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Selection and Study of Influence of Preparation "CMC" on the Process of Cooking of Blood

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Abstract

A study was carried out to identify a new structural fragment of coumarins from moldy clover (CMC) and to study the anticoagulant and toxicological characteristics.

The quantitative and qualitative composition of the coumarin mixture was studied using spectrophotometry, fluorescence analysis and high-performance liquid chromatography. Coumarins and coumarinic acids were identified by absorption spectra and retention time in comparison with standard samples. The specificity of the method of direct spectrophotometry was assessed from the absorption spectra of clover grass extracts, the standard solution of coumarin solutions of model coumarin mixtures. The general orientation of the change in the clotting process under the action of drugs was judged by the records of a thromboelastogram performed on a thromboelastography (Tromb-2).

The obtained fluorescence spectra as a function of the concentration of the extract showed a linear dependence of their intensity on the concentration, which made it possible to estimate the quantitative content of coumarins in CMC. The results show that the CMC really refers to the anticoagulants of indirect action, since the maximum CMC effect is manifested after 24 hours and is associated with a decrease in the content of procoagulants. By the effectiveness CMC is similar to the drug curantyl.

Keywords: coumarins, anticoagulants of indirect action, thromboelastography.

1. Introduction

One of the characteristic pharmacological properties of coumarin derivatives is the anticoagulant effect, the mechanism of which is to stop the normal formation of clotting factors-coagulation, which disrupt the synthesis in the liver of clotting factors-prothrombin and proconvertin, suppressing vitamin K-dependent synthesis of biologically active forms of calcium-dependent clotting factors blood II, VII, IX and X, as well as proteins C, S and Z in the liver (Shakhmatova et al., 2014).

At present, the number of isolated natural coumarins significantly exceeds 200 compounds that are found in both the free state and in the form of glycosides (Fedoseeva et al., 2013; Maksyutina et al., 1985). Structural fragments of coumarins have an anticoagulant effect. For example, dicumarol (*3,3'-methylene-bis-4-oxicoumarin*) interferes with blood clotting and causes painful bleeding in cattle caused by consumption of sweet clover (Knunyants, 1990). It was

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found that when feeding laboratory rats and mice with bait containing the extract of the moldy clover *Trifolieae* (EMC), depending on the dose, it caused severe bleeding in the animals for 5-8 days, which served as the basis for obtaining and studying its anticoagulant and toxicological characteristics.

The purpose of this work was to isolate a new structural fragment of coumarins from a moldy clover and study its anticoagulant and toxicological characteristics.

2. Materials and methods

To obtain the extract, clover specimens of the *Trifolieae* species were harvested at the time of flowering. Moldy clover was obtained leaving it in a cellophane bag in a dark place for 10 days. After the mold was formed, the moldy clover was crushed to a particle size of not more than 2 mm and subjected to extraction at a raw material / extractant ratio of 1: 100. The most complete extraction of coumarins (in free form and in the form of glycosides) was achieved when 45- 96 % ethyl alcohol was used both in the cold and during heating. At the same time, the yield of extractive substances was 32-36 % of the initial mass of the moldy clover.

To purify the amount of coumarins from the concomitant substances, the thick extract obtained after distillation of the extractant was treated with chloroform and a mixture of coumarins was recovered. After concentrating the extract, a mixture of coumarins in a crystalline state was obtained.

The quantitative and qualitative composition of the coumarin mixture was studied using spectrophotometry, fluorescence analysis and high-performance liquid chromatography, as described in (Sheluto et al., 2003).

Coumarins and coumarinic acids were identified by absorption spectra and retention time in comparison with standard samples. O-coumaric acid (Sigma, Cat.№. I2, 280-9), coumarin (Sigma, Cat.№.4261), scopolite (Sigma, Cat.№. S2500), umbelliferon (PhytoLab, Cat.№. 80098).

Based on the results of the research, the optimum conditions for preparation samples for the quantitative determination of coumarins in raw materials were determined.

