SYNTHESIS AND ANTIOXIDANT ACTIVITY OF ISOFLAVONES CONTAINING HYDROPHILIC AND LIPOPHILIC SUBSTITUENTS

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Isoflavones (3-arylchromones, 3-aryl-γ-pyrones) are known to play an important role in plant physiology [1]. Being phenolic compounds, isoflavones exhibit antioxidant properties and are capable of inhibiting the oxidation of lipid substances. The antioxidant activity of isoflavones has been most thoroughly studied in a group of compounds frequently encountered in plants, such as genistein, daidsein, formononetin, and biochanin A [1 – 4].

At the same time, it is known that flavones (2-arylchromones, 2-aryl-γ-pyrones), which are the closest relatives of isoflavones, possess P-vitamin activity and are capable of reducing the permeability of capillary vessel walls and decreasing their brittleness. The effect is explained by the ability of flavonoids to inhibit free-radical processes, thus protecting the walls of capillaries against the detrimental effect of free radicals formed in the course of lipid oxidation [5].

The antioxidant activity of flavonoids can be manifested via various mechanisms [6]. According to one of these, flavonoids may react as classical radical phenolic inhibitors, by interacting both with the lipid radicals and with the active forms of oxygen:

$$R^{\cdot} + In\text{–}H \rightarrow R\text{–}H + I\,n^{\cdot}$$

$$a)\ LOO^{\cdot} + In\text{–}H \rightarrow LOO\text{–}H + |In^{\cdot},$$

$$b)\ O_2^{-\cdot} + In\text{–}H \rightarrow H_2O + InO^{-\cdot},$$

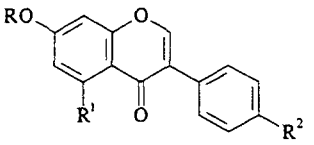
where $R^{\cdot}$ is the initiator of radical processes, In–H is the isoflavone inhibitor, and $LOO^{\cdot}$ is the lipid peroxide radical.

The antioxidant properties of flavonoids can be related to their ability either to inhibit lipoxygenase [7, 8] or to form complexes with Fe^{3+} ions – activators of free-radical lipid oxidation [9, 10].

In our opinion, it is possible to elucidate the mechanism of isoflavone action by studying the antioxidant activity of several compounds possessing different complex-forming ability with respect to Fe^{3+} ions, that is, isoflavones free of hydroxy groups and those containing the hydroxy groups with different reactivities. If the above mechanism of antioxidant action of isoflavones is valid, compounds forming strong complexes with Fe^{3+} ions would possess a higher antioxidant activity. The zone of interaction of isoflavone with a lipid-oxidizing agent can be determined provided derivatives that are soluble either in water or in lipids are available.

The purpose of this work was to obtain (by isolating from raw plant materials and by chemical synthesis) isoflavones containing substituents at positions 5, 7, or 4′, such as hydroxy groups, higher alkyls, carboxy groups, carbon residues, etc., and to study their antioxidant activity.

In the first step of the work, we have obtained samples of the following natural isoflavones and their analogs:



I – X

I: R = R^1 = H, R^2 = OCH_3 (formononetin);
II: R = H, R^1 = OH, R^2 = OCH_3 (biochanin A);
III: R = H, R^1 = OH, R^2 = β-*D*-glucopyranosyloxy (sophoricoside);
IV: R = *n*-C_8H_{17}, R^1 = H, R^2 = OCH_3;
V: R = –$CH(C_8H_{17})COOCH_3$, R^1 = H, R^2 = OCH_3;
VI: R = H, R^1 = OH, R^2 = Cl;
VII: R = tetra-O-acetyl-β-*D*-glucopyranosyloxy, R^1 = OH, R^2 = Cl;
VIII: R = β-*D*-glucopyranosyloxy, R^1 = OH, R^2 = Cl;
IX: R = $-CH_2-CH{=}CH_2$, R^1 = OH, R^2 = Cl;
X: R = *n*-C_8H_{17}, R^1 = OH, R^2 = β-*D*-glucopyranosyloxy (octylsophoricoside).

