

Biology

ANTIBACTERIAL AND ANTI-PHAGE ACTIVITY
OF *PLANTAGO MAJOR* L. RAW MATERIAL

N. Zh. SAHAKYAN *, M. M. GINOVYAN **, M. T. PETROSYAN ***, A. H. TRCHOUNIAN ****

Chair of Biochemistry, Microbiology and Biotechnology YSU, Armenia

Plantago major L. is a popular medicinal herb, which has been being used for treatment of various diseases in traditional health systems. The aim of present study was to examine the antibacterial and anti-phage activity of *P. major* ethanol extract. *P. major* fresh raw material extract was possessed of moderate antibacterial activity against *Escherichia coli* VKPM-M17, *Salmonella typhimurium* TA 100 and *Staphylococcus aureus* MDC 5233 bacterial strains. Anti-phage activity was evaluated by double overlay plaque assay against T4 phage of *E. coli* C-T4. Fresh, dried and frozen raw materials showed moderate antiviral activity: 1 mg/mL concentration of fresh raw material ethanol extract caused 0.927Log₁₀ reduction of phage units, while the frozen and dried plant ethanol extracts caused 0.875 Log₁₀ and 0.821 Log₁₀ reductions, respectively. Fresh raw material contains the highest concentration of vitamin C.

Keywords: *P. major*, antibacterial, anti-phage activity.

Introduction. Since time immemorial *Plantago major* L. leaves have been being used as a remedy all over the world in the treatment of a number of diseases, such as ones related to the skin, respiratory organs, digestive organs, reproduction, etc. According to some authors it has immune enhancing, hepatoprotective, anti-ulcerogenic, antioxidant and free radical scavenging effects [1]. It possesses anticancer and cytotoxic activity on some human transformed cells of colon, cervical, ovary and nasopharynx carcinoma [2]. Some authors point out hemato-poietic [3], wound-healing [4], anti-inflammatory, antigenotoxic and external poison detoxification effects [1] of this plant. Methanol extracts of *P. major* seeds demonstrate the inhibition of TNF- α , IL-1 β , IL-6 and IFN- γ production [5]. Some authors point out that *Plantago* extracts can also have some side effects, including bloating and allergic reactions [6].

P. major contains biologically active compounds such as polysaccharides, lipids, caffeic acid derivatives, flavonoids, iridoid glycosides, alkaloids and terpenoids [4]. Many authors also have reported the presence of different vitamins

* E-mail: sahakyannaira@ysu.am

*** E-mail: margaritpetrosyan@ysu.am

** E-mail: mikayel.ginovyan@ysu.am

**** E-mail: trchounian@ysu.am

such as ascorbic acid and carotenoids [7]. According to the Triple Quadrupole GC-MS examination of chemical composition of various extracts (petroleum ether, methanol, ethyl acetate, n-butanol and aqueous) of *P. major* leaf, the main constituents are phytol, benzofuranone, penthynediol and benzene propanoic acid in petroleum ether extract; group of diglycerol and glycol in methanol extract; glycerine, benzene and dibutyl phthalate in ethyl acetate extract; phthalic acid, benzene propanoic acid and group of phenol in n-butanol; phenol, diathiapentene, naphthalenone and glycerine in aqueous extract [8].

Armenia is positioned at the junction of several bio-geographical regions. Consequently the climatic and soil conditions have deep differences, which bring to the large variety in the plant chemotypes. *P. major* is abundant in various regions of Armenia and have been widely used in Armenian traditional medicine for treatment of many diseases including bronchitis, tuberculosis, blue cough, dysentery, gastric ulcer, wounds, tumors, etc. [9]. These findings give rise to new studies of natural content and biological activity of extracts obtained from *P. major* inherent for Armenian flora.

Thereby, the main goal of this work was to study the biological activity of extracts derived from *P. major*, described for Armenian flora and suggest as the potential source of biologically active substances.

Material and Methods.