The specificity of the method of direct spectrophotometry was assessed from the absorption spectra of clover grass extracts, the standard solution of coumarin solutions of model coumarin mixtures. The spectra were recorded on a spectrophotometer "SPEKOL 1300"

The experiments were carried out on 24 rats of males, females weighing 170.0 ± 15 g for 6 in each group. The study of the process of blood coagulation was carried out in dynamics after 24 hours and after 5 and 8 days from the moment of a single oral administration of the preparation "CMC" in doses of 0.04 and 0.06 mg/kg and curantyl in a dose of 50 mg/kg. Animal control group received distilled water orally in an equivalent volume.

To investigate the coagulation activity of the drugs, citrate plasma, taken from the blood of a rat prepared on sodium citrate 3.5 %, was used in a ratio of 1:9.

The general orientation of the change in the clotting process under the action of drugs was judged by the records of a thromboelastogram performed on a thromboelastography (Tromb-2) (Charnaya et al., 2010). The thromboelastogram included the following:

3. Results and discussion

Using spectrophotometry methods, it was found that in the UV spectra of the coumarin mixture, two characteristic maxima are observed for high-intensity dicoumarins in the range 220-380 nm, the first of which is of an oscillatory nature, and the second corresponds to the p-tt conjugation of the benzene ring (Figure 1).

Fluorescence analysis of the extract (a mixture of coumarins) showed that with a saturated bromine solution in an alkaline medium it was possible to identify them on the basis of equal excitation maxima (380 nm) and emission (480 nm) (Khabarov et al.,1980).

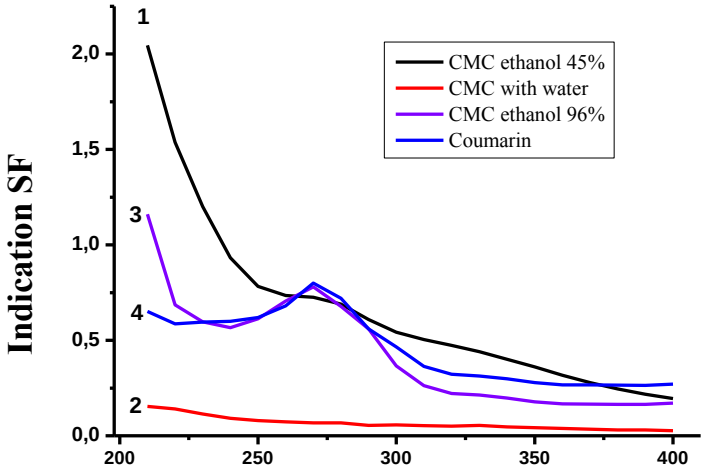

Fig. 1. Spectrophotometric readings of CMC and coumarin Electronic absorption spectrum of coumarin and CMC in 45-96 % alcohol extraction

The obtained fluorescence spectra as a function of the concentration of the extract showed a linear dependence of their intensity on the concentration, which made it possible to estimate the quantitative content of coumarins in this extract (Figure 2).

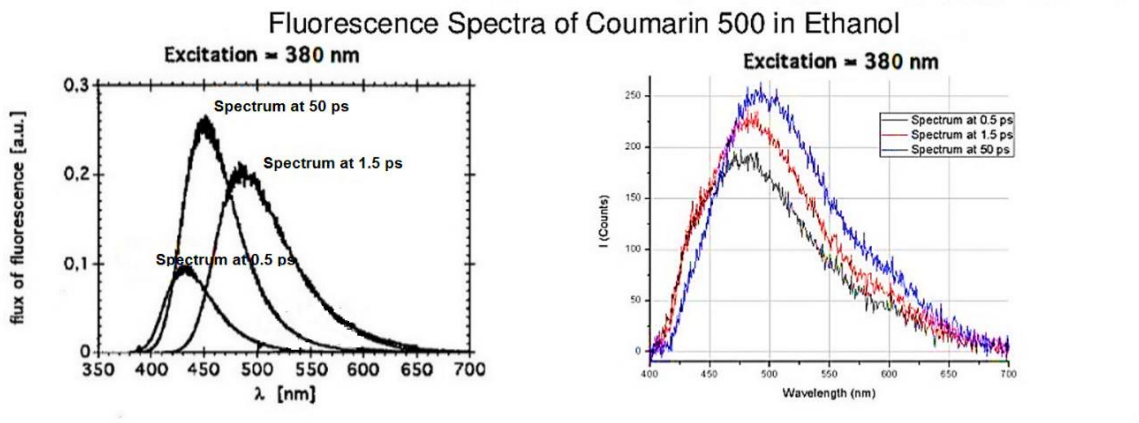

Fig. 2. Indications of intensity from the concentration of the fluorescence spectrum