Isoflavones I and II were synthesized by the action of a dimethylformamide – phosphorus pentachloride complex on α-(4-methoxyphenyl)-2,4-dihydroxy- and -2,4,6-trihydroxyacetophenones [11].

Sophoricoside III was extracted from the fruits of *Sophora Japonica* [12].

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Isoflavone IV was obtained by the reaction of isoflavone I with 1-iodooctane.

Isoflavone V was obtained by the interaction of isoflavone I with the methyl ester of α-bromodecanoic acid.

Isoflavone VI was synthesized by the reaction of α-(4-chlorophenyl)-2,4,6-trihydroxyacetophenone with acetoformic anhydride [13, 14].

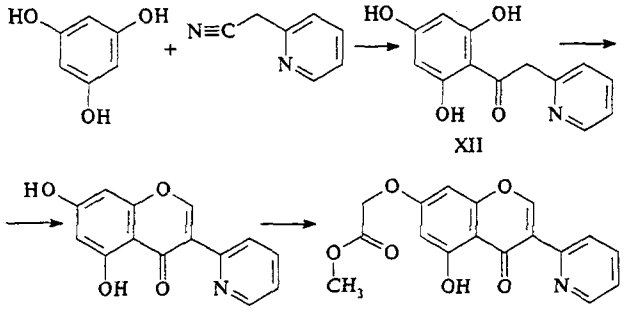
Compound VII was obtained by the reaction of α-bromo-*D*-glucopyranose tetraacetate with isoflavone VI.

Glucoside VIII was obtained by alkaline methanolysis of acetate VII.

Isoflavone IX was obtained by the reaction of 5,7-dihydroxy-4′-chloroisoflavone with 1-bromoprop-2-ene.

Isoflavone X was obtained by the interaction of sophoricoside III with 1-iodooctane.

5-Hydroxy-7-(methoxycarbonylmethocy)-2′-azaisoflavone XI was obtained by a three-stage synthesis proceeding from fluoroglucine and 2-pyridylacetonitrile, via α-(2-pyridyl)-3,4,6-trihydroxyacetophenone (XII) and 3-(2-pyridyl)-5,7-dihydroxychromone (XIII) [13, 14]. Alkylation of chromone XIII with methyl ester of α-bromodecanoic acid leads to the target azaisoflavone XI.



XII

XIII XI

It should be noted that compound IV contains no hydroxy groups, compound I has a hydroxy group weakly interacting (under the experimental conditions studied) with Fe^{3+} ions, and all the other compounds contain hydroxy groups in position 5, forming strong complexes of the chelate type with Fe^{3+} ions with participation of a keto group of the heterocyclic nucleus.

The effects of isoflavones on oxidation processes were investigated by two methods. The antiradical activity of isoflavones was studied on a model system of the production of superoxide anion-radical $O_2^{-\bullet}$, including the photosensitization of riboflavin [15]. The antioxidant activity was studied on the model of phosphatidylcholine liposome oxidation by evaluation of the degree of oxidation inhibition. This was monitored from the accumulation of malonic dialdehyde (MDA) as the main product of lipid oxidation [16].

The reference preparations were represented by the well-known antioxidants ionol and vitamin E, and allyloxytoluene (AOT).

The results of investigation of the effect of isoflavones on oxygen anion-radical production in the photosensitized system show different levels of the inhibiting action of the isoflavone derivatives studied (see Table 1). The maximum activity was observed for compounds IV and X, which contain octyloxy groups in position 7, whose effects were comparable with that of ionol. Compounds V and I, which contain an ester group, proved to be much less active (even though the ester group occupies a position adjacent to the octyl group). These results agree with the data [17] on the inhibiting action of 3,5,7,3′,4′-pentahydroxyflavone (quercetin) in the same model system, where quercetin at a concentration of 0.5×10^{-5} and 1×10^{-5} M decreased the concentration of $O_2^{-\bullet}$ ions by 24 and 40%, respectively. With respect to the antiradical effect, the flavonoids studied in our work are less efficient as compared to the of $O_2^{-\bullet}$ anion radical "trap" superoxide dismutase (which decreases the $O_2^{-\bullet}$ content in the same model system by 91% at an enzyme concentration of 10,000 units / ml [18]).

