolstikov [44]. In animal experiments, it was shown that composites of drug molecules with GA can significantly reduce the therapeutic doses of drugs and reduce or even completely eliminate undesired side effects; in some cases, they can enhance the atypical (so-called pleiotropic) properties of drugs [36]. Further studies showed that such significant and favorable changes in pharmacological characteristics occur due to the formation of so-called "supramolecular inclusion complexes" of drug molecules in GA self-associates [3, 45–47]. It is believed that the pharmacological effect of such structures is achieved due to several factors, the most important of which are increased solubility, membrane permeability, and bioavailability, as well as reduced metabolism caused by gastrointestinal enzymes.

The most detailed studies have been reported in the literature for the formation of GA micelles. The critical micelle concentrations (CMCs) determined by various methods are in good agreement. Kondo et al. performed a comparative study of α- and β-GA [48]. It was noted that although both compounds form micelles (the CMC values of the compounds were almost equal, ~$(2–3) \times 10^{-4}$ M), only β-GA forms a gel in aqueous solution in acidic medium (pH <4.5), even in a dilute solution (~1 mM). It was concluded from studies of gelation of several β-GA derivatives that gelation requires a free carboxyl group in the triterpene fragment, and at least one of the carboxyl groups and some of the hydroxyl groups of the glucuronic acid fragment should also be free. As for the shape of micelles, opinions differ. Thus, according to the data obtained by Matsuoka et al. by small-angle X-ray scattering [49], GA forms micelles with a rod-like structure (radius 1.5 nm, length 21 nm) in an aqueous solution at pH 5–6. Other authors believe that GA micelles have a round shape. Thus, Wang et al. showed, using dynamic light scattering and transmission electron microscopy, that GA micelles are spherical particles with a diameter of ~10 nm [50].

In studies on the formation of GA micelles and gels [48–51], it was emphasized that the interaction of GA molecules with each other is directly related to the state of carboxyl groups. This conclusion follows from the fact that micelles form only in an acidic medium, when the COOH groups of GA are not dissociated. This was investigated in more detail by nuclear magnetic resonance (NMR). High-resolution NMR offers good opportunities for studying the structure of GA aggregates due to the sensitivity of chemical shifts and line widths of GA protons to aggregation processes. Thus, Petrova et al. [52] analyzed the ^{1}H NMR spectra of GA solutions at different pH values and concentrations and measured the relaxation times T_2. It was found that the GA concentration determined from high-resolution NMR spectra did not correspond to the amount of the solute and reached the limiting value starting from a certain concentration depending on pH of solution. The authors assumed that this deviation was associated with gelation. Indeed, the NMR lines for a typical gel have a width of 3–8 kHz [53] due to fast dipole–dipole relaxation; i.e., because of the large line width of the gel, they cannot be observed in high-resolution NMR spectra. Thus, at a spectrum width typical for solutions, the observed lines of GA refer only to the structures present in solution (from monomers to micelles). The authors showed that at each pH value there is a certain critical concentration, above which a solid-like gel forms, while the concentration of the "labile" fraction remains approximately constant. When pH changes from 2 to 5, the critical gel concentration increases from 0.3 to 2.7 mM [52].

To understand the role of association in spectral transformations due to changes in the GA concentration, the authors measured the relaxation times T_2 of GA protons at pH 3–5. In all cases, the relaxation was well described only within the framework of the biexponential model, which is typical for slow (on the NMR scale) exchange between the micelle and all premicellar states. The fast component of relaxation has an almost constant time T_{21} = 3–4 ms, while the slow

component T_{22} changes considerably from 200 to 15 ms when the GA concentration changes. The authors showed that the decrease in the relaxation times T_{22} for pre-micellar states at increased GA concentrations is caused by a shift of equilibrium toward larger associates. The critical micelle concentrations of GA were determined while measuring the relaxation times at different GA concentrations. At pH 5, the CMC is ~2.3 mM, which agrees with the data of [49], while at lower pH (4 and lower), it is lower than 0.3 mM [52].

