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# ANTIMICROBIAL ACTIVITY OF ETHANOL EXTRACTS OF *RIBES NIGRUM* AND *RIBES RUBRUM* LEAVES

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Antimicrobial activity of *Ribes nigrum* L. and *Ribes rubrum* L. leaf extracts was determined by disk-diffusion method as well as specific growth rate and generation succeeding factor determination. Different Gram-positive (*Bacillus subtilis* WT-A, *Staphylococcus aureus* MDC 5233, *Enterococcus hirae* ATCC 9790) and Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* MDC1754, ampicillin resistant *E. coli* dhpα-pUC18 and kanamycin resistant *E. coli* pARG25) bacteria and yeasts (*Saccharomyces cerevisiae* ATCC 9804 and *S. cerevisiae* ATCC 13007) were used as test-microorganisms. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was applied to measure the radical scavenging capacity of extracts obtained from *Ribes* spp. *R. rubrum* and *R. nigrum* extracts antiradical activity expressed with $IC_{50}$ value of 91.2±1.69 $\mu g \cdot mL^{-1}$ and 66.01±1.65 $\mu g \cdot mL^{-1}$, respectively. The total flavonoid content in plant extracts was determined employing $AlCl_3$ colorimetric assay, and the values were 34.71±0.63 and 49.99±0.86 $\mu g$ QE$\cdot mg^{-1}$ for *R. rubrum* and *R. nigrum* extracts, respectively. Total phenolic content of studied extracts was investigated by Folin–Ciocalteu assay. The contents for *R. rubrum* and *R. nigrum* extracts were 133.12±6.65 and 167.15±7.29 $\mu g$ GAE$\cdot mg^{-1}$, respectively. Thus, these plants can be considered as potential sources of biologically active substances.

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***Keywords***: *Ribes nigrum*, *Ribes rubrum*, antimicrobial activity, ampicillin and kanamycin resistant *Escherichia coli*, antiradical activity, flavonoid, phenol.

**Introduction.** Plants have the ability to synthesize different compounds possessing biological activity [1–4]. Armenian flora is rich of plants with pharmacological significance [4, 5]. Plants species of *Ribes* genus (Grossulariaceae family) contain a wide range of secondary metabolic products with high biological activity and are widely used in folk medicine [6, 7]. More than 160 species of this genus are known and 6 of them are described for Armenian flora [8].

*Ribes nigrum* and *Ribes rubrum* are among the widely distributed plants of this genus. The most important products of these plants are berries due to their nutritional value and high contents of anthocyanins, flavonols, ellagitannins, vitamin

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C and other compounds. Some literature sources are describing the application of the leaves and buds of these plants as sources of biologically active substances for food and cosmetic industries. According to the number of authors, the main chemical constituents of leaves of *Ribes* genus plants are flavonoids (kaempferol and quercetin, myricetin and isorhamnetin glycosides, and proanthocyanidins), phenolic acids, anthocyanins, lignoids, lipids and essential oil components [3, 6, 9–13]. The leaf extracts obtained from *Ribes* spp. were reported to possess antioxidant, antimicrobial, anti-inflammatory and anti-hypertensive effects [3, 6, 14–16].

The leaves of *Ribes* spp. are also popular for their therapeutic values. They are useful also as flavoring agents in soups, tea blends as well as for food preservation. Our previous investigations showed the influence of *R. nigrum* leave extract on the activity of some of the main antioxidant enzymes [17].

*R. nigrum* leaves are known to be used in folk medicine for gastrointestinal system disorders, rheumatism, arthritis, respiratory problems, urinary complaints. Moreover, dried leaves have been used as a poultice for the healing of wounds, while fresh rubbed leaves for the treatment of insect bites [14, 18].

Plant origin substances are also able to inhibit the growth of different microorganisms via different mechanisms [19]. This feature is of great importance, because of the nowadays challenges derived from the problem of antibiotic-resistance [19, 20]. Despite our data stating the low antibiotic activity of *R. nigrum* leaf extracts against Gram-negative bacteria, there was some literature concerning other species of this genus possessing this activity [21].

The main aim of this study was to investigate some peculiarities of the antimicrobial activity of leaf extracts of *R. nigrum* and *R. rubrum*, represented in high altitude Armenian flora.

**Materials and Methods.**

***Chemicals and Reagents.*** Folin–Ciocalteu (FC) reagent, ethanol, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), kanamycin, ampicillin, fluconazole, Mueller–Hinton agar and catechin were purchased from Sigma-Aldrich GmbH (Taufkirchen, Germany).

