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Biochemical Mechanisms of Therapeutic and Prophylactic Effects of Bioflavonoids

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Abstract: In the present study to investigate the biochemical mechanisms of therapeutic and prophylactic action of bioflavonoids, carried out a comparative evaluation of antioxidant and antiproteinase properties of certain bioflavonoids standards *in vitro*. Therapeutic and prophylactic efficacy of individual bioflavonoids as well as herbal medicines with bioflavonoids, was examined at an experimental pathology (toxic hepatitis, dental caries, periodontitis, stomatitis, dysbiosis, diabetes Types 1 and 2, gastric ulcer, osteopenia) in Wistar line rats. Condition of organs and tissues was assessed by biochemical markers of inflammation, antioxidant and antimicrobial defense systems of animals. Research has shown the ability of bioflavonoids in varying degrees inhibit the formation of superoxide anion radicals and malondialdehyde, recover free radicals, bind ions of Fe^{2+}, inhibiting the activity of proteases, such as leukocyte elastase. Established partially competitive type of trypsin and elastase activity inhibition by bioflavonoids. Was revealed a positive effect of bioflavonoids in experimental pathology on animals. Therapeutic and prophylactic effects of bioflavonoids, in our opinion, are realized through a strong antioxidant and antiprotease properties of these compounds.

Key words: Bioflavonoids, antioxidant and antiprotease properties *in vitro*, experimental therapy.

1. Introdcution

Bioflavonoids are now seen as an important bio-regulators in the human body. These compounds are synthesized exclusively by higher plants [1-3]. Our interest in the study of bioflavonoids mechanisms of action caused by diversity of their biological effects.

As of today identified approximately 8000 different bioflavonoids. The structure of the molecule based on the flavan tricycle in which two benzene rings A and B are connected by propane bridge to oxygen forming heterocycle (Ring C) [4]. Despite the similarity of the structure, individual groups of bioflavonoids are significantly different from each other by their biochemical properties. This diversity is achieved due to connection of hydroxyl, methyl, and oxymethyl groups in different parts of the Rings A, B and C (Fig. 1).

Depending on the group and its connection zone, bioflavonoids are classified into eight classes: flavones, flavonones, flavonols, isoflavones, catechins, anthocyanidins, chalcones and leukoanthocyanidins.

Structural diversity of bioflavonoids defines a wide range of biological and pharmacological action: antioxidant, inhibition of proteinkinases, phospholipase, hyaluronidase, lipoxygenase, the ability to stabilize biomembranes, provide anti-inflammatory and trophic action, inhibit metastasis and growth of malignant tumors, stimulate osteogenesis, increase bone mineral density, prevent development of osteoporosis and dental caries [1, 4, 5]. This diversity posing a logical question of common mechanisms of bioflavonoids action existence, aimed at preventing and/or arresting the development of diseases with different pathogenesis. This circumstance has defined aim of the present work—study of leading biochemical mechanisms of therapeutic and prophylactic action of bioflavonoids *in vitro*, as well as in experimental pathology *in vivo*.

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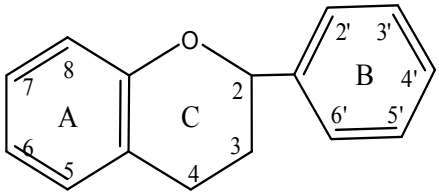

Fig. 1 Structure of flavan.

2. Methods

In the pathogenesis of the vast majority of diseases are disorders of antioxidant protection and intensification of inflammation. In its turn the development of inflammation is accompanied by activation of proteases. Based on this, we conducted a study of antioxidant and antiproteinase properties of bioflavonoids *in vitro*.

In the study was used a commercial bioflavonoids apigenin, luteolin, quercetin, hesperetin, naringenin, catechin, genistein, daidzein, phloretin, rutin ("Sigma", USA), and bioflavonoids, selected from plant raw materials: baikalin was isolated from the roots of Scutellaria baicalensis (Scutellaria baicalensis Georgi.), sophoricoside—from the fruits of Sophora japonica (Sophora japonica L.), flavolignans—from the fruits of Sylibum marinum (Sylibum marianum L. Gaerth).

