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РАЗРАБОТКА ПЕССАРИЕВ КОМБИНИРОВАННОГО СОСТАВА ДЛЯ ЛЕЧЕНИЯ ГЕНИТАЛЬНОГО ГЕРПЕСА

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DEVELOPMENT OF COMBINED COMPOSITION PESSARIES FOR GENITAL HERPES TREATMENT

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На сегодняшний день проблема лечения и создания эффективных лекарственных препаратов для лечения генитального герпеса (ГГ) является актуальной для современной гинекологии и фармации.

***Цель:** разработка комбинированного состава пессариев с ацикловиром и эфирными маслами чайного дерева и чабреца и подтверждение их фармакологической активности: противовирусной и антибактериальной.*

***Методы.** Анализ ассортимента препаратов для лечения ГГ проведенный на основании материалов Государственного реестра лекарственных средств Украины и Компендиума. Определение распадаемости и однородности пессариев проводили в соответствии с ГФУ 2.0. Концентрацию ацикловира в растворах в процессе высвобождения определяли методом абсорбционной спектрофотометрии при длине волны (265 ± 2) нм. Исследование противовирусного действия ацикловира, эфирных масел чайного дерева и чабреца, а также пессариев с ацикловиром и эфирными маслами проводили на базе Национальной академии медицинских наук Украины, в ГУ «Институт эпидемиологии и инфекционных болезней им. Л. В. Громашевского» (г. Киев), в*

лаборатории экспериментальной химиотерапии вирусных инфекций. Изучение антибактериальных свойств исследуемых образцов проводили на базе ГУ «Институт микробиологии и иммунологии им. И. И. Мечникова».

Результаты. В лечении ГГ ацикловир является одним из наиболее назначаемых и безопасным противовирусным препаратом. В результате проведенных исследований доказано, что разрабатываемый препарат в форме пессариев с ацикловиром и эфирными маслами чайного дерева и чабреца является активным ингибитором репродукции вируса герпеса 2 типа и эффективным профилактическим препаратом на экспериментальной модели герпетической инфекции гениталиев у морских свинок.

Выводы. Комбинированные лекарственные препараты с ацикловиром и растительными субстанциями, обладающие противовирусной активностью, на рынке Украины вообще отсутствуют. Учитывая, что пессарии имеют ряд преимуществ перед другими лекарственными формами, создание новых лекарственных препаратов с ацикловиром и эфирными маслами чайного дерева и чабреца является перспективным направлением для современной медицины и фармации

Ключевые слова: состав, пессарии, ацикловир, эфирные масла, герпесвирусная инфекция, генитальная форма

Recently, the problem of therapy, as well as creation of effective remedies applied for genital herpes (GH) treatment is relevant for modern gynecology and pharmacy.

Aim. *Development of the combined composition pessaries containing Acyclovir and Tea tree and Thyme essential oils, together with confirmation of their pharmacological activity: antiviral and antibacterial.*

Methods. *The analysis of the assortment of drugs for GH treatment was carried out on the basis of the materials of the State Register of Medicinal Products of Ukraine and the Compendium. The pessaries disintegration and homogeneity was performed in accordance with the SPhU 2.0 requirements. Absorption spectroscopy*

method at wavelength (265±2) nm was used for determination of Acyclovir concentration in the solutions during release. Study of the antiviral activity of both Acyclovir, and Tea tree and Thyme essential oils, as well as the pessaries containing Acyclovir and the essential oils, was carried out on the base of the National Academy of Medical Sciences of Ukraine, in the State Institution «Institute of Epidemiology and Infectious Diseases named after L. V. Gromashevsky» (Kiev), in the laboratory of viral infections experimental chemotherapy. The analysis of the antibacterial properties of the studied samples was carried out on the base of the State Institution «Mechnikov Institute of Microbiology and Immunology».

