

Current Issues in Pharmacy and Medical Sciences

Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: <http://www.curipms.umlub.pl/>



Development of standardization methods of gel with sapropel extract and their validation

OKSANA STRUS^{1*}, IVAN REVYATSKYY², SERHII HOLOTA^{3,4}, SERGEY GUSHCHA⁵

¹ Department of Drug Technology and Biopharmaceutics, Danylo Halytsky Lviv National Medical University, Ukraine

² Department of Management and Economy of Pharmacy, Medicine Technology and Pharmacoeconomics, Postgraduate Faculty, Danylo Halytsky Lviv National Medical University, Ukraine

³ Department of Organic Chemistry and Pharmacy, Lesya Ukrainka Volyn National University, Ukraine

⁴ Department of Organic, Bioorganic and Pharmaceutical Chemistry, Danylo Halytsky Lviv National Medical University, Ukraine

⁵ Department of Fundamental Research, State Institution "Ukrainian Research Institute of Medical Rehabilitation and Resort Therapy of Ministry of Health of Ukraine, Odessa, Ukraine

ARTICLE INFO

Received 15 December 2022

Accepted 8 February 2023

Keywords:

humic acids, validation, identification, gel, quantification, aqueous sapropel extract.

ABSTRACT

Humic acids (HA) are important natural compounds that are characterized by a wide range of biological activity and therapeutic impact on different pathological processes. Sapropels are natural healing resources that contain a large amount of HA. The pharmaceutical market of Ukraine needs domestic drugs with dermatotropic action based on natural compounds that have a combined antibacterial, wound healing and anti-inflammatory activity, as well low toxicity.

The aims of the research: standardization of the gel under the conditional name "Saprogel" for use in medicine as a wound-healing and anti-inflammatory agent, development of methods for identification and quantification, as well as their validation.

A linear dependence between the concentration of the total mass fraction of HA on the mass of the sample of aqueous sapropel extract (ASE) with a correlation coefficient of 0.9985 (≥ 0.9981) was observed, while the angular coefficient of linear dependence (b) was found to be 1.02, with the free member of linear dependence (a) being $-1.66 \leq 2.60$. The obtained results show that the method is precise because the value of the relative confidence interval is less than the critical value for the convergence of the results: $\Delta\% = 1.27 \leq 1.60$ and the criterion of the insignificance of systematic error: $\delta = 0.51$. As a result of the quantitative analysis, we found that the total mass fraction of HA in the gel samples from the wind farm is 1.302%.

INTRODUCTION

Treatment of wound processes is still one of the most pressing problems of modern medicine. The number of domestic and industrial injuries are rising [1], as are road traffic injuries [2]. In addition, the number of patients with purulent skin and soft tissue diseases, as well as postoperative complications, and injuries resulting from natural disasters and military conflicts have increased greatly [1-3].

Nowadays, treatment of dermatological skin diseases and wounds is most often carried out with the use of semi-solids (semi-solid dosage forms (SSDFs)), due to the need for local action of the drug on the skin surface [4].

Drugs for external use have certain advantages, including ease of use and monitoring of treatment. In addition,

most of these drugs do not enter the systemic circulation and, accordingly, do not cause complications in the body. Interaction with other drugs is also uncommon [5].

According to the analysis of the State Register of Medicines of Ukraine, as of June 2020, inside the group of D03 dermatological agents for the treatment of wounds and ulcers (D03AX – other drugs that promote wound healing) ointments predominate at the level of 36% of all registered drugs [6,7]. At the same time, gels make up 7% of all dermatological treatments for wounds [5].

Gels possess a number of advantages over other SSDFs, being easy to apply and distribute on the surface, and quickly absorbed into the skin. These attributes ensure high bioavailability of active substances and proper penetration of active substances. In addition, gels do not contaminate and do not leave a greasy shine, and are easy to produce [8,9].

* Corresponding author

e-mail: oxana.strus@ukr.net

The urgency of the search for new highly effective means of natural origin is influenced by resource factors because, in today's market conditions, the availability of raw materials determines the demand for the proposed drug.

