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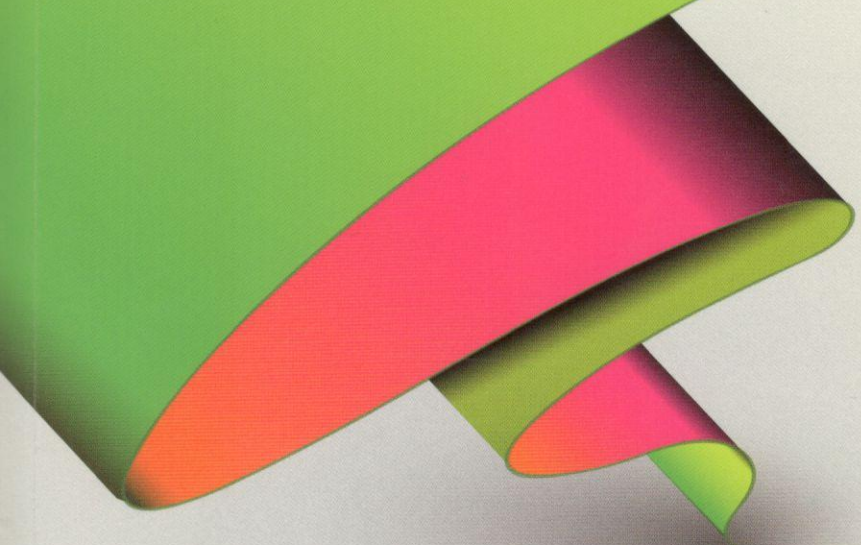

MICRO & NANO TECHNOLOGIES

ADVANCES IN BIOTECHNOLOGY

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MICRO AND NANO TECHNOLOGIES, ADVANCES IN BIOTECHNOLOGY

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OPTIMIZATION PROTOCOL FOR THE EXTRACTION OF ANTIMICROBIAL COMPONENTS FROM VEXIBIA ALOPECUROIDES ROOTS

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ABSTRACT

Quantity and activity of plant extracts are dependent on extraction protocol. Many factors such as solvent, temperature, number of extraction steps, liquid-to-solid ratio and the particle size contribute to the efficacy of the extraction process.

The objective of this study was to optimize the conditions (solvent: methylene chloride, ethanol; extraction time: from 12 to 36 hours; liquid-to-solid ratio: 4:1, 8:1, 10:1 ml/g; and particle size: 3, 5, 7 mm) for the extraction of antimicrobial compounds from *Vexibia alopecuroides* roots and to measure total flavonoid contents and antimicrobial activity of obtained extracts.

Results of this study shown the best extraction protocol: the solvent was methylene chloride; best extraction time were found 24 hours, the liquid-to-solid ratio were found 10:1 ml/g, and the particle size - 7 mm which rendered a total flavonoid content of 13.5 μg of QE/mg of extract with the highest antibacterial activity against *Staphylococcus aureus* (IC_{50} 3.05 μg/ml) and *Methicillin-resistant S. aureus* (IC_{50} 2.9 μg/ml).

This study can be useful in the development of pharmaceutical extraction processes, including further studies concerning the optimal number of sequential steps to enhance the efficacy of a large-scale extraction system.

Keywords: Extract, antimicrobial activity, roots

INTRODUCTION

The genus *Sophora* (syn. *Vexibia*) has a big scientific and practical interest. This genus occurs worldwide and includes 52 species; most of them are widely distributed in west and middle Asia. About fifteen species of this genus are widely used in traditional Chinese medicine. In recent decades, the use of this genus has led to the rapid growth of information on active substances and various pharmacological and therapeutic properties, the majority of *Sophora* alkaloids have been recognized by their main active chemical components, including matrine, sophocarpine, and others [1, 2]. As well as flavonoids, isoflavonoids [3], flavonostilbenes [4] flavones, flavonols, saponins, phospholipids and fatty acids. *Sophora* has various pharmacological properties

including antioxidant [5], anticancer [6], antibacterial [7], antiviral, antipyretic, cardiotonic, anti-inflammatory [8].

In Kazakhstan grows only two species of this genus, among them is widely distributed all over the Kazakhstan *Vexibia alopecuroides* (L.) (syn. *Sophora alopecuroides*) except the north part. *Vexibia alopecuroides* grows in river valleys, along rivers and ditches, in weedy places.

