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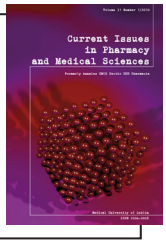

A comparative study of phenolic compound antioxidant activity by the polarography method, using microsomal lipid peroxidation *in vitro*

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ABSTRACT

For comparative purposes, a quantitative estimation of antioxidant activity of phenolic compounds of different classes was conducted by way of the polarography method, via the ADP-Fe^{2+} model of the induced ascorbate-dependent lipid peroxidation of rat liver microsomes within an *in-vitro* system. As a result, it was recognized that the antioxidant properties of phenolic compounds depend on the nature and chemical structure of several substances. In respect of such activity, leaders in the classes of investigated polyphenolic compounds are: Propyl gallate = Gallotannin (Phenolcarboxylic acids and their derivatives) > Quercetin = Myricetin (Flavonols) > Luteolin (Flavo n) = Mangiferin (Xanthones) > Kaempferol (Flavonols) = Catechin (Flavans). Thus, the assessment of the inhibition ability of the lipid peroxidation of microsomes by phenolic compounds can be used as an accessible test for the preliminary quantitative estimation of their antioxidant properties.

INTRODUCTION

Plant phenolic compounds hold a feature that is associated with many biological and pharmacological effects, including the antioxidant. This is their *in vivo* ability to undergo the reverse redox "phenol-semiquinone-quinone" reactions. Herein, their weak semiquinone radical appears to be a "free radical trap" [1-3].

The antioxidant action of such plant phenols is attributed to their ability to neutralize reactive oxygen species (ROS) and block free radical reactions. The reciprocal formation of oxocomplexes between the flavonoid molecules and oxygen (or oxygen radicals) allows them to change the local oxygen concentration in the cell, to protect its easily oxygenatable areas and reaction centers, or to supply oxygen in case of its total deficiency.

Due to the fact that plant phenols have the ability to inhibit the processes of free radical oxidation (FRO) and lipid peroxidation (LP), they can be used as natural antioxidants in the treatment of diseases induced by lipid peroxidation [4,5]. In works [6,7], for example, the phenolic compounds inhibitory effect on the lipid peroxidation processes of mitochondria and cells microsomes is shown. However, unfortunately, there is still a small number of works in which a comparative estimation of the antioxidant activity of phenolic compounds of different classes is conducted.

The goal of the work was to study the comparative antioxidant activity of individual plant polyphenolic compounds via the ADP-Fe^{2+} model of the induced ascorbate-dependent lipid peroxidation of rat liver microsomes within an *in-vitro* system.

MATERIALS AND METHODS

Solvents and Reagents

Herein, each sample came with a Certificate of Analysis that includes a purity determination, and all test material held purities higher than 97-98% (HPLC).

The following phenolic compounds were purchased from Sigma-Aldrich (Steinheim, Germany): Gallic acid (> 98%), Kaempferol (≥ 97.0%), (+)-Catechin (≥ 97%), Quercetin

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(≥ 98%), Quercitrin (≥ 97.0%), Frangula-emodin (≥ 97%), Myricetin (≥ 96.0%), Astragaloside (> 98.0%), Homoorientin (> 98%), Mangiferin (> 98%).

Tannic acid (> 98%) was purchased from Sigma-Aldrich (Steinheim, Germany). Luteolin was from Roth (≥ 98%) (TLC) (Karlsruhe, Germany). Rutin was from Alexis biochemicals (≥ 98%) (Lausen, Switzerland).

Other test compounds, such as 5-Hydroxykhellin and Licorice were a kind donation from the State Enterprise "Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines" (SE "Pharmacopoeial Center", Kharkiv, Ukraine).

All solvents and reagents, while from various suppliers, were of the highest purity needed for each application.

Procedure

The antioxidant properties of polyphenolic compounds were investigated *in-vitro*. In so doing, the microsomes were first separated from the liver of outbred white rats (males), weighing 200-240 g, by a high-speed centrifuge [8]. The microsomes precipitate was then suspended in a medium containing 125 mmol of KCl and 20 mmol of tris-HCl buffer, pH 7.4. The final concentration of protein in the microsomes suspension, which was determined by the Lowry method [9], was 40-60 mg/mL.