General TAA (antioxidant activity) was determined by the degree of inhibition by bioflavonoids ascorbate- and ferro-induced oxidation Tween-80 to malondialdehyde, the content of which was determined by thiobarbituric acid. ASA (antisuperoxideanion activity) was determined by the ability of bioflavonoids to compete with nitroblue tetrazolium in the reduction of superoxide radicals, that are formed in the system phenazine methosulphate—NADH. Antiradical activity was evaluated by the ability of bioflavonoids to give labile hydrogen atom to free radical diphenyl-picryl-hydrazyl. Chelating activity determined by the ability of bioflavonoids to bind ions of Fe^{2+}, the number of which was determined by ferrozine [6].

In the study of bioflavonoids antiproteinase activity was used a commercial proteolytic enzymes collagenase Type I A from Clostridium histolyticum ("Sigma"), trypsin ("Fluka"), chymotrypsin from porcine pancreas and leukocyte elastase ("Sigma"). AEA (antielastase activity) determined by hydrolysis of N-t-BOC-L-alanin-p-nitrophenyl ester; ATA (antitrypsin activity)—by hydrolysis of Benzoyl-DL-arginine-4-nitroanilide hydrochloride, AChA (antichymotrypsin activity) and ACA (anticollagenase activity)—by 2% casein hydrolysis. Method of determination and calculation bioflavonoids antiproteinase activity are described in Ref. [7].

The degree of antioxidant and antiproteinase activity of bioflavonoids expressed as IC_{50}. Its values were determined by regression analysis using the program "MS Excel", and expressed in molar concentration (*M*) [8]. Considered acceptable regression coefficient of determination R^2 is not lower than 0.95.

3. Results and Discussion

Results of research bioflavonoids antioxidat activity are presented in Table 1. The highest ASA (antisuperoxideanion activity) was achieved with quercetin. IC_{50} of quercetin in the system of generation superoxide radicals was lower than for phloretin, rutin and genistein. The lowest ASA had naringenin and catechin.

All investigated bioflavonoids possess ARA (antiradical activity). The highest ARA was observed with quercetin. Next in descending order of ARA is rutin, baikalin and apigenin. The lowest ability to repair free radical set for naringenin, genistein and daidzein (Table 1).

Our studies have shown that quercetin, which has the highest ASA and ARA, was also the most active ChA (chelating activity). IC_{50} in the ions binding system Fe^{2+} to 10 times was lower than other bioflavonoids. We could not find ChA from daidzein and phloretin.

The most pronounced ability to inhibit the oxidation of Tween-80 (TAA) had a catechin. Its IC_{50} 5.4 times higher than from quercetin. In addition to quercetin about the same high TAA had phloretin and baikalin. Lowest TAA was for sophoricoside, daidzein,

Table 1 The antioxidant activity of bioflavonoids, IC_{50}, *M*.

Bioflavonoid	ASA	ARA	ChA	TAA
Quercetin	0.099×10^{-3}	0.0047×10^{-3}	0.054×10^{-3}	0.254×10^{-3}
Apigenin	1.369×10^{-3}	0.0392×10^{-3}	1.521×10^{-3}	2.292×10^{-3}
Hesperetin	1.567×10^{-3}	0.8846×10^{-3}	1.336×10^{-3}	0.910×10^{-3}
Naringenine	3.453×10^{-3}	18.7119×10^{-3}	1.640×10^{-3}	3.635×10^{-3}
Catechin	3.545×10^{-3}	0.0099×10^{-3}	2.863×10^{-3}	0.047×10^{-3}
Genistein	0.992×10^{-3}	15.0740×10^{-3}	2.651×10^{-3}	2.200×10^{-3}
Daidzein	2.249×10^{-3}	16.9231×10^{-3}	Not detected	3.290×10^{-3}
Phloretin	0.760×10^{-3}	0.3127×10^{-3}	Not detected	0.573×10^{-3}
Rutin	0.587×10^{-3}	0.013×10^{-3}	0.174×10^{-3}	1.262×10^{-3}
Baikalin	1.928×10^{-3}	0.018×10^{-3}	0.229×10^{-3}	0.587×10^{-3}
Sophoricozid	1.557×10^{-3}	1.676×10^{-3}	3.985×10^{-3}	3.821×10^{-3}
Flavolignans	2.553×10^{-3}	0.657×10^{-3}	12.147×10^{-3}	2.496×10^{-3}

ASA—antisuperoxideanion activity;
ARA—antiradical activity;
ChA—chelating activity;
TAA—total antioxidant activity.

Table 2 The antiproteinase activity of bioflavonoids, IC_{50}, *M*.