***Plant Material Collection, Identification and Extraction.*** The plant material was collected from Lori Province (Armenia, 1600–1650 m a.s.l.) during the fruiting period (July 2019). The collected leafs were washed, dried in the shadow at room temperature and subsequently crushed to obtain the powder, which was stored in a dry and dark place until use. Plant material was extracted using ethanol, as described [17]. The obtained dried extracts were stored at 4°C until further use.

***Determination of Radical Scavenging Activity.*** Free radical scavenging potentials were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Catechin was applied as standard. Sample solution contained 125 $\mu L$ (1 *mM*) DPPH, 375 $\mu L$ ethanol and 500 $\mu L$ of test-solution (extract and catechin with different concentrations (1000, 500,100, 50, and 10 $\mu g \cdot mL^{-1}$, respectively). Test-solution was replaced by ethanol in the control sample. The absorbance was measured at the wavelength of 517 *nm* using spectrophotometer Genesys 10S UV-Vis ("Thermo Scientific", USA).

The radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100,$$

where $A_c$ is absorbance of control (DPPH without the addition of test solution), $A_s$ is sample absorbance.

$IC_{50}$ calculated denote the concentration of investigated samples required to decrease the DPPH absorbance at 517 *nm* by 50% [22, 23].

***Determination of Total Phenolic Content.*** The concentration of phenolics in plant extracts was determined using Folin–Ciocalteu assay. The reaction mixture consists of 0.5 *mL* of extract (1 $mg{\cdot}mL^{-1}$) and 0.1 *mL* of Folin–Ciocalteu reagent. After 5 *min*, 1 *mL* of 7% sodium carbonate ($Na_2CO_3$) was added. The volume was made up to 2.5 *mL* by adding distilled water. A set of standard solutions of gallic acid (5–100 $\mu g{\cdot}mL^{-1}$) were prepared in the same manner as described earlier. The mixtures were incubated for 90 *min* at room temperature and the absorbance for test and standard solutions was determined against the reagent blank at 765 *nm* with an Genesys 10S UV-Vis spectrophotometer. Total phenolic content was expressed as $\mu g$ of GAE$\cdot mg^{-1}$ of extract [24, 25].

***Determination of Total Flavonoid Content.*** The total flavonoid content in plant extracts was determined employing $AlCl_3$ colorimetric assay. The extract was dissolved in 80% ethanol to obtain a final concentration of 1 $mg{\cdot}mL^{-1}$. 0.5 *mL* of this extract solution was mixed with 0.1 *mL* of $AlCl_3$ (10%), 0.1 *mL* of sodium acetate (1 *M*) and 2.8 *mL* of distilled water. The sample was incubated for 15 *min* and the absorbance of the samples was measured at 415 *nm* against a blank consisting of distilled water utilizing a Genesys 10S UV-Vis spectrophotometer. Total flavonoid content was determined employing a calibration curve of quercetin (Q), as a reference flavonoid (0–1000 $\mu g{\cdot}mL^{-1}$) and results were expressed in terms of Q equivalents (QE) per *g* extract dry weight [18, 26].

***Investigation of Antimicrobial Activity.*** The antimicrobial activity of *R. nigrum* and *R. rubrum* leaf ethanol extracts was determined by the disk-diffusion method as well as specific growth rate ($\mu$) and generation succeeding factor ($g$) determination [18]. Mueller–Hinton agar and LB broth (pH 7.5) medium were used. Different Gram-positive (*Bacillus subtilis* WT-A, *Staphylococcus aureus* MDC 5233, *Enterococcus hirae* ATCC 9790) and Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* MDC1754, ampicillin resistant *E. coli* dhpα-pUC18 and kanamycin resistant *E. coli* pARG25) bacteria and yeasts (*Saccharomyces cerevisiae* ATCC 9804 and *S. cerevisiae* ATCC 13007) qwere used as test-microorganisms. The following concentrations of ethanol extracts were used: 1000, 500, 250, 125 $\mu g{\cdot}mL^{-1}$. Microorganisms were grown on Mueller–Hinton agar for 24 *h* at 37°C temperature. Ampicillin (25 $\mu g{\cdot}mL^{-1}$), kanamycin (25 $\mu g{\cdot}mL^{-1}$) and fluconazole (25 $\mu g{\cdot}mL^{-1}$) were used as a positive control. Data were expressed in minimal inhibitory concentrations (MIC) values.

