



## Antiviral properties of clinoptilolite

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### Abstract

The aim of this study was to evaluate the antiviral properties of clinoptilolite, a natural non-toxic zeolite. Herein, a fine powder of micronized zeolite (MZ) was obtained by tribomechanical micronization of natural clinoptilolite. Different viral suspensions were treated with MZ in concentrations ranging from 0.5 to 50 mg/ml. The viral proliferation was evaluated by optical microscope as percentage of cytopathic effect (CPE). Human adenovirus 5, herpes simplex virus type 1 (HSV 1) and human enteroviruses (coxsackievirus B5 and echovirus 7) were used in the antiviral assay. Concentrations of 0.5 and 5 mg/ml of MZ induced a very low antiviral effect or the antiviral was not observed at all, while concentrations of 12, 25 and 50 mg/ml of MZ induced a significant inhibitory effect upon viral proliferation. MZ inhibited the viral proliferation of HSV 1, coxsackievirus B5 and echovirus 7 more efficiently than adenovirus 5. The antiviral effect of MZ seems to be non-specific and is more likely based on the incorporation of viral particles into pores of MZ aggregates than ion exchange properties of clinoptilolite. Our preliminary results indicate a possibility of therapeutical application of MZ, either locally (skin) against herpesvirus infections or orally in cases of adenovirus or enterovirus infections. Furthermore, MZ could also be used in purification of drinking water from different viruses.

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### 1. Introduction

Clinoptilolite is a natural, non-toxic zeolite that has monoclinic crystal structure symmetry and strong adsorptive and ion exchange capacity [1]. These properties have been largely exploited in industrial, agricultural, environmental and biological technologies [2]. Zeolites also possess biological activities, either positive or negative. The best known and documented positive biological activity of natural clinoptilolite is its action as antidiarrheal drug [3]. Furthermore, some of them seem to have antibacterial property [4]. The clinoptilolite from Vranje, Serbia used in this study has antioxidative and immunostimulatory effects [5], and it has been used as an adjuvant to anticancer therapy [6–8].

Clinoptilolite administered by gastric intubation to mice injected with melanoma cells significantly reduced the number of melanoma metastases [2]. Clinoptilolite treatment of mice and dogs suffering from a variety of tumor types led to improvement in the overall health status, prolongation of life span, and decrease in tumor size. Local application of clinoptilolite to skin cancers of some dogs effectively reduced tumor formation and growth [6].

The major negative biological effect of clinoptilolite could be its toxicity in higher organisms (mammal) if the content of heavy metals (Pb, Cd, Zn, etc.) is high. Therefore, a classic acute, sub-chronic and chronic toxicity study of the clinoptilolite from Vranje, Serbia was performed on mice and rats [6,9]. Results clearly show that oral (in diet) administration of clinoptilolite to mice and rats for 6 and 12 months, respectively, caused no changes that could be considered a toxic effect of treatment.

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Based on these results we assumed that the adsorbent qualities and ion exchange properties of clinoptilolite could be effective on viruses too. Herein, we tested a natural clinoptilolite (Vranje, Serbia) [6] on in vitro viral replication of adenoviruses, herpesviruses and enteroviruses (coxsackievirus, echovirus).

## 2. Experimental

### 2.1. Natural clinoptilolite

A fine powder of natural clinoptilolite, i.e., micronized zeolite (MZ), was obtained by tribomechanical micronization [6] of natural clinoptilolite from Vranje, Serbia. Chemical composition and characteristics of MZ have been described previously [5,6].

### 2.2. Cell lines

Human cervical carcinoma cells (HeLa; ATCC number: CCL-2) and African green monkey kidney epithelial cells (BS-C-1; ATCC number: CCL-26) were used. Cells were propagated in Dulbecco's modified eagle medium (MEM; Gibco BRL, USA) supplemented with 10% inactivated foetal bovine serum (FBS; Gibco BRL, USA), 1% L-glutamine and 0.3% sodium bicarbonate at 37°C and 5% CO<sub>2</sub>.

