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ORIGINAL PAPER

Alkanna orientalis (L.) Boiss. plant isolated cultures and antimicrobial activity of their extracts: phenomenon, dependence on different factors and effects on some membrane-associated properties of bacteria

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Abstract Herbal medicine requires searching for new sources with antimicrobial activity. *Alkanna* sp. (Boraginaceae) is widely used in medicine due to detoxification and antibacterial effects. The aim of this study was to obtain *Alkanna orientalis* (L.) Boiss. plant callus extracts, to investigate antimicrobial activity of extracts against bacteria and yeast and to reveal responsible mechanisms. Callus tissue cultures have been obtained using different nutrient media. Antimicrobial activity and minimal inhibitory concentrations (MIC) were determined by the methods of extract diffusion in agar and dilution using different test-microorganisms. Quantity and quality of naphthoquinones were determined using spectrophotometric and high performance liquid chromatographic analyses. H^+/K^+ exchange by whole cells was assayed using selective electrodes, ATPase activity and SH-groups number of membrane vesicles—by spectrophotometric methods. It was revealed that callus extracts containing naphthoquinones (0.19 ± 0.01 %) possessed bacteriostatic activity against gram-positive bacteria including pathogenic ones (MIC were 125–750 $\mu g/mL^{-1}$), and bactericidal activity against lactic acid bacteria (*Lactobacillus acidophilus*, *L. rhamnosus*) (MIC was 250 μg/mL). MIC against *Enterococcus hirae* was 250 μg/mL of callus extract or 31.25 μg/mL of shikonin. It depended on the medium content, cultivation duration and the activity remained for 10–11 years. Moreover, intact root extracts inhibited H^+/K^+ exchange of *E. hirae* but callus extracts had a stronger effect. The inhibitor, *N*,*N*′-dicyclohexylcarbodiimide (DCCD)-sensitive H^+/K^+ exchange was changed; ATPase activity and SH-groups number were lowered by two and more fold under the influence of the extracts. Alkanet root and callus tissues extracts were concluded to have a high antimicrobial activity. So, they might directly affect the F_OF_1-ATPase which in turn regulates the bacterial growth. These results are useful for further investigation of alkanet extracts composition and their application as an alternative antimicrobial agent in pharmaceutical and food industry as well as in medicine.

Keywords Alkanet · Callus tissue · Naphthoquinones (shikonin) · Bactericide and bacteriostatic influence · Ion transport and F_OF_1-ATPase · *Enterococcus hirae*

Abbreviations

MS	Murashige and Skoog
BAP	6-Benzylaminopurine
DCCD	*N*,*N*′-dicyclohexylcarbodiimide
F_OF_1	Proton-translocating ATPase
IAA	Indole-3-acetic acid
$\Delta\mu_H^+$	Proton-motive force
MEP	Multidrug efflux pump
MIC	Minimal inhibitory concentration

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Introduction

The ever-increasing demand for herbal medicine is to search for new sources of biologically active compounds. Biotechnological methods to obtain secondary metabolits of isolated plant tissues are very promising: they will help to solve the above stated objectives without damaging natural plant sources. Besides, biotechnological techniques contribute to obtain a standardized product under certain specific conditions, regardless of weather (Hussain et al. 2012).

In recent years preparations based on biologically active compounds of plant origin possess antimicrobial and antiviral activity, have been widely used for the treatment of various infectious diseases (Schempp et al. 1999; Darbinian-Sarkissian et al. 2006; Dadi et al. 2009; Zare et al. 2010; Abdallah 2011). Some biologically active compounds of different plants including *Alkanna* sp. (Boraginaceae) possess detoxifying properties by means of inactivating the microbe toxins and have antioxidant and cytotoxic effects (Baronetz et al. 2001; Oktyabrsky et al. 2009; Gharehmatrossian et al. 2012; Sykłowska-Baranek et al. 2012; Bame et al. 2013). Quinones of plant origin are known to have antimicrobial activity among these compounds (Gafner et al. 1996; Nagata et al. 1998). Pigment shikonin ((±)-5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone) and its esters are the widely studied class of these substances which are used in medicine, cosmetics and food industry, and they are commercially very important (Chen et al. 2001, 2003). Gram-positive bacteria *Mycobacterium tuberculosis*, parasitic protozoa and filamentous fungi are the most sensitive to naphthoquinones. Yeast-like fungi (*Candida albicans*) and some Gram-negative bacteria (*Escherichia coli*) are relatively resistant to quinones (Sasaki et al. 2002; Shen et al. 2002). Plant origin quinones may suppress the activity of electron transport systems of many microorganisms and ATP production: they can serve as electron acceptors and cofactors of NADPH/H_2 oxidation in the respiratory chain. Moreover, they may interact with SH-groups of enzymes which are involved in energy supplying processes such as succinate dehydrogenase or the F_OF_1-ATPase (Dadi et al. 2009). It has been revealed that sarothrin from *Alkanna orientalis* (L.) Boiss. (Boraginaceae) as an antimicrobial agent can inhibit efflux pumps activity of *Staphylococcus aureus* (Bame et al. 2013).

There is only a little information concerning isolated cultures of Boraginaceae family plants (*Lithospermum*, *Arnebia*, *Alkanna*, *Anchusa*, *Echium* and *Onosma* genus). But isolated culture of *A. orientalis* as a source of naphthoquinones has not been examined yet (Malik et al. 2014). Therefore, obtaining isolated cultures of *A. orientalis*, developing conditions for their cultivation, as well as maintenance of a stable growth and active metabolism are of considerable interest to develop new cell lines possessing the ability to accumulate naphthoquinones. An isolated culture of *A. orientalis* which synthesizes naphthoquinones was obtained in this study. Alkanet callus tissue extract antimicrobial activity of against various groups of microorganisms was studied after pro-longed sub-cultivations. Furthermore, the dependence of isolated tissue antibacterial properties on dry material storage period (7 years) or long-term sub-cultivation (10–11 years) was established. This finding could have a great practical significance.