Let us now consider how the ability of GA to form self-associates and inclusion complexes is related to its antioxidant activity in combination therapy. Three independent physicochemical approaches can be distinguished here. The first approach is to increase the solubility and bioavailability of natural antioxidants through association with GA. As an example, we can cite works on the study of complexes of carotenoids and flavonoids with GA [41–43]. As is known, carotenoids are very effective natural antioxidants, but their use in practice in medicine is limited by their extremely low solubility in water and instability in the presence of light, transition metal ions, and other factors. It was shown that inclusion of carotenoids in GA complexes and micelles makes it possible to overcome most of these problems [32, 37, 39]. In particular, the solubility in water can be increased several thousand times, and the rate of carotenoid oxidation with iron ions can be decreased to a few tenths in the presence of GA and its disodium salt not only in water, but also in aqueous mixtures with organic solvents [37].

Interesting results were obtained in a study of GA complexes with the carotenoids xanthophylls (lutein, zeaxanthin, astaxanthin). In the presence of even small amounts of water in an organic solvent (<5%), these carotenoids form aggregates possessing significantly lower antioxidant activity. It was shown that the interaction with GA molecules destroys the self-associates of these carotenoids, thereby increasing their antioxidant activity [37]. Zeaxanthin and lutein play an important role in protecting the human and mammalian eye retina from oxidation by short-wave visible light and reactive oxygen intermediates (ROIs). The insufficient levels of these carotenoids in retinal tissues lead to eye damage and ultimately to age-related macular degeneration and irreversible blindness.

Another aspect of the effect of GA on the antioxidant activity of carotenoids was detected in [32]. Using EPR with spin traps, the authors showed that the complexation of some carotenoids with GA (even in nonaqueous media) significantly (several times, and in some cases even dozens of times) increased the reaction rate of some carotenoids with peroxide radicals. It was noted that the ability of various carotenoids to scavenge peroxide radicals correlated with their oxidation potentials, and it was assumed that complexation could affect the electrochemical potentials of carotenoids. Measurement of the current-voltage characteristics of carotenoids in the presence of GA confirmed this hypothesis [32]. The effect of GA increased with the oxidation potential of carotenoid. For carotenoids with the lowest potentials (beta-carotene and zeaxanthin, E_{ox} ~ 0.5 eV), the effect of GA was not observed.

Finally, the third approach is associated with inhibition of the formation of free radicals during the photodecomposition of phototoxic drug compounds. As is known, many drug molecules contain chromophoric groups capable of entering into photochemical reactions when a light quantum is absorbed. Their phototransformations can decrease the therapeutic effect and increase the toxicity of the compounds. In addition, other problems can arise because of damage to internal organs when the drug interacts with radiation. The reactions of biological systems under the influence of sunlight are of particular interest due to their wide applications [54]. One of the biological applications is photosensitization. Photosensitization reactions is an ever-growing area of research on desirable and undesirable processes induced in biological systems by light absorption. In general, photosensitization is anomalously high reactivity of a biological substrate under the action of artificial sources or natural sunlight. Here are some examples. The first example is nifedipine (NF; dimethyl 1,4-dihydro-2,6-dimethyl-4-(2'-nitrophenyl)-3,5-pyridinedicarboxylate), a drug used to treat hypertension; it is extremely sensitive to UV radiation and visible light up to 450 nm. The quantum yield of its photodegradation is ~0.5. This extreme photoinstability, coupled with the fact that NF is often prescribed for long-term therapy, was the reason for the start of studies on the mechanisms of its photoinduced transformations, including reactions with biological targets [55, 56]. In these works, it was found that exposure to UV-A and daylight gave the same photoproducts. In a phosphate buffer, the conversion is quantitative, the only photoproduct being the nitroso derivative of nifedipine. In the body, nifedipine forms a complex with an L-type calcium receptor binding site, consisting of six spatially separated amino acid residues, with its conformation corresponding to a closed channel. As a result of the development of detailed atomistic models of drug interaction with the receptor (QSAR analysis), it was shown that electron transfer is the most likely mechanism of NF interaction with the environment of the Ca^{2+} receptor binding site [57]. It was shown by optical spectroscopy, NMR, PAMRA (Parallel Artificial Membrane Permeability Assay), and molecular dynamics methods that NF forms strong complexes with GA in aqueous solutions, which are characterized by increased (35-fold) solubility and (fivefold) membrane permeability [38, 58, 59]. It was demonstrated, using NMR and chemically induced dynamic nuclear polarization (CIDNP), that the photoinduced interaction of NF with aromatic amino acids proceeds via a

radical mechanism. Complexation with GA completely blocks the stage of electron transfer between NF and amino acid [38].