### 2.3. Viruses

Adenovirus 5 (ATCC number: VR-5), herpesvirus type 1 (HSV 1; ATCC number: VR-733), and two enteroviruses, coxsackievirus B5 virus (ATCC number: VR-185) and echovirus 7 (ATCC number: VR-37) were included in this study. Adenovirus and herpesvirus were propagated on HeLa, while enterovirus were propagated on BS-C-1 confluent cell monolayers. The viral suspension consisted the cell-free supernatant collected after centrifugation (20 min, 4°C, 5000 × *g*) of infected media (MEM supplemented with 2% FBS) collected at maximal viral proliferation, i.e. 100% cytopathic effect (CPE) of whole cell monolayer. Five different relative viral titres ( $V^1$ – $V^{-4}$ ) obtained by serial dilution of viral suspension (1:2 for adenovirus and herpesvirus and 1:10 for enteroviruses) were treated with MZ prior to antiviral assay.

### 2.4. MZ treatment

Due to sedimentation of clinoptilolite in its water suspension, it is not possible to treat a cell culture with MZ and further follow up morphological changes of cells upon viral infection. For this reason, different viral titres ( $V^1$ – $V^{-4}$ ) and MEM supplemented with 2% FBS (negative control) were treated with MZ at concentrations

ranging from 0.5 to 50 mg/ml. After incubation (15 h, 4°C, constant rotation), the suspension (media and MZ) was centrifuged (10 min, 4°C, 3000 × *g*) to separate the liquid from the solid phase (MZ).

### 2.5. Antiviral assays

HeLa and BS-C-1 cell were seeded at  $2 \times 10^4$  cells per ml on 24-well flat-bottomed microtitre plates (Becton Dickinson, USA). The viral infection was performed on one-day-old confluent cell monolayers. The plates were incubated at 37°C and 5% CO<sub>2</sub> and the CPE were followed by optical microscopy every 24-h during 3–4 days (depending on the type of virus). Each assay was done four times. The inhibitory effect of viral proliferation was evaluated as percentage of CPE and was compared to CPE of similar dilutions of viral suspension also incubated at 4°C during 15-h but without MZ (positive control).

## 3. Results and discussion

Four different viruses were chosen on the basis of their morphology and biological characteristics: (a) with or without lipoprotein envelope acquired from the host cell, (b) DNA or RNA replicating viruses and (c) high infectivity and relatively rapid CPE in cell culture. The herpesviruses capsid is surrounded by a lipoprotein envelope, varying in size from 100–200 nm in diameter and their genome consists of double-stranded linear DNA. Adenoviruses and enteroviruses (coxsackieviruses and echoviruses) are non-enveloped and are relatively small (65–80 and 22–30 nm virion size, respectively) viruses, as compared to herpesviruses. The genome of adenoviruses consists of linear double-stranded DNA, while the one from enteroviruses consists of single-stranded RNA.

Enteroviruses are highly infective and specific CPE (cell lysis) appears in cell culture (BS-C-1) rapidly, within 24–48 h depending of the viral titre (1:10 serial dilution). Adenoviruses and herpesviruses are less infective than enteroviruses and specific CPE (cell rounding) appears in cell culture (HeLa), within 24–72 h depending of the viral titre (1:2 serial dilution).

The CPE of adenovirus 5 and herpesvirus type 1 (HSV 1) was observed on HeLa cells, while CPE of coxsackievirus B5 and echovirus 7 on BS-C-1 cells.

The influence of clinoptilolite on viral proliferation depends on both the concentration of MZ ( $C_{MZ}$ , ranging from 0.5 to 50 mg/ml) and the viral titre (ranging from  $V^1$  to  $V^{-4}$ ), i.e. antiviral effect (Figs. 1–4). The antiviral effect was highest with the highest concentration of clinoptilolite (50 mg/ml) and the lowest viral titre ( $V^{-4}$ ). The observed percentages of antiviral effect also depended on the type of virus (Tables 1–4).