Enterococcus hirae is known to have a role in human physiology, clinical relevance and promising application in medicine and technology. *E. hirae*, which is found in intestinal microbiota, similar to streptococci, lacks respiratory chain and generates a proton-motive force ($\Delta\mu_H^+$) under fermentation of glucose only by the hydrolysis of ATP via the F_OF_1-ATPase (Kakinuma 1998). *E. hirae* growth in anaerobic conditions at alkaline pH is caused by the changes in pH, $\Delta\mu_H^+$ and environmental oxidation–reduction potential (Poladyan et al. 2006; Vardanyan and Trchounian 2010, 2012). F_OF_1 has a crucial role in bacterial energetics: it mediates many energy-dependent processes including ion transport, and it regulates the enzymatic activity of the membrane. It was stated that F_OF_1 might have direct involvement in secondary solute transport systems such as K^+ uptake Trk-like or KtrI system forming H^+/K^+-exchanging pump. The energy of ATP is proposed to be transferred from F_OF_1 to Trk-like system through a dithiol–disulfide interchange (Poladyan and Trchounian 2006). Therefore, a role of enzymes thiol-groups during operation of H^+/K^+-exchanging pump in *E. hirae* was proved. F_OF_1 could be a target for antibacterials (Torgomyan and Trchounian 2013) determining also drug-resistance of pathogenic bacteria (Cook et al. 2014). Thus, the effects of plant extracts on F_OF_1 could establish mechanisms of antibacterial action.

The main aim of this study was also to reveal antimicrobial activity and content of naphthoquinones in *A. orientalis* cultures extracts and their action mechanisms in bacteria as well. The growth and membrane-associated activity of *E. hirae* were investigated in the presence of *A. orientalis* intact root (roots of native plants) and callus tissue extracts possessing marked antimicrobial activity against various microorganisms. The findings can be essential to search new natural sources with high antimicrobial activity.

Materials and methods

Isolation of *A. orientalis* cultures and preparation of extracts from callus tissues and intact roots

The investigated plant of *A. orientalis* (Alkanet) was collected in Syunik region in Armenia (1500–1600 m above sea level) during blossoming period (July, 2003). The identification of plant was carried out in the Institute of Botany, National Academy of Sciences (Yerevan, Armenia), which was included in the collection of the same institute herbarium. The callus cultures were obtained using Murashige and Skoog (MS) nutrient medium (Murashige and Skoog 1962). The sterilization process was carried out with solution of acetyl pyridinium chloride (660 mg/L) and mercuric chloride (330 mg/L). The Petri dishes with explants (approx. diameters of explants −0.8 to 1.2 cm) were placed in thermostat at 22–25 °C (for the initiation of proliferation processes). Afterwards the formed primary callus tissues were placed to the flasks (50 mL) and then replaced in thermostat (22–25 °C). Further stable growth of callus tissue was supported in different media. Medium N1 was mineral base and vitamins containing medium according to Murashige and Skoog (1962) supplemented with 2 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L indole-3-acetic acid (IAA) (control); medium N2—like medium N1, but it did not contain NH_4NO_3, copper concentration was increased to 0.2 mg/L; medium N3—like medium 2, only concentration of BAP increased to 3.0 mg/L. In all media 30 mg/L sucrose and 6 g/L agar (pH 5.8) were used. All nutrient media were sterilized by autoclaving at 121 °C for 20 min.

One gram of powdered dried plant material (intact roots and callus tissues of root origin after 60–63th and 145–148th sub-cultivations) was homogenized in 10–15 mL 70 % ethanol for 15 min and left overnight at ~10 °C to prepare extracts. The extract was centrifuged at 5000 rpm for 5 min, and the supernatant was isolated. The precipitate was extracted by 4-fold and the combined supernatant was dried by evaporation at room temperature. The evaporated mass was solved in 40 % ethanol, and the ethanol extracts in different dilutions were used.

Determination of naphthoquinones

Dry callus tissues were embedded in 80 % ethanol and left for 20 h at 8 °C to determine the naphthoquinones content. Subsequently, naphthoquinones were converted into chloroform, and then in 0.1 N KOH. Samples were analyzed by a spectrophotometer Ultraspec 1000 (Biochrom, USA) at 614 nm (0.1 N KOH was used as a control) (n = 4). Shikonin was used as a standard solution dissolved in 0.1 N KOH. The qualitative and quantitative analyses of ethanol extracts was determined by high performance liquid chromatography [Beckman System Gold, USA, Luna Su Column C18 (250 × 4.6 mm)]. The solvent was acetonitrile dissolved in 0.1 % trifluoroacetic acid; gradient −45 to 75 % acetonitrile; flow rate with 1 mL/min; excitation wavelength was 254 nm. The ethanol solution of shikonin was used as a standard (for comparison; Yamamoto et al. 2000).

Determination of extracts minimal inhibitory concentrations

The minimal inhibitory concentration (MIC) of extracts was determined using dilution method. Several tubes were examined, which contained the same volume (0.9 mL) of culture medium corresponding to each bacterial species (see hereafter the part "Investigation of antimicrobial activity of extracts"), inoculated with the test bacteria (10^3–10^6 bacterial cells per mL). The extracts (100 µL) with different concentrations were added into the above mentioned bacterial suspension, so the final concentration of the extract reached 500, 250, 125, 62.5, 31.25 and 15.625 µg/mL. The absorbance of bacterial suspension was measured after 24 h of incubation at 37 °C at the wavelength of 560 nm using the spectrophotometer Genesys 10S UV–Vis (Thermo Scientific, USA). MIC was determined as the lowest concentration of the investigated extract that completely inhibited the growth of the test microorganism (Bame et al. 2013). The desired concentration of the test organism suspension was obtained by serial dilutions, as described (Benson 2002). As a positive control, shikonin and ampicillin were used with the same concentrations as those of the extract.

