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BIOLOGICAL ACTIVITY OF THE OXIDIZED POLYSACCHARIDES

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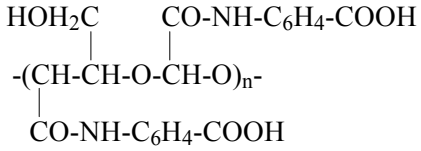
Bioreactive polysaccharides have been most commonly used as drugs or drug delivery systems. The present paper describes the biological activity of some artificial and natural polyanionic polysaccharides. Results regarding oxidized cellulose and carboxymethylcellulose modified with benzocaine or N – hydroxy – 3,4 – dihydroxybenzamide (Didox) complete the picture of antiviral and antitumoral effects of polysaccharides. The biological tests regarding antiviral and antitumor activity showed that the introduction of benzocaine as a spacer unit between the main chain and a CMC carboxylic group enhances the antiviral and antitumor activity of carboxymethylcellulose.



INTRODUCTION

Many natural polysaccharides participate in a variety of biochemical reactions *in vivo*. However, the mechanism of the biological activity of these natural carbohydrates is quite difficult to elucidate because of their complex chemical structure and the elevated content of impurities. In order to better understand the structure-activity relationship of polysaccharides, significant efforts have been made to chemically synthesize simpler polysaccharides.[1]

Similar types of polysaccharides have been shown to have various biological activities.[2,3] Glucans have been known for a long time to exhibit an enhanced effect on the immune system. For example, a glucan from the edible mushroom *Lentimus edodes* was found to exhibit a marked antitumor effect.[4-6]

Specifically, *lentinan*, a polysaccharide composed of β-1,3 and β-1,6 glucosidic linkages,along with other polysaccharides inhibited the growth of *Sarcoma-180* transplanted subcutaneously into mice. The antitumor activity of these polysaccharides was due to a host mediated reaction with participation of the thymus or thymus dependent cells (T cells).[7-9]

The present paper describes the biological activity of modified cellulose and carboxymethylcellulose (CMC) by selective oxidation and further modification with benzocaine and didox (N-hidroxy – 3,4 dihydroxybenzamide).

EXPERIMENTAL

Chemical reactions. Commercial cellulose powders (100-200 mesh) or carboxymethylcellulose (CMC) for column chromatography where treated with sodium metaperiodate at ambient temperature in the dark. The amounts of metaperiodate added were 1.4 fold as much as the required amount. Periodate oxidation was conducted in a reaction vessel for more than at least 150 hours until periodate reached a constant value; the amount of periodate consumed in the reaction process was determinated by UV at 290 nm. After decomposition of the excess periodate with ethylene glycol,

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oxidized products were separated into water-insoluble precipitate and supernatant liquid by centrifugation. The precipitate was recovered as colorless powder through washing with water, 50% ethanol and ethanol 99.9% and drying in vacuum. In this manner 2,3 – dialdehyde cellulose (DAC) or 2,3 – dialdehyde carboxymethylcellulose (DACMC) were obtained. DAC and DACMC were used for the synthesis of 2,3 – dicarboxycellulose (DCC) or 2,3 – dicarboxy-CMC (DCCMC). Aqueous suspension (300 mL) containing DAC (15 g) or DACMC (15 g) were further oxidized with sodium chloride and acetic acid in the conditions described in literature.[10,11]

CMC, DCC and DCCMC were modified with benzocaine (ethyl-p-amino benzoate) and N-hydroxy-3,4-dihydroxy benzamide (DIDOX) using the classical chemical reactions. The details of the chemical processes were given in the literature.[12-16]

The synthesis of CMC derivatives has been proven by IR spectra recorded on a *Perkin Elmer 1600* series Fourier transform IR spectrophotometer on KBr pellet and by elemental analysis of studied compounds.

Carboxyl groups content titrimetric determination

Free terminal carboxyl groups were quantitatively determinated by alkaline titration with a sodium hydroxide solution, 0,1 mol/L. The eight obtained compounds solutions were titrated with sodium hydroxide 0,1 mol/L solution from a automated burette, during 5 parallel determinations, in presence of 3-4 drops of phenolphthalein 0,1 % as indicator for each sample, with changing colour from colorless to pale pink persistent at equivalence point.[17] It was determinated the equivalence volume of sodium hydroxide solution which was consumed for. It has been used the average of 5 made measurements (Table 3), for accurate measurements $0.9 \leq f \leq 1.1$. The equivalence moment was indicated photometrically using a Mettler Toledo DP 660 phototrode, which measured the absorbance of the solution after each addition of titrant volume (V) and therefore, color changes at the equivalence point were perceived automatically as well as visually. The process was observed using a potentiometric unit. The equivalence point was automatically determined from the titration curve E = f(V) by non-linear regression analysis.[18,19]

The carboxyl group content[18] is obtained according to:

$$COOH = \frac{V \times C \times f}{m} \quad [mol/g]$$

V (mL) represent the consumption of titrating reagent (aqueous solution of NaOH, 0,1 mol/L), C is the molar concentration of titrating reagent NaOH (0,1 mol/L), f is the average factor of titrating reagent and m represents the weight of sample (g). Five titrimetric determinations for each weighted sample was made. All presented values are the mean value of 5 parallel measurements.