Today, sapropel reserves in Ukraine are over 86 million tons. Humic acids (HA) are the main group of bioactive natural compounds in sapropels. HA possess a pronounced biological activity, inhibit the development of malignant tumors, have antiviral, wound healing and anti-inflammatory activity [10-23]. Very low concentrations of HA are effective for physiological action. Beyond biological activity, HA are characterized by high sorption capacity, bacteriological and biostimulating activity [10,11].

The pharmaceutical market of Ukraine needs domestic drugs with dermatotropic action based on natural compounds that have a combined antibacterial, wound healing, and anti-inflammatory activity, as well low toxicity. That is why the development and standardization of SSDFs (gels) based on aqueous sapropel extract (ASE) containing HA is an actual and important task in the area of drug technology.

AIM

Standardization of the gel under the conditional name "Saprogel" for use in medicine as a wound-healing and anti-inflammatory agent, development of methods for identification and quantification and their validation.

MATERIALS AND METHODS

Materials

Material of the study was the gel under the conditional name „Saprogel”, which contained an ASE in the amount of 15%, carbopol Ultrez 10, potassium sorbate, glycerol, purified water. „Saprogel” – tested gel with ASE was prepared according to the following technology: potassium sorbate (preservative) was dissolved in purified water, the pre-weighed carbomer Ultrez 10 was added and left to swell for 30-60 min. During the swelling, the mixer was periodically switched on and run at a speed of 60-90 rot/min. Gradually, the ASE and glycerol were added and homogenized at a speed of 60-90 rot/min for 5-10 min until obtaining a homogeneous gel [24].

Reagents

The following reagents were used to develop the qualitative and quantitative analysis methods for the determination of HA in the ASE gel.

0.1 M alkaline solution of sodium diphosphate ($\text{Na}_4\text{P}_2\text{O}_7 \times 10\text{H}_2\text{O}$). Here, 44.6 g of sodium diphosphate and 4.0 g of sodium hydroxide were first placed in a 1000 ml volumetric flask, a portion this was then dissolved in 500 ml of purified water P and the solution made up with purified water P to the mark.

0.4 M solution of chromium mixture. Firstly, 40.0 g of finely divided potassium dichromate was placed in a 1000 ml volumetric flask. This was then dissolved in 500 ml of purified water P and made up to the mark with purified water P. The solution was subsequently placed in a conical flask with a capacity of 3000 ml and sulfuric acid concentrated

in an amount of 1000 ml was added in 100 ml portions carefully, at intervals of 10-15 min. After cooling, the mixture was poured into a dark glass vessel.

0.2 M solution of Mohr's salt ($(\text{NH}_4)_2\text{SO}_4 \times \text{FeSO}_4 \times 6\text{H}_2\text{O}$) 80.0 g of Mohr's salt was dissolved in 500 ml of purified water P, and filtered into a volumetric flask with a capacity of 1000 ml. Afterwards, 20 ml of concentrated sulfuric acid was added and then the solution was made up with purified water P to the mark. To check the concentration of Mohr's salt: in three conical flasks with a capacity of 100 ml, 10 ml of Mohr's salt solution is placed, 1 ml of concentrated sulfuric acid is then added and the solution is titrated with 0.1 M potassium permanganate solution to a pale pink color that does not disappear within 1 min. The three test results are averaged.

0.2% N-phenylanthranilic acid solution. Initially, 0.20 g of N-phenylanthranilic acid was triturated with a small amount of 0.2% sodium carbonate solution, transferred to a volumetric flask with a capacity of 100 ml, mixed and made up to the mark with 0.2% sodium bicarbonate solution.

Methods

The following indicators were selected for standardization of the developed gel with ASE: description, identification of HA, pH value, structural viscosity, microbiological purity, quantitative determination of the total mass fraction of HA.

Description

European Pharmacopoeia (EPH) 10th edition, Vol. 1, "Semi-solid preparations for cutaneous application" (EPH, 2018). p.935 [25].

Odour

EPH 10th ed., Vol. 1, 2.3.4., p.133 [25].

Homogeneity

Homogeneity was determined using the method of State Pharmacopoeia of Ukraine (SPhU) 1st edition p. 511 [26].

Ph value

Ph value was determined using the method of EPH 10th ed., Vol. 1, 2.2.3., p.24-25. [25].