Another example of a drug whose photoinduced radical degradation is inhibited by glycyrrhizin is the diterpene alkaloid lappaconitine (LC). As is known, LC exhibits antiarrhythmic and hypotensive activities [60]. However, because of its toxicity and side effects, its clinical use is very limited. Another disadvantage of LC that limits its use is its high photochemical sensitivity [61–65]. Due to wide applications of diterpene alkaloids in chemistry and pharmacology [61], the study of the structure and properties of their paramagnetic intermediates is certainly of interest. It was demonstrated by CIDNP, EPR, and laser flash photolysis that under the action of UV radiation ($\lambda < 350$ nm), LC can undergo radical photodecomposition as a result of both monomolecular electron transfer and interaction with biological electron donors [62–66]. In a series of NMR and CIDNP studies [67–69], it was shown that glycyrrhizinic acid can significantly change the efficiency and direction of the phototransformation of lappaconitine due to both its solubilization in GA micelles [37] and protonation of the amino nitrogen of LC in aqueous alcoholic solutions. As a result, the intra- and intermolecular pathways of the reaction are blocked. Recall that GA micelles form in aqueous solutions at concentrations of ~1 mM, which depend on both pH of the medium and organic solvent additions [52]. At lower concentrations, GA forms stable supramolecular complexes with LC molecules with a composition of 1 : 1 and a stability constant of $\sim 2 \times 10^5$ M^{-1} s^{-1} [66]. It should be emphasized that solubilization of LC in GA micelles and complexes significantly affects its therapeutic activity. The use of GA complexes in in vivo experiments made it possible to reduce the therapeutic dose of LC dozens of times [44].

The third example of a drug whose photoinduced radical degradation is inhibited by glycyrrhizin is ketoprofen (KP), a non-steroid anti-inflammatory agent. As is known, KP is the most light-sensitive compound among non-steroid anti-inflammatory agents, which can cause phototoxic and photoallergic reactions [70–73]. The short-lived paramagnetic species formed under the action of UV irradiation in homogeneous solutions are considered to be the main source of phototoxicity of KP. The formation of these species was proved by chemical nuclear polarization in addition to other methods [72, 73]. Selyutina et al. [74] attempted to use glycyrrhizin to increase the photostability of KP. To study the photolysis of ketoprofen in micelles and gel nanoparticles of GA, they also used CIDNP and NMR. It was shown that inclusion of ketoprofen in GA micelles or gel nanoparticles significantly reduced the rate of photodegradation. For the mechanism of KP photostabilization, the authors proposed isolation of KP molecules from water molecules in micelles and gel nanoparticles, as the presence of water significantly accelerates the photodegradation. The results obtained in this study may be useful in the development of ketoprofen formulations for external application.

INTERACTION OF GLYCYRRHIZIN WITH LIPID MEMBRANES

An important aspect of the antioxidant activity of GA is its ability to be incorporated into cell membranes and affect their physical and functional properties. First, due to its lipophilicity, GA can penetrate into the lipid bilayer and work not only as a hydrophilic, but also as a lipophilic antioxidant, protecting lipid molecules and built-in proteins from being damaged by free radicals. Second, by changing the lipid mobility, phase transition temperature, transmembrane potential, and other physical parameters of the membrane, GA can produce a lipid-mediated effect on the functioning of the cell's own antioxidant systems [3]. Interest in the membrane-modifying ability of GA has especially increased in recent years in view of the discovery of the virus-inhibitory effect of GA on SARS-CoV-2, the causative agent of COVID-19 [13, 14, 75, 76]. This is associated with the fact that one of the possible mechanisms of the antiviral action of GA is considered to be prevention of fusion of the virus shell with the plasma membrane of the host cell [12, 16, 17].

Another reason for increased interest of researchers in the membrane-modifying ability of GA is associated with the fact that GA improves the absorption and bioavailability of various drugs [58, 59, 77–79]. During transport, a drug molecule has to overcome many barriers in the form of single- and multilayer membranes. Although the cell structures are not all identical, the factors of action and drug pathways are similar for different cells, which allows the use of model lipid membranes (liposomes and bicelles) to elucidate the physical mechanisms of transport of small molecules through cell membranes. Some results indicate that GA can enhance drug penetration into cells by affecting the properties of cell membranes [80–84]. In particular, it was established in the cited works that GA can increase the permeability of erythrocytes and K562 cells for formate ions. The authors showed that increased permeability might be associated with the membrane-modifying activity of GA. To prove this hypothesis, they used NMR and molecular dynamics (MD) methods. The interaction of GA with the liposomes of palmitoyl-oleoyl-phosphatidylcholine (POPC), dioleyl-phosphatidylcholine (DOPC), and dipalmitoyl-phosphatidylcholine (DPPC) was studied. It was found that in the presence of glycyrrhizin, the transport of formate ions across the erythrocyte membrane was accelerated twofold compared with the transport in untreated cells [84]. Molecular-dynamic modeling showed that GA molecules are predominantly located in the "outer" half-layer of the membrane and can be freely transferred between the