Bioflavonoid	ATA	AEA	AChA	ACA
Quercetin	0.041×10^{-3}	0.509×10^{-3}	23.944×10^{-3}	Not detected
Apigenin	1.167×10^{-3}	1.552×10^{-3}	Not detected	24.232×10^{-3}
Luteolin	0.937×10^{-3}	1.974×10^{-3}	Not detected	20.318×10^{-3}
Hesperetin	22.759×10^{-3}	Not detected	12.943×10^{-3}	13.710×10^{-3}
Naringenine	26.859×10^{-3}	Not detected	6.868×10^{-3}	0.905×10^{-3}
Catechin	4.297×10^{-3}	$35.873 \times \cdot 10^{-3}$	14.169×10^{-3}	Not detected
Genistein	4.086×10^{-3}	4.248×10^{-3}	27.581×10^{-3}	5.510×10^{-3}
Daidzein	2.845×10^{-3}	3.052×10^{-3}	92.664×10^{-3}	5.028×10^{-3}
Phloretin	9.511×10^{-3}	13.476×10^{-3}	Not detected	5.669×10^{-3}
Rutin	16.116×10^{-3}	2.233×10^{-3}	Not detected	29.670×10^{-3}
Baikalin	0.385×10^{-3}	11.680×10^{-3}	Not detected	Not detected
Sophoricozid	2.258×10^{-3}	1.355×10^{-3}	4.951×10^{-3}	16.025×10^{-3}
Flavolignans	9.311×10^{-3}	0.770×10^{-3}	3.776×10^{-3}	14.254×10^{-3}

ATA—antitrypsin activity;
AEA—antielastase activity;
AChA—antichymotrypsin activity;
ACA—anticollagenase activity.

flavolignans, apigenin and genistein (Table 1). TAA of bioflavonoids is carried, probably due to the ability to neutralize free radicals in the initial stages of their formation, as well as to bind the ions Fe^{2+}, in the presence of which are formed secondary free radicals.

IC_{50} determination results in the interaction of bioflavonoids with trypsin, elastase, chymotrypsin and collagenase are summarized in Table 2. Character of manifestation of degree ATA and AEA of the most studied bioflavonoids is similar. The most active inhibitor for trypsin and elastase was quercetin. Less active, but with low IC_{50} values in the reaction with trypsin and elastase, was apigenin and luteolin. Along with high-ATA and AEA quercetin, apigenin and luteolin had a very low ability to inhibit chymotrypsin and collagenase. The manifestation of antiproteinase activity of hesperitin and naringenin was an inverse relationship. Hesperidin and naringenin poorly inhibits trypsin and were not active against elastase, but has a very high inhibitory activity to chymotrypsin and

collagenase. Phloretin, genistein and daidzein can be attributed to substances with an average inhibitory activity against proteinases, except chymotrypsin. Catechin highly inhibits only with trypsin. Studied bioflavonoids, except hesperitin and naringenin, was active inhibitors of trypsin (Table 2).

Thus, our study showed the ability of bioflavonoids in varying degrees inhibit some proteinases. Quercetin, apigenin and luteolin were very active inhibitors of elastase and trypsin, but little or inactive relative to chymotrypsin and collagenase. Hesperidin and naringenin, conversely, was active inhibitors of chymotrypsin and collagenase on the background of a low inhibitory activity to the trypsin and the absence of such relative to elastase. Genistein and daidzein have approximately the same medium inhibitory effect on elastase, trypsin and collagenase with weak inhibit activity of chymotrypsin. Inhibitory activity of plant bioflavonoids also varies in relation to the study proteinases. Probably, a different degree of bioflavonoids inhibition of proteases depends on the specific structure of the binding region in the molecule of enzyme. In general, the results show a very broad spectrum of inhibitory effect of bioflavonoids against proteinases. Established fact can attribute bioflavonoids to the low specific inhibitors of these enzymes.

How does the interaction of bioflavonoids with proteinases realise? To answer this question, conducted determination the Michaelis constant, calculated using regression equations on dependence reciprocals of the reaction rate on the substrate concentration in the presence and absence of bioflavonoid (graphics Lineweaver-Burke). Fig. 2 shows an example of the interaction of quercetin with elastase and in Fig. 3 quercetin with trypsin. Similar calculations are made for all available bioflavonoids and proteinases (Table 3). In all cases the interaction of bioflavonoid with proteinase point of intersection abscissa axis shifted right, and the Michaelis constant increases as compared with the index calculated in absence of bioflavonoid. This indicates the type of competitive inhibition. But at the same time reduces the maximum reaction rate, which is a typical for non-competitive type.