Structural viscosity

Structural viscosity was determined using the method of EPH 10th ed., Vol. 1, 2.2.3., p.28-30 [25].

Microbiological purity

Studies were performed according to the requirements of the EPH 10th ed., Vol. 1, 2.6.12. p. 201, 2.6.13, p. 205.

EXPERIMENTAL PART

Identification

Identification of HA in the gel with ASE was performed after extraction from ASE by alkaline hydrolysis, by using the method of quantification with follow-up precipitation of HA with a sulfuric acid concentrated solution.

Quantitative determination of the HA total mass fraction in the ASE gel

The total mass fraction of HA was determined by the method described in the literature, after oxidation of HA by the method after I.V. Tyurin in the modification after B.A. Nikitin, and subsequent titration with Mora salt solution [27].

Extraction of humic acids from a portion of the gel

Firstly, 5,000 g of gel (exact portion) was placed in a 250 ml conical flask (flask A), 100 ml of alkaline sodium diphosphate solution was then added, and the solution was stirred for 1 hour on a shaker.

The resulting suspension was transferred to a centrifuge tube and centrifuged for 15 min at 2000 rpm. The solution above the precipitate was subsequently decanted into a conical flask with a capacity of 1000 ml (flask B). The precipitate was then washed twice with 100 ml of 1% sodium hydroxide solution, centrifuged each time and the solution was poured into flask B.

To the precipitate in the centrifuge tube, 100 ml of 1% sodium hydroxide solution was added and the resulting solution was transferred to flask A. The flask with suspension was then heated for 2 h in a boiling water bath and cooled to room temperature. The suspension was subsequently centrifuged and the solution was poured over the precipitate into flask B.

The remaining precipitate was washed twice with 100 ml of 1% sodium hydroxide solution and centrifuged, the solution was then poured into flask B. The precipitate was washed three times with 100 ml of purified water P, centrifuged and the solution was also poured into flask B.

The contents of flask B were subsequently filtered through a blue strip filter into a 1000.0 ml volumetric flask, made up to the mark with purified water P and mixed (solution to determine the total mass fraction of HA).

Sedimentation of humic acids (identification of HA)

From the 1000 ml flask containing the alkaline HA solution, 50.0 ml of the solution was transferred to a conical flask with a capacity of 100 ml, and, while stirring, sulfuric acid concentrated was added dropwise to develop a pH of 2.0 to 3.5. The pH value of the solution was monitored potentiometrically. The flask was then heated in a boiling water bath for 30 minutes, cool, and left for 16 hours to settle completely.

HA residue was subsequently filtered through a "blue stripe" filter. The precipitate on the filter and the flask in which the HA was precipitated were then washed with 0.05 M sulfuric acid solution 3 times in 10 ml to obtain a clear filtrate.

The funnel with the precipitate on the filter was subsequently inserted into a volumetric flask with a capacity of 100.0 ml and the precipitate was dissolved with hot 0.05 M sodium hydroxide solution. Afterwards, the filter was washed with hot sodium hydroxide solution until the precipitate was completely dissolved and a clear filtrate was obtained. The resulting alkaline HA solution was then

cooled to room temperature and the volume of the flask was adjusted to 0.05 M with sodium hydroxide solution.

Oxidation of HA by the method after I.V. Tyurin in the modification after B.A. Nikitin and titration with Mora salt solution

20.0 ml of an alkaline solution of HA was placed in a conical flask with a capacity of 100 ml and then evaporated in a boiling water bath to dryness without overdrying. Subsequently, 10 ml of 0.4 M solution of chromium mixture was added to the conical flask with the dried residue. The solution was then topped up slowly, with the walls of the flask being thoroughly washed off. The flask was then closed with a funnel and placed in an oven for 20 minutes at a temperature of 150 - 160° C. After cooling, the funnel was removed from the flask, and the flask was rinsed with purified water, 6 drops of 0.2% N-phenylanthranilic acid solution was then added, and the solution was titrated with 0.2 M Mora salt solution until the color changed from cherry-red to green.

The control study was done in parallel.