Thus, the data obtained from the first series of experiments suggest that, in the absence of lipids, isoflavones interact directly with the active forms of oxygen.

In the second series of experiments, the antioxidant activity of compounds I – XI was studied on the model of phosphatidylcholine liposome oxidation by measuring the MDA concentration 10, 15, 20, and 30 min after the onset of reaction. As is seen from Fig. 1, isoflavones decrease the MDA content on the average by 20 – 60%, albeit the degree of oxidation inhibition is lower as compared to that of ionol or vitamin E. The most pronounced antioxidant activity was observed for compound X containing carbohydrate and alkyl (*n*-C_8H_{17}) fragments. The other compounds can be arranged in the following order of decreasing antioxidant activity: IV → VII → VIII → IX → V → XI → III → II → VI → AOT → I.

This sequence agrees with the data on the antiradical activity of isoflavones in the riboflavin-sensitized system of superoxide radical production.

The above results indicate that Fe^{3+} (and Fe^{2+}) ions chelated by isoflavones under the experimental conditions studied have no significant effect on the intensity of lipid oxidation, because compound IV (incapable of forming complexes with iron ions in the absence of hydroxy groups) exceeds all other isoflavones (forming most strong complexes of the chelate type with iron ions) with respect to inhibition

TABLE 1. Antiradical Activity of Isoflavones

Compound	Percentage inhibition of $O_2^{-\bullet}$ production
Ionol	30.6
III	17.5
IV	23.2
V	13.9
X	28.5
XI	10.3

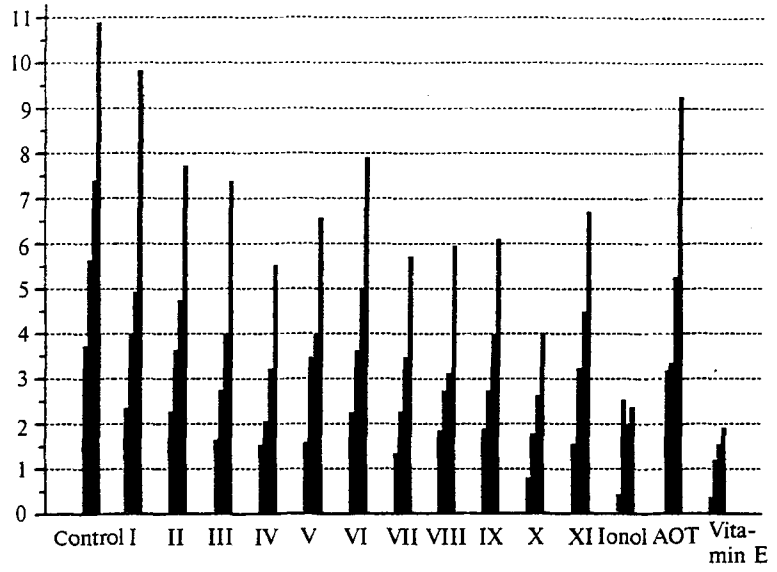


Fig. 1. Effect of isoflavones on malonic dialdehyde (MDA) accumulation. For each compound the first column corresponds to a 10-min incubation, second – 15 min, third – 20 min, and fourth – 30 min. In all cases $n = 5$; $p < 0.05$. Ordinate shows the MDA concentration, nmole / ml.

activity. Another evidence is the low antioxidant activity of 2′-azachromone XI which is capable of forming chelates with iron ions both via the C=O-group oxygen – 5-OH system and via the C=O-group oxygen – pyridine nitrogen system. Thus, either the chelate complex is insufficiently strong to remove iron ions from the reaction zone, or the complex itself is as capable of lipid oxidation as do the free iron ions. In any case, the experimental data confirm that the mechanism of the antioxidant action of isoflavones in the phosphatidylcholine – ascorbic acid – $FeCl_3$ – oxygen system does not involve the step of isoflavone binding to iron ions.