Using HPLC in isocratic mode, a qualitative and quantitative analysis of the extract of moldy clover with standard dicumarol was performed – 15.22 min. (Figure 3). The amount of dicumarol in the CMC was calculated by comparing the peak areas in the chromatogram of the standard sample and in the test sample. As a result of the studies, it was found that in a dense extract the amount of dicumarol was 0.64 %, and in the crystalline fraction the sum of coumarins obtained with chloroform was 30.5 %.

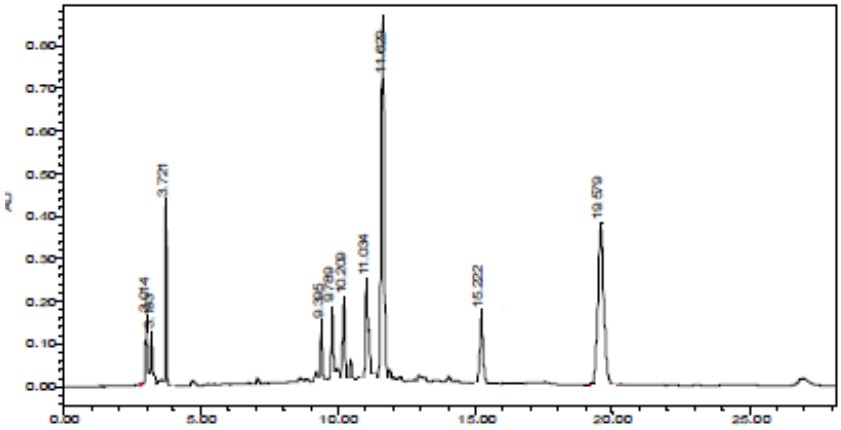

Fig. 3. Chromatogram CMC

Since the preparation CMC contains a mixture of dicumarols, which refers to vitamin K-dependent indirect anticoagulants, we investigated the effect on the blood coagulation process with its single administration compared with the effectiveness of curantyl.

The results of the effect of CMC on blood coagulation are shown in Table 1.

Table 1. Effect of the preparation "CMC" and curantyl on the indices of thromboelastograms of rats with oral administration (M ± m; n = 6)

TEG indicators	Time of study through			
	control	24 hours	5 day	8 day
Preparation CMC 0,04 mg/kg				
Blood reaction time R, mm	35±2,1	65±4,0*	53±1,0*	48±1,0*
Clot formation time, K, mm	10±1,0	20±2,0*	12±1,0	10±1,0
Coagulation constant R + K, mm	45±3,5	85±6,0*	65±5,0*	58±4,6
The constant of using prothrombin R / K	3,5±0,2	3,3±0,1	4,4±0,1*	4,8±0,1*
Maximal amplitude MA, mm	96±1,5	77±1,0*	82±2,0*	97±3,4
Coagulation constant t, mm	100±10	100±10	100±10	100±10
The syneresis constant S, mm	110±11	120±10	112±11	110±12
Total clotting time T, mm	145±14	185±14*	175±15*	158±13
Hypercoagulation index Ci (MA / R + K)	2,1±0,1	0,9±0,1*	1,1±0,1*	1,7±0,14*
Coefficient of elasticity of the bunch E, (MAX100 / 100-MA)	2400±190	334±16,0*	446±15,0*	3233±14
thrombohemorrhagic potential of ITP (E / S)	21,8±2,0	2,8±0,2*	4,0±0,3*	29,4±2,1
Preparation CMC 0,06 mg/kg				
Blood reaction time R, mm	35±2,1	67±4,0*	50±1,0*	48±1,0*
Clot formation time, K, mm	10±1,0	17±1,1*	15±1,0*	13±1,0*
Coagulation constant R + K, mm	45±3,5	84±3,0*	63±3,0*	61±3,0*
The constant of using prothrombin R / K	3,5±0,2	3,59±0,1	3,3±0,1	4,0±0,1
Maximal amplitude MA, mm	96±1,5	80±1,0*	82±1,0*	93±1,
Coagulation constant t, mm	100±10	100±10	100±10	100±10
The syneresis constant S, mm	110±11	117±10	115±10	112±10
Total clotting time T, mm	145±14	184±14*	163±15*	164±16*
Hypercoagulation index Ci (MA / R + K)	2,1±0,1	0,9±0,1*	1,4±1,0*	1,55±1,0*
Coefficient of elasticity of the bunch E, (MAX100 / 100-MA)	2400±190	400±20*	456±30*	1329±100*