The HA total mass (X), in mg in the 1.0 g of gel was calculated by applying the formula:

$$X = \frac{(V_1 - V_0) \times K \times 82.72}{m} \quad (1),$$

where:

V_1 – volume of Mora salt solution for titration of control experiment, ml;

V_0 – volume of Mora salt solution for titration of analytical sample, ml;

K – correction factor for Mora salt solution;

m – portion of the tested sample, g.

The amount of HA in 1 g of gel should be at least 12.5 mg.

The approach to the validation of BAS in herbal preparations, described in the literature [28] was used to study the validation characteristics of ASE gel. The specificity of this analytical procedure was proved by determining the number of milligrams of the amount of HA in the studied gel with ASE by volumetric titration. The control experiment was performed to increase the specificity of the redox technique in parallel.

Statistical analysis

Statistical analysis of the data was performed using the Shapiro-Wilk test via the software package GraphPad Prism 5.04 (GraphPad Software Inc., USA). The result was calculated using two-way analysis of variance with the Bonferroni test post hoc. The arithmetic mean (M) and the standard error of the arithmetic mean (m) were determined for each result obtained. The difference between the means was considered statistically significant if p was less than 0.05.

RESULTS

Identification of ASE

HA have a dark brown color, soluble in weak alkalis, slightly soluble in water.

The presence of ASE was determined by the total mass fraction of HA in the test gel. Extraction of HA from the gel sample, precipitation, oxidation of HA and titration with Mora salt (as conducted by the method of I.V. Tyurin in the modification of B.A. Nikitin) and titration with Mora salt, were developed on the basis of DSTU 7083: 2009 [27].

The results of quantitative determination of the HA total mass fraction in the ASE gel samples and metrological characteristics of the average results are presented in the Table 1.

Linear dependence between the concentration of the total mass fraction of HA on the mass of the sample of the gel with ASE was the criterion of acceptability for the proposed method. Each sample in a concentration of from 80% to 120% of the selected was titrated at least three times when determining the linearity. The results are given in Table 2 and shown in Figure 1.

Table 1. Metrological characteristics of the average results of quantitative determination of the HA total mass fraction in the ASE gel (n=6)

The № of set	HA total mass fraction, mg	S ²	S	S _x	Δx	Δx̄	ε̄, %	ε, %
1	13.28	0.0429	0.2073	0.0846	0.5329	0.2176	1.67	4.10
2	12.80							
3	12.92							
4	13.05							
5	13.23							
6	12.81							

Table 2. Calculation of linearity parameters of the method of quantitative determination of the mass fraction of HA

% of working concentration	Weight of the gel sample, g	Total mass fraction of HA, mg
80	4.0007	9.92
85	4.2508	10.70
90	4.5011	11.39
95	4.7492	11.84
100	5.0027	12.57
105	5.2513	13.23
110	5.5009	13.84
115	5.7504	14.39
120	6.0015	15.16

The linear dependence between the concentration of the total mass fraction of HA on the weight of the sample of concentrated ASE was observed with a correlation coefficient of 0.9985 (≥ 0.9981), the angular coefficient of linear dependence (b) is 1.02, the free member of the linear dependence (a) – $1.66 \leq 2.60$.

Precision and accuracy were investigated in nine determinations in the range of concentrations from 80 to 120% according to the chosen method (Table 3).

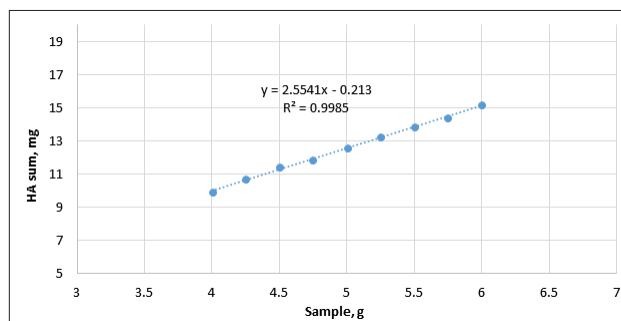


Figure 1. Dependence of the concentration of the HA total mass fraction on the mass of the sample of the tested gel

The obtained results (Table 3), reveal that the method is precise, because the value of the relative confidence interval was less than the critical value for the convergence of the results: $\Delta\% = 1.27 \leq 1.60$ and the criteria of insignificance of systematic error was $\delta = 0.51$. As a result of the quantitative analysis, we found that the total mass fraction of HA in the ASE gel samples is 1.302%.