The oxygen intake rate by the microsomes suspension was determined by the polarographic method, using a Clark's standard closed platinum electrode at 30°C in 100 mmol of tris-HCl buffer, pH 7.4 [10].

The medium of the 1 mL volume polarographic cell, was sequentially filled with 1.0 to 1.5 mg of microsome protein, 4 mmol of ADP, 0.8 mmol of sodium ascorbate, and, after 1-2 minutes – 0.012 mmol of $Fe(NH_4)_2\times(SO_4)_2\times6H_2O$. Subsequently, solutions of substances were added by titration. Phenolic compounds, which were not solved in distilled water or 100 mmol of tris-HCl buffer at pH 7.4, were solved in an aqueous solution of borax or ethanol. The used concentrations of the solutions of borax and ethanol, in which the substances were solved, did not affect the activity of the microsomes lipid peroxidation. Finally, the concentrations of substances inhibiting microsomes oxidation by 50% (ID_{50}), were calculated. The smaller the ID50, the greater the activity of the substance.

RESULTS AND DISCUSSION

Due to the presence of hydroxyl groups and the peculiarities of the electronic structure of the benzene ring, polyphenols have the properties of anti-radical inhibitors [1,2]. The results obtained from the study of the polyphenols effects on ADP-Fe^{2+} induced ascorbate-dependent lipid peroxidation of intact rat liver microsomes within an *in-vitro* system are listed in Table 1.

As can be seen in the table, compounds belonging to different classes of polyphenols do not equally inhibit the lipid peroxidation processes. Herein, relatively high activity is detected by *esters of gallic acid*; among the flavonoid compounds – quercetin, myricetin and luteolin; and among the xanthones – mangiferin.

Table 1. Influence of polyphenolic compounds on the ADP-Fe^{2+} induced ascorbate-dependent lipid peroxidation of intact rat liver microsomes, n=5

Items	Compounds class and substance name	ID_{50} μg/mL
Flavonols		
1	Kaempferol	5
2	Robinin	50
3	Quercetin	2,5
4	Quercetin pentaacetate	23
5	Quercetin pentamethyl ether	25
6	Avicularine	30
7	Quercitrin Quercetin-3-rhamnoside	30
8	Hyperoside	32
9	Rutin	33
10	Myricetin	2,5
11	Astragaleroside	45
Flavons		
12	Luteolin	3
13	Homoorientin	34
Flavans		
14	(+)-Catechin	5
Chalcones		
15	Licorice	25
Xanthones		
16	Mangiferin	3
Furanocromons		
17	Khellin	30
18	5-Hydroxykhellin	35
Anthraquinones		
19	Frangula-emodin	25
Phenol carbonic acids an their derivatives		
20	Gallic acid	30
21	Propyl gallate (Propyl 3,4,5-trihydroxybenzoate)	2
22	Gallotannin	2

Notes

Structural characteristics of compounds:

1 - 3,5,7,4'-Tetraoxyflavone (3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one),

2 - Kaempferol-3-O-robinoside-7-O-rhamnoside (5-hydroxy-2-(4-hydroxyphenyl)-7-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-3-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-[[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one),

3 - 3,3',4',5,7-Pentahydroxyflavone (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one),

4 - Quercetin pentaacetate (2-(3,4-Diacetoxyphenyl)-4-oxo-4H-chromene-3,5,7-triyl triacetate),

5 - Quercetin pentamethyl ether (2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxychromen-4-one),

6 - Avicularine (3-[(2S,3R,4R,5S)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy-2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one),

7 - Quercetin-3-rhamnoside (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxychromen-4-one),

8 - Quercetin 3-O-beta-D-galactopyranoside (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxychromen-4-one),

9 - Quercetin-3-O-rutinoside (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one),

10 - 3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone,

11 - Isorhamnetin-3,7-di-β-D-glucopyranoside (5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one),

12 - 3',4',5,7-tetrahydroxyflavone (2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone),

13 - Luteolin-6-C-b-D-glucopyranoside,

14 - Trans-(+)-3,3',4',5,7-Flavanpentol ((2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol),