A study of growth kinetics was also carried out by observing the growth specificity of *E. coli* ATCC 25922 bacteria under the influence of *R. nigrum* and *R. rubrum* extracts. The specific growth rate constant and generation succeeding factor in the growth log phase ($t_0 = 0$, $t = 1.5$ *h*) were determined by the following formulas:

$$\mu = \frac{(\log_{10}N_t - \log_{10}N_0)2.303}{t - t_0},$$

$$g = \frac{0.693}{\mu},$$

where $N$ is the number of cells.

**Results and Discussion.** The study showed that the extracts of *R. nigrum* and *R. rubrum* have no expressed antimicrobial activity. We observed that in case of ampicillin and kanamycin resistant *E. coli, B. subtilis*, *S. aureus* and *E. hirae* strains both investigated extracts did not show any suppressing activity. Anyway, the *R. rubrum* extract possess some inhibiting activity against Gram-negative bacteria. So, the MIC value against *S. typhimurium* was 250 $\mu g \cdot mL^{-1}$, but in case *E. coli* it was twice higher (500 $\mu g \cdot mL^{-1}$). The investigated extracts did not show any growth inhibiting activity against tested yeast strains.

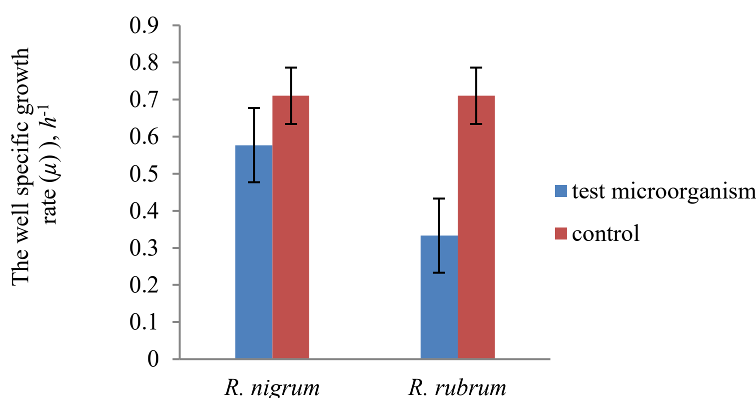


Fig. 1. The $\mu$ ($h^{-1}$) values for *E. coli* ATCC 25922 treated with of *R. nigrum* (p>0.05) and *R. rubrum* (p=0.05) ethanol extracts.

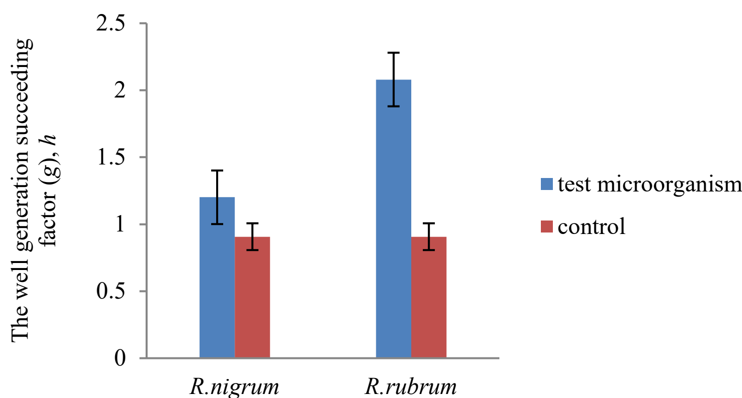


Fig. 2. The $g$ ($h$) values for *E. coli* ATCC 25922 treated with of *R. nigrum* (p>0.05) and *R. rubrum* (p=0.05) ethanol extracts.

According to our investigation data, under the influence of *R. nigrum* extract the specific growth rate of non-resistant *E. coli* strain decreased by 1.23 fold ($\mu$=0.577 $h^{-1}$, $g$=1.201 $h$), but these data were not statistically significant. These results as well as data obtained from disk-diffusion assay can serve as a basis for stating the absence of any antibacterial activity of *R. nigrum* leaf extracts against Gram-negative bacteria (Figs. 1 and 2).

The opposite result we got with *R. rubrum*, where we observed that this plant extract influences the specific growth rate of bacteria by decreasing it by 2.13 fold ($\mu$=0.333 $h^{-1}$, $g$=2.08 $h$), compared to the control sample. In control medium $\mu$=0.71 $h^{-1}$ and $g$ value was 0.977 $h$ (see Figs. 1 and 2). This data again confirm the statement, that plant origin products, even when they are extracted from the same genus plants, can have different effects and influence on test-organisms' metabolism by different mechanisms. This is due to the differences between the chemical composition of investigated extracts, as well as the interaction modes of these components with each other [23].

