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ELUCIDATION OF DPPH ANTIRADICAL AND PHOTODYNAMIC ACTIVITIES OF *HYPERICUM PERFORATUM* EXTRACTS

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The antioxidative potentials of extracts of *Hypericum perforatum* from Tavush region of Armenia were evaluated by DPPH assay with simultaneous monitoring of the total flavonoids content. It was shown that H. perforatum ethanolic and aqueous extracts express significant antiradical activity with near inhibition capacities that vary significantly in their ratio index (RI). The DPPH scavenging activity in ethanolic extract correlates with flavonoids content. Ethanolic extract has been shown to have photohemolytic activity that is less than that of pure hypericin. It was revealed the protective effect of quercetin on hypericin induced photohemolysis. This certifies the possible and antiradical activity and the reduced phototoxicity of ethanolic extract expressed by flavonoid compounds.

Hypericum perforatum – flavonoids – DPPH – photodynamic activity

Ուսումնասիրվել է ՀՀ Տավուշի մարզում աճող *Hypericum perforatum*-ի էքստրակտների հակաօքսիդիչ ակտիվությունը (DPPH) և ֆլավոնոիդների ընդհանուր պարունակության մոնիտորինգը: Բացահայտվել է, որ *H. perforatum*-ի էթանոլային և ջրային էքստրակտները ցուցաբերելով բավականաչափ տարբեր ինդեքսներ (RI)՝ ունեն հակառադիկալային մոտ ակտիվություն: DPPH-ի հանդեպ հակառադիկալային ակտիվությունը կորելացվում է էթանոլային էքստրակտի ֆլավոնոիդների պարունակության հետ: Ցույց է տրվել, որ էթանոլային էքստրակտի ֆոտոդինամիկ ակտիվությունը հիպերիցինի համեմատ ավելի թույլ է: Հայտնաբերվել է քվերցետինի պաշտպանիչ դերը հիպերիցինով մակածված ֆոտոհեմոլիզում: Ենթադրվում է, որ էթանոլային էքստրակտների և ֆոտոտոքսիկության նվազումը և հակաօքսիդիչային ակտիվությունները պայմանավորված են ֆլավոնոիդային միացություններով:

Hypericum perforatum – ֆլավոնոիդներ – DPPH – ֆոտոդինամիկ ակտիվություն

Исследован антиоксидантный потенциал экстрактов зверобоя продырявленного (*Hypericum perforatum*) из Тавушского марза Армении анализом антирадикальной (DPPH) активности с одновременным мониторингом общего содержания флавоноидов. Показано, что этанольный и водный экстракты *H. Perforatum* обладают близкой антирадикальной активностью при значительной отличающемся индексе (RI). Антирадикальная активность против DPPH спиртового экстракта коррелирует с содержанием флавоноидов. Этанольный экстракт обладает более слабой фотодинамической активностью по сравнению с гиперицином. Выявлено протекторное действие кверцетина на фотогемолиз, индуцированный гиперицином. Возможно, что как антирадикальная активность, так и низкая фототоксичность этанольного экстракта обусловлены флавоноидными компонентами.

Hypericum perforatum – флавоноиды – DPPH – фотодинамическая активность

Hypericum perforatum (St. John's wort) has a wide range of medicinal applications, including skin wounds, eczema, burns, diseases of the alimentary tract and psychological disorders. The particular biological activity of plant extracts could be connected with the presence of a single [4; 10; 17] and several compounds that collectively provoke a variety of physiological responses. One of the most active components of extracts of *H. perforatum* is hypericin (HY) (1,3,4,6,8,13-Hexahydroxi-10,11-dimetylfenantro [1,10,9,8-*opqra*]perylen-7,14-dion), that is known as photodynamic agent [1]. HY is considered as the most powerful natural photosensitizer with high quantum yield of singlet oxygen ($^1O_2$), bright fluorescence and minimal dark toxicity [3]. *H. perforatum* has significant anti-tumor activity with low toxicity that was shown both on cell cultures and in vivo. This effect is connected mainly with HY that selectively accumulates in transformed cells [1; 13]. Besides, antitumor effect of *H. perforatum* could be due to flavonoids, i.e. quercetin (1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthroperylene-7, 14-dion) – the most widely distributed flavonoid that acts not only neutralizing a variety of cancer-causing agent, e.g. reactive oxygen species (ROS), but also can inhibit many types of cancer cells growth and metastasis. Thus the role of *H. perforatum* flavonoids content in the fight against cancer cannot be ignored [12]. There is an increasing interest in natural antioxidants, namely phenols, present in medicinal and dietary plants, that might help prevent oxidative damage [7].