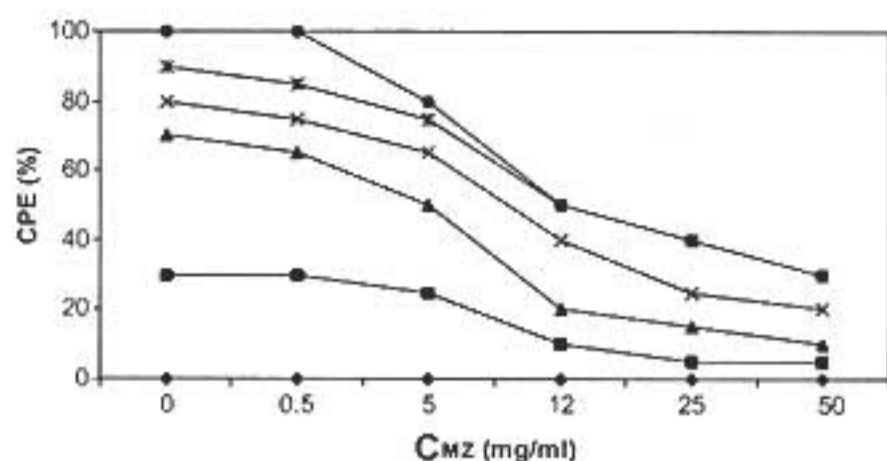


Fig. 1. Influence of different concentrations of clinoptilolite ( $C_{MZ}$ ) on HSV 1 proliferation on HeLa cell line (percentage of cytopathic effect—CPE) for viral titre  $V^1$  (●),  $V^{-1}$  (\*),  $V^{-2}$  (x),  $V^{-3}$  (▲),  $V^{-4}$  (■) and negative control—culture media without virus (◆).

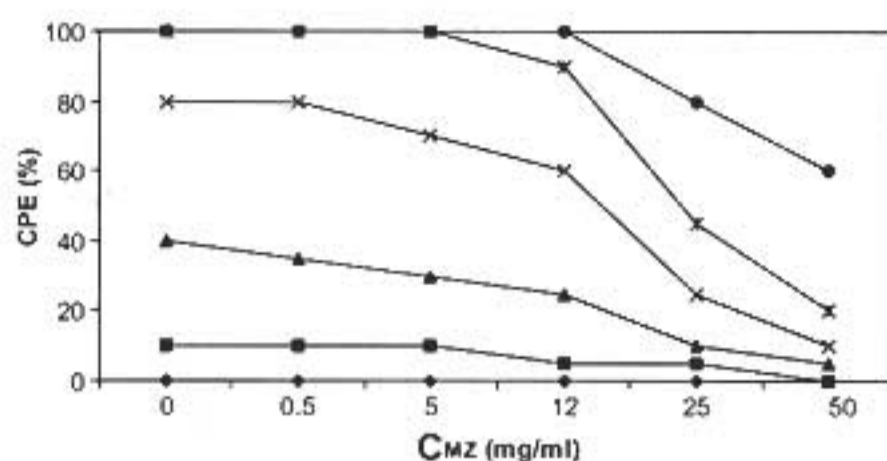


Fig. 4. Influence of different concentrations of clinoptilolite ( $C_{MZ}$ ) on echovirus 7 proliferation on BS-C-1 cell line (percentage of cytopathic effect—CPE) for viral titre  $V^1$  (●),  $V^{-1}$  (\*),  $V^{-2}$  (x),  $V^{-3}$  (▲),  $V^{-4}$  (■) and negative control—culture media without virus (◆).