Plant Material. *P. major* plants were collected from Armavir region, 850–900 m above sea level (June, 2017). The identification of plant was carried out at the Department of Botany, Yerevan State University, Armenia, and plant samples were deposited and are available there.

Harvested plant leaves had been dried in ventilated rooms at 25–30°C until steady weight to determine the dry matter weight. Another leaves had been frozen down to –20°C and stored until using.

Extract Preparation. 1–5 g of plant row (fresh, frozen and dried) material was homogenized in 10 to 15 mL 40% ethanol and left overnight at ~10°C. Extract was centrifuged for 5 min at 5000 rpm, and the supernatant was isolated. The precipitate was extracted by 4-folds, and the combined supernatant was dried by evaporation at room temperature. The evaporated mass was dissolved in ethanol, and the extracts in different dilutions were used.

Antibacterial Activity Determination. The minimal inhibitory concentrations (MIC) of plant extracts were determined by agar-diffusion method [10]. For this purpose different Gram-positive bacteria, viz. *Staphylococcus aureus* WDCM 5233 from Microbial Depository Center, Armbiotechnology Scientific and Production Center, Armenia (Laboratory control strain), and *Bacillus subtilis* WT-A1, isolated from metal polluted soils of Kajaran, Armenia; Gram-negative bacteria, viz. *Escherichia coli* VKPM M-17 from Russian National Collection of Industrial Microorganisms at the Institute of Genetics and Selection of Industrial Microorganisms, Russia (Laboratory control strain), and *Salmonella typhimurium* TA 100 (Laboratory control strain of Islamic Azad University, Iran) were used.

Test-organisms were grown on the meat-peptone agar (MPA). Plates containing MPA were inoculated with the test-bacteria mentioned above. Then

several wells (diameter 8 mm) were made on the agar. The extracts (100 μL) with different concentrations (125, 250, 500, 1000 and 1500 $\mu\text{g}/\text{mL}$) were added into these wells. Note that the 40% ethanol extract does not possess antibacterial activity.

The lowest concentration of the investigated extract which had caused well defined growth inhibition zone around the well, considered as MIC [10]. In the control ethanol was used. Ampicillin was used as standard antibacterial agent.

The medium samples from the growth inhibition zones were cut off and sub-cultured in a liquid medium. After 24 h incubation the absence of growth indicated about bactericidal activity of the test plant extract.

Anti-phage Activity Determination. Anti-phage activity was determined by double agar overlay plaque assay [11]. Firstly, phage suspension was prepared. The concentration of plaque-forming units (PFU) was determined by the same method. In experimental group 30 μL of phage suspension at 10^{11} PFU/mL concentration and 30 μL of tested extracts (at 2 mg/mL concentration) were poured in a tube, while in the control group 30 μL ethanol was used instead of the extracts. The mixtures were incubated for 90 min at 37°C. Then phage mixtures were diluted down to 10^{-8} in Mueller-Hinton Broth (MHB) medium by serial ten-fold dilutions.

E. coli C-T4 was pre-cultivated on slant agar then washed with MHB and transferred into sterile tubes. The concentration of cells was determined measuring OD at 595 nm wavelength light. Appropriate dilution of phage (in volume of 1 mL) was poured into a tube. *E. coli* C-T4 (100 μL) was added and then 5 mL of MHB containing 0.7% agar at 50°C was added on top. The mixture was shaken and poured in plates containing 20 mL MHB with 1.5% agar. The plates were swirled, left to dry for 10 min at room temperature and incubated for 24 h at 37°C. Viable phages had form plaques on the seeded plates which could be enumerated. The number of viable phage particles in stock solution was determined by multiplying plaque numbers with dilution factor. The efficiency of the preparations was determined by comparing the amount of viable phage particles of experimental group with that of control group.

Ascorbic Acid Determination by Iodometric Titration. This method determines the vitamin C concentration in a solution by a redox titration with potassium iodate in the presence of potassium iodide. Data were expressed as mg% in investigated extracts [12]. Fresh extracts of *P. major* of different types of raw material was used.