15 - 2,4'-Dihydroxy-trans-halcon-4-0-beta-D-glucopyranosyl-(2'')-0-b-D-apiosofuranoside,

16 - (1S)-1,5-Anhydro-1-(1,3,6,7-tetrahydroxy-9-oxo-9H-xanthen-2-yl)-D-glucitol,

17 - 2-Methyl-5,8-dimethoxyfuro [4',5':6,7]-chromone,

18 - 2-Methyl-5,8-dimethoxy-furo-3,2:6,7-chromone,

19 - *1,3,8*-Trihydroxy-*3-methyl-9,10-anthraquinone (1,3,8-Trihydroxy-6-methyl-9,10-anthrachinon),*

20 - 3,4,5-Trihydroxybenzoic acid (3,4,5-trihydroxybenzoic acid),

21 - Propyl gallate (Propyl 3,4,5-trihydroxybenzoate),

22 - 1,2,3,4,6-Pentagalloyl glucose (1,2,3,4,6-Pentakis-O-(3,4,5-trihydroxybenzoyl)-β-D-glucopyranose),

n - the number of observations in each of the determined indicators,

ID_{50} - the concentration of substances in μg/mL which inhibits the activity of the microsomes lipid peroxidation by 50%.

Following the analysis of the results of the study of the antioxidant activity of flavonoid substances, the possible conclusion is that the degree of activity is significantly influenced by the amount of hydroxyl groups in the B ring of the flavon core. Thus, in the series kaempferol-quercetin-myricetin, which have, respectively, one, two and three hydroxyl groups at once in the B ring, kaempferol (OH group at C_4) was the least active. The OH group at C_3 also has a certain influence. Hence, the presence of a hydroxyl group in quercetin (luteolin) leads to a decrease in its antioxidant activity by 17%, while the renewal in quercetin of the pyron ring to the pyrene (catechin) decreases its activity by 50%. Furthermore, the glycation of flavonols leads to a sharp decrease in their inhibitory activity. Acetylated and methylated derivatives of quercetin (pentaacetate quercetin and pentamethyl quercetin), for example, practically did not differ in their antioxidant activity and were 10 times less active than their aglycone (quercetin). In addition, unlike flavon-C-glucoside (homoorientin), xanthon-C-glucoside (mangiferin) is more active.

Given the fact that the activity of flavones is influenced by the number of oxygroups in the B ring, we experimented with both gallic acid and its esters: propyl gallate and gallotannin. Gallic acid itself inhibits oxidation by 50% at a concentration of 30 mg/mL (ID_{50} = 30 mg/mL), but the esterification of the carboxyl group leads to a sharp increase in the antioxidant activity of the gallic acid, The ID_{50} of propyl gallate and gallotannin is 2 mg/mL, for example, which is almost equal to the activity of quercetin (ID_{50} = 2.5 mg/mL).

The hydroxyl group at C_5 flavonoid substances and furanochromones (5-hydroxykelin) does not significantly affect the antioxidant properties of compounds. Hence, the decrease in effectiveness, apparently, is due to the strong hydrogen bond with the carbonyl of the pyrone cycle. This is made evident from the weak activity of frangula emodin. Herein, the replacement by sugars or other substituents of oxygroups in the 3 and 7 or 3 and 4'-positions of the flavonoid core leads to the inactivation of the molecule (robinin, astragaloside).

We also found that glycosylated chalcones (licorice) are close in activity to flavonol-3-glycosides (avicularine, quercitrin, hyperoside, rutin).

The obtained results are in agreement with the data given in the literature concerning the antioxidant properties of flavonols, flavones, phenolcarboxylic acids and their esters in relation to the microsomes lipid peroxidation, due to the accumulation of the final reaction products of the lipid peroxidation – the TBC reactants [6].

According to the results of chemiluminescence studies [2] of individual biological active substances of plants of different groups (the phenol acids, phenol alcohols, flavonoids and terpenoids) in the model system, with the participation of superoxide anion radicals and peroxide radicals of glycyltriptophane (which are formed in reactions of riboflavin molecules in an excited triplet state with a peptide and oxygen), the increase in the number of phenolic hydroxyl groups in a number of benzoic acid – salicylic acid – gallic acid reactions leads to a sharp increase in antioxidant effect. Herein, reducing the amount of phenolic hydroxyls and esterification abates the effectiveness of vanillin acid as compared with gallic acid [2]. As tannins [6] interact with lipid peroxide radicals, they are effective inhibitors of LPO in the mitochondria and liver microsomes (similar to the phenol carboxylic acid effect in our studies).