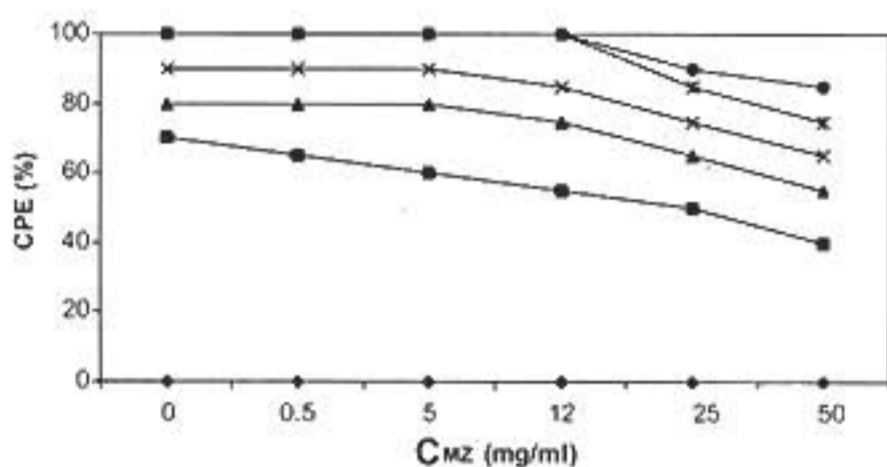


Fig. 2. Influence of different concentrations of clinoptilolite ( $C_{MZ}$ ) on adenovirus 5 proliferation on HeLa cell line (percentage of cytopathic effect—CPE) for viral titre  $V^1$  (●),  $V^{-1}$  (\*),  $V^{-2}$  (x),  $V^{-3}$  (▲),  $V^{-4}$  (■) and negative control—culture media without virus (◆).

Table 1  
Percentage of inhibition of HSV1 proliferation upon treatment with MZ

MZ (mg/ml)	Viral titre				
	$V^{-4}$	$V^{-3}$	$V^{-2}$	$V^{-1}$	$V^1$
0	0	0	0	0	0
0.5	0	7.1	6.3	5.6	0
5	16.7	28.6	18.8	27.8	0
12	66.7	71.4	50	44.4	20
25	83.3	78.6	68.8	55.6	50
50	83.3	85.7	75	66.7	60

Table 2  
Percentage of inhibition of adenovirus 5 proliferation upon treatment with MZ

MZ (mg/ml)	Viral titre				
	$V^{-4}$	$V^{-3}$	$V^{-2}$	$V^{-1}$	$V^1$
0	0	0	0	0	0
0.5	7.1	0	0	0	0
5	14.3	0	0	0	0
12	21.4	6.3	5.6	0	0
25	28.6	18.8	16.7	15	10
50	42.9	31.3	27.8	25	15

Table 3  
Percentage of inhibition of coxsackievirus B5 proliferation upon treatment with MZ

MZ (mg/ml)	Viral titre				
	$V^{-4}$	$V^{-3}$	$V^{-2}$	$V^{-1}$	$V^1$
0	0	0	0	0	0
0.5	50	0	0	0	0
5	100	20	5.6	0	0
12	100	80	77.8	45	0
25	100	90	88.9	70	10
50	100	100	100	80	20