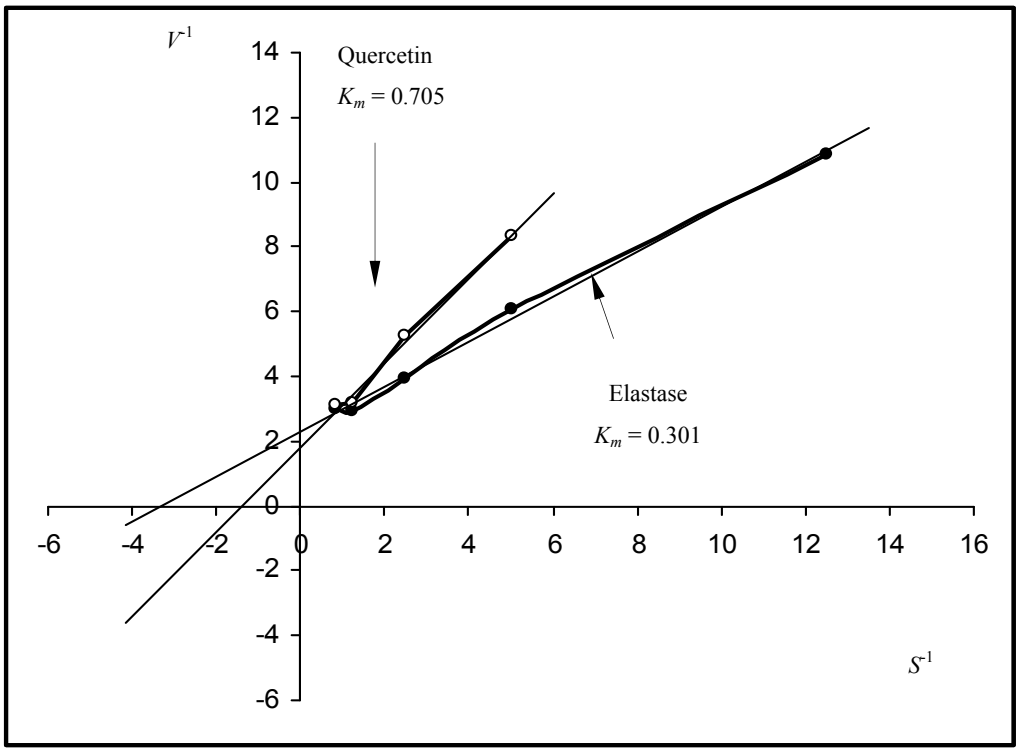

Fig. 2 Schedule Lineweaver-Burk in the interaction elastase with quercetin.