Literature data suggest that in some cases plant-origin metabolites are able to act as protecting agents for microbial cells against oxidative stress and even support the growth of these organisms [27]. Based on this information it was meaningful to test the antioxidant capacity of the tested plant extracts. DPPH assay is a widely used and simple method for checking the ability of compounds to act as free radical scavengers or active reducing agents. Our investigations revealed that the antiradical activity of *R. nigrum* and *R. rubrum* extracts possess the ability to scavenge the DPPH radicals *in vitro*. Although it is hard to extrapolate data obtained for *in vitro* tests to *in vivo* conditions, anyway these investigation data allowed as comparing the antioxidant capacity of the investigated plant extracts.

So, according to obtained results the $IC_{50}$ value for the positive control (catechin) was determined to be 13.08 $\mu g \cdot mL^{-1}$ ($R^2$ = 0.93) (data not shown). In case of *R. rubrum* and *R. nigrum* this parameter had the following values: 91.2±1.69 $\mu g \cdot mL^{-1}$ ($R^2$ = 0.99) and 66.01±1.65 $\mu g \cdot mL^{-1}$ ($R^2$ = 0.98), respectively (Fig. 3). These data indirectly support the known facts that antioxidant potential is important in the manifestation of antimicrobial activity of plant metabolites [27].

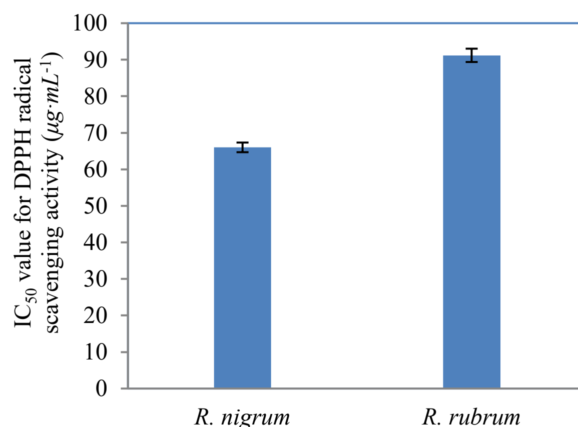


Fig. 3. The radical scavenging activity of *R. nigrum* and *R. rubrum* extracts.

Both antioxidant capacity and antimicrobial activity of plant-origin metabolites and their complexes can be connected to the quantitative and/or qualitative compositions of phenolic compounds [6]. The results of total phenolic content of *R. nigrum* and *R. rubrum* extracts expressed as $\mu g$ of GAE$\cdot mg^{-1}$ (see Table) (the standard curve equation: $y = 0.005x + 0.048$, $R^2 = 0.979$) (data not shown). The results of total flavonoids content of *R. nigrum* and *R. rubrum* extracts expressed as $\mu g$ QE$\cdot mg^{-1}$ (see Table) (the standard curve equation: $y = 0.002x + 0.070$, $R^2$=0.989) (data not shown). The qualitative composition of *R. nigrum* extract phenolic fraction was represented in one of our recently published article [6], the *R. rubrum* extract composition is now under investigation.

*Total phenolic and the total flavonoid contents of R. nigrum and R. rubrum extracts*

| Extract | Total phenolic content, $\mu g$ of GAE$\cdot mg^{-1}$ | Total flavonoid content, $\mu g$ QE$\cdot mg^{-1}$ |
|---|---|---|
| *R. nigrum* | 167.15±7.29 | 49.99±0.86 |
| *R. rubrum* | 133.12±6.65 | 34.71±0.63 |

**Conclusion.** In conclusion, we can suggest the *R. nigrum* and *R. rubrum* leaves as potential sources for biologically active substances.