A statistical parameter was determinated: $\mathbf{C_V}$ **%** – the coefficient of variance, defined as $c_v = \frac{S}{\overline{x}} \cdot 100$; where S = standard deviation and $\overline{\mathbf{X}} = (x_1 + x_2 + ...+ x_n) / n$ – average of values.The coefficient of variance intra- and inter-day must be $\mathbf{C_V \leq 5\%}$. [17,20]

Benzocaine content of related compounds was calculated as number of benzocaine moles per structural unit and benzocaine percentage of studied compounds (wt%).

Biological evaluation. The antiviral activity of the compounds DCC, DCCMC, BMCMC, DMCMC, BMDCC, DMDCC and DMDCCMC was studied during two experiments. The first experiment was realized on 7 groups of 30 mice each (2 days old, 5 g weight) from the Hygiene and Public Health Institute, Virusology Department. Each 30 mice group received 0,4 mL of *COXSAKIE A4* virus in sterile saline solution by sub-occipital injection. Then the mice were placed to the cages and maintained on food and water *ad libitum*. One hour after the virus administration, the mice were injected with 8 mg/ Kg of the above compounds (1-7). It was evaluated death rate expressed as number of dead mice per day, compared with control group, at 24, 48, 72 and 96 hours. *IUT group (inoculated and untreated group) which is the control group, also received the same amount of *COXSAKIE A4* virus but it was not injected with studied compounds.

In the second experiment, two different groups of mice were selected: the first group of 20 and the second of 45 healthy mice including *IUT groups, which received 0.4 mL of *COXSAKIE A4* virus in sterile saline solution in the same conditions. After one hour of virus administration, the experimental groups excepted the control groups, received 8 mg/ Kg of studied compounds and it was calculated death rate after 24, 48, 72 and 96 hours for each treated group (Table 4).

Statistical study

To confirm the results obtained in these two experiments, a statistical study based on Kaplan-Meier survival test and ROC evaluation curves was made.

Kaplan-Meier evaluation is used to analyze how a given population evolves with time*. It estimate the fraction of subjects living for a certain amount of time after treatment. Kaplan-Meier test was carried out on three mice groups (30, 20 and 45 mice) treated with the studied compounds as mentioned above. It was used StatsDirect version 2.7.9 software.[21,22]

The ROC test was used for verifying the antiviral activity effectiveness of these substances among three mice groups treated with synthetized compounds, in comparison with control group (*IUT group). ROC analysis was conducted on total amount of 95 mice resulted from the fusion of three mice groups.[23]

Antitumor activity. The antitumor activity was evaluated on 90 male adult Wistard rats, 11 week old, free from chronic disease obtained from National Cancer Institute, Bucharest, Roumania.

The animals were treated daily, during 9 days with cell tumor suspensions. Experimental *Okker solid tumors* were obtained by subcutaneous injection of cell suspensions, according to Pollak's modification of the published procedures,[24] to afford a greater reproducibility and easier routine screening. The tumor bearing rats were divided in 9 experimental groups (10 rats per group).[25]

Starting one day after tumor inoculation, each group received 1,5 mg / day / rat of synthetized compounds, during 9 days and it were sacrificed under general anesthesia induced by chloroform at the end of each day. The tumor weight was determined for both control group and studied groups. For each sacrificed 10 rats group it was calculated every day, tumoral regression value $TR = (m_C - m_T)/m_C \times 100$, where m_C and m_T are tumoral weight of control group and treated rat groups. The antitumor activity of studied compounds (1-7) finally was elucidated by calculating average tumoral

regresssion (ATR%): $ATR = \frac{M_C - M_T}{M_C} x100$, where M_C and M_T represents average tumoral weight of the control group and average tumoral weight of the treated groups, calculated after nine days of compounds administration.[26] ATR % is the mean of daily tumoral regression values was calculated by raport of average tumoral weight of injected and untreated control groups.

ANOVA single factor test was conducted, to find the statistical significance between groups, whether the difference between the tumoral regression values of the nine samples calculated daily are statistically significant.[20] If $p \leq 0.05$, the values obtained have statistical significance; $p \leq 0.01$ – a high statistical significance and $p \leq 0.001$ – a very high statistical significance.[20] P values were calculated using Microsoft Office Excel 2007.

RESULTS

a) Chemical reactions

IR spectrometry and elemental analysis of studied compounds

The elemental analysis and IR spectra for all synthesized compounds show the characteristic data which prove the chemical structures suggested by us. The synthesis of CMC-Didox, BMCMC, DCCMC and BMDCCMC is described in experimental part. The modified polymers (formulas 3-7) and 2,3 – dicarboxy-CMC (DCCMC- formula 2) were characterized by elemental analysis, FTIR, carboxyl groups content and molecular weight.

