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H. R. Vardapetyan[1], S. S. Blbulyan[1], S. G. Tiratsuyan[2], corresponding member of NAS RA A. H. Trchounian[2]

Elucidation of Antiradical and Antibacterial Properties of *Hypericum Perforatum* Extracts

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Keywords: *Hypericum perforatum, flavonoids, DPPH, antibacterial activity.*

Introduction. Many plants used in herbal preparation have been found to possess sufficient antioxidant compounds that can be used in the battle against cellular damage and disease [1]. In situations of increased free radical generation the reinforcement of endogenous antioxidants via intake of dietary antioxidants may be of particular importance in attenuating the cumulative effects of oxidatively damaged molecules. It has been determined that the antioxidant effect of plant products is mainly due to radical scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes . The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [2].

Plants of the *Hypericum* genus are well known for their use in traditional medicine due to their therapeutic efficacy. *H. perforatum* extracts has been found to contain several classes of compounds common to most plants, including polyphenolics, flavonoids (quercetin, rutin), etc, as well as two very active classes of unique constituents – the phloroglucinols (hyperforin, adhyperforin) and the naphthodianthrones (hypericin, pseudohypericin) [3, 4]. 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 different biological activities of *H. perforatum* ethanolic and aqueous extracts, e.g. cytotoxic and free radical scavenging activities, determine the relationship of flavonoids composition, antiradical and antimicrobial activities in chemical and biological systems.

Materials and methods. *H. perforatum* plants (flowers, including 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 [5]. To study the antibacterial activity extracts were pre-sterilized through antibacterial filters with 0.2 μm pore size.

Aluminum chloride colorimetric method was used for flavonoids determination [6] based on the property of flavonoids and flavones glycolsides form internal yellow complexes of chelate type with Al^{3+}. As a flavonoid standard the quercetin (1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthroperylene-7, 14-dion) (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 have revealed. 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):

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

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:

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

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 of tested extracts compounds was assessed in a chemical model, i.e. DPPH (2,2-diphenyl-picrylhydrazyl) stable radical system as described previously [7] , occurring by the DPPH radical transformation into its reduced form, which is accompanied with its bleaching at 517 nm at 25°C [8]. DPPH solution which absorbance was not changed in the time of the experiments was used as a negative control. Quercetin and rutin (3, 3´,4´,5,7-pentahydroxyflavone-3-L-ramnopyranoside) (0.1-10 μg/ml) were used as a positive control. A remnant optical density of DPPH at 517 nm was detected even after its complete scavenging and thus the percentage of the radical scavenging activity (RSC) was calculated by the following equation:

$$RSC\% = [(A_{control} - A_{sample})/(A_{control} - A_{remnant})] \times 100.$$

The inhibition concentration (IC_{50}) values that denote the concentration of sample, required for scavenging the 50% of DPPH free radicals were calculated. 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 [9].

Antibacterial activities of *H. perforatum* aqueous extract were studied on polyresistant strain *Staphylococcus aureus* № 906 and wild type *Escherichia coli* K-12 cultures (Collection of the Department of Epidemiology of Yerevan State Medical University) by the method of serial double dilutions with different content of extracts [10]. From dose-depending curves the concentration of extract required for the inhibition of the bacterial survivability on half (IS_{50}) was determined.

The antibacterial activities of both extracts of *H. perforatum* were also in-

vestigated by the disc-diffusion method [11]. As an indicator of antibacterial activity the inhibition zone (in millimeters) formed after 24 – 48 hours of bacteria development was taken. *E. coli* K-12 wild type strain was grown on nutrient media in Petri dishes with 1.5% agar and minimal salt media [12]. Microbial loading on a Petri dish was about 10^8 cells. The sample holes were gained by removal of 8 mm core of agar from the seeded Petri dish agar and 100 µl of each extract (10 - 100 mg/ml), as well as pure quercetin and rutin (0.01 - 0.1mg/ml) was applied. The filled hole was then covered with pure agar. The plates were incubated overnight at 37° C. Cultures incubated with ethanol were the negative control.

Experimental results are expressed as means ± SD (standard deviation). All measurements were replicated five 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. The property of compounds of flavonoid origin involved in complex formation with metals e.g. with aluminum [13] 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 (Table).