Investigation of extract antimicrobial activity

Antimicrobial activity of extracts was detected by the agar diffusion method (Müller-Hinton agar was used). This method allows revealing the spectrum of studied material influence (Scorzoni et al. 2007; Bame et al. 2013). The extracts under investigation (100 µL) were introduced to the wells in the agar with test microorganisms. Different Gram-positive (*Bacillus mycoides*, *B. mesentericus*, *B. megaterium*, *B. subtilis*, *Brevibacterium flavum*, *E. hirae*, *Micrococcus luteus*, *St.aureus*, *St. citreus*, *St. roseus*, *Lactobacillus acidophilus*, *L. rhamnosus*) and Gram-negative (*E. coli*, *Salmonella typhimurium*) bacteria and yeast (*Debaryomyces hansenii*, *Pichiaguillier mondii*) were used. Most bacterial cultures were grown on meat-peptone agar, lactic acid bacteria and *D. hansenii* on MRS medium. The testing of *A. orientalis* callus tissues extracts was carried out in the Center of Preventing Dangerous Infections, Ministry of Health (Yerevan, Armenia) to reveal antibiotic activity against

some pathogenic bacteria causing dangerous diseases (*Vibrio cholerae*, *Yersinia enterocolitica* and *Bacillus anthracoides*). Ampicillin (50 μg/mL) as a positive control and ethanol (40 %) as a negative control were used.

The selected pieces of nutrient medium from the zones of microorganism growth absence were transferred to the nutrient medium corresponding to each microorganism and then they were incubated for 2–3 days at 37 °C to determine the bacteriostatic or bactericidal action of the extracts. The action of extracts is evaluated as bacteriostatic in case of renewed growth of test-microorganisms after the re-cultivation.

E. hirae growth, preparation of membrane vesicles

Enterococcus hirae wild-type strain ATCC9790 (Trchounian and Kobayashi 1998) was used to determine the action mechanisms of the extracts on bacterial cells. The strain was kindly provided by Prof. H. Kobayashi (Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan). Bacteria were grown under anaerobic conditions at 37 °C in the medium containing 1 % tryptone, 0.5 % yeast extract, 1 % K_2HPO_4 (pH 8.0); 0.2 % glucose was added as described previously (Trchounian and Kobayashi 1998; Poladyan and Trchounian 2006). *E. hirae* growth was monitored by changes in optical density of bacterial suspension using a Spectro UV–Vis auto spectrophotometer (Labomed, USA) at a wave length of 600 nm. Membrane vesicles were isolated from lysozyme treated cells using the buffers lacking K^+, as described previously (Poladyan and Trchounian 2006; Vardanyan and Trchounian 2012).

Determination of ions fluxes across the cell membrane, accessible thiol-groups number and ATPase assay

H^+ and K^+ fluxes across the cell membrane were determined by the changes in H^+ and K^+ external activities using appropriate selective electrodes (HJ1131B, Hanna Instruments, Portugal; and PVC, Cole Parmer Instrument Co., USA), as detailed before (Poladyan and Trchounian 2011; Vardanyan and Trchounian 2012). Electrode readings data were processed automatically by LabView computer program (National Instruments Co., USA). Using this program the electrode readings were calibrated by titration of the assay medium (200 mM Tris–phosphate buffer (pH 8.0) containing 0.4 mM MgSO4, 1 mM NaCl and 1 mM KCl) with small quantities of 0.01 N HCl and 0.01 M KCl. Ions fluxes were expressed in mMol/min per number of cells in a unit of volume.

Accessible thiol-groups of membrane vesicles were determined by Ellmann's reagent (5,5′-dithiobis-2-nitrobenzoic acid) using spectrophotometric method, as described previously (Poladyan and Trchounian 2006); and glutathione was used as a standard. Corrections were made for blanks with different reagents used. The assay mixture was 200 mM Tris–phosphate containing 0.4 mM $MgSO_4$ and 1 mM KCl and 1 mM NaCl (pH 8.0). The level of thiol-groups was expressed in nmol per mg protein.

ATPase activity was measured by the amount of liberated inorganic phosphate (P_i) after adding 5 mM ATP by the method of Taussky and Shorr, as described before (Poladyan and Trchounian 2011; Vardanyan and Trchounian 2012). P_i was determined spectrophotometrically; corrections were made for blanks without membrane vesicles and with different reagents used. The assay mixture contained 50 mM Tris–HCl (pH 8.0), 0.4 mM $MgSO_4$ and 100 mM KCl. The ATPase activity was expressed in nmol P_i per min and mg protein.

Whole cells and membrane vesicles were pre-incubated with *A. orientalis* intact root and callus tissue extracts for 10 min. The concentrations of alkanet extracts were calculated based on MIC of intact roots and callus tissue extracts, respectively. Different concentrations of extracts were used by dilutions.

Other conditions, data processing and reagents

The number of cells in a volume unit was calculated by the number of colonies after plating the diluted bacterial suspension on solid nutrient media. Protein was measured by the method of Lowry as described before (Vardanyan and Trchounian 2010) using bovine serum albumin as a standard. Whole cells or vesicles were incubated with 0.2 mM DCCD for 10 min for *N*,*N*′-dicyclohexylcarbodiimide (DCCD) inhibition studies. All assays were done at 37 °C.

Data were averaged by duplicate or triplicate independent measurements. 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 (p) using Microsoft Excel 2010 with the help of T test function, as before (Vardanyan and Trchounian 2010, 2012), $p < 0.05$ (if not indicated).

Tryptone, yeast extract and Tris (aminomethan) were obtained from Roth (Germany), acetyl pyridinium chloride, agar, ATP (Tris salt), DCCD, mercuric chloride, shikonin (5,8-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-1,4-naphthoquinone) and Müller-Hinton agar were received from Sigma (USA), glucose acquired was from Borisov Medical Preparations Plant (Belarus), ampicillin—from Kyiv Plant of Medicinal Preparations (Ukraine).