polar part of the half-layer and its hydrophobic inner part. They also capture several water molecules. It was also found that GA can penetrate bilayers of different types of lipids. However, GA can penetrate into the "inner" half-layer only for the DPPC membrane, which is most rigid among the three studied types. Membrane thinning occurs at the site of localization of GA molecules, which can lead to the formation of pores, making the bilayer permeable for ions and small molecules [83]. Sapra et al. demonstrated the ability of GA to create small pores on the surface of the bilayer and destroy the structure of the lipid bilayer in rat epidermis in vitro [85]. Note that this ability may be involved in the mechanism of transdermal drug delivery. Also, the observed pore formation and transfer of water molecules by GA molecules can facilitate the passive transport of molecules across the lipid membrane in the supramolecular complex with GA. The enhancement of ion transport across the membrane in the presence of GA can also affect the transmembrane potential. This assumption was confirmed in experiments with rat thymocyte cells [78]. The effect of GA on the transmembrane potential of rat thymocytes was studied using the potential-sensitive fluorescent probe 4-(*p*-dimethylaminostyryl)-1-methylpyridinium (DSM). Incubation of cells with micellar GA leads to a decrease in the amplitude of the observed fluorescence kinetics of DSM, which indicates a decrease in the transmembrane potential. The proposed mechanism consists in an increase in the permeability of the plasma cell membrane for ions (passive ion transport) due to the inclusion of GA.

INTERACTION OF GLYCYRRHIZIN WITH FREE RADICALS AND SOLVATED ELECTRON

The available in vitro and in vivo studies emphasize the presence of the intrinsic antioxidant activity of GA [23, 25, 86–88]. The results of the in vitro and in vivo studies were described in detail in reviews [3, 89–91]. These works have demonstrated the antioxidant activity with respect to reactive oxygen species such as hydroxyl radicals and peroxide and superoxide ions, which play an important role in the development of diseases associated with reactive oxygen species (ROS) or in aging mechanisms. Glycyrrhizic acid can also activate the nuclear factor Nrf2 by means of redox regulation by Keap1, which can affect the cellular levels of ROS via additional mechanisms.

Considerable attention in in vitro and in vivo studies has been paid to the antioxidant role of GA in photoinduced processes, in particular, in processes associated with the development and treatment of skin diseases. For treatment of tumors caused by UV-B radiation in the case of skin cancer, GA is considered to be a natural antioxidant agent that protects the mitochondrial functions under the oxidative stress conditions [92]. The results of the work of Lee et al. showed that the effect of GA as an anticancer agent may be associated with increased formation of ROS and a decrease in the GSH concentration, which cause changes in the permeability of the mitochondrial membrane, leading to a release of cytochrome *c* and activation of caspase-3 [93]. Other authors showed that GA inhibits proliferation of HepG2 cells in the case of the liver cancer and also increases the formation of ROS and production of NO, and reduces the potential of the mitochondrial membrane [94]. The physicochemical aspects of the interaction of GA with cell membranes were considered in the previous chapter of the present review.

Taking into account the participation of antioxidants in various processes of living systems, it can be assumed that the antioxidant activity is an important aspect of the action of glycyrrhizin in the complex therapy of various diseases. It should be emphasized that, despite the abundance of examples of the antioxidant activity of glycyrrhizin in vivo and in vitro, there is still no consensus on the molecular mechanism of this activity of GA. Moreover, discussions on this topic continue today [25–35]. Some authors argued that glycyrrhizin does not capture hydroxyl radicals or superoxide radical anions, but reacts with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals [27–29]. In contrast, other authors showed that glycyrrhizin neutralizes ROS radicals, but does not remove the DPPH radicals [30–35]. Thus, the relative rate constants of the reaction of OOH radicals with glycyrrhizin and a number of antioxidants (carotenoids) were measured using EPR spin trapping, and it was shown that the antioxidant ability of glycyrrhizin is even higher than that of the widely used antioxidants beta-carotene and zeaxanthin [32]. A pulsed radiolysis study showed that glycyrrhizin provides radiation protection by capturing the free radicals and solvated electrons formed during irradiation [33]. The authors measured the rate constants of the reaction of glycyrrhizin with the hydroxyl radical and solvated electron (1.2×10^{10} and 3.9×10^{9} M^{-1} s^{-1}, respectively). We believe that the radio- and photoprotective properties of glycyrrhizin can also be useful in practice. D. Farmanzadeh et al. calculated the O–H bond dissociation enthalpies and the ionization potential of GA by the DFT method and showed that the antioxidant nature of GA may be determined by the hydrogen atom transfer mechanism [26].