It is well known that *Vexibia alopecuroides* contains various chemical components such as alkaloids [9]: sparteine, sophoridine, sophocarpine, oxymatrine, oxysophocarpine, sophoridine, matrine, sophocarpine, cytisine, nicotine, flavonoids [10]: quercetin, rutoside, isobavachin, glabol, trifolirhizin, ammthamnidin, vexibinol, vexibidin, flavonostilbenzenes [4]: alopecurones A-F. They determine a variety of its biological activity.

In traditional Chinese medicine, this plant is used as a pain reliever, antibacterial agent, for the treatment of eczema, colitis. Also this plant has anticancer and anti-inflammatory, antibacterial and antiviral activities [9, 11].

However, in Kazakhstan *Vexibia alopecuroides* is not in the list of official medicine, which makes it a priority for Pharmacognostic studies and inclusion among medical herbs.

The most convenient way to obtain biologically active substances is extraction. In the process of extraction of medicinal plants should be taken into account that the process is exposed to a large variety of factors including type of solvents, the temperature, the pH, the number of extraction steps, liquid-to-solid ratio and the particle size. Currently, in the literature there are few data about the influence of the extraction conditions on the extraction process of flavonoids and information in this regard is often fragmentary and contradictory.

The aim of this study was to optimize the conditions for the extraction of *Vexibia alopecuroides* to achieve high flavonoids content and high antimicrobial capacity.

MATERIALS AND METHODS

Plant material

The roots of *Vexibia alopecuroides* were collected during flowering stage on May 23, 2013 in the foothills of Trans-Ili Alatau, in Kazakhstan at location coordinates: N = $43^0$09'391", E = $076^0$30'974". The plant was identified by Dr. Nadejda Gemejieva. A voucher specimen is deposited in the herbarium of the Institute of Botany and Phytointroduction, Almaty, Kazakhstan, under the number 2997a/25.

Extracts preparation

The roots were chopped and dried at room temperature for 10 days and used as raw material.

In the first series of experiment was chosen proper solvent using sequential extraction and single-stage extraction with methylene chloride (CH_2CL_2) and 95% ethanol. The ground roots (100 g) of *Vexibia alopecuroides* were extracted at room temperature with 1.7 L of methylene chloride for at least 24 h, provided 2.2 g of extractable after evaporation of solvent. 1.9 L of 95% ethanol providing 8.7 g of extractable following evaporation of solvents.

In the second series of experiment was optimized the extraction conditions. The dried roots were ground using miller into a particle size of 3, 5, 7 mm, extraction time was 12, 24 and 36 hours, solvent ratios used were 1:4, 1:8, 1:10.

Antimicrobial assay

Microorganisms were obtained from the American Type Culture Collection and included the bacteria *S. aureus* ATCC 29213, MRSA ATCC 33591. All microorganisms were tested using modified versions of the CLSI (formerly NCCLS) methods. Drug control, ciprofloxacin (99.3%, ICN Biomedicals) was included in each assay.

Total Flavonoid Content

Total flavonoid contents were measured with the aluminum chloride colorimetric assay [12]. Methylene chloride extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distill water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

Statistical analysis

Data obtained was analyzed using Statistica 6.0 software. The results obtained (samples run in triplicates) were expressed as means ± standard deviation. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

An extraction solvent system is generally selected according to the propose of extraction, polarity of the interested components, overall cost, safety and environmental concern [13]. Extraction yield and extraction activity are also dependent on the solvent. Therefore, in the first series of the experiment was used sequential extraction with methylene chloride (CH_2CL_2) and 95% ethanol. In the experiments was used ground roots 100 g of *Vexibia alopecuroides*, the extraction was conduct at room temperature using 1.7 L of methylene chloride and 1.9 L of 95% ethanol.

The antimicrobial activity and amounts of the crude extracts obtained from *Vexibia alopecuroides* using different extraction solvents are presented in Table 1. The highest extraction yield of 8.7 was obtained using 95% ethanol, but in spite of that the extract did not showed antimicrobial activity. Methylene chloride extraction solvent was more effective isolating compounds with antimicrobial properties.

For comparison, an independent experiment was conducted in which the sequential extraction has been replaced by a single-stage, using the same solvents. As in the previous experiment, the dried raw materials were weighed (100 g) and placed in a glass container for extraction for 24 hours with occasional stirring (maceration method). The data are presented in Table 2.