Results

Antimicrobial activity of *A. orientalis* callus tissues and intact roots extracts, accumulation of naphthoquinones and effects of pro-longed cultivation

Alkanna orientalis callus cultures of root origin only possessed the ability to synthesize shikonin (retention time was 14.98 min). They produced the highest concentration of naphthoquinones (0.19 ± 0.01 %) on medium 3 in comparison to medium 2 where this parameter was 0.14 ± 0.01 %. The concentrations of naphthoquinones in callus cultures of leaf and stem origin on medium 2 were 0.11 ± 0.01 and 0.09 ± 0.01 %, respectively. Under the influence of components of nutrient medium 3 their concentrations reached 0.55 ± 0.04 and 0.81 ± 0.03 %, respectively. The retention times of non-identified naphthoquinone intense peaks were 11.00, 13.50, 13.94, 14.20, 16.36, 16.79, 17.59 and 20.73 min. On the basis of these data subsequent studies were performed using the callus cultures of root origin.

The inhibitory effects of *A. orientalis* callus extracts against various microorganisms were identified clearly. Gram-positive bacteria including *E. hirae* were the most sensitive to biologically active compounds of alkanet extracts whereas the tested yeast showed low sensitivity (Fig. 1). Gram-negative bacteria (*E. coli* and *S. typhimurium*) were even less sensitive to the extracts (see Fig. 1). Note that biologically active compounds synthesized in an isolated culture possessed greater antibacterial activity than those of intact plants. The high-inhibiting effect of the extracts was also detected against some pathogenic bacteria but no significant differences were obtained between Gram-positive (*V. cholerae*, *B. antracoides*) and Gram-negative (*Y. enterocolitica*) bacteria (data not shown).

To determine the nature of alkanet callus tissues biologically active compounds action, their bacteriostatic effects on the test microorganisms were established. It proved that the growth of the test microorganisms was renewed after placing of nutrient agar slices from the growth absence zone in the proper nutrient medium. Extracts exhibited a bactericidal effect in the concentration of 250 μg/mL in case of lactic acid bacteria (*L. acidophilus*, *L. rhamnosus*). The extracts inhibited the fermentation of milk by lactic acid bacteria, and the re-cultivation of these strains in fresh milk without plant extract did not cause clot formation.

Minimal inhibitory concentration of extracts was established using dilution method (see "Materials and methods" section). The latter helped to avoid the disparity which occurred when relative antimicrobial potency was determined by the comparison of inhibition zone sizes around extracts. Indeed, the absorbance of bacterial suspension was not changed at 250 μg/mL concentration of

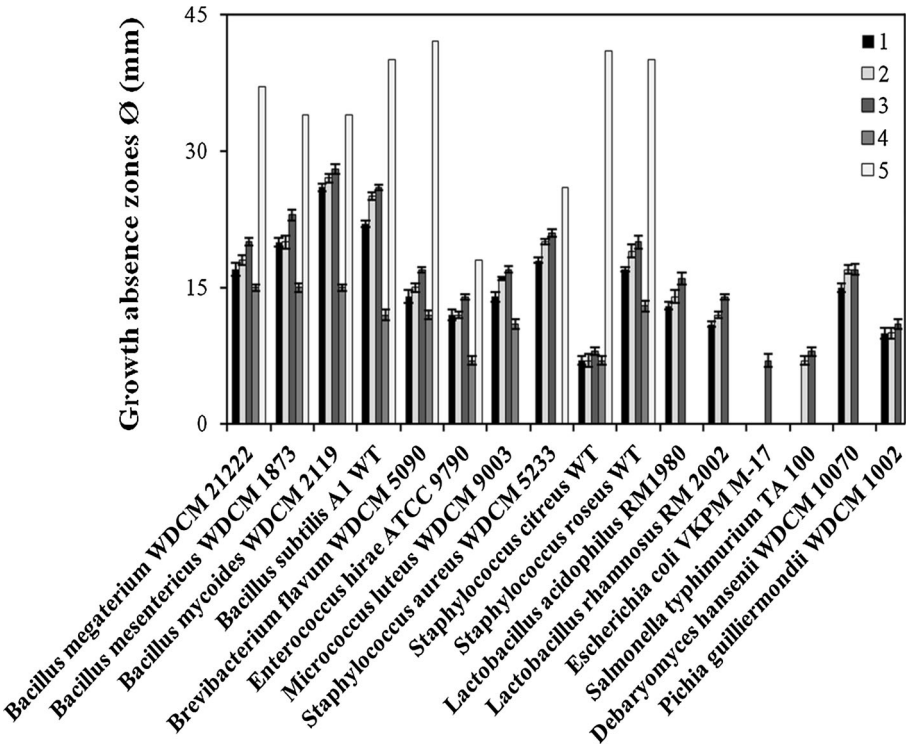

Fig. 1 Sizes of the test microorganism's growth absence zones under the influence of *A. orientalis* callus tissues and intact plant extracts: *1* callus tissue extracts growing on medium N1 (control); *2* callus tissue extracts growing on medium N2; *3* callus tissue extracts growing on medium N3; *4* Intact plant root extracts; *5* positive control (ampicillin 50 μg/mL). $p < 0.05$, in case of *S. typhimurium*, *P. guilliermondii*, *L. acidophilus*, *L. rhamnosus*, *E.coli*, *D. hansenii*, *M. luteus P* values were not calculated

Table 1 Minimal inhibitory concentrations of *A.orientalis* callus tissues extracts, shikonin and ampicillin against some microorganisms