Results. *In GH therapy, Acyclovir is the most prescribed and safe anti-viral drug. In result of research it was proved that the developed remedy in pessaries form containing Acyclovir, and Tea tree and Thyme essential oils inhibits type 2 herpevirus reproduction and is effective preventive drug on the experimental model of genital herpes infection in guinea pigs.*

Conclusion. *Combined remedies containing Acyclovir and herbal substances and showing anti-viral activity are absent on Ukrainian market. Considering the fact that pessaries have a number of advantages over the other dosage forms, development of the new remedies containing Acyclovir and Tea tree and Thyme essential oils is a promising direction for modern medicine and pharmacy*

Keywords: *composition, pessaries, Acyclovir, essential oils, herpeviral infection, Genital form*

1. Introduction

Among sexually transmitted infections, Genital herpes (GH) is the second most common disease after trichomoniasis. According to the statistics data, only in the USA, every 4-6 people of this country are carriers of this virus [1]. Thereby, in the world the active search for anti-herpetic drugs is being carried out; it led to the creation of the anomalous nucleosides group – Acyclovir and its derivatives [2].

2. Formulation of the problem in a general way, the relevance of the

theme and its connection with important scientific and practical issues

Acyclovir is one of the most frequently prescribed antiviral drugs (AVD), the "gold" standard of GH treatment. Among the existing synthetic AVD, it is the safest, but at the same time its bioavailability is rather low (about 20%) [3]. It is well-known that the use of Acyclovir combination with other drugs is one of the ways to increase its bioavailability and efficiency. The best results are shown when using drugs with different mechanisms of action.

3. Analysis of recent studies and publications in which a solution of the problem and which draws on the author

On the modern Ukrainian pharmaceutical market, anti-herpetic remedies are mainly represented by synthetic mono composition drugs, which together with high therapeutic activity have an impressive range of adverse effects [4].

Anti-herpetic therapy includes both the use of topical drugs (to stop the spread of infection to other tissues), as well as systemic drugs that help to eradicate the viral infection after absorption into the bloodstream [5].

The modern assortment of the combined anti-herpetic drugs is extremely limited, and there are no remedies for GH local treatment. Therefore, development of new combined drugs on the basis of Acyclovir and herbal compounds applied for the genital form of herpetic infection local treatment is expedient to create new combination drugs based on acyclovir and plant components for topical treatment of the genital form of herpetic infection [6].

Among herbal substances, there are some of them with proved antiviral activity, but they are not referred to officinal drugs in the given pharmacotherapeutic group. Tea tree and Thyme essential oils, having wide spectrum of pharmacological activity (bactericidal, antiviral, immunostimulant and anti-inflammatory) are the most promising among herbal substances [7–9].

4. Allocation of unsolved parts of the general problem, which is dedicated to the article

Scientific researchers from different countries have shown that the combination of essential oils with synthetic substances in a single dosage form increases their antiviral and antibacterial activity [10]. Besides, essential oils components can serve as penetrators, due to their ability to accelerate the active substances penetration into the skin by changing the lipids fluidity in the stratum corneum.

This approach, that combines potential therapeutic properties of chemotherapeutic agents and natural substances, is promising in drug technology [11, 12]. In addition, replenishment of Ukrainian pharmaceutical market with new medicinal products will change the market structure in favor of Ukraine.

5. Formulation of goals (tasks) of Article

Therefore, development of the combined remedy containing Acyclovir and Tea tree and Thyme essential oils in the form of pessaries for local GH treatment is relevant for modern pharmacy.

6. Statement of the basic material of the study (methods and objects) with the justification of the results

Materials and methods. Acyclovir substance «Guimica Sintetica, S.A.», Spain was used for the pessaries composition development.

It is known, when creating drugs for GH local therapy, firstly it is necessary to select a suppository base, since it is a carrier and is in constant contact, both with active substances and with vaginal mucosa. At development of the pessaries containing Acyclovir and the essential oils, in accordance with the medical and biological GH treatment requirements, fatty bases for suppositories (Witepsol, Hard fat, etc.) were used. Well-known hydrophilic bases, namely Macrogols having high osmotic activity, are unacceptable in case of the given disease due to the mentioned above requirements.