Flavonoid, hypericin contents, IC_{50} values for DPPH radical scavenging and IS_{50} antibacterial activities of *H. perforatum* extracts, quercetin and rutin. Represented values are means of five independent experiments (mean ± SD, p<0.05)

H.perforatum extracts	Flavonoid content (mg/g)	Hypericin content (µM)	IC_{50} (mg/ml)	IS_{50} g/ml	
				E.coli	*S.aureus*
Eth-Ex	0.59 ±0.031	30.0 ± 0.5	0.68 ± 0.036	-	-
Aqua-Ex	0.073 ±0.004	7.0 ±0.05	0.75 ± 0.045	0.45±0.010	0.30±0.015
Quercetin	-	-	0.01 ± 0.003	-	-
Rutin	-	-	0.03 ± 0.003	-	-

The evaluation of the free radical scavenging capacity of *H. perforatum* extracts, quercetin and rutin was based on the DPPH consumption elicited by their addition. Hydrogen donating ability was dose-dependent. From the dose-activity curves IC_{50} of each extract, quercetin and rutin was determined graphically. Both extracts of *H. perforatum* showed high approximately the same free radical scavenging activities by DPPH assay (Fig 1). The standard quercetin shows an IC_{50} at 0.01±0.003 mg/ml, and rutin 0.03± 0.003 mg/ml.

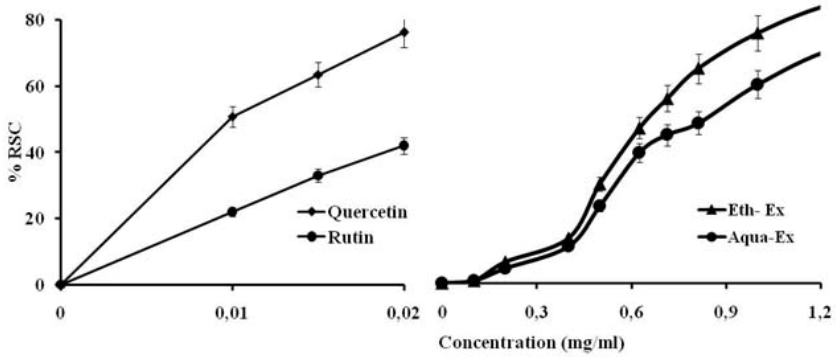

Fig 1. DPPH radical consumption induced by quercetin and rutin (left) and ethanolic and aqueous extracts of *H. perforatum* (right) (mean ± SD of five independent experiments, $p<0.05$).

The antibacterial activity of aqueous extract of *H. perforatum* was assessed by cultivation of bacteria in media containing different concentrations of extracts. It was shown that the extract has expressed inhibiting action on gram-positive (*S. aureus* № 906) and gram-negative (*E. coli* K-12) bacteria. *S. aureus* and *E. coli* become susceptible to *H. perforatum* extract beginning from the concentration of 0.18±0.005 and 0.28±0.009 g/ml, respectively. The concentration of extract that is needed to inhibit the survivability on half (IS_{50}) is 0.3±0.010 and 0.45±0.015 g/ml for *S. aureus* and *E. coli*, respectively. These data demonstrate the sizeable antimicrobial activity of aqueous extracts of *H. perforatum* (Fig. 2).

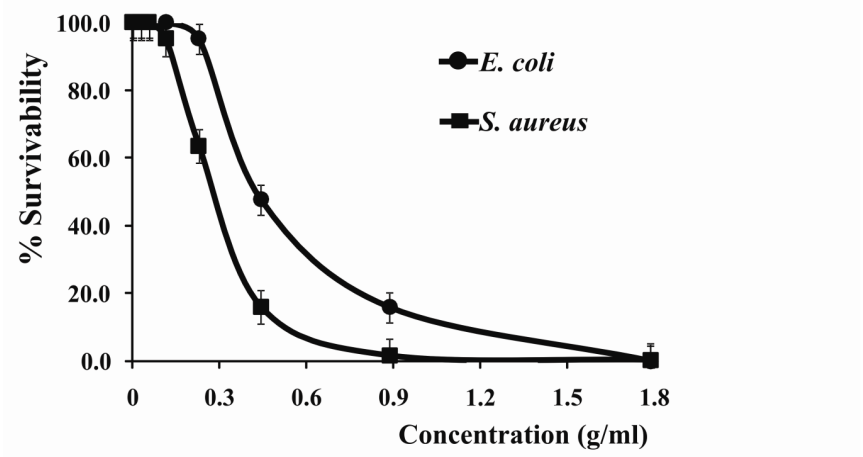

Fig. 2. Survivability (in %) of *S. aureus* № 906 and *E. coli* K-12 depending on concentration of aqueous extract of *H. perforatum* (mean ± SD of four independent experiments, $p<0.05$).

Antibacterial activities of extracts of *H. perforatum* and quercetin on *E. coli* K-12 were also studied by disc-diffusion method. It was shown that etha-

nolic extract and quercetin possess antimicrobial activity with the close values diameters of inhibition zone (with the core diameter), within the 24 h period. Rutin possess a less inhibition on *E. coli* K-12, and the aqueous extract action is negligible. After 48 h a slight increase of the inhibition zone was observed. The ethanol alone did not show any zone of inhibition (Fig. 3, 4).