Test microorganisms	MIC (μg/mL)[a]		
	A. orientalis extract	Shikonin	Positive control (ampicillin 50 μg/mL)
B. megaterium WDCM 2122[b]	125	15.625	12.5
B. mesentericus WDCM 1873	125	15.625	12.5
B. mycoides WDCM2119	125	15.625	12.5
B. subtilisA1 WT	125	15.625	12.5
Br. flavum WDCM 5090	250	31.25	12.5
E. hirae ATCC 9790	250	31.25	12.5
M. luteus WDCM 9003	250	31.25	NT
St. aureus WDCM 5233	125	15.625	12.5
St. citreus WT	500	62.5	25
St. roseus WT	125	15.625	12.5
E. coli VKPM M-17	500	62.5	25
S. typhimurium TA 100	750	125	NT
D. hansenii WDCM 10070	250	31.25	NT
P. guilliermondii WDCM 1002	500	62.5	NT

NT not tested

[a] See "Materials and methods" section

extract (in case of *E. hirae*) (see "Materials and methods" section). It was determined that MIC corresponded to the 250 μg/mL concentration of the extract, at which the absorbance of the suspension after 24 h of incubation remained unchanged. MIC of shikonin, as a positive control, was 31.25 μg/mL (Table 1). For the extracts of *A. orientalis* callus culture, MIC was 250 μg/mL and positive control was purified shikonin, for which MIC was 31.25 μg/mL. Therefore, *A. orientalis* callus culture can be considered as a promising source of naphthoquinones.

The positive control with ampicillin showed only by 2-fold higher activity in comparison with the examined extracts (see Fig. 1), the negative control with ethanol in the used concentration showed no activity against test-microorganisms (data not shown). Moreover, the results indicated that a composition of the nutrient medium exerted a significant influence on the synthesis of naphthoquinones in the isolated alkanet culture (Ye et al. 1991). It was found that the elimination of NH_4NO_3 from the medium, the increased content of copper to 0.2 mg/L and the concentration of BAP to 3.0 mg/L led to an increase of extract antimicrobial activity compared with the control—the growth absence zones of test microorganisms (namely *B. subtilis*, *S. roseus*, *D. hansenii*) were 3–5 mm greater (see Fig. 1, media N 2 and N 3).

It was revealed that long-time cultivation as well as storage of callus tissues (dried) did not change the antimicrobial activity of *A. orientalis* tissue extracts. The antibacterial activity was again stronger against Gram-positive bacteria (see Table 2).

A. orientalis intact root and callus tissues extracts effects on *E. hirae* whole cells H^+/K^+ exchange, accessible thiol-groups number and ATPase activity of membrane vesicles

As it was mentioned above the marked bacteriostatic effect was with Gram-positive bacteria including *E. hirae* (see Fig. 1). In order to determine the action mechanisms of these extracts, some membrane-associated activity of *E. hirae* was examined in the presence of *A. orientalis* intact root and callus tissue extracts. Indeed, the kinetics of H^+ secretion and K^+ accumulation by *E. hirae* ATCC9790 whole cells was determined when 0.2 % glucose added into the assay medium (data not shown). Parallel experiments were done with DCCD, specific inhibitor of F_OF_1 and other H^+ translocation mechanisms (Trchounian and Kobayashi 1998; Vardanyan and Trchounian 2010, 2012). Both H^+ and K^+ fluxes were DCCD-sensitive; there was ~2.6-fold inhibition of H^+ efflux in the presence of 0.2 mM DCCD ($p < 0.05$) (Fig. 2) suggesting the participation of F_OF_1 in H^+ secretion.

Different concentrations of *A. orientalis* intact root and callus tissue extract influence on H^+ and K^+ fluxes rates has been investigated (see Fig. 2). Bacteria were treated by plant extract for 10 min in assay medium before glucose supplementation. The effect had a concentration-dependent manner: 10 and 20 μL/mL (the final concentrations calculated for dry weight) intact root extracts inhibited H^+ efflux by ~1.27- and ~1.47-fold and K^+ influx by ~1.25- and ~1.36-fold, respectively. The differences between

Table 2 Sizes of the test microorganisms growth absence zones under the influence of *A. orientalis* extracts of callus tissue of different age (cultivations)

Test-microorganism	The zones of growth absence (Ø mm)		
	60th sub-cultivation	145th sub-cultivation	Positive control (Ampicillin 50 μg/mL)
B. subtilis A1WT	30 ± 0.5	20 ± 0.8	40
B. mesentericus WDCM 1873	27 ± 0.6	19 ± 0.7	34
B. mycoides WDCM 2119	27 ± 0.5	24 ± 0.7	34
B. megaterium WDCM 2122	18 ± 0.3	16 ± 0.7	37
Br. flavum WDCM 5090	15 ± 0.4	13 ± 0.6	42
St. aureus WDCM 5233	24 ± 0.7	17 ± 0.2	26
St. citreus WT	7 ± 0.5	6 ± 0.4	41
S. typhimurium TA 100*	19 ± 0.5	0	NT
P. guilliermondii WDCM 1002*	14 ± 0.5	8 ± 0.4	0