To substantiate the suppository base choice, the samples of pessaries containing Acyclovir and Tea tree and Thyme essential oils on fatty bases were prepared under laboratory conditions. Disintegration analysis of the pessaries was

carried out according to the SPhU 2.0 requirements. Acyclovir concentration in the solutions was determined by the absorption spectroscopy method at a wavelength of (265 ± 2) nm [13].

At the excipients selection, namely the emulsifier, the following criteria were used: the emulsifier should be natural product and it should be included into the list of surfactants allowed for use in pharmacy practice. Lecithin, the natural product which is included in the remedies for intravenous and intramuscular injections, infusions, ointments, creams, suppository bases, was proposed as an emulsifier. Besides, lecithin composition includes phosphatidylcholines and other substances (glycerin, di-, triglycerides) that activate Acyclovir transport through mucous membranes [14].

To substantiate the rational concentration of Acyclovir and Tea tree and Thyme essential oils in the developed pessaries, the experimental samples of the remedy were obtained for the further study of their specific activity.

Study of the antiviral activity of both Acyclovir, and Tea tree and Thyme essential oils, as well as the pessaries containing Acyclovir and the essential oils, was carried out on the base of the National Academy of Medical Sciences of Ukraine, in the State Institution «Institute of Epidemiology and Infectious Diseases named after L. V. Gromashevsky» (Kiev), in the laboratory of viral infections experimental chemotherapy. The cells culture and the virus from the Museum of viral strains of Ivanovsky Institute of Virology (RAMS, Moscow) were used. 25 non-linear male guinea pigs (weighing 250-300 g) from the «Glevaha» kennel, which were kept under the standard vivarium conditions, were used [15, 16].

The analysis of the antibacterial properties of the studied samples was carried out on the base of the State Institution «Mechnikov Institute of Microbiology and Immunology». According to the WHO requirements, test-strains of microorganisms: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Basillus subtilis* ATCC 6633, *Proteus vulgaris* ATCC 4636, *Candida albicans* ATCC 885/653 were used to estimate the drugs activity.

Densi-La-Meter (PLIVA-Lachema, Czech Republic; wavelength 540 nm)

equipment was used for microbial microorganisms' suspension preparation. The suspension was prepared according to the manual, which is attached to the device and the information letter about health care system innovation № 163-2005 «Microbial suspension preparation standardization». The strains synchronization was carried out under low temperature (4° C). The microbial load was 10^7 microbial cells per 1 ml of medium and was set using the McFarland standard. 18-24 hours microorganisms' culture was used for research. Mueller-Hinton agar («HIMedia Laboratories Pvt. Ltd India», shelf life till Sept. 2015) was used. Sabouraud agar («HIMedia Laboratories Pvt. Ltd India», shelf life till Sept. 2015) was used for *Candida albicans*. The «wells» method was carried out for the drug diffusion into agar.

Results and discussion. The results of Acyclovir quantitative determination in the process of its release from the samples of pessaries with hydrophobic bases are shown in Figure 1 [17, 18].

As it is shown in Figure 1, about 70-72% of Acyclovir released into the solution in 30 minutes from the pessaries on Hard fat and Suppocire bases; about 98% of Acyclovir released from the pessaries on Witepsol base. Also, Witepsol-based pessaries showed the best disintegration results.

Therefore, in result of the research, produced in Germany Witepsol base was chosen as a suppository base for pessaries containing Acyclovir and the essential oils.