The antioxidant activity of GA was also studied by CIDNP technique [74, 95], which is one of the most informative experimental methods for studying free radical reactions in complex chemical and biochemical processes [96–98]. The authors traced the effect of GA on the behavior of paramagnetic species formed during UV irradiation of xenobiotics, including drug molecules (naproxen and ketoprofen), and found that the concentration of free radicals in solutions decreased in the presence of GA [74, 95]. In addition,

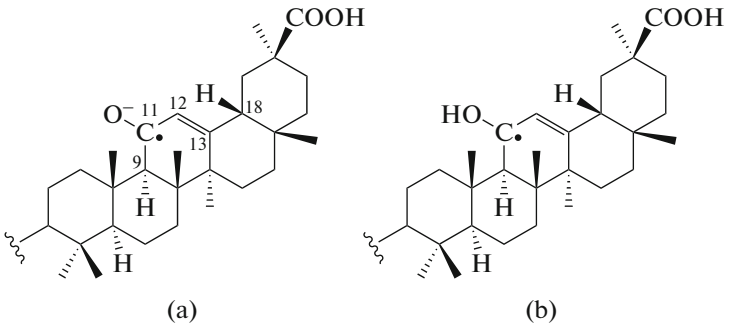

Fig. 2. Paramagnetic intermediates of glycyrrhizin: (a) is the radical anion; (b) the neutral radical.

it was reliably established by CIDNP method that the GA molecule captures the solvated electron generated by UV irradiation of naproxen and ketoprofen. An analysis of CIDNP effects in combination with DFT calculations made it possible to determine the nature of the radical intermediates of GA formed during the capture of a solvated electron (Fig. 2).

The DFT calculations predicted a significant difference in the spin density distribution between the radical anion and the neutral GA radical (Table 1) [95].

The observation of CIDNP effects of the same sign (emission) on the 9-H, 12-H, and 18-H protons of glycyrrhizin during UV irradiation of naproxen in the presence of GA led to the conclusion that the GA radical anion in solution undergoes fast protonation, forming a neutral radical.

CONCLUSIONS

Thus, the physicochemical studies performed using a wide range of physical methods made it possible to establish the possible molecular mechanisms of the antioxidant action of GA, including both its own antioxidant activity and the ability to potentiate the action of other antioxidants. The mechanisms can be conventionally divided into three groups. The first mechanism is the reaction of the GA molecule itself with reactive oxygen species, solvated electron, or radical forms of xenobiotics. Pulsed radiolysis, EPR spin trapping, and chemical nuclear polarization studies showed that for some paramagnetic species, the efficiency of their capture by GA molecules exceeds that of known natural antioxidants. The second mechanism is inhibition of the formation of free radicals involving drug molecules in dark and photoinduced redox reactions due to encapsulation of the drug molecule in micelles or gel nanoparticles of GA. Finally, the third mechanism is associated with the ability of GA to potentiate the therapeutic (including antioxidant) activity of other drugs and antioxidants. The effect of GA is an increase in the solubility and bioavailability of natural antioxidants and other lipophilic molecules due to their incorporation into GA micelles and complexes. Bioavailability is further increased due to the membrane-modifying ability of GA, which facilitates the passive transport of biomolecules across the lipid membrane. The membrane-modifying ability of GA, according to some authors, may also have an indirect effect on the functioning of the intrinsic antioxidant enzymes of living cells. In conclusion, it can be stated that glycyrrhizin has excellent prospects for use in combination therapy due to its own biological activity and ability to potentiate other drugs as a delivery system.

Table 1. HFI constants (mT) in the radical anion (A) and neutral radical (B) of glycyrrhizin calculated by DFT according to the data of [95]

Position	A	B
9-CH	+0.266	+0.676
12-CH	–0.069	+0.072
18-CH	+0.147	+0.206

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