The data given in Table 1 confirm the synthesis of DCC, DCCMC and its derivatives.

The CMC derivatives structure which has been proven by IR spectra has shown the followed IR band frequencies: DCC: 3,478.0, 2,737.2, 2,450.4, 1,772.5, 1,780.6, 1,478.4, 1,287.3, 1,235.5, 1,224.5, 1,179.8, 1,078.3; BMCMC: 3,460.5, 2,936.4, 1,757.3, 1,640.0, 1,255.1, 1,131.3, 1,065.8, 818.3 and 694.6, cm^{-1}; BMDCC: 3,471.3, 2,955.1, 1,756.3, 1,639.5, 1,263.1, 1,121.0, 1,038.8, 813.4 and 536.1 cm^{-1}; Didox-CMC (DMCMC): 3,471.3, 2,955.1, 1,795.5, 1,639.1, 1,268.1, 1,121.6, 1,068.8, 807, 753.9, 684.2, 584.3 cm^{-1}; Didox modified DCC (DMDCC): 3,473.2, 2,956.1, 1,755.4, 1,642.4, 1,264.5, 1,123.4, 1,039.9, 813.4, and 537.4 cm^{-1}; DCCMC (2,3 – dicarboxy-CMC): 3,480.0, 2,734.2, 2,450.4, 1,774.5, 1,780.6, 1,480.4, 1,290.3, 1,235.5, 1,180.8, 1,080.3, 1,060.6 cm^{-1}. Didox modified DCCMC (DMDCCMC): 3,484.5, 2,957.3, 1,820.3, 1,641.5, 1,271.3, 1,126.2, 1080, 810, 665.4, 600.7 and 570.6 cm^{-1}. IR spectra values specific for obtained compounds (3-7) were compared with DCC, DCCMC and CMC IR spectra. The research of above compounds (3-7) by elemental analysis (calculated per structural unit) led to following results: DCC calc. C 58%, H 4%, O 34%, det. C 57.8%, H 3.6%, O 33.7%; BMCMC calc. C 52.5%, H 5.1%, O 38.3%, N 4.1%, det. C 52.2%, H 5.0%, O 38%, N 4.0%; BMDCC calc. C 57%, H 3.9%, O 31.6%, N 6.9%, det. C 57.1%, H 4.1%, O 31.8%, N 6.7%; Didox-CMC calc. C 49.4%, O 44.3%, H 4.95%, N 3.38%, det. C 49.1%, O 44.7%, H 4.92%, N 3.42%; Didox modified DCC (DMDCC) calc. C 57%, H 3.9%, O 32.62%, N 6.9% , det. C 56.6%, H 4.12%, O 33%, N 6.8%; DCCMC calc. C 61.5%, H 5.9%, O 35.1%, det. C 61.8%, H 5.80%, O 35.5%; Didox modified DCCMC (DMDCCMC) calc. C 59.0%, H 4.9%, O 32%, N 3%, det. C 58.3%, H 4.7%, O 32.4%, N 3.2%.

Table 1

Physico-chemical characteristics of CMC and CMC derivatives

Compound	Carboxyl content n. mole/g		Benzocaine content				Molecular weight M_{GPC}*
			mole/structural unit		wt%		
	Found	Calc.	Found	Calc.	Found	Calc.	
DCC	4.30	4.54	-	-	-	-	46500
BMCMC	2.80	3.12	1.73	2.05	23.33	26.60	43000
DCCMC	4.65	4.88	-	-	-	-	41000
BMDCC	3.67	4.01	3.94	4.30	46.45	59.45	39000
CMC-Didox (DMCMC)	-	-	0.72[xx]	1.00[xx]	20.85[xx]	22.30[xx]	45100
DMDCC	3.01	3.35	3.63	3.90	24.52	28.48	46200
DMDCCMC	-	-	1.34	1.65	32.30	35.42	70300

Table 2

Carboxyl free groups content of studied compounds, measured by alkaline titration method

Compound name	Found average COOH content (mol/g)	NaOH, 0.1 mol/L consumed	average V (mL) NaOH, 0.1 mol/L	C_V %
DCC	4.30	7.7, 7.7, 7.7, 7.9, 8.0	7.80	1.81
BMCMC	2.80	5, 5.05, 5.1, 5.2, 5.2	5.11	1.75
DCCMC	4.65	8.44, 8.44, 8.53, 8.62, 8.62	8,53	1,05
BMDCC	3.67	6.7, 6.7, 6.8, 6.8, 6.8	6.76	0.81
DMDCC	3.01	5.7, 5.7, 5.75, 5.8, 5.8	5.75	0.87

CV % is the coefficient of variance = standard deviation/average of values.

Carboxyl groups content titrimetric determination. Terminal carboxyl groups of the related compounds were found by alkaline titration method in a sodium hydroxide solution (NaOH, 0.1 mol/L). Molarity mean factor of titrant reagent was calculated: $\bar{f}$ (NaOH) = 0.9533. It was determinated also the carboxyl groups content of CMC and for synthetized compounds, expressed in mol/g (Table 2).

b) Biological evaluation

Antiviral activity. This activity of studied compounds is described in Tables 3 and 4.