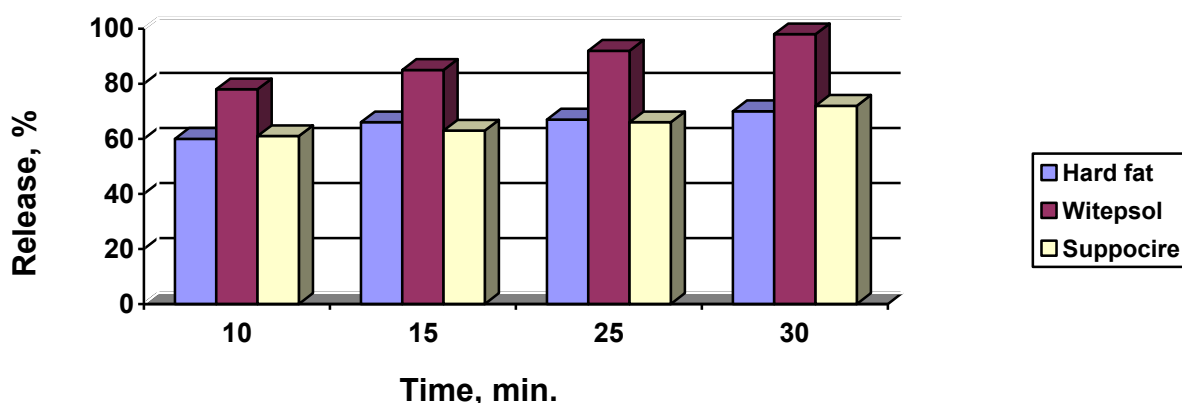


Fig. 1. The influence of suppository base on Acyclovir release from the pessaries

In the laboratory of experimental chemotherapy of viral infections, for

determination of the antiviral activity of Acyclovir, Tea tree and Thyme essential oils, cytotoxic concentration (CC_{50}), effective dose (ED_{50}), maximum tolerated dose (MTD), minimum effective concentration (MEC) and chemotherapeutic index (HTI) of the mentioned substances were analyzed. In result of the study, MTD (the highest dose that does not cause cell death) for the substances was: Tea tree essential oil (1:1000) – 0.1 %, Thyme essential oil (1:400) – 0.25 %, Acyclovir (1:800) – 0.125 %.

Results of the MEC (the minimum amount of a drug that delays the virus-specific cytopathogenic action (CPA) development by 50%). To determine the effective dose (ED_{50}), test-virus in the dose of $TCD_{50} / 0.1$ ml (infectious titer of CPA in cell culture is 6.0-9.0 lg) was put into *Vero* cells culture and incubated for 60 minutes at 37° C. After the virus absorption, its residues were removed, the cells were washed with a nutrient medium, and the remedies in different concentrations were put into a supporting medium (RPMI-1640 with 2 % fetal serum) [15]. Results of the study are shown in Table 1.

Table 1

Minimum effective concentration of the studied samples of the substances on the model of herpetic infection

Samples	Infectious titer in lg ED_{50} at dilutions of the remedies						MEC
	50000	100000	200000	400000	800000	VC	
Tea tree essential oil	0.0	2.0	2.0	2.0	2.0	2.0	>1:50000 0.002 %
Thyme essential oil	1.5	1.0	1.5	1.0	2.0	2.0	32000 0.0031 %
Acyclovir	0.0	0.0	0.0	0.0	2.0	2.0	>1:400000 >0.25 μ g/ml

Besides, a chemotherapeutic index of the substances (IS) was carried out by

MTD and MEC correlation. Results of the study are shown in Table 2.

Table 2

Chemotherapeutic index of the samples of the studied substances

Samples	MTD	MEC	Chemotherapeutic index (IS)
Tea tree essential oil	0.1 %	> 0.002 %	50.0
Thyme essential oil	0.25 %	0.0031 %	80.0
Acyclovir	125 µg/ml	0,25 µg/ml	500.0

In vitro analysis of the antiviral activity of the pessaries containing Acyclovir and the essential oils for genital herpetic infection therapy was carried out using daily transplantable cell culture, which formed a continuous layer on the substrate. The cells were grown in the bottles (Nunclon, Surface, Denmark) in RPMI-1640 medium with 10% fetal serum addition at a temperature of 37° C in a thermostat with carbon dioxide (CO₂).

In accordance to the results of Herpes virus type 2 infectious titre determination, all the studied samples effectively inhibited the reproduction of the herpes virus (Tables 3, 4).