$p < 0.05$

NT Not tested

* p values were not calculated

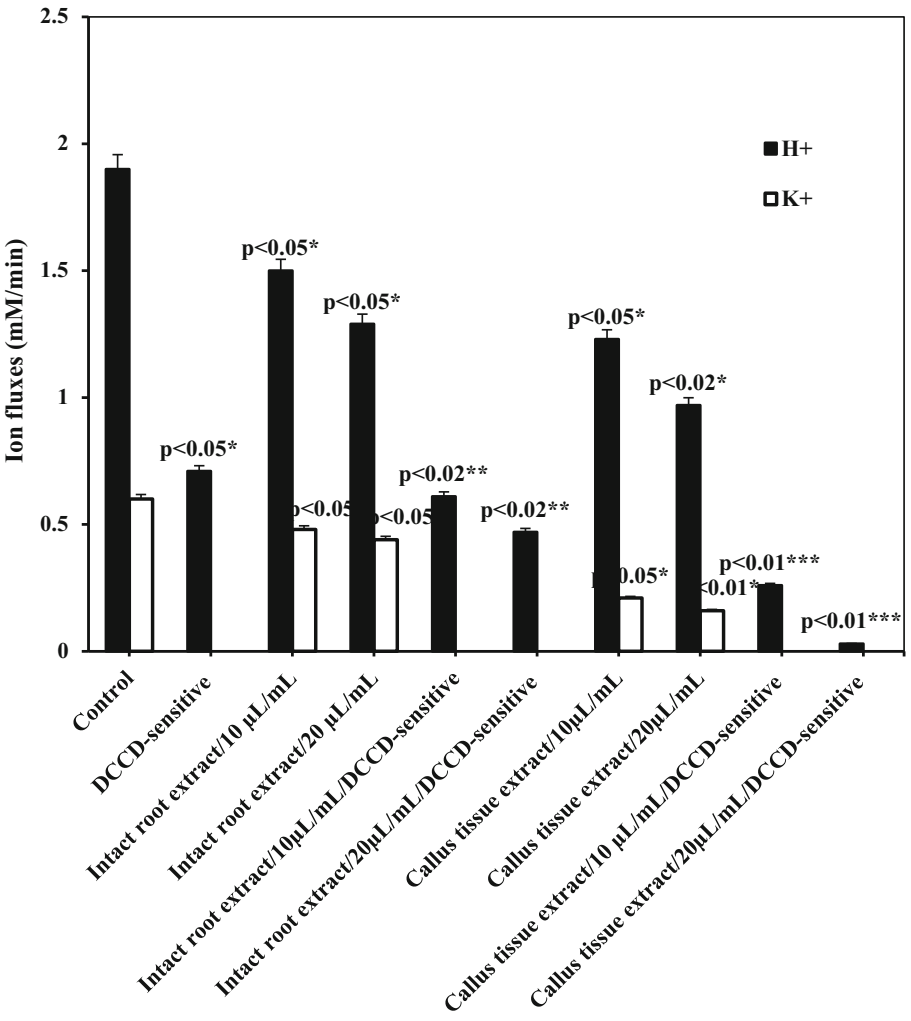

Fig. 2 H^+/K^+ exchange fluxes by *E. hirae* ATCC9790 in the presence of *A. orientalis* intact root and callus tissues extracts. Glucose (0.2 %) was added into the assay medium before bacteria. Control was without extracts supplementation. Ion fluxes calculated per 10^{10} cells/mL. DCCD-sensitive fluxes are the differences between fluxes in parallel experiments in the absence and presence of 0.2 mM DCCD. For p, *control is the sample without any additions; **control is the sample where only intact root extract was added in appropriate quantities; ***control is the sample where only callus tissue extract was added in appropriate quantities. For the others see "Materials and methods" section and legends to Fig. 1

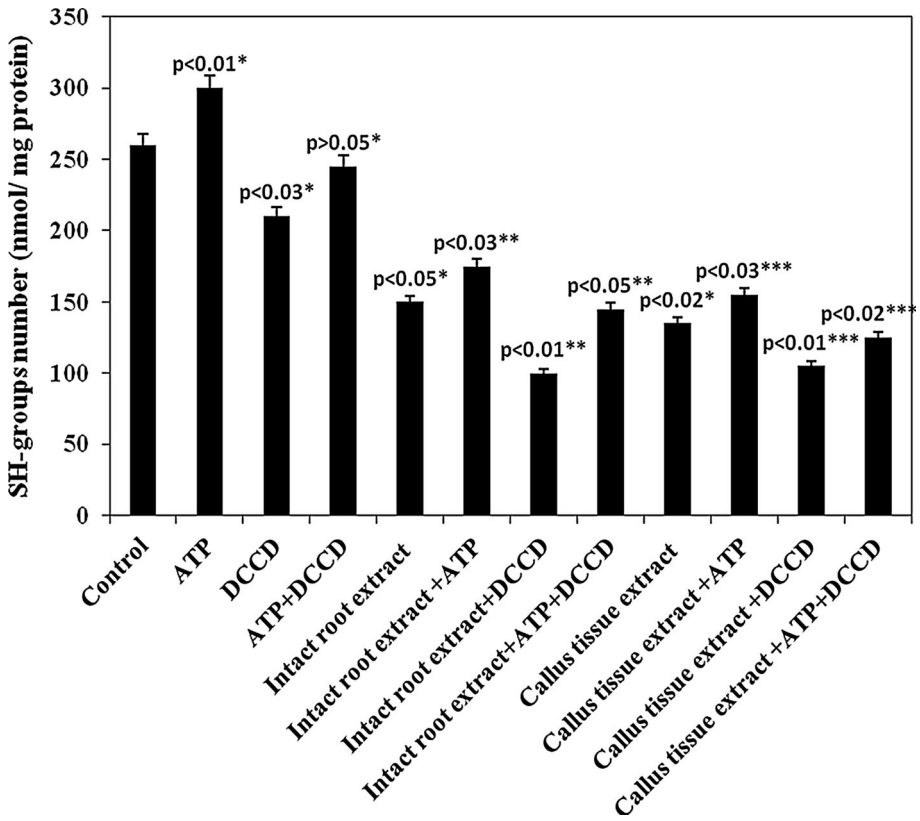

Fig. 3 The changes in the number of accessible SH-groups of *E. hirae* ATCC9790 membrane vesicles in the presence of *A. orientalis* intact root and callus tissue extracts. 3 mM ATP and 0.2 mM DCCD were added into the medium when indicated. For the others, see "Materials and methods" section and legends to Fig. 2

average data of control values and appropriate experiments with extracts were 0.40 ± 0.03, 0.61 ± 0.04 mM/min per 10^{10} cells/mL for H^+ fluxes and 0.12 ± 0.01, 0.16 ± 0.01 mM/min per 10^{10} cells/mL for K^+ fluxes, respectively ($p < 0.01$). Callus tissue extract effect on ions fluxes was stronger: 10 and 20 μL/mL callus tissue extract suppressed H^+ efflux by ~1.54 and ~2-fold and K^+ influx by ~2.8- and ~3.8-fold, respectively and the differences between average data of control values and appropriate experiments with extracts were 0.67 ± 0.04, 0.93 ± 0.05 mM/min per 10^{10} cells/mL for H^+ fluxes and 0.39 ± 0.03, 0.44 ± 0.03 mM/min per 10^{10} cells/mL for K^+ fluxes, respectively ($p < 0.02$).