In spite of this intense research activity, the antioxidant potential of *H. perforatum* extracts has been poorly studied. The aim of current work was to elucidate photodynamic and free radical scavenging activities of *H. perforatum* extracts, determine the relationship of flavonoids composition.

Materials and methods. *H. perforatum* plants (flowers, approx. 3-5 cm of stem) were collected in 2010 from Tavush region of Armenia and the biomass was dried on the same day for the preparation of ethanolic and water extracts [16].

Aluminum chloride colorimetric method was used for flavonoids determination [5]. As a flavornoid standard the quercetin (0.1–10 μg/ml) dissolved in ethanol was used. The calibration curves traced for standard solutions of quercetin/Al^{3+} complexes at 430 nm. From the calibration curve the dependence of quercetin concentration on absorption (A_{430}) was revealed that is expressed in the equation with a coefficient of determination (R^2):

$$C_{quercetin} = -0.19+14.64\times A_{430}; \qquad R^2 = 0.9834 \qquad (1)$$

To calculate the quantity of total flavones (expressed as quercetin equivalents as mg/g of dry weight of plant material) in *H. perforatum* extracts the dilution factor was quantified and the concentration [mg flavonoid / g sample] was calculated by equation:

$$C_{Flav.} = V\,(ml) \times 10^{-3} / m\,(g) \times F \times C_{det}\,(mg/ml), \qquad (2)$$

where: V is the volume of solution prepared from the plant material, m is the mass of plant material, F is the dilution factor that is equal to 10, C_{det} is the flavonoid content for each extract, quantifies by formula (2).

Antiradical activity or hydrogen donating ability of tested extracts compounds was assessed in a chemical model, i.e. 2,2-diphenyl-picrylhydrazyl radical (DPPH) system, occurring by the DPPH radical transformation into its reduced form (DPPH-H), which is accompanied by discoloration of the solution from red to yellow [11; 9]. DPPH solution was used as a negative control. Quercetin solution was used as a positive control. A remnant optical density of DPPH at 517 nm (14.5% in average) was detected even after its complete scavenging and thus the percentage of the radical scavenging activity (RSA) was calculated by the following equation:

$$RSA\% = [(A_{control} - A_{sample})/(A_{control} - A_{remnant})] \times 100, \qquad (3)$$

The IC_{50} values that denote the concentration of sample, required for scavenging the 50% of DPPH free radicals were calculated and expressed as means ± SD. The IC_{50} values were calculated by a linear regression analysis. To show the DPPH discoloration reaction kinetics the IC_{50} values were converted to a ratio by dividing them to a value, where a total DPPH reduction is in its steady state. This ratio was named reduction index (RI), a kinetic parameter that measures the velocity of the reaction of DPPH reduction.

To reveal the photodamaging effect on red blood cells (RBCs) the extract was standardized by HY content (0.46 μM). Photodynamic activity of *H. perforatum* ethanolic extract and HY with/without quercetin was studied by photo-RBC test. RBCs suspensions in the presence of photosensitizer – extract or HY were irradiated for 10 min by visible light [5]. Evaluation of RBC hemolysis degree was conducted at 680 nm and calculated by formula:

$$\% \text{ hemolysis} = (1 - A_x/A_0) \times 100\%, \qquad (4)$$

where A_0 and A_x – optical densities of RBC suspension initial and after irradiation during x time, respectively.

The influence of quercetin (up to 5 μM) on HY induced photohemolysis was studied by 10 min irradiation of RBC suspension. Long lasting incubation of 5% ethanol with RBCs did not cause hemolysis in dark, as well as after irradiation.

Experimental results are expressed as means ± SD (standard deviation). All measurements were replicated three times. The data were analyzed by a one-way analysis of variance (ANOVA) and the values of $p < 0.05$ were considered as significant.

Results and Discussion. Most of the naturally occurring compounds of flavonoid origin contain one or more common structural features that can be involved in complex formation with metals, e.g. with aluminum. This property of flavonoids was used to reveal their content in H. perforatum extracts. The data received in this research indicate that the flavonoids content in ethanolic extracts of H. perforatum exceeds that of aqueous extracts more than 8 times (tab. 1).

Table 1. Flavonoid and hypericin contents of *H. perforatum* extracts. IC_{50} and RI values for DPPH radical scavenging activities of extracts and quercetin. Values are the mean of five independent experiments (mean±SD, $p<0.05$).