Statistical analysis: Kaplan-Meier test which includes calculation of average, confidence interval, survival probability and hazard is applied for all three mice groups and the results are exposed in Table 5.

ROC evaluation test is presented in Table 6.

Antitumor activity. Antitumor activity of studied compounds reflected by tumoral regression calculated per day is described in Table 7.

The average tumoral regression values of rat groups treated with synthesized compounds are given in Table 8.

Table 3

Antiviral activity of the compounds – first experiment

Compounds	Death Rate expressed as number of dead mice per day			
	24h	48h	72h	96h
*IUT	30	-	-	-
DCC	25	3	1	1
^{1}DCCMC	21	3	3	2
^{3}BMCMC	20	4	2	1
DMCMC	25	3	2	-
^{2}BMDCC	21	3	3	1
DMDCC	23	3	3	1
DMDCCMC	26	2	2	-

*IUT = inoculated and untreated group; exponents indicate the number of mice that survived after 96 hours of treatment.

Table 4

Antiviral activity studied on two different mice groups treated similarly in different days – second experiment

Compounds	Death Rate expressed as number of dead mice per day for each group			
	24h	48h	72h	96h
*IUT_{20}	20	-	-	-
*IUT_{45}	45	-	-	-

Table 4 (continues)

DCC_{20} $^{1}DCC_{45}$	17 39	1 3	1 1	1 1
$^{2}DCCMC_{20}$ $^{3}DCCMC_{45}$	15 35	1 2	1 2	1 3
$^{4}BMCMC_{20}$ $^{6}BMCMC_{45}$	11 29	3 6	1 3	1 1
$DMCMC_{20}$ $DMCMC_{45}$	18 41	1 2	1 1	- 1
$^{3}BMDCC_{20}$ $^{4}BMDCC_{45}$	13 32	2 3	1 3	1 3
$^{1}DMDCC_{20}$ $^{2}DMDCC_{45}$	16 37	1 2	1 2	1 2
$DMDCCMC_{20}$ $DMDCCMC_{45}$	19 42	1 2	- 1	- -

*IUT_{30} = inoculated and untreated 30 mice group; *IUT_{45} = inoculated and untreated 45 mice group; exponents indicate the number of survival mice from both groups.

Table 5

Description of survival parameters according to administered compounds

Compound	Studied group	Survival time		Survival probability (SE)				Hazard			
		average	CI 95%	24h	48h	72h	96h	24h	48h	72h	96h
DCC	30 20 45	30.4 31.9 29.9	24-36 23-40 25-35	0.17 (0.07) 0.15 (0.08) 0.13 (0.05)	0.07 (0.05) 0.10 (0.07) 0.07 (0.04)	0.03 (0.03) 0.05 (0.04) 0.04 (0.03)	0 0 0.02 (0.02)	1.79 1.90 2.01	2.71 2.30 2.71	3.40 3.00 3.11	infin. infin. 3.81
DCCMC	30 20 45	38.4 38.4 36.8	29-47 26-50 29-44	0.30 (0.08) 0.25 (0.10) 0.22 (0.06)	0.20 (0.07) 0.20 (0.09) 0.17 (0.06)	0.10 (0.05) 0.15 (0.08) 0.13 (0.05)	0.03 (0.03) 0.10 (0.07) 0.07 (0.04)	1.20 1.39 1.50	1.61 1.61 1.73	2.30 1.90 2.01	3.40 2.30 2.71
BMCMC	30 20 45	40.0 48.0 41.6	31-49 34-62 34-50	0.33 (0.09) 0.45 (0.11) 0.36 (0.07)	0.20 (0.07) 0.30 (0.10) 0.22 (0.06)	0.13 (0.06) 0.25 (0.10) 0.16 (0.05)	0.10 (0.05) 0.20 (0.09) 0.13 (0.05)	1.10 0.80 1.03	1.61 1.20 1.50	2.01 1.39 1.86	2.30 1.61 2.01
DMCMC	30 20 45	29.6 27.6 27.7	25-34 22-33 24-32	0.17 (0.07) 0.10 (0.07) 0.09 (0.04)	0.07 (0.05) 0.05 (0.05) 0.04 (0.03)	0 0 0.02 (0.02)	0 0 0	1.79 2.30 2.42	2.71 3.00 3.11	infin. infin. 3.81	infin. infin. infin.
BMDCC	30 20 45	38.4 43.2 40.0	30-47 30-56 32-48	0.30 (0.08) 0.35 (0.11) 0.29 (0.07)	0.20 (0.07) 0.25 (0.10) 0.22 (0.06)	0.10 (0.05) 0.20 (0.09) 0.16 (0.05)	0.07 (0.05) 0.15 (0.08) 0.09 (0.04)	1.20 1.05 1.24	1.61 1.39 1.50	2.30 1.61 1.86	2.71 1.90 2.42

Table 5 (continues)

