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EFFECT OF HEAT TREATMENT ON ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS OF SOME ARMENIAN HERBS

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The goal of this research was to explore the thermostability of active antimicrobial constituents of some Armenian herbs. The following herbs were used: *Agrimonia eupatoria*, *Hypericum alpestre*, *Rumex obtusifolius* and *Sanguisorba officinalis*. Thermal stability was determined by exploring plant extracts antimicrobial activity against *Staphylococcus aureus* MDC 5233 before and after heat treatment. Antimicrobial activity of the plant extracts was tested by agar well diffusion assay. Broth microdilution assay was used for determination of minimal inhibitory concentrations of the plant extracts. According to obtained data, some of the plant crude extracts retained their antimicrobial activity even after 121°*C* heat exposure. In contrast, other plant extracts have lost their activity after treating just with 60°*C*. Thus, obtained results about thermostability of compounds responsible for plant extracts antimicrobial activity will allow to choose appropriate temperature ranges during processing of the tested plant materials in order to maintain their antimicrobial activity.

***Keywords*:** plant material, thermal stability, plant crude extract, antimicrobial activity, minimum bactericidal concentration.

Inroduction. Phytochemicals have high potential as a new source of antimicrobials due to their direct antimicrobial action or their possible synergistic interactions with antimicrobials, which can work as enhancers of microbes susceptibility to antibiotics. The interest toward plant materials as source of new antimicrobials has particularly increased recently taking into account an emerging challenge of antibiotic resistance. Currently this phenomenon is considered as one of the largest problems facing humanity [1–3].

It is well known that the use of plants in traditional medicine has started from ancient times and continues till now. Moreover, in many developed and developing countries the use of plants in medicine has been increasing recently [4–6]. In recent decades screening programs have been implemented in various regions of the world aiming to find plants possessing high antimicrobial activity as a potential source of new therapeutic compounds [2].

One of the critical factors to pay attention during processing of plant materials is the stability of phytochemicals. This is due to possibility of losing their

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bioactivity. For example, heat treatment of plant materials (including sterilization and pasteurization) can lead to decomposition of active phytochemicals in plant materials [6]. Hence, it is important to keep their activity during processing. There are different methods for processing of plant materials which includes various temperature ranges. In order to choose appropriate methods, attention should be given to sensitivity of plant active compounds to heat treatment.

The aim of this research was the investigation of effect of the heat treatment on the antimicrobial activity of crude extracts from some Armenian herbs, which possess high antimicrobial activity according to already conducted research. We also aimed to explore their antimicrobial activity against some bacterial strains.

Materials and Methods.

Collection, Identification and Drying of Plant Materials. The collection, identification and preparation of plant materials were done according to already established protocol [7]. The following plant materials were used: *Agrimonia eupatoria* L. (whole plant) (ERCB 13207: numbers of Voucher specimens, which were deposited to the Herbarium of YSU), *Hypericum alpestre* subsp. *polygonifolium* (Rupr.) Avet. & Takht. (aerial part) (ERCB 13206), *Rumex obtusifolius* L. (seed) (ERCB 13208), *Sanguisorba officinalis* L. (aerial part) (ERCB 13205) [7]. These plants species and their most active parts have been chosen based on initial screening of various Armenian herbs [7, 8].

Preparation of Plant Crude Extracts. Plant crude extracts were prepared by maceration technique using methanol (98%) and acetone (99.8%), according to the method described previously [7].

Used Microorganisms and Growth Conditions. The following bacteria were used as test strains: *Escherichia coli* VKPM-M17, *Staphylococcus aureus* WDCM 5233, ampicillin-resistant *E.coli* dhpα-pUC18, kanamycin-resistant *E. coli* PARS-25. Mueller–Hinton broth medium and Mueller–Hinton agar medium ("Liofilchem", Italy) were used for cultivation of bacteria.

Determination of Thermal Stability of Plant Active Constituents. Thermal stability of plants crude extracts bioactive compounds was tested using the method described by Simlai and Roy [9] with some modifications. Dried methanol and acetone extracts of all four plant materials were kept under 60, 80, 100, 121º*C* for both 30 and 60 *min*. Then the samples were cooled at room temperature and stored in a freezer under –18º*C* till further use. Afterwards, residual antimicrobial activity of plant extracts was tested by agar diffusion assay against *S. aureus* MDC 5233 strain. The results were compared with control samples, which were kept under room temperature.