Fig. 3. The formation of inhibition zones after injection of *H. perforatum* ethanolic extract (1) and quercetin (2) into the agar holes. Represented values are means of five independent experiments (mean ± SD, $p<0.05$).

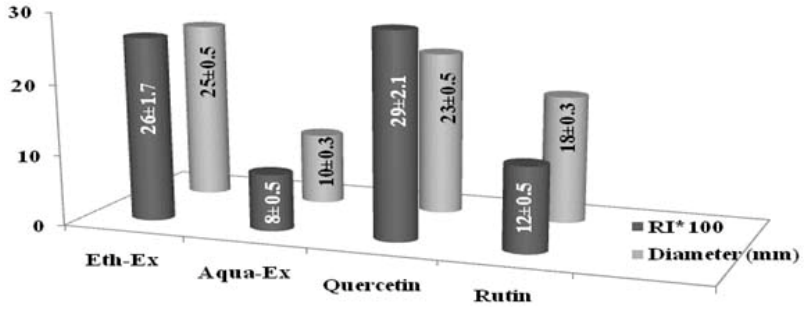

Fig. 4. RI and inhibition zone diameter (with core diameter) values for *H. perforatum* extracts, quercetin and rutin. Represented values are means of five independent experiments (mean ± SD, $p<0.05$).

Discussion. Medical plants are therapeutic resources of modern drugs for the treatment of common diseases. The screening of medicinal plants for active compounds has become very important as potential sources of novel antibiotics

[14]. *H. perforatum* plants are reported to have multiple biological activities including vasodilator, anti-depressive, anti-inflammatory, anticancer, antiviral, antibacterial etc [1, 3, 9] due a number of phenolic compounds, including hypericin, hyperforin, rutin, hyperoside, quercetin, flavonols, flavones, etc [15]. Several compounds in *H. perforatum* could be attributed to pharmacological activities associated with free radicals. Overproduction of free radicals causes oxidative damage to biomolecules such as lipids, proteins and DNA, eventually leading to many chronic diseases [1, 2]. However, antioxidants are known to reduce the possibility of occurrence of these adverse events.

The results of this study have revealed the antioxidant potential of *H. perforatum* aqueous and ethanolic extracts in a chemical model. 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 [1]. The aqueous extract of *H. perforatum* has a low content of hypericin and flavonoids that suggests that the expressed antiradical activity is more likely caused by water-soluble non-flavonoid origin antioxidant compounds. The high RSC 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 (table). Quercetin exhibits a better scavenging activity, supporting the role of 3-OH group in free radical quenching. The relatively low scavenging activity of rutin could be attributed to the O-rutinose substituted 3'–position of the C-ring confirms that in flavonols with thrihydroxyl the hydroxyl groups function as effective radical scavengers [16]. In this test system quercetin was more potent antioxidant, than rutin.

The IC_{50} values give little information about the reaction kinetics, in terms of velocity [17]. Therefore, we used an additional parameter, the reduction index (RI) that characterize the DPPH bleaching rate (Fig. 4). Both extracts induced a moderate decrease in the free DPPH radical. It was shown that inserted RI values differ significantly for ethanolic and aqueous extracts (more than 3 times), but RI for ethanolic extract is equal quercetin RI. It is possible that high velocity of DPPH bleaching is due to antioxidant compounds of flavonoid origin, whereas slow velocity of DPPH decolorization by aqueous extract is due to antioxidant compounds of non-flavonoid origin. Besides, synergism of phenolic compounds in an extract may contribute to the overall antioxidant activity

The antibacterial activity of *H. perforatum* aqueous extracts showed growth inhibition against *S. aureus* and *E. coli*, where the stronger activity was found on *S. aureus* (Fig. 2). The activity of the extract was found to be higher on Gram-positive bacteria compared with the Gram-negative strains. The latter effect was not surprising as it is known that Gram-negative bacteria are more resistant to plant extracts [12]. Data received in this study allow concluding that *H. perforatum* aqueous extracts act on cytoplasmic targets. It should be noted that aqueous extract showed antiradical activity (IC_{50} = 0.75 ± 0.045 mg/ml) in much more less concentrations than is needed for the expression of antibacterial activity (IS_{50} = 0.3±0.010 and 0.45±0.015 g/ml) for *S. aureus* and *E. coli*, respectively.