Table 3

Antiviral activity of the pessaries against Herpes virus (Preventive administration mode)

Dilution of the drug (pessaries)	Pessaries containing Acyclovir and the essential oils, their influence on Herpes virus infectious titre in lg ED ₅₀			
	Pessary №1	Pessary №2	Pessary №3	Acyclovir

1:4000	0	0	0	3.0
1:8000	0.5	0	0	4.0
1:16000	2.5	0	1.0	4.0
1:32000	2.5	1.5	1.5	3.0
1:64000	2.0	2.5	3.0	5.0
Virus control	9.0	9.0	9.0	9.0

Table 4

Antiviral activity of the pessaries against Herpes virus (Therapeutic administration mode)

Dilution of the drug (pessaries)	Pessaries containing Acyclovir and the essential oils, their influence on Herpes virus infectious titre in lg ED ₅₀			
	Pessary №1	Pessary №2	Pessary №3	Acyclovir
1:4000	4.0	3.0	4.0	2.0
1:8000	4.0	3.0	3.0	3.0
1:16000	2.0	3.0	2.0	3.0
1:32000	3.0	3.0	3.0	1.0
1:64000	1.0	4.0	3.0	3.0
Virus control	9.0	9.0	9.0	9.0

According to the results of the study, all substances of the pessaries were effective inhibitors of Herpes virus type 2 reproduction in preventive and therapeutic experimental schemes. ED₅₀ for the pessary № 1 - the dilution was 1: 64000, while Herpes virus type 2 reproduction inhibition was 6.0 to 9.0 lg ID₅₀.

Anti-herpetic activity study of the pessaries containing Acyclovir and the essential oils, and the pessaries' suppository mass for the genital form of herpes viral infection treatment was carried out *in vivo* on the model of genital herpes in guinea pigs (Marennikova S.S. model) [15]. The results of research are presented in Table. 5.

Table 5

Efficiency of the pessaries on the model of genital herpes in guinea pigs

Influence	Duration of the disease (days)	P	OIS, points	TEI %
Herpes virus	15.0		51.0	
The pessaries containing Acyclovir and the essential oils (therapeutic administration mode)	9.0	< 0.05	22.0	56.0
The suppository mass containing Acyclovir and the essential oils (therapeutic administration mode)	5.0	< 0.05	12.0	76.4
The pessaries containing Acyclovir and the essential oils + the suppository mass (therapeutic administration mode)	5.0	< 0.05	18.0	64.7
The pessaries + HSV (preventive administration)	0	< 0.05	0	100.0

mode)				
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Notes: OIS – overall index of symptoms;

TEI –therapeutic effect index;

HSV – Herpes simplex virus.

The studies have shown that the single use of the pessaries containing Acyclovir and the essential oils within 5 days reduces the severity of symptoms to 22.0 points, which corresponds to therapeutic effect index (therapeutic effect) at 56.0% and reliably shortens the disease duration.

The use of the suppository mass containing Acyclovir and the essential oils also decreases the symptoms severity to 12.0 points, and therapeutic effect is 76.4%. The disease duration in animals was 5 days, which is statistically reliable for all parameters and indicates the efficiency of this dosage form of the remedy. Thus, the basis (Witepsol) and the emulsifier (Lecithin) in the suppository mass and pessaries composition cannot significantly affect OIS, but increase the therapeutic action index. At the combined therapeutic scheme using both the pessaries and the suppository mass, the disease duration was 5 days, and the therapeutic effect index was 64.7%.

In the preventive treatment scheme – administration of the pessaries 24 hours before Herpes virus infection – the disease did not occur during the whole study period (10 days).

In result of the studies it was proved that the developed remedy in the form of pessaries containing Acyclovir and Tea tree and Thyme essential oils inhibits type 2 herpevirus reproduction and is effective preventive drug on the experimental model of genital herpes infection in guinea pigs.