In addition, the experimental data show that the antioxidant activity of isoflavones depends on the lipophilicity of their molecules: the presence of a lipophilic residue (*n*-octyl, methoxycarbonylnonyloxy, tetraacetylglucosyl, and even allyl) in compounds IV, V, VII – X enhances antioxidant activity, while compounds I – III and VI (containing no lipophilic residues) show minimum activity. This fact is rather surprising, because isoflavones containing no lipophilic substituents can be expected to more strongly inhibit the oxidation process. Indeed, a high hydrophilicity must facilitate their concentration in the aqueous phase (where the $O_2^{-\bullet}$ anion-radicals are formed) rather than in liposomes. However, the experimental data show that oxidation processes are suppressed in the lipid phase (liposomes) and not in the aqueous phase. Therefore, in the presence of phosphatidylcholine isoflavones interact primarily with the lipid radicals rather than with the oxygen anion-radicals penetrating into the lipids from the aqueous phase.

These considerations do not contradict the high activity of compound X, which contains a glucose residue besides the octyloxy group. This compound is amphiphilic (surface-active) and its molecules are localized on the surface of liposome, while their carbohydrate parts are in contact with the aqueous phase. This orientation is optimum for the antioxidant, since it provides a maximum concentration of the active agent in the zone of development of the free-radical oxidation processes.

By the same token we can explain the large difference between the activities of compounds III and VIII, which have similar structures. In contrast to compound VIII, sophoricoside III has no pronounced lipophilic and hydrophilic parts and, hence, cannot be localized at the interface of aqueous and lipid phases; this accounts for the lower antioxidant activity.

Thus, the results obtained in the second series of experiments indicate that isoflavones, in the presence of phosphatidylcholine, interact primarily with lipid radicals rather than with oxygen anion*n*-radicals. Should the latter be the case, the oxidation would be more efficiently suppressed by water-soluble isoflavones.

EXPERIMENTAL CHEMICAL PART

The course of the reactions was monitored and the purity of the products was checked by thin-layer chromatography on Silufol UV-254 plates eluted in chloroform – methanol (98 : 2, 90 : 10, or 85 : 15) systems. The IR spectra were measured on a Pye Unicam spectrophotometer using samples pelletized with KBr. The ^1H NMR spectra were obtained with a Bruker WP-100 spectrometer using DMSO-d_6 or $(CD_3)_2CO$ as solvents and TMS as the internal standard. All reactions were performed using dry components of the reagent grade.

Isoflavones IV – XI appear as fine-crystalline colorless substances. The data of elemental (C, H, N) analyses agree with the results of analytical calculations. The IR spectra of compounds IV, V, X, XI contain absorption bands at 2850 and 2925 cm^{-1} (corresponding to stretching vibrations of the CH_3 groups of the alkyl fragment), intense bands at 1250 cm^{-1} (ν_{C-O}), 1615 and 1620 cm^{-1} ($R^1 = H$), or 1625 and 1650 cm^{-1} ($R^1 = OH$), ($\nu_{C=C}$ and $\nu_{C=O}$ of the chromone nucleus). The stretching vibrations of the C=O ester groups of compounds V, VII, X, and XI are manifested at 1750 cm^{-1}.

7-Hydroxy-4′-methoxyisoflavone (I) and 5,7-dihydroxy-4′-methoxyisoflavone (II). Compounds I and II were obtained with a yield of 80 – 90% by the interaction of a dimethylformamide – phosphorus pentachloride complex with borofluoride complexes of α-(4-methoxyphenyl)-2,4-dihydroxy- and -2,4,6-trihydroxyacetophenones [11]. The products were purified by recrystallization from a water – DMF mixture. Compound I: m.p., 265°C; compound II: m.p., 216°C.

5,7-Dihydroxy-4′-(β-*D*-glucopyranosyloxy)isoflavone (III). Compound III was obtained by extraction from the fruits of *Sophora Japonica* [12] and purified by conversion

into hexaacetate, followed by recrystallization from an ethanol – acetone mixture and deacetylation with an equivalent amount of NaOH on boiling in ethanol; m.p., 296°C.