Investigation of Plant Crude Extracts Antibacterial Activity. The antimicrobial activity of the crude plant extracts were initially evaluated by modified agar well diffusion assay [10]. Determination of minimum inhibitory and bactericidal concentrations (MIC and MBC) of the samples was done according to method described in previous research work [7]. Gentamicin (range: 0.03–2.0 $\mu g/mL$) was used as positive control; whereas 1% dimethyl sulfoxide (DMSO) (which was present in extracts with 2048 $\mu g/mL$ concentration) was used as negative control.

Data Processing. All experiments were independently repeated three times. Obtained data were processed; standard deviations were calculated using GraphPad Prism 5.03 (GraphPad Software, Inc.; USA) software.

Results and Dicussion.

Determination of Plant Crude Extracts Antimicrobial Activity. Antimicrobial activity of tested four plants crude methanol and acetone extracts were initially tested by agar well diffusion assay and then their MIC and MBC values were determined against three gram-negative and one gram-positive strains (see Tabs. 1 and 2). Antimicrobial properties of tested plants were investigated against *E. coli* PARS-25 and *E. coli* dhpα-pUC18 for the first time, whereas *S. aureus* MDC 5233 and *E. coli* WKPM-M17 have already been used as test strains for evaluation of the test plants antimicrobial properties in previous works [7, 8]. However, in this research work tested concentration range was different. Obtained data showed that methanol and acetone extracts from all tested plants at 500 $\mu g/mL$ concentration exhibited antimicrobial activity against *S. aureus* and *E. coli* WKPM-M17. None of the tested plant extracts inhibited the growth of *E. coli* dhpα-pUC18 at 500 $\mu g/mL$ concentration. *E. coli* PARS-25 was only susceptible to the acetone extract of *H. alpestre* at tested concentration.

According to obtained data, plant crude extracts had high MIC values against *E. coli* PARS-25 and *E. coli* dhpα-pUC18 compared with MICs against *S. aureus*. These two strains are also less sensitive than *E. coli* WKPM-M17. Both *E. coli* PARS-25 and *E. coli* dhpα-pUC18 are resistant strains toward ampicillin and kanamycin respectively. This can be the reason of their lower susceptibility to tested plants crude extracts.

Table 1

Evaluation of antimicrobial activity of the crude extracts from four Armenian herbs by agar well diffusion assay at 500 μg/mL concentration

Plant species	Tested part	Extract	Growth inhibition zone with standard deviation, *mm*			
			E. coli AMP [a]	*E. coli* KN	*E. coli* M-17	*S. aureus*
Agrimonia eupatoria	whole plant	methanol	– [b]	–	10±0.6	9±0.6
		acetone	–	–	10±0.6	11±0.6
Hypericum alpestre	aerial part	methanol	–	–	10±0.6	10±0.6
		acetone	–	9±0.6	9±0.6	15±0.6
Rumex obtusifolius	seed	methanol	–	–	11±0.6	11±0.6
		acetone	–	–	10±0.6	12±0.6
Sanguisorba officinalis	aerial part	methanol	–	–	10±0.6	12±0.6
		acetone	–	–	11±0.6	13±0.6
PC [c]			18±0.6	18±0.6	19±0.6	20±1

[a] Tested bacterial strains: ampicillin-resistant *E. coli* dhpα-pUC18 (*E. coli* AMP), kanamycin-resistan *E. coli* PARS-25 (*E. coli* KN), *E. coli* WKPM-M17 (*E. coli* M-17), *S. aureus* MDC 5233 (*S. aureus*);
[b] the absence of growth inhibition zone;
[c] PC – positive control (gentamicin, 10 $\mu g/mL$).

Obtained data showed that only crude methanol extract of *S. officinalis* had bactericidal activity against one of the tested gram-negative strains at 2048 $\mu g/mL$ concentration: ampicillin-resistant *E. coli*. Two out of four tested plants crude extracts had bactericidal activity against *S. aureus*. MBC value of the methanol extract of *S. officinalis* against *S. aureus* was 2048 $\mu g/mL$, whereas it was 512 $\mu g/mL$ in case of the methanol extract of *H. alpestre*.