thrombohemorrhagic potential of ITP (E / S)	21,8±2,0	3,4±1,4	4,0±2,5	11,9±1,1
Curantyl, 50 mg/kg				
Blood reaction time R, mm	35±2,1	72±3,2	61±6,6*	53±5,5
Clot formation time, K, mm	10±1,0	28±2,0*	39±2,2*	31±2,2*
Coagulation constant R + K, mm	45±3,5	100±8,3	100±11*	94±6.0*
The constant of using prothrombin R / K	3,5±0,2	2,6±0,2	1,7±0,2	2,0±0,2
Maximal amplitude MA, mm	96±1,5	82±2,0	77±1,0*	87±2,0
Coagulation constant t, mm	100±10	105±10	105±10	105±10
The syneresis constant S, mm	110±11	132±12	144±13	136±12
Total clotting time T, mm	145±14	232±16*	205±16*	199±16*
Hypercoagulation index Ci (MA / R + K)	2,1±0,1	0,83±0,6	0,77±0,06 *	0,6±0,04 *
Coefficient of elasticity of the bunch E, (MAX100 / 100-MA)	2400±190	456±30	334±16*	669±12*
thrombohemorrhagic potential of ITP (E / S)	21,8±2,0	3,5±0,1*	2,3±0,2*	4,9±0,1*

* P≤ 0,05 in relation to the control

As can be seen from the data in Table 1, 24 hours after the administration of CMC, at a dose of 0.04 mg/kg on a thromboelastogram, we observed hypocoagulation, which is expressed by an increase in the R, K, and R + K values by a factor of 2, prothrombin (R) and the concentration of thrombin formed and the amount of fibrinogen (K). Also, the Ci-index of hypercoagulation decreased 2-fold.

The maximum effect of the drug on the indicators E and ITP, where the coefficient of elasticity of the coagulum E decreases from 2400-190 to 334 16.0 or 7 times, and the thrombohemorrhagic potential of the ITPs is 21.8-2.0 to 2.8-8.2 or 7.8 times.

Gradually, the effect of the drug decreases, respectively, after 8 days is close to control.

An increase in the therapeutic dose of CMC to 0.06 mg/kg does not increase the effect after 24 hours, but it increases the duration of the drug.

As can be seen from the data in Table 1, such parameters as the elasticity coefficient of the clot E and the thrombohemorrhagic potential of the ITP, remained 8 days after the administration of the drug, decreased 2-fold with respect to the control group of animals (respectively, from 2400±190 to 1329±100 21.8±2.0 to 11.9±1.1).

As can be seen from the data in Table 1, a single administration of quarantine after 24 hours resulted in an increase in the reaction time of blood R by 100%. The K indicator increased by 69.6 %, and the MA maximum amplitude decreased by 13.6 %, which indicates a decrease in the formation of both thrombin and fibrinogen. Moreover, the hypercoagulation index Ci decreased by 52 %, the elasticity of the clot E by 33 %, and the thrombohemorrhagic potential index by 46 %.

After 8 days of the recovery period after the abolition of the curantyl, the blood coagulation indexes studied remained significantly altered towards hypocoagulation, although the effect was somewhat weaker.

4. Conclusion

The conducted studies made it possible to conclude that CMC is indeed an anticoagulant of indirect action, since the maximum effect of the drug is manifested in 24 hours and is associated with a decrease in the content of procoagulants. It is effective in comparison with the drug curantyl.

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