In the assays DCCD in 0.2 mM concentration was added and 5 min later DCCD-sensitive fluxes were calculated (see Fig. 2): intact root extract (10 and 20 μL/mL) inhibited DCCD-sensitive H^+ efflux by ~1.16- and ~1.5-fold, respectively, and the differences between average data of DCCD-inhibited values and appropriate experiments with extracts were 0.10 ± 0.02 and 0.34 ± 0.03 mM/min per 10^{10} cells/mL, respectively ($p < 0.05$). 10 μL/mL callus tissue extract inhibited the fluxes by ~2.7-fold, while 20 μL/mL caused almost complete inhibition ($p < 0.001$ for the difference between average data of DCCD-inhibited value and experiment with extracts). These results pointed out that *A. orientalis* intact root and callus tissue extracts affected F_OF_1 enzyme activity.

Membrane-associated ATPase activity of *E. hirae* was determined to be K^+-dependent (Trchounian and Kobayashi 1998; Poladyan and Trchounian 2011) and markedly inhibited by DCCD (Trchounian and Kobayashi 1998; Vardanyan and Trchounian 2010, 2012). The interaction of F_OF_1 with K^+ uptake system could be due to dithiol–disulfide interchange in which the changes in the number of accessible thiol-groups by ATP had been established before (Poladyan and Trchounian 2006). In fact ATP in the 3 mM concentration increased the number of accessible thiol-groups in membrane vesicles: the level of these groups was 1.2–1.3-fold higher ($p < 0.01$) (Fig. 3). However, similar effects were not detected when DCCD was present in the medium (either containing ATP or not, $p > 0.5$, see Fig. 3). These results indicated that the changes in the number of thiol-groups in the presence of ATP might be associated with F_OF_1, as describes previously (Poladyan and Trchounian 2006). The *E. hirae* membrane vesicle number of thiol-goups was determined in the presence of 20 μL/mL *A. orientalis* tissues extracts as well. The addition of extracts into the assay medium decreased the number of these groups (see Fig. 3): it was lowered by ~1.7-fold and by ~2-fold in the presence of intact root and callus tissue extracts, respectively, compared with the control ($p < 0.005$). The strongest effects were obtained with 0.2 mM DCCD (see Fig. 3). These results proved the specific role of F_OF_1 in the observed

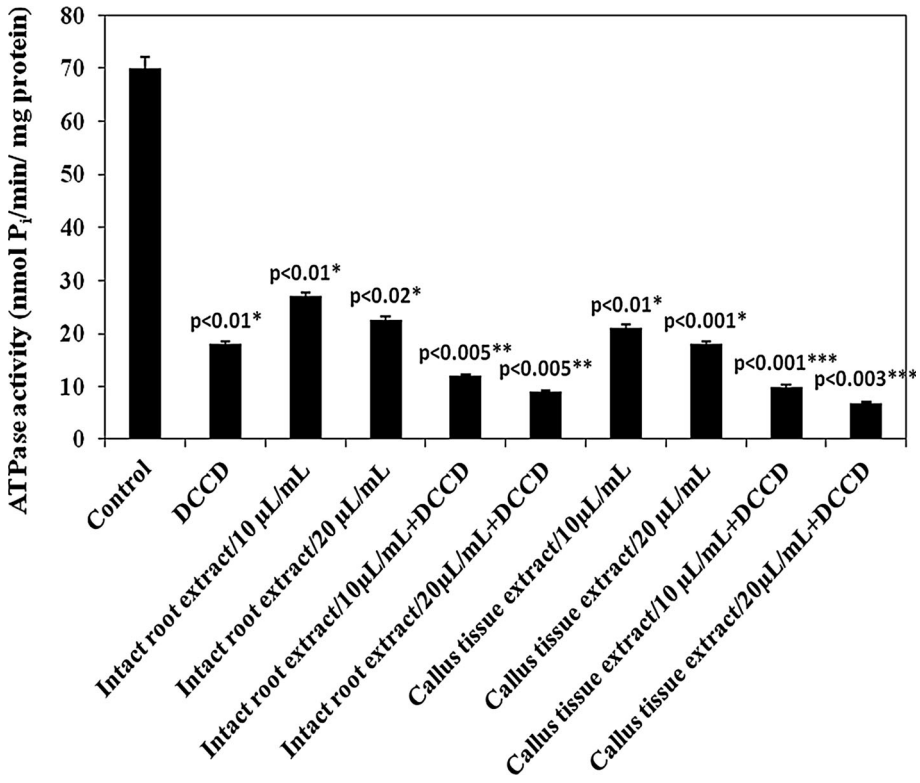

Fig. 4 The changes in ATPase activity of *E. hirae* ATCC9790 membrane vesicles in the presence of *A. orientalis* intact root and callus tissue extracts. 0.2 mM DCCD was present in the assay medium when mentioned. For the others, see "Materials and methods" section and legends to Fig. 2

effects. It was not ruled out that conformational changes of the enzyme might occur in the presence of *A. orientalis* root and callus tissue extracts.

To ascertain the role of F_OF_1, the ATPase activity was determined in the presence of *A. orientalis* root and callus tissue extracts. The ATPase activity was assayed in the presence and absence of 0.2 mM DCCD: strong inhibition by DCCD was revealed (Fig. 4). Moreover, the ATPase activity was decreased by ~3.1-fold and by ~3.5-fold compared with the control in the presence of 20 µL/mL intact root and callus tissue extracts, respectively ($p < 0.01$) (see Fig. 4). The maximum inhibition was detected when intact root and callus tissue extracts were mixed with 0.2 mM DCCD simultaneously each separately. The effect had a concentration-dependent manner (data not shown).