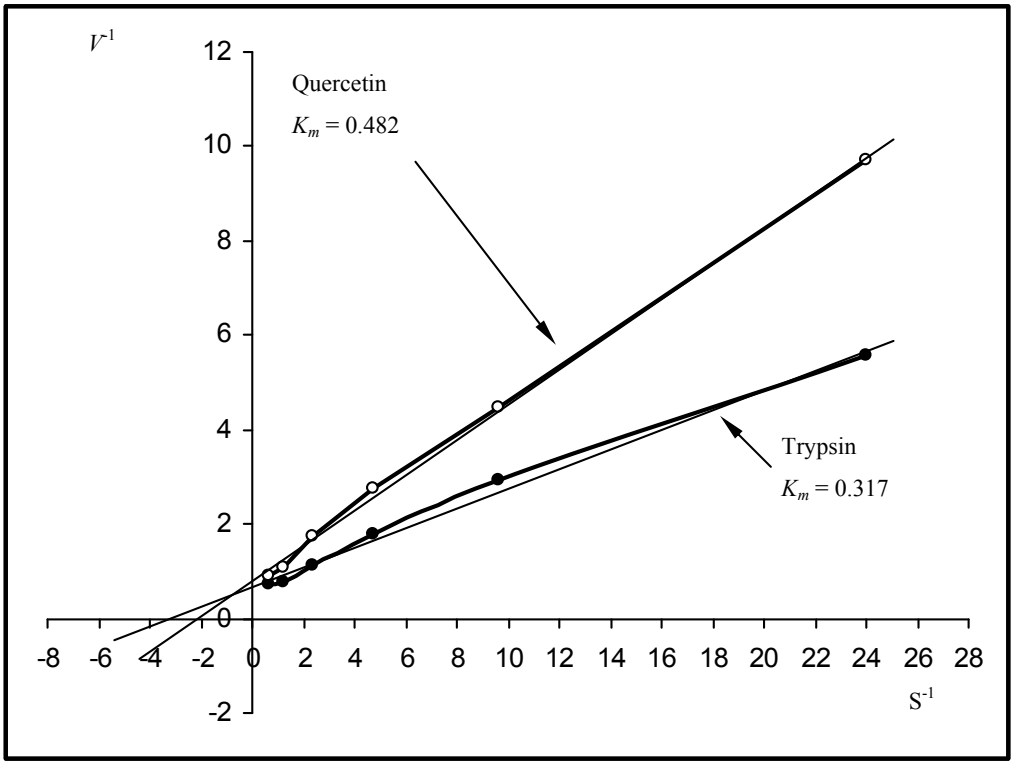

Fig. 3 Schedule Lineweaver-Burk in the interaction trypsin with quercetin.

Table 3 Michaelis constant reactions elastase-bioflavonoid and trypsin-bioflavonoid.

Bioflavonoid	Leukocyte elastase	Trypsin
Without flavonoid	0.301	0.317
Quercetin	0.705	0.482
Genistein	0.669	1.243
Daidzein	0.509	0.732
Baikalin	0.379	0.650
Flavolignans	0.492	0.920
Sophoricozid	Not detected	0.812

Construction of schedules corresponds to a situation where the bioflavonoid associated with the "protease-substrate" complex stronger than a proteinase as many times as the value of the Michaelis constant rise after interaction with bioflavonoid. Thus, bioflavonoids have effect on the proteinase partially competitive inhibition, i.e., they do not bind to the active site of the enzyme, but its effect on the structure. Possibly, bioflavonoid joins to proteinase in the area close to the active center, its deformation occurs, whereby the enzyme affinity for the substrate is reduced and the reaction rate decreases. Inhibition may also occur due to the chelation of metal ions of bioflavonoids which are in the active site of proteinases (Zn^{2+}) or activating proteases (Ca^{2+} and Mg^{2+}).

In general, conducted researches in vitro have shown the ability to separate bioflavonoid in varying degrees to inhibit proteolysis, inhibit at different stages of production of active oxygen species and lipid peroxides, i.e., basic biochemical units of destructive and inflammatory processes. Investigated effects of bioflavonoids are realized by a combined mechanism. These compounds are non-specific antioxidants and protease inhibitors, capable in a different degree inhibit main stages formation of active oxygen forms, lipid peroxides and inhibit proinflammatory proteinase.

Antioxidant and anti-inflammatory efficacy of bioflavonoids confirmed by us in numerous (more than 100) experimental studies in vivo. In Wistar line rats was simulated toxic hepatitis, dental caries, periodontitis, stomatitis, dysbiosis, diabetes Types 1 and 2, gastric ulcer, osteopenia. As a source of bioflavonoids was used quercetin, rutin, hesperetin, genistein, as well as plant extracts from soy beans, blueberries, grape leaves contain bioflavonoids. Condition of organs and tissues was assessed by biochemical markers of inflammation, antioxidant and antimicrobial defense systems.

As a result, due to the significant volume of the results, we can confidently conclude that bioflavonoids ability to prevent the development of numerous pathologies due to manifestations of antioxidant and anti-inflammatory action. According to the classification [9] bioflavonoids can be attributed to adaptogenes—substances that increase the body's non-specific resistance in extreme conditions. In addition, our results are consistent with the previous work in Refs. [10, 11], where bioflavonoids are considered as promising anti-inflammatory drugs and antioxidant action.

On the basis of experimental studies suggested the formulation of new effective therapeutic and preventive medicines containing bioflavonoids. These are tablet forms, powders for oral administration, mouthwashes, oral mucous-adhesive gels.

4. Conclusions

The complex of conducted *in vitro* studies showed bioflavonoids expressed ability to act as an active antioxidant and antiprotease agents. High antioxidant and antiproteinase activity of these compounds, in our opinion, causes expressed *in vivo* anti-inflammatory and adaptogenic effects of drugs containing bioflavonoids.

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