Table 1 – Effects of extracting solvents on the extract yields and antimicrobial activity (sequential extraction)

Solvent	Yield, g dry weight	Growth inhibition,% (the investigated sample concentration of 50.0 μg / ml)	
		S. aureus	MRSA
100% methylene chloride	2.2 ± 0.3	99 ± 1,0	100
95% ethanol	8.7 ± 0.6	45 ± 2,1	38 ± 3,8

The samples with the growth inhibition <50 % were considered - not active

The comparison experiment showed that the antimicrobial activity of 100% methylene chloride extract significantly ($p<0.05$) higher from other solvent. But still the highest extraction yield was obtained in 95% ethanol. Possibly, the difference in polarity of selected solvents leads to the fact that with the antimicrobial compounds can isolate with ballast compounds. Due to the results, further work was carried out using as solvent - 100% methylene chloride.

Table 2 – Effects of extracting solvents on the extract yields and antimicrobial activity (single-stage extraction)

Solvent	Yield, g dry weight	Growth inhibition,% (the investigated sample concentration of 50.0 μg / ml)	
		S. aureus	MRSA
100% methylene chloride	2.5 ± 0.2	97 ± 1,2*	100*
95% ethanol	7.9 ± 0.4	59 ± 3,3	51 ± 2,6

The samples with the growth inhibition <50 % were considered - not active

*Significant differences ($p<0.05$) within the antimicrobial activity

Solid-to-liquid ratio was another important parameter influencing on yield. Table 3 showed that the highest extraction yield was obtained at 1:10, solid: liquid ratio. It was observed that yield for 1:4 and 1:8 were about 48% less than ratio 1:10.

Table 3 – Yield of crude *Vexibia alopecuroides* roots extracts

Solvent	Ratio (solids : solvents)	Yield, g dry weight
100% methylene chloride	1:4	1,0±0,3
	1:8	1,3±0,2
	1:10	2,9±0,5

The next step of optimizing process of extraction was effects of ground raw materials (3, 5, 7 mm) on antimicrobial activity of the extracts. The results shown in Table 4 indicate that all extracts have potential to inhibit the test microorganisms (*S. aureus* and MRSA) despite the size of the particles. These negligible differences in antimicrobial activity of the crude *Vexibia alopecuroides* roots extracts could be linked to their different composition of bioactive compounds. But among the extracts, extract obtained using particle size 7 mm exhibited the significant stronger antimicrobial activity against MRSA (2.9±0.20 μg/ml). However, the antimicrobial activity of the all extracts was lower than the standard antibiotic, this can be explained by the fact that it is crude extract and after separation into individual substance activity could be similar to positive control data.

Table 5 – Effect of particle size on antimicrobial activity

Solvent	Particle size, mm	IC_{50} - 50% of grows inhibition, μg/ml	
		S. aureus	MRSA
100% methylene chloride	3	5.98 ± 1.87	6.34 ± 2.7
	5	4.56 ± 0.80	5.43 ± 2.4
	7	3.05 ± 1.74	2.9±0.20*
Positive control (Ciprofloxacin)	-	0.1±0.02	0.1±0.01

Extraction time was the final step in a series of experiments. Table 5 showed that flavonoids increased gradually with increasing of extraction time from 12 hours (8.7 ±1.9 mg Quercetin/100 g DW) up to 24 hours (13.5 ±2.3 mg Quercetin/100 g DW) and begun to decline sharply until reaching a minimum (6.3 ± 1.6 mg Quercetin/100 g DW). This process could be explained by the Fick's second law of diffusion, predicting that a final equilibrium between the solute concentrations in the solid matrix and in the solvent might be reached after a certain time, leading to deceleration in the extraction yield [14]. Thus, extraction time of 24 hours was selected as the optimum point for the subsequent step due to the practical considerations.

Table 5 – Extraction time of crude *Vexibia alopecuroides* roots extracts

Solvent	Extraction time, hours	Total flavonoids content, mg Quercetin/100 g DW
100% methylene chloride	12	8.7 ±1.9
	24	13.5 ±2.3
	36	6.3 ± 1.6

CONCLUSION

Thus, the experimental optimum conditions that allow fast, quantitative and maximum extractions of antimicrobial compound and optimum amount of flavonoids from roots of *Vexibia alopecuroides* were obtained through the effective classical solvent extraction method as well as single factor experiments. 100% methylene chloride was found to be the best solvent for the extraction. Subsequently, the optimum conditions for the extraction was found to be 100% methylene chloride, extraction time 24 hours, the liquid-to-solid ratio were found 10:1 ml/g, and the particle size - 7 mm with content of 13.5 μg of QE/mg of extract with the highest antibacterial activity against *Staphylococcus aureus* (IC_{50} 3.05 μg/ml) and *Methicillin-resistant S. aureus* (IC_{50} 2.9 μg/ml). These optimum conditions can be useful for further optimization of the extraction using response surface methodology (RSM) in conjuction with central composite rotatable design (CCRD). Also, further research is needed to identify active individual compounds with antimicrobial properties and develop application in pharmaceutical systems.

