Extract of the birch bark suppresses reproduction of the hepatitis C virus (HCV) in cells cultures and in infected mice

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The viral hepatitis C is one of the most dangerous and wide-spread infectious diseases of viral nature. More than 3% of the world's population is infected with HCV, which was discovered in 1989. Since its discovery the active studies of that type of hepatitis, the main cause of development of chronic diffuse liver diseases, cirrhosis and primary liver cancer, have begun. The distinctive feature of the hepatitis C virus is its ability to long persistence in organism which makes this infection highly chronicle (50-80% of cases). Both the HCV persistence phenomenon and high mutation level, resulting in development of some immunologically different types which enable the virus to avoid immune surveillance, are interdependent processes in infected organism. Taking into consideration the low therapeutic efficacy of existing drugs (α-interferon and α-interferon in combination with ribavirin) and high hepatitis C virus sickness rate there is need in research and creation of new preparations for therapy of the viral hepatitis C. Besides, the research of new remedies is important because of expensiveness of the used drugs and side effects such as development of anemia, neutropenia, leukopenia, and some dyspeptic effects.

BIRCH BARK EXTRACT CONTAINING BETULIN

The present work aims at evaluating antiviral activity of a new product of Birch World Ltd., a birch bark extract, containing betulinol (betulin 72%), by the sample of an infection caused by hepatitis C virus (HCV) in infected mice.

The task of the present research is to examine the birch bark extract as antiviral preparation for treatment of hepatitis C. The natural origin of the active substances of the birch bark extract solves a number of negative problems arising when taking chemicals. The ground acting substance of the extract is triterpenoid alcohol betulinol (betulin).

Using sublimation in 1788 Russian academician T.E. Lovits, chemist and pharmacist, extracted from birch bark a white substance named afterwards Betulin. Its molecular formula ($C_{30}H_{50}O_2$) was determined in the 1820's, and structural formula in the beginning of the 1950's. Betulin is a pentacyclic triterpenic alcohol in a range of lupan.

Betulin is a protective agent of the birch tree and it is basically contained in outer layer of the bark, giving it the white color. Many researches are devoted to hepatoprotective activity of betulin. The applicability of betulin derivatives on hepatitis was studied at experimental hepatitis of non-virus origin caused by CCl_4, tetracycline and acetone [Flechter, O.B. "Synthesis of triterpenoids esters of a range of lupan and their hepatoprotective activity", *Bioorganic Chemistry* **2000**, *26*, 3, 215-223].

The results of the researches proved antiviral activities of the birch bark extract. This effect was shown at experimental infection caused by hepatitis C virus of a human (genotype 1b) in cells cultures and at experimental infection caused by HCV in mice.

EXPERIMENTAL HCV INFECTIONS ENABLE SCREENING OF ANTIVIRAL DRUGS

Screening of new drugs that can be active against hepatitis C virus became

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Table I – Antiviral activity of the birch bark extract containing betulin 98% against infection caused by the Hepatitis C virus in pig embryo's kidney cells cultures

Extract's dilutions	HCV titres in medium tests on the 4-th day after the pig embryo's kidney cells cultures were infected ($lgTCD_{50}$)		
	Extract right after infection	In 24 hours after infection	24 hours before infection
1:10	3.3	5.5	2.5
1:20	1.3	5.5	3.4
1:40	5.0	4.6	3.2
1:80	4.8	5.0	3.3
Without extract (control)	5.5	5.5	5.4

a reality due to the received experimental infections caused by HCV in cells cultures of laboratory mice. As a result we received acute and chronic infection caused by HCV in cells cultures. There were isolated and identified high-yield variations of HCV for cells cultures of a various origin. The simulation of infection caused by various genotypes of HCV was shown to be possible not only in cells cultures, but also in laboratory animal (mice). There was received acute and chronic infection caused by HCV in cells cultures, susceptible to reproduction of flavi-viruses. Thus, it became possible to evaluate antiviral activity of drugs at experimental infection caused by HCV in cells cultures. Isolated types of HCV showed ability to cause chronic infection in laboratory mice and rabbits that gave chance to use those animals in evaluation of antiviral activity of drugs.