DMDCC	30 20 45	33.6 34.8 33.6	27-41 24-45 27-40	0.23 (0.08) 0.20 (0.09) 0.17 (0.06)	0.13 (0.06) 0.15 (0.08) 0.13 (0.05)	0.03 (0.03) 0.10 (0.07) 0.09 (0.04)	0 0.05 (0.05) 0.04 (0.03)	1.46 1.61 1.73	2.01 1.90 2.01	3.04 2.30 2.42	infin. 3.00 3.11
DMDCCMC	30 20 45	28.8 25.2 26.1	24-34 23-28 24-29	0.13 (0.06) 0.05 (0.05) 0.07 (0.04)	0.07 (0.04) 0 0.02 (0.02)	0 0 0	0 0 0	2.01 3.00 2.71	2.71 infin. 3.81	infin. infin. infin.	infin. infin. infin.

CI 95% represents the confidence interval; infin. = infinity

Table 6

Comparative ROC analysis of administered compounds effectiveness reported to the control group

Compound	Relative risk	CI95%	p	PPV (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
DCC	0.93	0.88-0.98	0.021	48.1	100.0	6.9	53.4
DCCMC	0.86	0.80-0.94	0.001	46.3	100.0	12.0	56.0
BMCMC	0.77	0.69-0.86	0.001	43.5	100.0	18.8	59.4
DMCMC	0.95	0.90-0.99	0.030	48.6	100.0	5.0	52.5
BMDCC	0.81	0.74-0.89	0.001	44.8	100.0	15.9	58.0
DMDCC	0.88	0.82-0.95	0.002	46.9	100.0	9.5	54.7
DMDCCMC	0.97	0.93-1.0	0.244	49.2	100.0	3.1	51.5

PPV% represents the predictive value of a positive test

Table 7

Tumoral Regression values of administrated compounds

Compound	Tumoral Regression calculated daily (TR %)								
	24 h	48 h	72 h	96 h	120 h	144 h	168 h	192 h	216 h
DCC	19.0	21.0	25.0	27.0	30.0	32.0	36.0	39.0	41.0
DCCMC	25.0	27.0	30.5	33.3	35.5	37.3	40.4	42.0	44.0
BMCMC	43.3	46.0	49.2	52.4	55.3	58.2	61.4	63.2	66.0
DMCMC	46.0	49.0	52.0	55.0	58.0	61.0	64.0	67.0	70.0
BMDCC	48.0	51.0	54.0	57.0	60.0	63.0	66.0	69.0	72.0
DMDCC	42.5	45.0	48.0	51.5	54.5	57.5	60.0	62.0	65.0
DMDCCMC	47.0	50.0	53.0	56.0	59.0	62.0	65.0	68.0	71.0

Table 8

Average tumoral regression of the studied compounds

Compounds	*ATR (%)
DCC	30 35
DCCMC	55
BMCMC	58
DMCMC	60
BMDCC	54
DMDCC	59
DMDCCMC	

*ATR = average tumoral regression

DISCUSSION

a) Chemical reactions

IR spectrometry and elemental analysis

With reference to the CMC derivatives synthesis, the IR spectra, as well as the carboxyl groups content and molecular weight determination, has proved the structure of the synthetized compounds.

IR spectra of these compounds have shown characteristic bands of the carboxyl groups from acid or ester at 1,757.3 cm^{-1} for BMCMC, 1,756.3 cm^{-1} for BMDCC. DMDCC characteristic carboxyl groups IR frequency were found at 1,755.4 cm^{-1} and ester groups were alocated at 1,820.3 cm^{-1} for DMDCCMC and 1,795.5 cm^{-1} assigned to DMCMC. The amide groups were found at 1,639.5 cm^{-1} for BMDCC and 1,640.0 for BMCMC and 1,639.1 cm^{-1} for Didox-CMC (DMCMC). The corresponding amide IR frequencies 1,642.4 and 1,641.5 cm^{-1} have been assigned to DMDCC respectively DMDCCMC and ether IR frequencies at 1,264.5 cm^{-1} and 1,123.4 cm^{-1} for DMDCC. IR ether frequencies were found at 1,126.2, 1080 cm^{-1} for DMDCCMC and at 1,121.6 cm^{-1} assigned to CMC-Didox (DMCMC). DCCMC carboxyl IR frequencies were found at 1,774.5 and 1,780.6 cm^{-1} and ether DCCMC groups were found at 1,235.5, 1,180.8 cm^{-1}. Similarly, Didox modified CC (DCC) presented significant IR carboxyl group frequencies at 1,772.5, 1,780.6 cm^{-1}, ether groups at 1,179.8, 1,078.3 cm^{-1} and hydroxyl groups at 1,235.5 cm^{-1}, 1,224.5 cm^{-1}. Aromatic nucleus was present at 818.3 cm^{-1} for BMCMC, 753.9 and 807 cm^{-1} for DMCMC; 813.4, 810 cm^{-1} assigned to DMDCC , BMDCC respectively DMDCCMC.