Tested samples	Flavonoid content, mg/g	hypericin content, μM	IC_{50}, mg/ml	RI
Ethanolic extracts	0.59 ± 0.031	30.0 ± 0.5	0.68 ± 0.036	0.26 ± 0.017
Aqueous extracts	0.073 ± 0.004	7.0 ± 0.05	0.75 ± 0.045	0.08 ± 0.005
Quercetin	–	–	0.01 ± 0.003	0.29 ± 0.021

Antiradical activities of *H. perforatum* ethanolic and aqueous extracts were detected by their ability to reduce the DPPH radicals. On the fig. 2 shows the radical scavenging activities of the extracts expressed as quercetin equivalents (mg quercetin/ml sample). The obtained quenching activities against DPPH radicals were dose-dependent. From the dose-activity curves concentration of each extract causing radical scavenging on 50% or inhibition concentration (IC_{50}) against 460 μM DPPH was determined graphically. Both extracts of *H. perforatum* showed high free radical scavenging activities by DPPH assay (fig. 1).

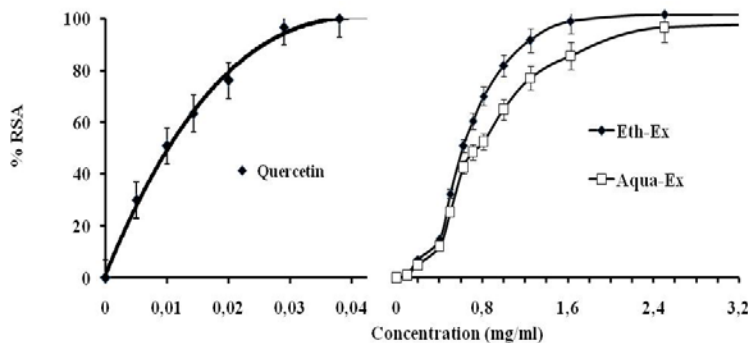

Fig. 1. DPPH radical scavenging activity (RSA) of quercetin (left) and ethanolic and aqueous extracts of *H. perforatum* (right) (mean ± SD of five independent experiments, $p<0.05$).

The major photosensitizing compound in *H. perforatum* extract is HY. Considering the high amount of the latter in ethanolic extracts (tab. 1) further experiments on elucidation of photodynamic properties were conducted with ethanolic extract. It was shown that HY causes RBCs hemolysis upon irradiation, which depends on HY concentration [16]. The comparison of the photodynamic properties of HY with ethanolic extract revealed that RBCs are more resistant in the case of extract with the same amount of HY in it (fig. 2). This fact allows concluding that there are several components within the extract that prevent the photoinduced destruction of RBCs. Flavonoids and quercetin in particular can serve as protectors due to their antioxidative properties [12].

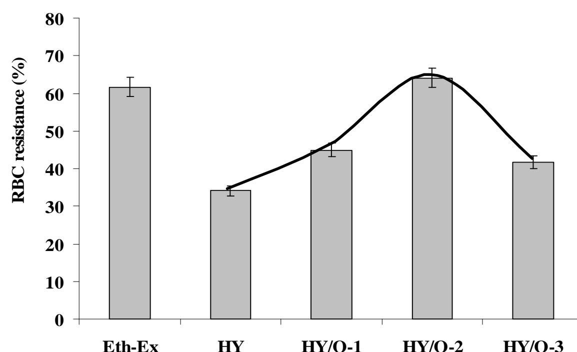

Fig 2. RBC resistance (in %) under the photodynamic (10 min irradiation) influence of H. perforatum ethanolic extract and HY (0.46 μM) alone, as well as under the joint action of HY with quercetin (Q), where HY/Q -1; -2; -3 are the molar ratios of HY and Q of 1/0.1; 1/1 and 1/10, respectively (mean ±SD of four independent experiments, $p<0.01$).

To ascertain the input of quercetin in RBCs protection the RBCs were irradiated with HY and quercetin in different concentrations (fig. 2). Results show that in concentrations of 0.05 – 1 μM quercetin had strong protective effect on photohemolysis, induced by HY with a maximal effect of 64 % in about 0.5 μM. This effect certifies that quercetin act as antioxidant and quenches the generated ROS, mainly the 1O2 that forms during the photosensitization by HY in used concentration. Quercetin itself, despite its concentration and irradiation time, did not lead to RBCs destruction.

H. perforatum plants are reported to have multiple biological activities including vasodilator, anti-inflammatory, anticancer, antiviral, antibacterial etc [4, 5]. Several compounds in *H. perforatum* could be attributed to its wound healing and other pharmacological activities associated with free radicals. Ethanolic extracts of *H. perforatum* are known to contain a number of phenolic compounds, including HY, hyperforin and their derivatives, rutin, hyperoside, quercetin, chlorogenic acid, flavonols, flavones, etc [6].