EVALUATION OF ANTIVIRAL ACTIVITY OF THE BIRCH BARK EXTRACT CONTAINING BETULIN 98% IN CELLS CULTURES INFECTED WITH HCV

A cytopathogenic strain of the hepatitis C virus (HCV), genotype 1b and cells cultures of pig embryo's kidney were used in this research. The birch bark extract containing betulin 98% (produced by Birch World Ltd.), as suspension in physiological solution with 1% of Twin 80, was diluted in nutrient medium 199. 30 of each extract dilutions were placed in holes containing cells cultures of pig embryo's kidney. In 4-6 days the cytotoxic properties of the extract were determined. The extracts in dilutions 1:10 and more showed no cytotoxic properties that made possible to use a number of concentrations of the extracts for studying of antiviral activity. For that purpose the extract in various concentrations was introduced into cells cultures of pig embryo's kidney 24 hours before the infection with HCV, during infection and in 24 hours after introduction of the virus into cells. The tests of the nutrient medium were selected and infectious activity of the virus in cells cultures of pig embryo's kidney was evaluated on the 4th day after the cells were infected. The results were considered on the 7th day when there were observed the maximal virus induced cells deaths in cells cultures infected with HCV, and control not infected pig embryo's kidney cells cultures remained healthy.

From Table I you can see that highest activity of the extract was noticed in introduction into cells cultures of pig embryo's kidney in dilution 1:20 right after the cells were infected with HCV. In this case the HCV titers in medium tests, selected on the 4th day after being infected with the virus, reduced by 4,2 lg. Being introduced in 24 hours after the cells had been infected with the virus, the extract did not result in decrease of infectious properties of HCV. At the same time the extract did not stimulate virus producing activity of cells. The most stable antiviral properties of the extract were noticed at prophylactic application, i.e. 24 hours before the cells were infected with the virus.

In this case the titres of the virus in tests of medium, selected from infected cells cultures of pig embryo's kidney, reduced by 2,1 2,9 $lgTCD_{50}$ under various dilutions of the extract.

EVALUATION OF ANTIVIRAL ACTIVITY OF THE BIRCH BARK EXTRACT CONTAINING BETULIN 72% IN MICE INFECTED WITH HCV

White outbred mice intraperitoneally infected with HCV were used in the tests. In 10 days after that the extract was perorally introduced into mice in doses 50, 100 and 150 mg/kg within 4 days. In 3 days after the last extract introduction the blood, liver and brain of mice were examined for infectious hepatitis C virus and its antigens. As you can see from Table II, the mice received the extract in dose 50 mg/kg, showed apparent tendency to HCV hemagglutinin titers reduction, and HCV infectious titers in blood serum reduced almost in 1000 times by 2,6 lg (average data). Significant decrease of antigenic and infectious activities of HCV in blood serum was noticed in the group of mice received the extract in dose 100 mg/kg daily. In this case HCV infectious activity titers decreased more than 1000 times (by 3,4 lg) and more than 2 times the antigenic activity of HCV in blood serum tests of the mice reduced. To a lesser degree the extract showed activity using the dose 150 mg/kg. Antigenic activity of the virus decreased, and almost in 100 times concentration of the virus (by 1,8 lg) was reduced.

Some other results proving efficiency of the birch bark extract were received during studies of the liver tissues in all groups of mice (Table II). In particular, maximum decrease of HCV antigens and its infectious activity was noticed while using single doses 50 and 100 mg/kg at 4-times introduction of the extract. HCV antigenic activity reduced in 2 and more times and up to 100 times HCV infectious activity decreased in the liver tissues. The liver size of HCV infected mice, received the extract, was also reduced.