7-Octyloxy-4′-methoxyisoflavone (IV). To a solution of 5.2 g (9.2 mmole) of formononetin I in 20 ml of dry DMF was added 1.2 ml (15 mmole) of iodooctane and 4 g (29 mmole) of freshly calcined potassium carbonate. The reaction mass was stirred for 4 h at 80°C and poured into 100 ml of water. Carbonate was neutralized with acetic acid. The precipitate was filtered, washed with water, dried, washed with hexane, and recrystallized from isopropyl alcohol. Yield of compound IV, 2.06 g (59%); m.p., 97°C; $C_{24}H_{28}O_4$; ^{1}H NMR spectrum, $CDCl_3$ (δ, ppm): 7.91 (s, H-2), 8.23 (d, J 8.0 Hz, H-5), 6.98 (m, H-6), 6.76 (d, J 2.0 Hz, H-8), 7.49 (d, J 8.0 Hz, H-2′, H-6′); 6.98 (m, H-3′, H-5′), 3.81 (s, CH_3-4′), 2.00, 1.28, 0.87 (m, *n*-C_8H_{17}).

5-Hydroxy-7-(1-methoxycarbonylnonyloxy)-4′-methoxyisoflavone (V). To a solution of 5.64 g (20 mmole) of biochanin A (II) in 100 ml of dry acetone was added 6.36 g (24 mmole) of methyl ester of α-bromodecanoic acid and 8.28 g (60 mmole) of freshly calcined potassium carbonate. The reaction mass was stirred for 14 h at 60 – 70°C and poured into 200 ml of cold water. Carbonate was neutralized by acetic acid. The oily precipitate was filtered, washed with water, dried, washed with hexane, and recrystallized from hexane. Yield of compound V, 4.8 g (40%); m.p., 85°C; $C_{27}H_{32}O_7$; ^{1}H NMR spectrum, $CDCl_3$ (δ, ppm): 7.87 (s, H-2), 12.87 (s, HO-5), 6.33 (d, J 2.0 Hz, H-6), 6.38 (d, J 2.0 Hz, H-8), 7.44 (d, J 8.0 Hz, H-2′, H-6′); 6.98 (d, J 8.0 Hz, H-3′, H-5′), 3.84 (s, CH_3-4′), 3.78 (s, $COOCH_3$), 4.71 (t, J 6.0 Hz), 1.98, 1.51, 1.28, 0.88 (m, *n*-$C_8H_{17}CH$).

5,7-Diydroxy-4′-chloroisoflavone (VI). Compound VI was obtained by the action of a complex reagent comprising boron trifluoride – DMF – PCl_5 on α-(4-chlorophenyl)-2,4,6-trihydroxyacetophenone [11]. Yield of the product after recrystallization from isopropyl alcohol 85.5%; m.p., 228°C [13, 14].

5-Hydroxy-7-(tetraacetyl-β-*D*-glucopyranosyloxy)-4′-chloroisoflavone (VII). To a solution of 6.72 g (24 mmole) of isoflavone VI and 3.36 ml (24 mmole) of triethylamine in 20 ml of toluene was added 8.24 ml (20 mmole) of tetraacetyl-α-bromo-*D*-glucopyranose. The homogeneous solution was treated at 101 – 105°C for 4 h and cooled. The precipitate was filtered and washed with toluene. The mother liquor was evaporated in vacuum and recrystallized from ethyl acetate to yield 1.3 g (9%) of compound VII; m.p., 212°C; $C_{29}H_{27}ClO_{13}$.