Thus, according to [2], with regard to the flavonoids (flavonols quercetin, routine, luteolin flavon and flavonone dihydroquercetin), the activity of these compounds is practically the same, which can be explained by the similarity of mechanisms of their antioxidant action. As stated in [2], for all the tested flavonoids (in which an O-dihydroxy group is found in positions 3 and 4, within the ringform), the probable reaction mechanism involves the oxidation of the O-dihydroxy groups of the B ring in superoxide to form quinone derivatives. This prediction is consistent with the results of the determination of oxidation-reducing potentials of various flavonoids. This indicates that the presence of free hydroxyl groups in positions 3 and 4' increase the restorative ability of flavonoids.

In accordance to the article [11], it was also found that quercetin exhibits a more pronounced antioxidant activity on the NADPH model of enzymatic polyol in rat microsomes than does rutin. In addition, our slightly lower comparative routine activity may be associated with the use of another model of LP.

Consequently, we can conclude that the antioxidant properties of phenolic compounds considerably depend on the chemical structure and nature of substances (Figure 1).

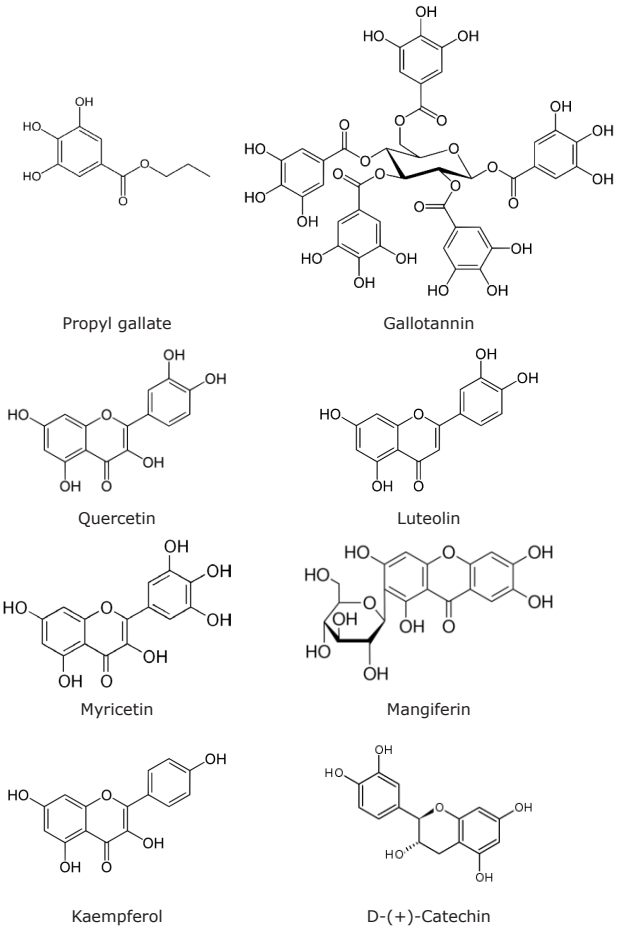

Figure 1. Chemical structural formulas of the most active phenolic compounds

CONCLUSIONS

It is shown that the antioxidant properties of phenolic compounds considerably depend on the chemical structure and nature of substances. In respect of the antioxidant activity, leaders in the classes of investigated polyphenolic compounds can be arranged in the following order: Propyl gallate = Gallotannin (Phenolcarboxylic acids and their derivatives) > Quercetin = Myricetin (Flavonols) > Luteolin (Flavon) = Mangiferin (Xanthones) > Kaempferol (Flavonols) = Catechin (Flavans).

The inhibition of the lipid peroxidation by substances that reveal the properties of antioxidants on the ADP-Fe^{2+} model of the induced ascorbate-dependent microsomes lipid peroxidation can be used as an accessible test for the preliminary quantitative estimation of their antioxidant properties.

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