Data Processing, Chemicals and Reagents. Experimental data ($n = 4$) were expressed as means \pm SD. The standard error did not exceed 3% (if not indicated). The validity of differences between experimental and appropriate control data were evaluated by Student's criteria using MS Excel 2010 with the help of T test function. The differences were taken to be reliable if $p < 0.05$ (if not indicated).

The main chemicals and reagents were purchased from "Sigma Chemical Co." (USA) and ampicillin were purchased from Kyiv Plant of Medicinal Preparations (Ukraine).

Results and Discussion. Agar-diffusion method allows to state that in suppressing the growth of test-microorganisms the responsibility had lain solely

with *P. major* fresh leaves extracts. But they had low or moderate antimicrobial activity. The MIC of extracts was 1000 $\mu\text{g/mL}$ against *E. coli* VKPM-M 17, *S. typhimurium* TA 100, *S. aureus* MDC 5233. *B. subtilis* WT-A1 had expressed no sensitivity to the extracts of *P. major* at tested concentrations (data not showed).

Several literature data confirm our results with some exceptions. Particularly, Velasco et al. [5] have shown that different extracts (aqueous, methanol, chloroform and hexane) of *P. major* leaves and seeds exhibit antibacterial activity against *E. coli*, *B. subtilis* and *Candida albicans*. Moderate antimicrobial activity of *P. major* leaf extracts against *B. subtilis*, *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* has been reported in [13]. However, contrary to literature data that *P. major* extracts possess moderate antibacterial activity against *B. subtilis*, we found no activity of this kind at tested concentrations. It may be due to high resistance of *B. subtilis* WT-A1 strain, which was isolated from soils contaminated with heavy metals.

Fresh, dried and frozen raw materials have specific antiviral activity: ethanol extract of fresh raw material of 1 mg/mL concentration cause 0.927 Log_{10} reduction of viable phage units, dried and frozen raw materials cause 0.875 Log_{10} and 0.821 Log_{10} reductions, respectively (Tab. 1).

Table 1

P. major leave extracts anti-phage activity against T4 phage of *E. coli* C-T4
(the reduction of viable phage units is represented by Log_{10} units)

Sample	Reduction of phage units	Standard deviation
Fresh leaves	0.927	0.063
Frozen leaves	0.875	0.056
Dried leaves	0.821	0.065

Obtained data show that storage conditions of *P. major* raw materials did not considerably affect its anti-phage activity.

Slight antiviral activity of *P. major* aqueous extract against herpes virus was reported [11]. In other research weak activity of aqueous extracts of *P. major* against HSV-2 virus was detected [11].

Table 2

Concentration of vitamin C in *P. major* raw material

Sample	Concentration of vitamin C (mg%) with standard deviation
Fresh leaves	61.6 \pm 1.1
Frozen leaves	33.6 \pm 0.8
Dried leaves	41.0 \pm 0.9

A number of medicinal plants contains vitamin C, which have been found to augment both humoral and cell-mediated immune function, reducing risk of infections by enhancing the antigenic surveillance of the immune system [14].

Vitamin C is considered to be also an active antioxidant agent, which can affect the action and activity of organism endogenous antioxidants.

The obtained data showed that dried leaves preserve the high level of vitamin C concentration during the preservation, but the lowest content is observed in the frozen leaves (Tab. 2).

Conclusion. Fresh extracts of *P. major* were found to exhibit slight antibacterial activity against *E. coli* VKPM-M17, *S. typhimurium* TA 100 and *S. aureus* MDC 5233 test strains. Fresh, dried and frozen raw materials showed moderate antiviral activity: 1 mg/mL concentration of fresh raw material ethanol extract caused 0.927Log₁₀ reduction of phage units, while the frozen and dried plant ethanol extracts caused 0.875 Log₁₀ and 0.821 Log₁₀ reductions, respectively. Fresh raw material contains the highest concentration of vitamin C.