5-Hydroxy-7-(β-*D*-glucopyranosyloxy)-4′-chloroisoflavone (VIII). To a solution of 0.5 g (1.78 mmole) of acetate VII in 0.9 ml of DMF was added dropwise on cooling in a nitrogen atmosphere 1.6 ml (10.7 mmole) of a 10% potassium hydroxide solution. The mixture was allowed to stand at room temperature for 15 min and neutralized with acetic acid. The precipitate was filtered, washed with water, dried, and recrystallized from ethyl alcohol. Yield of compound VIII, 0.34 g (93%); m.p., 247°C; $C_{21}H_{19}ClO_9$; ^{1}H NMR spectrum, DMSO-d_6 (δ, ppm): 8.54 (s, H-2), 12.85 (s, OH-5), 6.47 (d, J 2.0 Hz, H-6), 5.39 (d, J 6.0 Hz), 5.08 m, 4.58 m, 3.80 – 3.00 m (glucopyranose fragment), 6.73 (d, J 2.0 Hz, H-8), 7.55 (m, H-2′ – H-6′).

5-Hydroxy-7-(allyloxy)-4′-chloroisoflavone (IX). Compound IX was obtained similarly to compound IV proceeding from a mixture of 1.2 g (4 mmole) isoflavone VI, 0.52 ml (6 mmole) allylbromide, and 3 g (22 mmole) K_2CO_3 in 15 ml DMF, heated for 22 h. The product was recrystallized from isopropyl alcohol to obtain 0.8 g (60%) of ice-cream-colored crystals of compound VIII; m.p., 125°C; $C_{18}H_{13}ClO_4$; ^{1}H NMR spectrum, DMSO-d_6 (δ, ppm): 8.54 (s, H-2), 12.85 (s, HO-5), 6.45 (d, J 2.0 Hz, H-6), 6.00, 5.65, 5.30, 4.70 (m, CH_2=$CHCH_2$), 6.71 (d, J 2.0 Hz, H-8), 7.51 (m, H-2′ – H-6′).

5-Hydroxy-7-octyloxy-4′-(β-*D*-glucopyranosyloxy)isoflavone (X). Compound X was obtained similarly to compound IV proceeding from a mixture of 8.08 g (18.8 mmole) sophoricoside III, 4.67 ml (58 mmole) *n*-iodooctane, and 16.7 g (121 mmole) K_2CO_3 in 100 ml DMF, heated for 3 h. The product was recrystallized from ethyl alcohol to obtain 3.82 g (38%) of compound X; m.p., 184°C; $C_{29}H_{36}O_{10}$; ^{1}H NMR spectrum, DMSO-d_6 (δ, ppm): 8.45 (s, H-2), 12.89 (s, HO-5), 6.40 (d, J 2.0 Hz, H-6), 4.09 (t, J 7 Hz), 1.73, 1.29, 0.86 (m, *n*-C_8H_{17}), 6.65 (d, J 2.0 Hz, H-8), 7.52 (d, J 10.0 Hz, H-2′, H-6′); 6.10 (d, J 10.0 Hz, H-3′, H-5′), 4.91 (d, J 8.0 Hz), 3.80 – 3.00 m (glucopyranose fragment).

5-Hydroxy-7-(methoxycarbonylmethoxy)-2′-azaisoflavone (XI). Compound XI was obtained similarly to compound V, proceeding from a mixture of 6 g (23.5 mmole) azaisoflavone XIII [13], 4.78 g (28.3 mmole) methyl ester of α-bromoacetic acid, and 3.7 g (27 mmole) K_2CO_3 in 100 ml acetone, boiled for 4 h. The product was recrystallized from ethyl alcohol to obtain 7.2 g (93%) of compound XI; m.p., 160°C; $C_{17}H_{13}NO_6$; ^{1}H NMR spectrum, DMSO-d_6 (δ, ppm): 8.93 (s, H-2), 12.88 (s, HO-5), 6.74 (d, J 2.0 Hz, H-6), 6.49 (d, J 2.0 Hz, H-8), 8.65 (d, J 5.0 Hz, H-3′); 7.40 (dd, J 8.0 and 5.0 Hz, H-4′), 7.88 (t, J 8.0 Hz, H-5′), 8.20 (d, J 8.0 Hz, H-6′); 3.73 (s, OCH_3), 4.99 (s, $\underline{CH_2}COOCH_3$).