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## *RIBES NIGRUM* ԵՎ *RIBES RUBRUM* ԲՈՒՅՍԵՐԻ ՏԵՐԵՎՆԵՐԻ ԷԹԱՆՈԼԱՅԻՆ ԼՈՒԾԱՄԶՎԱԾՔՆԵՐԻ ՀԱԿԱՄԱՆՐԷԱՅԻՆ ԱԿՏԻՎՈՒԹՅՈՒՆԸ


*Ribes nigrum* L.-ի և *Ribes rubrum* L.-ի տերևներից ստացված լուծամզվածքների հակամանրէային ակտիվությունը որոշվել է սկավառակ–դիֆուզիոն մեթոդով, ինչպես նաև աճի տեսակարար արագության և սերունդների միջին քանակի որոշմամբ: Որպես փորձնական միկրոօրգանիզմներ օգտագործվել են տարբեր գրամ-դրական (*Bacillus subtilis* WT-A, *Staphylococcus aureus* MDC 5233, *Enterococcus hirae* ATCC 9790), գրամ-բացասական (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* MDC1754, ամպիցիլին կայուն *E. coli* dhpα-pUC18 և կանամիցին կայուն *E. coli* pARG25) բակտերիաներ և խմորասնկեր (*Saccharomyces cerevisiae* ATCC 9804 և *S. cerevisiae* ATCC 13007): *Ribes* ցեղի տարբեր տեսակներից ստացված լուծամզվածքների հակառադիկալային ակտիվությունը գնահատելու համար կիրառվել է 1,1-դիֆենիլ-2-պիկրիլհիդրազիլը՝ ԴՖՊՀ (DPPH): *R. rubrum*-ի և *R. nigrum*-ի լուծամզվածքների հակառադիկալային ակտիվությունն արտահայտվել է $IC_{50}$-ի արժեքներով, որոնք կազմել են 91,2±1,69 *մկգ·մլ*$^{-1}$ և 66,01±1,65 *մկգ·մլ*$^{-1}$, համապատասխանաբար: Ֆլավոնոիդների ընդհանուր պարունակությունը որոշվել է $AlCl_3$ գունաչափական վերլուծության միջոցով, և արժեքները *R. rubrum*-ի և *R. nigrum*-ի համարեղել են 34,71±0,63 և 49,99±0,86 *մկգ* ԿՀ·*մգ*$^{-1}$, համապատասխանաբար: Ուսումնասիրված լուծամզվածքների ընդհանուր ֆենոլային միացությունների պարունակությունը որոշելու համար կիրառվել է Ֆոլին–Չեոկալտեուի մեթոդը: Ընդհանուր ֆենոլային միացությունների պարունակությունը *R. rubrum*-ի և *R. nigrum*-ի լուծամզվածքներում կազմել է 133,12±6,65 և 167,15±7,29 *մկգ* ԳԹՀ·*մգ*$^{-1}$, համապատասխանաբար: Այսպիսով, այս բույսերը կարելի է համարել կենսաբանական ակտիվ նյութերի պոտենցիալ աղբյուրներ:

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# АНТИМИКРОБНАЯ АКТИВНОСТЬ ЭТАНОЛЬНЫХ ЭКСТРАКТОВ ЛИСТЬЕВ РАСТЕНИЙ *RIBES NIGRUM* И *RIBES RUBRUM*

Антимикробную активность экстрактов листьев *Ribes nigrum* L. и *Ribes rubrum* L. определяли диск-диффузионным методом, а также с помощью определения удельной скорости роста и фактора преемственности поколений. Различные грамположительные (*Bacillus subtilis* WT-A, *Staphylococcus aureus* MDC 5233, *Enterococcus hirae* ATCC 9790) и грамотрицательные (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* MDC1754, ампициллин резистентная *E. coli* dhpα-pUC18 и канамицин резистентная *E. coli* pARG25) бактерии и дрожжи (*Saccharomyces cerevisiae* ATCC 9804 и *S. cerevisiae* ATCC 13007) использовались в качестве тест-микроорганизмов. Для определения антирадикальной активности экстрактов, полученных из различных видов *Ribes,* использовали 1,1-дифенил-2-пикрилгидразила (DPPH). Антирадикальная активность экстрактов *R. rubrum* и *R. nigrum* выражалась значениями $IC_{50}$, которые составили 91,2 ± 1,69 и 66,01 ± 1,65 *мкг·мл*$^{-1}$ соответственно. Общее содержание флавоноидов в растительных экстрактах определяли с помощью колориметрического анализа $AlCl_3$, их значения составили 34,71 ± 0,63 и 49,99 ± 0,86 *мкг* КЭ·*мг*$^{-1}$ для экстрактов *R. rubrum* и *R. nigrum* соответственно. Общее количество фенольных соединений в исследуемых экстрактах определяли методом Фолина–Чеокальтеу. Их содержание в *R. rubrum* и *R. nigrum* составило 133,12 ± 6,65 и 167,15 ± 7,29 *мкг* ГАЕ·*мг*$^{-1}$ соответственно. Таким образом, эти растения можно рассматривать как потенциальные источники биологически активных веществ.