Table 2

Determination of MIC and MBC values of four tested plants' methanol crude extracts against some bacterial strains

Plant species	Tested part	MIC and MBC values, *μg/mL*							
		E. coli AMP [a]		*E. coli* KN		*S. aureus*		*E. coli* M17	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Agrimonia eupatoria	whole plant	1024	– [b]	1024	–	256	–	512	–
Hypericum alpestre	aerial part	1024	–	1024	–	128	512	512	–
Rumex obtusifolius	seed	1024	–	1024	–	256	–	512	–
Sanguisorba officinalis	aerial part	512	2048	1024	–	256	2048	512	–
PC [c]		0.5	NT [d]	0.5	NT	0.25	0.5	0.5	2

[a] Tested bacterial strains: ampicillin-resistant *E. coli* dhpα-pUC18 (*E. coli* AMP), kanamycin-resistant *E. coli* PARS-25 (*E. coli* KN), *E. coli* WKPM-M17 (*E. coli* M-17), *S. aureus* MDC 5233 (*S. aureus*);
[b] the value was not detected under tested concentrations;
[c] PC – positive control (gentamicin);
[d] NT – not tested.

The use of two different antimicrobial activity testing methods (agar diffusion assay and broth microdilution method) allowed us to compare results obtained by both methods. Generally, results gained by both methods were similar except one case. MIC value of *S. officinalis* against ampicillin-resistant *E. coli* determined by broth microdilution method was 512 *μg/mL*, while we did not find any growth inhibition zone with agar diffusion method at the same concentration.

Determination of Thermostability. It is important to explore the nature of active constituents of plants crude extracts, including their stability to heat treatment. Particularly, it will allow choosing convenient method for handling of these plant materials, including methods of extraction, drying and sterilization.

Table 3

Determination of thermostability of active phytochemicals of the crude extracts from studied herbs

Plant species	Extracts	Temperature, °*C*							
		60		80		100		121	Control 22°*C*
		Exposure time, *min*							
		30	60	30	60	30	60	30	60
Agrimonia eupatoria	methanol	– [a]	–	–	–	–	–	–	+
	acetone	+	+	+	+	–	–	–	+
Hypericum alpestre	methanol	+	+	+	+	+	+	+	+
	acetone	+	–	–	–	–	–	–	+
Rumex obtusifolius	methanol	+	+	+	+	–	–	–	+
	acetone	+	+	+	+	+	–	–	+
Sanguisorba officinalis	methanol	+	+	+	+	+	+	+	+
	acetone	+	+	+	+	+	+	+	+

[a] "–" Absence of activity; "+" presence of activity.

Acquired data showed that some of the plant crude extracts were very sensitive to heat treatment. Particularly, methanol extract of *A. eupatoria* lost its

antimicrobial activity against *S. aureus* after just 30 *min* exposure to 60º*C* temperature. Acetone extracts of the same plant kept its activity even at 60 *min* exposure to 80º*C*. Acetone extract of *H. alpestre* was also very sensitive to heat treatment. It has lost antimicrobial activity after keeping dried crude extract at 60º*C* for 60 *min*.

Three of tested plant extracts (methanol extract of *H. alpestre*, methanol and acetone extract of *S. officinalis*) retained their antimicrobial activity even after 30 *min* treatment with 121º*C*. This can mean that constituents responsible for their antimicrobial activity were thermo-labile compounds.

It is interesting to mention that there was difference in thermostability of the active constituents of acetone and methanol extracts of the same plants. This can lead to speculation that different compounds are responsible for the antimicrobial activity of acetone and methanol extracts from the tested three plants: *A. eupatoria*, *H. alpestre*, *R. obtusifolius*.

Conclusion. Thus, investigation of effect of heat treatment of antimicrobial activity of methanol and acetone extracts from four Armenian herbs was reaveled, that some of them were very sensitive to heat treatment, whereas others were stable even at 121º*C*. These results will allow choosing appropriate methods during processing of plant materials in order to maintain their antimicrobial activity. Moderate antimicrobial activity of tested plants against some gram-negative strains was also shown.

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