The additional study of antibacterial activity was carried out using the samples of pessaries containing Acyclovir and pessaries containing Acyclovir and Tea tree and Thyme essential oils in ratio (1:1): 2.5: 5.0 and 7.5%. Earlier studies carried out by the authors showed that Tea tree essential oil shows its antimicrobial activity even at concentration of 2%. To substantiate the essential oils rational concentration (Tea tree and Thyme essential oils), microbiological research was carried out. For the oils

antibacterial sensitivity determination, the samples of pessaries with 2.5%, 5% and 7.5% essential oils concentrations in the ratio (1: 1) were obtained; then they were tested on the strains of the cultures shown in Table. 6.

Table 6

Antimicrobial activity of the samples of pessaries containing Tea tree and Thyme essential oils in ratio (1:1)

Essential oils concentration, the sum, %	St. aureus ATCC 25923	E. coli ATCC 25922	Ps.aerugi nosa ATCC 27853	Basillus subtilis ATCC 6633	Proteus vulgaris ATCC 4636	C.albicans ATCC 885/653
	Diameter of growth retardation, mm					
2.5	18.7±1.8	16.8±2.3	growth	12.0 ±1.5	17.2±2.5	14.2 ± 1.6
5.0	22.7±1.6	18.2±1.2	growth	13.0 ±1.4	20.2±1.2	14.7 ± 1.4
7.5	21.8±1.7	18.5±2.3	growth	13.1 ±1.3	22.2±1.8	14.9 ± 1.7

Notes: n=5; P=95 %

The given samples of pessaries containing essential oils show pronounced antimicrobial activity, but the maximum diameters of the microorganisms' growth retardation zones were observed for the samples of pessaries containing the essential oils concentration 5% in the ratio (1: 1). The further essential oils concentration rise does not lead to a significant increase in antimicrobial activity. The optimal concentration of essential oils in pessaries is 5% concentration (1: 1 ratio); they can effectively inhibit the growth of such pathogens as Staphylococcus, Pneumococcus, Gonococcus, E. coli, as well as some pathogenic fungi of C. Albicans genus.

During antimicrobial activity studying for № 1 test sample (pessaries containing Acyclovir) and № 2 test sample (pessaries containing Acyclovir and Tea tree and

Thyme essential oils) it was found that test sample № 1 does not show antibacterial properties. Test sample № 2 has antibacterial properties since it shows the maximum diameters of the microorganisms growth retardation zones (Table 7).

Table 7

Antimicrobial activity of the samples of pessaries containing Acyclovir and pessaries containing Acyclovir and Tea tree and Thyme essential oils in ratio (1:1)

Pessaries	St. aureus ATCC 25923	E. coli ATCC 25922	Ps.aerugi nosa ATCC 27853	Basillus subtilis ATCC 6633	Proteus vulgaris ATCC 4636	C.albicans ATCC 885/653
	Diameter of growth retardation, mm					
Test sample № 1 Pessaries containing Acyclovir (5 %)	15.6± 1.7	14.3±. 1.7	growth	growth	16.4±1.7	13.4±1.9
Test sample №2 Pessaries containing Acyclovir (5 %) and the essential oils (5 %)	22.9±1.6	18.2±1.5	growth	13.0±1.3	20.8±1.9	14.7 ± 1.4

Notes: $n=5$; $P=95\%$

7. Conclusion

On the basis of pharmaco-technological, physico-chemical, and pharmacological studies, the composition of combined composition pessaries containing Acyclovir and Tea tree and Thyme essential oils was substantiated. The novelty of the research is protected by the Patent of Ukraine № 107464.

Anti-herpetic activity study for the substances (Acyclovir, Tea tree essential oil, and Thyme essential oil) and pessaries containing Acyclovir and the essential oils have shown that they can effectively inhibit Herpes virus type 2 reproduction.

Results of the pessaries antibacterial activity research point to antibacterial effect of Tea tree and Thyme essential oils in concentration 5%.

It was found that the original pessaries containing Acyclovir and the essential oils are effective preventive and therapeutic remedy, which was confirmed by the experimental model of herpetic infection in guinea pigs.

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