Discussion

Growing resistance of pathogenic-harmful microorganisms to antimicrobial agents led to screening of medicinal plants to reveal the active compounds which have become very important as potential sources of novel antibiotics nowadays (Gibbons 2008; Zare et al. 2010; Abdallah 2011; Calo et al. 2015). In our study, *A. orientalis* intact root and isolated culture (callus tissue) extract antimicrobial activity against the different groups of microorganisms was established; it varied not only by the degree of expressiveness but also by its stability, in favor of isolated cultures. Moreover, the impact of callus tissue storage on the biological activity of secondary metabolites was revealed: the dry powder (during 7 years the callus tissue of 62–65th sub-cultivations) did not lose its antimicrobial activity on some tested microorganisms and was even more active than the tissue of the 148th sub-cultivations (see Fig. 1; Table 2).

Plant cells are successfully used to produce valuable secondary metabolites. Since Tabata and Fujita (1985) first described the production of shikonin by *Lithospermum erythrorhizon* (Boraginaceae) callus cultures; intensive efforts have been made to identify the regulatory factors controlling shikonin biosynthesis. This naphthoquinone exhibits various pharmacologicals properties including anti-inflammatory and antitumor activity (Tabata et al. 1974), and as a result, it represents the first example of industrial production of a plant-derived pharmaceutical (Chen et al. 2003). Naphthoquinones (shikonin) isolated from different plants have strong antibacterial activity which is comparable with that of *A. orientals* callus culture extract. Therefore, *A. orientalis* culture could be a good source for naphthoquinones. This culture also contains unidentified substances of quinone nature (the retention times are given above); this makes it interesting for further

study. However, naphthoquinones in combination with other secondary metabolites are not ruled out to be responsible for antimicrobial activity of *A. orientalis* extracts. The latter would be preferable due to its natural origin. Using callus culture could be beneficial to protect plants in nature.

The mechanism of plant antimicrobials action has not been completely elucidated. Efflux mechanisms like multidrug efflux pumps (MEPs) have become broadly recognized as major components of resistance to many classes of antibiotics (Stavri et al. 2007; Gibbons 2008; Bame et al. 2013). A novel and promising approach to deal with multidrug resistance is to improve the clinical performance of various antibiotics by employing MEPs inhibitors. Plants have widely been explored, as potential sources of these inhibitors (Fiamegos et al. 2011).

Furthermore, to reveal the mechanisms of antibiotic actions of different substances it should be mentioned that the most prevalent synthetic chemical reaction in bacteria is the synthesis of ATP by F_OF_1 (Kakinuma 1998). Clearly, this ATPase has the central role in energy metabolism, and perturbations of ability of F_OF_1 to produce ATP or to pump H^+ have profound effects on cellular bioenergetics and might determine antibacterial action of plant extracts. In fact, recent studies suggested that F_OF_1 might be a primary target for heavy metal ions (Vardanyan and Trchounian 2010, 2012), antibiotics and environmental factors such as extremely high frequency electromagnetic fields (Torgomyan and Trchounian 2013; Cook et al. 2014). Moreover, it was proposed that discharging $\Delta\mu_H^+$, generated by F_OF_1 under fermentation conditions of bacteria; instability in proton-coupled ions transport, alterations in appropriate transport systems and lowering of ATP level (Trchounian 2004) as well as differences in membrane proteome (Xu et al. 2006) can increase sensitivity to antibiotics and different chemicals (Torgomyan and Trchounian 2013).

Multidrug resistant microbial infections caused by Gram-positive bacteria represent an exponentially growing problem affecting communities worldwide. In our study, *A. orientalis* isolated culture extracts bacteriostatic activity against Gram-positive *E. hirae* was established; some membrane-associated properties were determined: *A. orientalis* intact root and callus tissue extracts decreased F_OF_1-associated H^+ efflux and K^+ influx; membrane vesicles ATPase activity and number of thiol-groups were also markedly decreased upon supplementing extracts. Importantly, callus tissue extract had the strongest effect. Thereby the effect of *A. orientalis* intact root and callus tissue extracts on H^+/K^+ exchange by *E. hirae* whole cells, thiol-groups number and ATPase activity of membrane vesicles could be explained by direct effects of these substances on F_OF_1. Conformational changes might occur in the enzyme modulating its activity and disturbing the dithiol–disulfide interchange between the membrane proteins.

However, targets and mechanisms of antimicrobial action of alkanet extracts are complex and require a further study.

Conclusions

The isolated culture of *A. orientalis* was obtained; it was capable to biosynthesis of secondary metabolites like naphthoquinones. Shikonin was detected in callus culture of root origin only. This process could be controlled, directed and regulated by the composition of nutrient medium and other factors.

Alkanet root and callus tissue extracts had a high antimicrobial activity. Callus extracts possessed bacteriostatic activity against Gram-positive bacteria including pathogenic ones and bactericidal activity against lactic acid bacteria. MIC of these effects were determined; MIC against *E. hirae* ATCC 9790 was 250 μg/mL of callus extract or 31.25 μg/mL of shikonin. The antibacterial activity depended on medium content and cultivation duration and remained for 10–11 years.

Intact root extract inhibited H^+/K^+ exchange of *E. hirae* whole cells; callus extract had a stronger effect. The F_OF_1-ATPase inhibitor, DCCD-sensitive H^+/K^+ exchange was changed, ATPase activity and accessible SH-groups number of membrane vesicles were lowered 2-fold and more by the extracts. The latter might directly affect F_OF_1 which in turn regulates the bacterial growth.

All the above mentioned findings could be significant for using these plant extracts as an alternative antimicrobial agent in pharmaceutical and food industry as well as in medicine.

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Conflict of interests The authors report no declarations of interests.

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