Received 19.09.2018

Reviewed 31.01.2019

Accepted 05.03.2019

REFERENCES

1. **Nazarizadeh A., Mikaili P., Moloudizargari M., Aghajanshakeri Sh., Javaherypour S.** Therapeutic Uses and Pharmacological Properties of *Plantago Major* L. and Its Active Constituents. // *J. Basic. Appl. Sci. Res.*, 2013, v. 3, No. 9, p. 212–221.
2. **Ozaslan M., Karagoz I., Kiliç I., Cengiz B., Kalender E., Güldür E., Karagoz A., Zümürüdal E.** Effect of *Plantago Major* sap on Ehrlich Ascites Tumours in Mice. // *African J. Biotech.*, 2009, v. 8, No. 6, p. 955–959.
3. **Velasco-Lezama R., Tapia-Aguilar R., Román-Ramos R., Vega-Avila E., Pérez-Gutiérrez S.** Effect of *Plantago Major* on Cell Proliferation *in vitro*. // *J. Ethnopharm.*, 2006, v. 103, p. 36–42.
4. **Samuelsen B.** The Traditional Uses, Chemical Constituents and Biological Activities of *Plantago Major* L. // *J. Ethnopharm.*, 2000, v. 71, p. 1–21.
5. **Kartini A., Suratsawadee P., Thongpraditchote S., Siripong P., Vallisuta O.** Effects of *Plantago Major* Extracts and Its Chemical Compounds on Proliferation of Cancer Cells and Cytokines Production of Lipopolysaccharide-Activated THP-1 Macrophages. // *Pharmacogn. Mag.*, 2017, v. 13, No. 51, p. 393–399.
6. **Haddadian K., Haddadian K., Zahmatkash M.** Review of *Plantago* Plant. // *Indian J. Tradit. Knowl.*, 2014, v. 13, No. 4, p. 681–685.
7. **Adom M., Taher M., Mutalabisin M., Amri M., Kudos M., Sulaiman M., Sengupta P., Susanti D.** Chemical Constituents and Medical Benefits of *Plantago Major*. // *Biomed. Pharmacotherapy.*, 2017, v. 96, p. 348–360.
8. **Jamilah J., Sharifa A., Sharifah N.** GC-MS Analysis of Various Extracts from Leaf of *Plantago Major* Used as Traditional Medicine. // *World Appl. Sci. J.*, 2012, v. 17, p. 67–70.
9. **Torosyan A.** The Armenian Medical Herbs. Yerevan: Hayastan, 1983, 298 p. (in Armenian).
10. **Patel J.B., Cockerill F.R., Alder J., Bradford P.A., Eliopoulos G.M., Hardy D.J., Hindler J.A., Jenkins S.G., Lewis J.S., Miller L.A., Powell M., Swenson J.M., Traczewski M.M., Turnidge J.D., Weinstein M.P., Zimmer B.L.** Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. M100-S24. 2014, v. 34, No. 1, p. 226.

11. **Chiang L.C., Chiang W., Chang M.Y., Lin C.C.** *In vitro* Cytotoxic, Antiviral and Immunomodulatory Effects of *Plantago Major* and *Plantago Asiatica*. // The American J. Chinese Med., 2003, v. 31, No. 02, p. 225–234.
12. GOST 24556 – 89. Products of Fruit and Vegetable Processing. Methods for Determination of Vitamin C (in Russian).
13. **Stanisavljević I.T., Stojičević S.S., Veličković D.T., Lazić M.L., Veljković V.B.** Screening the Antioxidant and Antimicrobial Properties of the Extracts from Plantain (*Plantago Major* L.) Leaves. // Sep. Sci. Tech., 2008, v. 8, No. 43(14), p. 3652–3662.
14. **Mahima Ingle A.M., Verma A.K., Tiwari R., Karthik K., Chakraborty S., Deb R., Rajagunalan S., Rathore R., Dhama K.** Immunomodulators in Day to Day Life. // Pak. J. Biol. Sci., 2013, v. 16, p. 826–843.