Based on the obtained data, we proposed to introduce requirements for the quantitative content of the HA total mass fraction not less than 1.25% to the draft SSDFs of the medicinal product. The specification and study results are presented in Table 4.

Table 3. The results of the study of the precision of the method of quantitative determination of the HA total mass fraction

Parameters	Value	Criteria 1	Criteria 2	Conclusion
Precision	ΔZ	1.27	≤1.60	Sustained by the Criteria 1

Table 4. Specification and research results of quality ASE indicators

Indicator	Permissible norms	Analysis results
Description	Brown color gel, without order	Meets
HA identification	A dark brown precipitate of HA is formed (precipitation of HA) after acidification of the solution with sulfuric acid to pH=2-3	Meets
Homogeneity	The drug must be homogeneous and 3 of the 4 samples should be free of visible particles	Meets
pH	6.5-8.0	7.0 ± 0.2
Microbiological purity	The total number of aerobic microorganisms (TAMC) is 102 CFU/g. The total number of yeasts and molds (TYMC) is 101 CFU/g. Absence of <i>Pseudomonas aeruginosa</i> in 1 g. Absence of <i>Staphylococcus aureus</i> in 1 g	Meets
Quantitative determination	The content of the HA total mass fraction should be not less than 12,5 mg/1 g	13.02 ± 0.22
Packing	100 g in a plastic tube in a pack. Packs are placed in group containers	
Marking	According to the original layout of the package	
Storage	In the original packaging at a temperature not exceeding 25±2°C	
Expiration date	2 years	

DISCUSSION

Research carried out in various countries suggests that the efficacy of humic acids depends on its extremely complex chemical structure, which makes biochemical investigations elaborate, costly and difficult to reproduce [29]. Many fundamental questions relating, in particular, to the

physicochemical characteristics of humic and fulvic molecules are yet to be answered [30,31]. Differences in the obtained values have been attributed to either the variability of humic substances or the intrinsic limitations of methods when applied to poly-disperse humic systems [32,33].

The traditional method of determining humic content (humic and fulvic acids) in commercial fertilizers, biostimulants and organic materials is based on the oxidation of the organic carbon contained in the basic-soluble but acid-insoluble fraction (humic acids) and the basic-acid soluble fraction (fulvic acids) of their alkaline water extracts. This methodology, merely operational, makes it impossible to distinguish if the quantified carbon corresponds to substances with chemical nature or to non-humic organic matter but with similar solubility properties to those of humic matter [34].

In Spain, the current official analytical determination of the humic content in commercial products is based on the quantification of the total organic carbon soluble at alkaline pH (total humic content) or in the humic content of the acid-insoluble residue remaining in the alkaline extract (content in humic acids) by oxidation with potassium dichromate. Weaknesses of this method are linked to the need to assume a specific percentage of carbon in humic matter and an oxidation factor, as well as to its low selectivity [34].

There is a method based on the International Humic Substances Society (IHSS) humic fractionation, but quantifying humic and fulvic fraction by gravimetric methods has been proposed. This method determines HA and fulvic acids (FA) concentrations gravimetrically on an ash-free basis. This new method solved the inaccuracy associated with humic carbon concentration and oxidation factor assumptions— but still has potential low selectivity [35].

In the experimental work of Laurynas Jarukas *et al.*, humic substances were analyzed according to the recommendations of agricultural chemical analysis. Here, the quantity of fulvic acid was determined spectrophotometrically, while humic acid and humin were analyzed by thermogravimetric studies [36].

The experimentally developed method of determination of the HA total content described in this paper allows to use it to determine the active substances in SSDFs and to standardize the gel under the conditional name “Saprogel” for use as an anti-inflammatory agent.

This method of determination of the HA total content is easy to perform, and the evaluation neither requires expensive equipment nor is based on absolute variables that are equipment dependent.