The results of this study have revealed the antioxidant potential of H. perforatum aqueous and ethanolic extracts. DPPH radical scavenging effect of both extracts had dose-dependent character. The calculated IC_{50} values indicate that the antioxidant activity of ethanolic extract of *H. perforatum* is a little higher than its aqueous extract. It is known that the flavonoids content in extracts plays a significant role in their antioxidant capacity [8]. The aqueous extract of *H. perforatum* has a low content of HY and flavonoids that suggests that the expressed antiradical activity is more likely caused by water-soluble non-flavonoid origin antioxidant compounds. The high radical scavenging activity value of ethanolic extracts is likely connected with content of flavonoids and quercetin in particular the content of which was shown to be 8 times more in ethanolic extracts (tab. 1).

The IC_{50} values give little information about the reaction kinetics, in terms of velocity [2]. Therefore, we used an additional parameter, the reduction index (RI) that measures the velocity of the reaction of DPPH reduction to DPPH-H – characterize the DPPH bleaching rate (tab.1). This parameter gives an idea of the reactivity of tested extracts in bleaching the DPPH free radical. Both extracts induced a moderate decrease in the free DPPH radical (when compared with quercetin reactivity). It was shown that inserted RI values differ significantly for ethanolic and aqueous extracts (3.2 times), but practically similar for ethanolic extract and quercetin. Thus, despite the practically similar IC_{50} values, the ethanolic extract of *H. perforatum* was more reactive. It is possible that slow velocity of DPPH decolorization by aqueous extract is due to antioxidant compounds of non-flavonoid origin.

It was evidenced that DPPH scavenging could be separated in two parts: a first rapid one and a second in which the radical was being scavenged at a very slow rate. The stoichiometries of the rapid and slow stages are basic elements for explanation of the antiradical activity, as they could reveal the contribution of different functional groups to scavenging reactions. The rapid stage could be possibly attributed to the very reactive o-hydroxyls of B-ring as quercetin. In such case a reaction between the flavonoid and DPPH takes place to produce less active quinine [15]. The high activity of the extract therefore, could be attributed to the presence of phenolic compounds in it. This may also be associated with the presence of soluble alkaloids and phenolic compounds in substantial amounts as observed in the phytochemical screening [1]. Besides, synergism of phenolic compounds in an extract may contribute to the overall antioxidant activity.

The ethanolic extract of *H. perforatum* revealed also photodynamic activity. The mechanism currently thought to be responsible for the phototoxicity of H. perforatum extracts and HY in particular involves the production of $^1O_2$ and superoxide radicals upon light-activation. The production of ROS by HY upon light activation can cause oxidative damage leading to cell death, inducing apoptosis at lower concentrations of HY and light energy, whereas higher concentrations tend to induce necrotic cell death [13; 7]. The phototoxicity of HY on RBCs model has been shown to be concentration and light dose-dependent [16]. Ethanolic extract of *H. perforatum* exhibited phototoxic effect that is lower in comparison with pure HY. The fewer phototoxic side effects of *H. perforatum* extracts may be due in part to the antioxidant properties of other constituents present within the extract, such as flavonoids, phenolic acids, porphyrins. These constituents may be able to elicit cellular protection by reducing the amount of ROS generated by photo-induced HY, thus decreasing oxidative damage. In model experiments in vitro on RBCs a constituent of flavonoids origin – quercetin when supplemented with HY lowers the phototoxicity of the latter on about 30%, that correlate well with the clinical evidence suggesting that administration of *H. perforatum* extracts containing the photo-activated HY compounds may cause fewer skin photosensitization reactions than administration of pure HY. Based on these studies, it seems as though HY administered via *H. perforatum* extracts may be less toxic than administration of pure HY [14]. This attenuation may have occurred by multiple mechanisms including scavenging the free radicals produced by HY or competing for light energy, reducing the occurrence of triplet state HY. This property of *H. perforatum* extracts allow joint application HY with certain antioxidant components of the extract in photodynamic therapy in more beneficial conditions comparing with pure HY that can prevent side effects, such as RBCs photodestruction. Therapy using free-radical scavenging antioxidants has potential to prevent, delay or ameliorate many of disorders.

Data gathered in this study demonstrated the ability of ethanolic and aqueous extracts of H. perforatum to scavenge the DPPH stable radical with near IC50 that varies significantly in their RI. In aqueous extract the DPPH decolorization activity seems to be connected with contribution of soluble components of non-flavonoid origin. The DPPH stable radical scavenging activity correlates with flavonoids content in ethanol extract.

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