Elemental analysis presented in results part of this paper, has proven carbon, oxygen, hydrogen content of studied compounds.

Benzocaine content

Regarding chemical analysis of related compounds, it have found that BMDCC had the highest benzocaine content and DMCMC (CMC-Didox) presented the lowest value. DCC and DCCMC compounds had no benzocaine in their molecules. The decreased rate (wt%) of benzocaine content varied as follows: BMDCC > DMDCCMC > DMDCC > BMCMC > DMCMC (Table 1).

Carboxyl groups content titrimetric determination

The average parameter obtained $\bar{f}$ (NaOH, 0.1 mol/L) = 0.9533, which is the correct value situated between 0.9 and 1.1. From results expressed in table 2, it have noticed that DCCMC compound presented the highest carboxyl content which represented 4.65 mol/g and BMCMC had the lowest carboxyl content between all studied compounds, only 2.80 mol/g. CMC-Didox (DMCMC) and DMDCCMC had not at all free carboxyl groups in their molecule, which means that it had ester and amide groups, according to IR spectral analysis. The decrease rate of carboxyl groups content confirmed by coefficient of variance values is represented as follows: DCCMC > DCC > BMDCC > DMDCC > BMCMC (Table 2).

b) Biological evaluation

Antiviral activity. The COXSACKIE viruses which include echoviruses and polioviruses are RNA viruses, part of the *Picornaviridae* family *Enterovirus* genus that live in the human digestive tract. COXSACKIE A4 virus infect host cells and cause lyse of cells.[27,28] This virus determine a wide range of serious infections to mice, similarly with human infection diseases, that occur in a short time and are very well defined. In the case of experimental animals, these infections generates paralysis usually followed by death.[27]

To human subjects, COXSAKIE A4 virus determine serious infections of the skin and mucous membranes, and cause aseptic meningitis and mild paralysis, pleural pain, intercostal pain, herpangina (mouth blisters), respiratory diseases, hemorrhagic conjunctivitis, hand, foot and mouth diseases.[28] Also COXSACKIE A4 virus cause many kinds of infections, such herpes and throat heating type, common cold disease,cardiac muscle inflammation, and acute intestinal tract infection disease.[27,28] No vaccine was discovered yet against COXSACKIE viruses.

In the current experiment, COXSACKIE A4 virus caused simultaneous generalized paralysis of striated muscle to mice studied groups, followed by death within the first 24 hours after infection. In the presence of synthetized compounds, paralysis appeared to begin more gradually and started from the rear part of the animal. Based on the data given in Table 3, some preliminary conclusions can be made with respect to the structure – antiviral activity relationship: the presence of carboxyl groups appear to be essential for the antiviral activity. The intensity of the antiviral effect which is enhanced by the number of carboxyl groups per structural unit and by the insertion of benzocaine

as a spacer unit, seems to depend on the presence of lipophilic moieties in the compounds.

As shown in Table 3, the death rate of the infected groups treated with DMCMC, DCC and DMDCCMC reached the highest value compared to other compounds (25 dead mice after the first 24 hours and 26 dead mice respectively). This rate decreased after 48, 72 and 96 hours of treatment. The groups treated with DMDCCMC and DMCMC compounds had all the 30 mice dead after 72 hours of compounds administration and presented the highest death rate (26 and 25 dead mice) within 24 hours of treatment.

Also it was observed that two infected groups treated with BMCMC and BMDCC (Table 3), were characterized by the lowest death rate after the first 24 hours of treatment (20 and 21 dead mice respectively). The number of death mice decreased after 48, 72 and 96 hours. After 96 hours of compounds administration, 30 mice groups treated with BMCMC presented 3 mice alive and those treated with BMDCC had 2 survival mice. These two compounds were the most effective and had the strongest antiviral activity compared to another five administrated compounds. Death rate values obtained for DCCMC was situated nearly of those assigned to these two compounds. DCCMC death rate was represented by 21 dead mice after 24 hours of treatment; the group injected with this compound had 2 mice still alive after 96 hours of compound administration (Table 3).

În the second experiment which was made on other two mice groups in the same conditions, death rate values was very approached of those resulted from the first one and confirmed the obtained values (Table 4).

Statistical study: Kaplan-Meier survival test. The results obtained from Kaplan-Meier analysis led to a complex study of mice groups survival parameters.

For the first mice group (30 mice) which received DCC compound (Table 5), the average survival time was approximately 30 hours, the survival probability decreased below 17% after 24 hours. Risk factor increased more than 2.7 times at 48 hours after compound administration. The average survival time of the second mice group (20 mice) which reveived DCC was 31 hours. Survival probability decreased below 15% after 24 hours from the start of experiment. Risk factor increased more than 2.3 times after 48 hours of compound administration. The third mice group (45 mice) which received DCC presented a average survival time about 30 hours. Survival probability fell below after 24 hours from start of the test. Risk factor (hazard) increased more than 2.7 times after 48 hours of compound administration (Table 5).