The process of mixing multiple chemical compounds reduces the credibility of the active substance and the extent of its effect. The core of our study consists in obtaining a complex of natural biologically active compounds and developing methods for their qualitative and quantitative determination. The next stage of our research will be the separation and identification of HA, as well as the determination of their possible pharmacological activities. We are also planning to support this study with field tests and to take real samples of different infections. However, these studies will be of fundamental nature, which is beyond the scope of this article.

CONCLUSIONS

Methods for identification and quantification of humic acids in a gel with sapropel extract have been developed and described in this paper. Validation of the developed methods on linearity and precision was also carried out. The gel with ASE was standardized according to the following indicators: description, identification of HA, pH value, structural viscosity, microbiological purity, quantitative determination of HA total mass fraction.

FUNDING

No external funding was provided.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGMENTS

We are thankful to “Zander – Ukraine” LTD for providing free samples of sapropel.





ETHICAL APPROVAL

The authors declare that ethical approval was not required.

INFORMED CONSENT

The authors declare that informed consent was not required.

ORCID iDs

Oksana Strus  <https://orcid.org/0000-0003-1638-1162>
 Ivan Revyatskyy  <https://orcid.org/0000-0002-4726-3639>
 Serhii Holota  <https://orcid.org/0000-0002-9892-437X>
 Sergey Gushcha  <https://orcid.org/0000-0003-3097-5258>

REFERENCES

1. Borowy I. Road Traffic injuries: Social change and global development. *Med Hist.* 2013;57(1):108-38.
2. Jeong BY, Lee S, Lee JD. Safety and health at work. Workplace accidents and work-related illnesses of household waste collectors. *Saf Health Work.* 2016;7(2):138-42.
3. Vynnyk VS. Modern methods of treatment of purulent wounds. *Sybirskoie Medicinskoie Obrazovanie.* 2013;1:18-24.
4. Hilman A, Khardman D, Limberd L. *Clinical pharmacology by Hudman and Hilman.* Moscow: Praktika; 2006.
5. Strus O, Polovko N. The composition and technology development of the gel dermatotropic product with sapropel extract. *WJPPS.* 2020;9(3):209-24.
6. *State Register of Medicinal Products of Ukraine:* Information Fund. State Expert Center of the Ministry of Health of Ukraine. [<http://www.drz.kiev.ua/>]
7. *Compendium. Medicines. ATX-classification.* [https://compendium.com.ua/use_atc/]
8. Swarbrick J (ed). *Encyclopedia of pharmaceutical technology.* 3rd ed. New York: In-forma Healthcare USA; 2007:4372.
9. Gad SC. *Pharmaceutical Manufacturing Handbook. Production and Processes.* Hoboken: John Wiley & Sons; 2008. [<https://www.uv.mx/personal/izcamacho/files/2012/02/Pharmaceutical-Manufacturing-Handbook-Production-and-Processes-Wiley-2008.pdf>]