REFERENCES

[1] Liu M., Liu X.Y., Cheng J.F., Advance in the pharmacological research on matrine, Zhongguo Zhong Yao Za Zhi, vol. 28, pp. 801-804, 2003.

[2] Zhang Y.F., Wang S.Z., Li Y.Y., Xiao Z.Y., Hu Z.L., Zhang J.P., Sophocarpine and matrine inhibit the production of TNF and IL-6 in murine macrophages and prevent cachexia-related symptoms induced by colon 26 adenocarcinoma in mice, International Immunopharmacology, vol. 8, pp. 1767-1772, 2008.

[3] Iinuma M., Ohyama M.;Tanaka T., Flavonoids in roots of *Sophora prostrata*, Phytochemistry, vol. 38, pp. 539-543, 1995.

[4] Iinuma M., Ohyama M., Tanaka T. Six flavonostilbenes and a flavanone in roots of *Sophora alopecuroides*, Phytochemistry, vol.38, pp. 519-525, 1995.

[5] Piao X.L., Piao X.S., Kim S.W., Park J.H., Cai S.Q., Identification and characterization of antioxidants from *Sophora flavescens*, Biological and Pharmaceutical Bulletin, vol. 29, pp.1911-1915, 2006.

[6] Xingming Ma., Luo Y., Yu H., Cui Y., Ethanolic extracts of *Sophora moorcroftiana* seeds induce apoptosis of human stomach cancer cell line SGC-7901 in vitro, African Journal of Biotechnology, vol. 5, pp. 1669-1674, 2009.

[7] Yamaki M., Kashihara M., Takagi S., Activity of Kushen compounds against *Staphylococcus aureus* and *Streptococcus mutans*, Phytotherapy Research, vol. 4, pp. 235-236, 1990.

[8] Wen M., Mao X.J., Research progress on chemical composition, antiinflammatory and antiallergic activity of *Sophora viciifolia*, Yunnan Journal of Traditional Chinese Medicine and Materia Medica, vol. 27, pp. 63-64, 2006.

[9] Zhang L., Li G., Houghton P.J., Jackson S., Twentyman P.R., Alkaloids in *Sophora alopecuroides* seed and relevant tests for activity, Zhongguo Zhong Yao Za Zhi, vol.22/issue 12, pp.740-743, 1997.

[10] Syrov V.N., Yuldashev M.P., Mamutova M.I., Khushbaktova Z.A., Batirov É.Kh., Isolation, Chemical Analysis, and Study of the Hepatoprotector and Biligenic Activity

of Total Flavonoid Preparations from *Thermopsis dolichocarpa* and *Vexibia alopecuroides,* Pharmaceutical Chemistry Journal, vol. 35/issue 1, pp. 30-34, 2001.

[11] Kucukboyaci N., Ozkan S., Adiguzel N., Tosun F., Characterization and antimicrobial activity of *Sophora alopecuroides* L. var. alopecuroides alkaloid extracts. Turkish Journal of Biology, vol. 35, pp. 379–385, 2011.

[12] Patel A., Patel A., Patel N.M, Estimation of Flavonoid, Polyphenolic Content and In-vitro Antioxidant Capacity of leaves of *Tephrosia purpurea* Linn. (Leguminosae), International Journal of Pharma Sciences and Research, vol.1/issue 1, pp. 66-77, 2010.

[13] Wang J., Sun B.G., Cao Y., Tian Y., Li X.H., Optimization of ultrasound-assisted extraction of phenolic compounds from wheat bran, Food Chemistry, vol. 106, pp. 804-810, 2008.

[14] Tan M.C., Tan C.P., Ho C.W., Effects of extraction solvent system, time and temperature on total phenolic content of henna (*Lawsonia inermis*) stems, International Food Research Journal, vol. 20/issue 6, pp. 3117-3123, 2013.