The average survival time of first mice group which received DCCMC compound was about 38 hours and the risk factor increased more than 2.3 times after 72 hours of compound injection. Survival probability decreased less than 30% after 24 hours from the start of this study. The second group of mice receiving DCCMC was characterized by a mean survival time over 38 hours. Risk factor increased more than 1.9 times after 72 hours from the start of the test. The survival probability decreased below 25% after 24 hours of compound administration, reaching 10% at 96 hours after the experiment. The average survival time for the third mice group which received DCCMC was about 37 hours. Risk factor (hazard) was over 2 times higher after after 72 hours of the start. Survival probability fell below 22% after 24 hours from the start of this study (Table 5).

The average survival time of the first group of mice which received BMCMC was 40 hours. Risk factor induced by this compound was 2 times higher after 72 hours of dosing. Survival probability fell bellow 33% after 24 hours and under 13% at 72 hours after compound administration. The second mice group which received BMCMC presented a average survival time of 48 hours. The survival probability was less than 30% after 48 hours from the start of the test and risk factor increased over 1.6 times after 96 hours of treatment. The average survival time of third mice group which received BMCMC was 41.6 hours. After 96 hours of compound administration, risk factor increased 2 times over. Survival probability decreased below 36% after 24 hours from the initiation of the experiment.

The first group of mice which received DMCMC, mean survival time was approximately 30 hours. Risk factor increased 2.7 times over after 48 hours of compound adminsitration. Survival probability fell under 17% after 24 hours from the start of the experimen and became almost zero after 48 hours. The average survival of the second mice treated group time was 27.6 hours. After 48 hours of compound administration, risk factor induced by DMCMC increased more than 3 times. Survival probability decreased under 10% in the first 24 hours after initiation of the experiment and

became almost zero after 48 hours (Table 5). The mean survival time of the third mice group which received DMCMC was 27.7 hours. Risk factor increased 2.4 times more after 24 hours of treatment. Survival probability fell under 8% in the first 24 hours after initiation of the experiment.

The first mice group which received BMDCC had a average survival time about 48 hours. Risk factor induced by this compound increased 2.3 times over in the 72 hours of compound injection. Survival probability was less than 30% from the start of the experiment and was null after 96 hours of treatment. The second mice group which received BMDCC presented a mean survival time aproximately 43 hours. Risk factor increased more than 1.9 times after 96 hours of compound administration. The survival probability was under 35% after 24 hours from the start of the experiment and reached 15% after 96 hours. Average survival time of the third mice group receiving BMDCC was 40 hours. Risk factor increased more than 2.4 times after after 96 hours of treatment. Survival probability fell under 29% after 24 hours from the start of this experiment (Table 5).

The first mice group which received DMDCC had a mean survival time about 33.6 hours. Hazard induced by this compound increased more than 2 times after 48 hours of administration. Survival probability decreased under 23% after 24 hours of compound injection. The 20 mice group receiving DMDCC had a mean survival team of approximately 35 hours. Risk factor was over 2.3 times higher than at 72 hours of compound administration. Survival probability was situated under 20% after 24 hours from the start of the experiment. Average survival time of 45 mice group which received DMDCC was 33.6 hours. Risk factor increased more than 2 times after 48 hours of DMDCC administration and became over 3 times higher after 96 hours of the beginning. Survival probability decreased under 18% after 24 hours from the compound administration (Table 5).

The first mice group which received DMDCCMC had a average survival time about 29 hours. Risk factor increased more than 2 times after 24 hours of compound adminsitration. Survival probability decreased under 13% after 24 hours from the start of this experiment. The 20 mice group which received DMDCCMC had a average survival time about 25 hours. Risk factor increased more than 3 times after the first 24 hours of compound administration. Survival probability fell under 5% in the first 24 hours after experiment initiation. Average survival time of third mice group which received DMDCCMC was about 26 hours. Risk factor increased 2.7 times over after the first 24 hours of administration. Survival probability decreased under 7% after 24 hours of compound injection (Table 5).

ROC evaluation test. The relative risk (RR) (Table 6) was nil in all experiments and showed the protective antiviral role of administered compounds. The most antiviral protective compound was BMCMC (RR = 0.77), followed by BMDCC (RR = 0.81). The compounds represented by the lowest antiviral activity were DMCMC (RR = 0.95) and DMDCCMC (RR = 0.97).

The average survival time recorded under 48 hours values (Table 6) which entitles us to follow antiviral protective role of administered compounds according to lethality at 48 hours after administration, registered on 95 tested mice.

The lowest lethality was observed to BMCMC mice treated groups which presented a positive predictive value of 43.5%. The most increased lethality value was observed to the groups treated with DMDCCMC which presented a positive predictive value of 49.2% (Table 6).

ROC statistical analysis revealed a 100% sensitivity for each studied compound, with the highest BMCMC specificity (18.8%) followed by BMDCC (15.9%) and DCCMC (12%) for whom the acuracy of the experiment was more than 56% (Table 6). The lowest specificity value was represented by DMDCCMC (3.1%) with corresponding acuracy value of only 51% (Table 6).