4-Allyloxytoluene. To a solution of 4.32 g (40 mmole) of 4-methylphenol in 30 ml of dry acetone was added 5.2 ml (7.26 g, 60 mmole) of allyl bromide and 8.28 g (60 mmole) of freshly calcined potassium carbonate. The reaction mass was stirred for 20 h at 60 – 70°C. Then the precipitate was filtered, the acetone distilled off, and the residue washed with 20 ml of a 30% aqueous NaOH solution and water. The washing liquids were extracted, and the extracts combined with the above residue and dried over sodium sulfate. Then the solvent was distilled off and the residue distilled to yield 3.5 g (59%) of a colorless liquid product; b.p., 213°C (760 Torr); ^{1}H NMR spectrum, DMSO-d_6, (δ, ppm): 2.24 (s, CH_3), 6.95 (m, H-2 – H-6), 6.04, 5.36, 4.48 (m, CH_2=$CHCH_2$).

EXPERIMENTAL BIOLOGICAL PART

Determination of the Antiradical Activity of Isoflavones. To an incubation medium composed of 0.5 ml riboflavin (2×10^{-5} M), 0.2 ml tetramethylenediamine (1×10^{-2} M), 0.2 ml of *p*-nitrophenyltetrazolium chloride (8.5×10^{-4} M), and 1 ml ethanol was added an ethanol solution of isoflavone with a concentration of 1×10^{-3} M. The reaction was initiated by irradiation by a luminescent lamp (power, 40 W; distance, 10 cm; duration, 5 min) and terminated by switching off the lamp and adding 0.5 ml of a 10% potassium iodide solution. The optical density of the solution was measured (against a control solution) on a Specord-11 spectrophotometer operated at 470 nm. In the control medium, all the above procedures were performed in the absence of isoflavones. The inhibition was calculated using the formula

$$\text{Inhibition (\%)} = \frac{K_{470} - \bar{A}_{470}}{K_{470}},$$

where K_{470} and $\bar{A}_{470}$ are the optical densities of the control solution and the system with isoflavone, respectively.

Determination of the Antioxidant Activity of Isoflavones. To a test solution containing 0.4 ml DMSO, 0.2 ml $FeCl_3$ (1×10^{-4} M), 0.2 ml ascorbic acid (5×10^{-4} M), and 0.1 ml isoflavone solution (1×10^{-2} M) was added 0.01 ml of a 10% ethanol solution of phosphatidylcholine. The mixture was vigorously agitated for 2 min and incubated at 37°C for 10, 15, 20, or 30 min. The reaction was terminated by cooling the solution and adding EDTA (1×10^{-3} M). Phosphatidylcholine was removed from the reaction medium by adding 0.4 ml of a chloroform – ethanol mixture (1 : 1) and centrifuging for 10 min at 3000 rpm. Then 1 ml of the upper layer was taken and replaced by 1 ml of a thiobarbituric acid (5×10^{-3} M) solution in 10% acetic acid. The resulting solution was heated for 15 min at 100°C, and the optical density was measured at 530 nm. The control samples were obtained by performing the same procedures without adding isoflavones. The MDA content was determined by the following formula:

$$C_{MDA} = \frac{\bar{A}_{530} \times 1000(V_{in} + V_{chl\text{-}alc} + V_{TBA})}{152 V_{in}},$$

where $\bar{A}_{530}$ is the optical density of the solution; V_{in} is the volume of the incubation medium; $V_{chl\text{-}alc}$ is the volume of the chloroform – alcohol medium; and V_{TBA} is the volume of the thiobarbituric acid solution.

Interaction of isoflavones with MDA. To 0.05 ml 1,1,3,3-tetramethoxypropane was added 0.1 ml of a 0.1 N HCl and the solution was kept for 1 – 2 min at 50°C until the appearance of a yellow color. To this mixture was added 0.1 ml of an 0.1 N NaOH and 2.93 ml of a KCl-Tris-buffer (pH 7.4). Then 0.1 ml of this solution was removed and 0.1 ml DMSO added. The resulting working solution and an isoflavone solution in DMSO (1×10^{-3} M) were added to 1.0 ml DMSO. The resulting solution was incubated for 30 min at 37°C and then the MDA content was determined as described above.

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