The most HCV titers (up to 7.0 lg TCD_{50} / 20 μl) were observed in brain tissues of HCV infected mice. However, the mice received the extract in doses 50 and 100 mg/kg showed 100-times and more decrease of titers of HCV infectious activity. At the same time the HCV antigen reduction was noticed in all groups of mice received the extract. The group of mice received the extract

Table II – Antiviral activity of the birch bark extract, containing betulin, in HCV infected mice on the 17th day after being infected and on the 3rd day after the 4th introduction of the extract

Group of mice that received the extract	Indices of HCV infection					
	in blood serum tests		in liver homogenates		in brain tissues	
	HAT [1]	$lgTCD_{50}$/20μl	HAT [1]	$lgTCD_{50}$/20μl	HAT [1]	$lgTCD_{50}$/20μl
50.0 mg/kg	1:288	3.0 ± 0.2	1: 290	2.0 ± 0.3	1:220	4.7 ± 0.1
100.0 mg/kg	1:192	2.2 ± 0.2	1: 400	1.7 ± 0.3	1:180	4.2 ± 0
150.0 mg/kg	1:256	3.7 ± 0.1	1: 400	4.3 ± 0.3	1:280	7.1 ± 0.1
No preparation	1:468	5.6 ± 0.2	1: 800	3.7 ± 0.6	1:480	6.75 ± 0.5
Not infected mice	1:25	0	1: 50	0	1:50	0

1. HAT – hemagglutination test

in dose 150 mg/kg, showed the tendency towards increase of HCV infectious activity titers in cerebrum.

Thus the results received in studies of the mice organs and tissues on the 17th day after being HCV infected and on the 3rd day after the 4-times application of the birch bark extract, proved the high antiviral activity of the extract. Peroral administration of the birch bark extract to mice resulted in reduction of infectious and antigenic HCV activities in all the examined organs and tissues, including brain of infected mice.

In studies of prophylactic activity of the extract on mice the preparation on physiological solution containing 1% of Twin80 in doses 50, 100 and 150 mg/kg was perorally introduced into different groups of mice within 4 days. In 2 days after the 4th introduction of the extract, the mice were intraperitoneally infected with hepatitis C virus. Three groups of mice were taken as control groups – mice intraperitoneally infected with hepatitis C virus but were not given the extract; not infected mice; not infected mice that received the extract in dose 150 mg/kg within 4 days. In a week after being infected the mice were dissected. Liver, brain, and blood were extracted.

Table III – Results of studies obtained while prophylactic application of the birch bark extract containing betulin in mice infected with the hepatitis Ñ virus

Group of mice that received the extract	Indices of HCV infection					
	in blood serum tests		in liver homogenates		in brain tissues	
	HAT [1]	$lgTCD_{50}/20\mu l$	HAT [1]	$lgTCD_{50}/20\mu l$	HAT [1]	$lgTCD_{50}/20\mu l$
50.0 mg/kg	1:6.4	4.0 ± 0.2	1:220	3.0 ± 0.3	1:120	4.2 ± 0.1
100.0 mg/kg	1:8	4.5 ± 0.1	1:450	3.7 ± 0.3	1:280	4.8 ± 0
150.0 mg/kg	1:58	5.5 ± 0.1	1:600	4.5 ± 0.3	1:420	7.2 ± 0.1
No preparation	1:109	6.4 ± 0.2	1:850	4.6 ± 0.6	1:480	6.15 ± 0.5
Not infected mice	0	0	1:50	0	1:50	0

1. HAT – hemagglutination test

From Table III you can see that prophylactic application of the extract, as a rule, resulted in decrease of the infection caused by HCV in infected mice. In particular, studies of blood serum tests showed that the extract in dose 50 mg/kg resulted in considerable (18-times) decrease of hemagglutinating activity of the virus, and infectious HCV titers in blood serum of mice reduced by 2,4 lg. Application of the extract in dose 100 mg/kg resulted in 13-times decrease of the HCV antigens in blood, and infectious titers of HCV reduced almost in 2 times.

The similar results were obtained while analyzing liver tissues taken from different groups of mice. Studying of brain tissues of HCV infected mice showed that prophylactic application of the extract resulted almost in 100-times decrease of infectious activity of HCV and 4-times decrease of HCV antigens titers. To a lesser degree the extract acted at the daily dose 100 mg/kg. The results were considered as negative at prophylactic application of the extract in dose 150 mg/kg.

Thus, the received results prove that the birch bark extract containing betulin is able to suppress reproduction of hepatitis C virus both in cells cultures infected with HCV and in laboratory animals (mice) at therapeutic and prophylactic application of the extract. These results offer the challenge to apply the extract in clinical trials on HCV infected persons.