Antitumor activity. As shown in Table 7, daily development of the tumor under the treatment with the synthesized compounds is variable. The results showed in Table 8 have certified that oxidized and derivatized polysaccharides exhibit a significant antitumoral activity. It can be seen clearly that two Wistard rats groups injected with cell tumor suspensions presented the highest average tumor regression value (ATR%) (Table 8). These groups are those who were treated with BMDCC and DMDCCMC compounds (ATR = 60% and 59% respectively). On second place was situated DMCMC (ATR = 58%). Average tumoral regression of BMCMC was ATR = 55% and DMDCC with ATR = 54% (Table 8).

Between studied compounds, DCCMC had a lower tumor cells influence (ATR = 35%) and the rat group treated with DCC presented the lowest tumor regression value (ATR = 30%). It appears that the number and distribution of the carboxyl

groups in compounds chemical structure are not at all the decisive factors that determine the tumor inhibiting effect.

According to ANOVA single factor test which was made in Microsoft Office Excel 2007, the statistical significance between tumoral regression daily values of the nine rat groups was predicted by p value: $p = 0.011255$ ($p \leq 0.01$).

CONCLUSIONS

In this study, antiviral and antitumor activity of polycarboxylic polymers was achieved by derivatizing cellulose and carboxymethylcellulose with benzocaine and Didox.

The chemical structure of seven synthesized compounds was confirmed by analytical methods. BMDCC showed the highest benzocaine content (46.45%). The lowest benzocaine content was represented by DMCMC (20.85%).

The highest carboxyl group content was assigned to DCCMC (4.65 mol/g) and the lowest carboxyl group content was represented by BMCMC (2.80 mol/g).

Antiviral activity. The antiviral effect is enhanced by insertion of benzocaine as a spacer unit associated with the number of carboxyl groups per structural unit. The presence of the lipophylic phenyl moiety appears to enhance the hidrophobicity and the biocompatibility of the studied compounds.

According to Kaplan-Meyer survival test applied for studied compounds, BMCMC is the most effective compound with the highest average survival time (40-48 hours) and the lowest antiviral protective compound was DMDCCMC represented by a average survival time of 20-29 hours. After 24 hours survival probability decreased significantly in all mice groups, especially to those which received DMDCCMC and DMCMC, without no survivors after 72 hours of administration.

ROC evaluation test has ordered the studied compounds depending on sensitivity and specificity of the experiments and confirmed the results obtained during two mice experiments. The antiviral activity of studied compounds decreased as follows: BMCMC > BMDCC > DCCMC > DMDCC > DCC > DMCMC > DMDCCMC.

Benzocaine modified CMC (BMCMC) and benzocaine modified DCC (BMDCC) are benzocaine derivatives. BMDCC is a 2,3-dicarboxycellulose modified with benzocaine derivative which have the highest benzocaine content and presents carboxyl groups, while BMCMC is a carboxymethylcellulose derivative modified with benzocaine; both of these two compounds have an significant content of phenyl lipophilic moieties. The presence of benzocaine as spacer unit seems to play an important role in antiviral activity of these compounds.

DMCMC (Didox modified carboxymethycellulose) and DMDCCMC (Didox modified 2,3-dicarboxy-carboxymethylcellulose and benzocaine) are Didox (N-hydroxy-3,4-dihydroxy-benzamide) derivatives. DMCMC and DMDCCMC were the less effective and had the lowest antiviral activity compared to the other five studied compounds. The presence of Didox in their molecules could play an role in a weak antiviral activity manifested.

DMCMC have the lowest benzocaine content among the seven studied compounds. DMDCCMC and DMCMC have no carboxyl groups in their molecules. These aspects are essential and highlights the lowest antiviral capacity of these two compounds.

Antitumor activity. The rat groups injected with BMDCC and DMDCCMC presented the highest average tumor regression values (ATR = 60% and ATR = 59% respectively). These compounds contain a significant benzocaine content and also, a significant lipophylic phenyl moiety. It is certain that the presence of benzocaine associated with lipophylic phenyl content enhance antitumor activity of above compounds and carboxyl group content seems to plays an insignificant role in this matter.

The lowest average tumor regression value is attributed to DCC and DCCMC compounds (ATR = 30% and 35% respectively) which have a significant content of carboxyl groups but they do not contain benzocaine as a spacer unit in their molecules.

The results obtained in the experiment were confirmed by ANOVA single factor statistical study according to which it was found a high statistical significance between daily tumoral regression values of nine groups of rats treated daily with studied compounds ($p \leq 0.01$, $p = 0.01125$).

The antitumor activity of administrated compounds decreased as follows: BMDCC > DMDCCMC > DMCMC > BMCMC > DMDCC > DCCMC > DCC.

Experimental data show that tumor inhibition is enhanced by similar structural characteristics as in the case of antiviral activity. The only difference

seems to be that antitumor effect is enhanced especially by the benzocaine content. The presence of the lipophylic phenyl moiety also enhances the hidrophobicity and the biocompatibility of the studied compounds.

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