10. Van Rensburg CEJ. The antiinflammatory properties of humic substances: A mini review. *Phytother Res.* 2015;29(6):791-5.
11. Aeschbacher M, Graf C, Schwarzenbach RP, Sander M, Sander M. Antioxidant properties of humic substances. *Environ Sci Technol.* 2012;46(9):4916-25.
12. Zhao Y, Paderu P, Delmas G, Dolgov E, Lee MH, Senter M, et al. Carbohydrate-derived fulvic acid is a highly promising topical agent to enhance healing of wounds infected with drug-resistant pathogens. *J Trauma Acute Care Surg.* 2015;79:121-9.
13. Kala KJ, Prashob PKJ, Chandramohanakumar N. Humic substances as a potent biomaterials for therapeutic and drug delivery system – a review. *Int J App Pharm.* 2019;11(3):1-4.
14. Aykac A, Becer E, Okcanoglu TB, Guvenir M, Suer K, Vatansver S. The cytotoxic effects of humic acid on human breast cancer cells. *Proceedings.* 2018;2:1565.
15. Jurcsik I. Possibilities of applying humic acids in medicine (wound healing and cancer therapy). In: N. Senesi (ed). *Humic Substances in the Global Environment.* T.M. Milano. Amsterdam; London: Elsevier; 1994:1331-6.
16. Bernacchi F, Ponzanelli J, Minunni M, Falezza A, Loprieno N, Barale R. In vivo cytogenetic effects of natural humic acid. *Mutagenesis.* 1996;11(5):467-9.
17. Çalıřır M, Akpınar A, Talmaç AC, Alpan AL, Göze OF. Humic acid enhances wound healing in the rat palate. *Evid Based Complementary Altern Med.* 2018;1783513:6.
18. Ji Y, Zhang A, Chen X, Che X, Zhou K, Wang Z. Sodium humate accelerates cutaneous wound healing by activating TGF- β /Smads signaling pathway in rats. *Acta Pharm Sin B.* 2016;6(2):132-40.
19. Van Rensburg CEJ, Naude PJW. Potassium humate inhibits the production of inflammatory cytokines and complement activation in vitro. *Inflammation.* 2009;32 (4):270-6.
20. Peña-Méndez EM, Havel J, Patočka J. Humic substances – compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine. *J Appl Biomed.* 2005;3: 13-24.
21. Klöcking R, Sprössig M, Wutzler P, Thiel K-D, Helbig B. Antiviral wirksame Huminsäuren und huminsäureähnliche Polymere. *Zeitschrift für Physiotherapie.* 1983;33:95-101.
22. Klöcking HP, Dunkelberg H, Klöcking R. Substances of the humic acid type prevent U 937 cells from heat-induced arachidonic acid release. In: W. E. Müller (ed). *Modern Aspects in Monitoring of Environmental Pollution in the Sea.* Erfurt: Akademie gemeinnütziger Wissenschaften; 1997:156-8.
23. Klöcking R, Helbig B. The antiviral potency of humic substances. Humic Substances – Linking Structure to Functions. *Proceedings of the 13th International Humic Substances Society.* Karlsruhe; 2006: 397-400.
24. Strus O, Polovko NP. The influence of „Saprogel” in the wound healing process on rats with a full-thickness wound model. *Čes Slov Farm.* 2020;69:75-82.
25. *European Pharmacopoeia* 10th Edition, Volume I. Council of Europe. Strasbourg Cedex, France; 2019:4370.
26. *State Pharmacopoeia of Ukraine.* 1st Edition. Tom 4. SC “Scientific and Expert Pharmacopoeial Center”. Kharkiv, Ukraine; 2011:536.
27. SSTU 7083: 2009. *Organic and organo-mineral fertilizers. Methods for determination of humic acids.* Kyiv; 2011:8.
28. Lichtenthaler HK, Wellburn AR. Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem Soc Trans.* 1983;11(5):591-2.
29. Gomes de Melo BA, Motta FL, Santana MHA. Humic acids: Structural properties and multiple functionalities for novel technological developments. *Mater Sci Eng.* 2016;62:967-74.
30. Klućáková M. Characterization of pH-fractionated humic acids with respect to their dissociation behavior. *Environ Sci Pollut Res.* 2016;23:7722-31.
31. Tarasevich YI, Tryfonova MY, Dolenko SA, Aksenenko EV. Adsorption-based approach to determine the size and mass of humic acids molecules. *Adsorpt Sci Technol.* 2016;34:125-33.
32. Piccolo A. The supramolecular structure of humic substances. *Soil Sci.* 2001;166:810-32.
33. Klućáková M, V’ežníková, K. Micro-organization of humic acids in aqueous solutions. *J Mol Struct.* 2017;1144:33-40.
34. Fuentes M, Baigorri R, González-Gaitano G, García-Mina JM. New methodology to assess the quantity and quality of humic substances in organic materials and commercial products for agriculture. *JSS.* 2018;18(4):1389-99.
35. Lamar R, Olk DC, Mayhew L, Bloom PR. A new standardized method for quantification of humic and fulvic acids in humic ores and commercial products. *J AOAC Int.* 2014;97(3):721-30.
36. Jarukas L, Ivanauskas L, Kasparaviciene G, Baranauskaite J, Marksa M, Bernatoniene J. Determination of organic compounds, fulvic acid, humic acid, and humin in peat and spropel alkaline extracts. *Molecules.* 2021;26:2995.