Ethanolic extract of *H. perforatum* and quercetin showed practically equal antibacterial activities against *E. coli* K-12 by the disc diffusion method. Considering the fact that the flavonoid content in ethanolic extract is equal to quercetin content expressed in equivalents (Table), it could be concluded that the main input in antibacterial activity of this extract belongs to quercetin, although routin can introduce antibacterial activity of extract. The aqueous extract did not show antibacterial activity by this method, which may be connected with the low amounts of extracts used that was less from 20 to 30 times compared with the amounts, taken for the method of serial double dilutions. The differences in antibacterial activities of ethanolic and aqueous extracts within the same concentrations could be explained by the low quantity of flavonoids in the latter. These results agree well with the published data on antimicrobial activities of *H. perforatum* extracts on various bacteria [17]. In the present work it was shown correlation between RI and antibacterial activity, determined by disc-diffusion method that certify both antibacterial activity and velocity of DPPH bleaching is due to the same antioxidant compounds (Fig. 4).

Data gathered in this study demonstrated the ability of ethanolic and aqueous extracts of *H. perforatum* to scavenge the DPPH stable radical. It is shown, that both extracts express the significant antiradical activity with near IC_{50} values 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. Both extracts of *H. perforatum* reveal antibacterial action in a dose-dependent manner. The result of this study justifies the use of these extracts of *H. perforatum* for the treatment of infectious diseases caused by bacteria.

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[1]Russian-Armenian (Slavonic) University
[2]Yerevan State University

H. R. Vardapetyan, S. S. Blbulyan, S. G. Tiratsuyan, corresponding member of NAS RA A. H. Trchounian

Elucidation of Antiradical and Antibacterial Properties of *Hypericum Perforatum* Extracts

The antioxidative potentials of extracts of *Hypericum perforatum* were evaluated by 2,2-diphenyl-l-picrylhydrazyl radical (DPPH) assay with simultaneous monitoring of the total flavonoids content. The antibacterial effects of these extracts were also investigated. It was shown that *H. perforatum* ethanolic and aqueous extracts express significant antiradical activity with near inhibition capacities that vary significantly in their reduction index (RI). Aqueous extract reveals cytotoxic action on *E.coli* and

S.aureus bacteria in a dose-dependent manner that correlates with antiradical activity, which is seems to be connected with components of non-flavonoid origin. The DPPH scavenging activity in ethanolic extract correlates with flavonoids content.

Հ. Ռ. Վարդապետյան, Ս.Ս. Բլբուլյան, Ս. Գ. Տիրացույան,
ՀՀ ԳԱԱ թղթ. անդամ Ա.Հ. Թոչունյան

Hypericum perforatum-ի էքստրակտների հակառադիկալային և հակաբակտերիալ հատկությունների ուսումնասիրությունը

Ուսումնասիրվել են ծակոտկեն սրոհունդի (*Hypericum perforatum*) էքստրակտների հակաօքսիդիչ ակտիվությունը 2,2-դիֆենիլ-լ-պիկրիլհիդրազիլ (ԴՖՊՀ) կայուն ռադիկալների մարմամբ և ֆլավոնոիդների ընդհանուր պարունակության մոնիթորինգը: Հետազոտվել է նաև էքստրակտների հակաբակտերիալ ակտիվությունը: Բացահայտվել է, որ *H. perforatum*-ի էթանոլային և ջրային էքստրակտները, ունենալով հակառադիկալայինին մոտ ակտիվություններ, ցուցաբերում են բավականաչափ տարբեր վերականգնման ինդեքսներ (RI): Ջրային էքստրակտը *E. coli* և *S. aureus* բակտերիաների նկատմամբ ցուցաբերում է ցիտոտոքսիկություն՝ կախված կոնցենտրացիայից, որը կորելացվում է հակառադիկալային ակտիվությամբ՝ պայմանավորված ոչ ֆլավոնոիդային բնույթի բաղադրիչներով: Էթանոլային էքստրակտի ԴՖՊՀ -ի հանդեպ հակառադիկալային ակտիվությունը կորելացվում է ֆլավոնոիդների պարունակության հետ:

Г. Р. Вардапетян, С. С. Блбулян, С. Г. Тирацуян,
член-корреспондент НАН РА А. А. Трчунян

Исследование антирадикальных и антибактериальных свойств экстрактов *Hypericum perforatum*

Исследован антиоксидантный потенциал экстрактов зверобоя продырявленного (*Hypericum perforatum*) тушением стабильного радикала 2,2-дифенил-л-пикрилгидразил (ДФПГ) с одновременным мониторингом общего содержания флавоноидов. Исследована также антибактериальная активность экстрактов. Показано, что этанольный и водный экстракты *H. perforatum* обладают близкой антирадикальной активностью при значительно отличающемся индексе восстановления (RI). Водный экстракт обладает цитотоксичностью в отношении бактерий *E.coli* и *S.aureus*, зависящей от концентрации, что коррелирует с антирадикальной активностью, связанной с компонентами нефлавоноидной природы. Тушение радикала ДФПГ этанольными экстрактами коррелирует с содержанием флавоноидов.

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