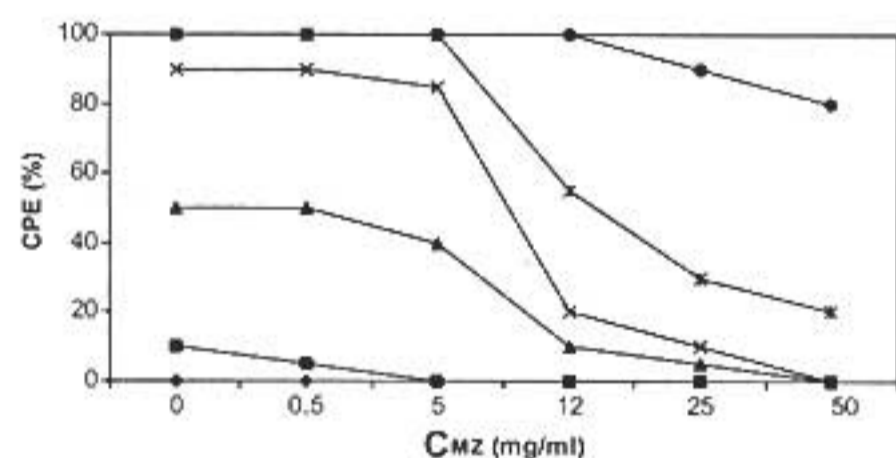


Fig. 3. Influence of different concentrations of clinoptilolite ( $C_{MZ}$ ) on coxsackievirus B5 proliferation on BS-C-1 cell line (percentage of cytopathic effect—CPE) for viral titre  $V^1$  (●),  $V^{-1}$  (\*),  $V^{-2}$  (x),  $V^{-3}$  (▲),  $V^{-4}$  (■) and negative control—culture media without virus (◆).

Viral suspensions, regardless of viral titre, treated with MZ at concentrations of 0.5 and 5 mg/ml prior to antiviral assay induce very low (5.6–28.6%) inhibition of specific CPE, or inhibition of adenovirus 5, HSV 1 and echovirus 7, except for coxsackievirus B5 which was inhibited completely at viral titre  $V^{-4}$  (Tables 1–4).

Concentrations of 12 mg/ml of MZ induced a maximum of 21.4% ( $V^{-4}$ ), 50% ( $V^{-4}$ ), 71.4% ( $V^{-3}$ ) and

100% ( $V^{-4}$ ) inhibition of CPE of adenovirus 5, echovirus 7, HSV 1 and coxsackievirus B5, respectively (Tables 1–4).

Table 4  
Percentage of inhibition echovirus 7 proliferation upon treatment with MZ

MZ (mg/ml)	Viral titre				
	V <sup>-4</sup>	V <sup>-3</sup>	V <sup>-2</sup>	V <sup>-1</sup>	V <sup>1</sup>
0	0	0	0	0	0
0.5	0	12.5	0	0	0
5	0	25	12.5	0	0
12	50	37.5	25	10	0
25	50	75	93.8	55	20
50	100	87.5	87.5	80	40

Concentrations of 25 and 50 mg/ml of MZ induced a significantly higher inhibition of CPE of most treated viruses, except adenovirus 5 (Figs. 1–4). The maximum of 28.6% and 42.9% inhibition of CPE of adenovirus 5 was observed at the lowest viral titre (V<sup>-4</sup>) treated with 25 and 50 mg/ml of MZ, respectively (Table 2).

Concentrations of 25 and 50 mg/ml of MZ induced a high inhibitory effect of CPE of HSV 1 of 83.3% (V<sup>-4</sup>) and 85.7% (V<sup>-3</sup>), respectively (Table 1, Fig. 1). Similarly, concentrations of 25 and 50 mg/ml of MZ induced the highest inhibitory effect of CPE, up to 100% (Tables 3 and 4) of coxsackievirus B5 (Fig. 3) and echovirus 7 (Fig. 4).

Our study indicates an inhibitory effect of MZ upon viral proliferation. The inhibitory effect was represented by the inhibition of specific viral CPE on cell culture compared to the same without treatment with MZ. As mentioned previously, the inhibitory effect of MZ depends on the concentration of MZ (0.5–50 mg/ml), the type and the concentration of virus (viral titre ranging from V<sup>1</sup> to V<sup>-4</sup>) (Figs. 1–4 and Tables 1–4). A significant inhibition of viral proliferation over 50% was observed with concentration of MZ over 12 mg/ml.

Treatment of viral suspension of adenovirus 5 with MZ did not induce any significant inhibition of viral proliferation contrary to HSV 1, coxsackievirus B5 and echovirus 7. The inhibition of viral proliferation must probably be unspecific and independent of virion size, structure and genome type. As MZ consists of a mixture of particles of approximately 1 µm in diameter and a internal pore size of 0.35 nm, virions ranging from 20 to 200 nm in size were probably incorporated within the mesoporous zeolite aggregate and/or adsorbed on the surface of their crystalline microstructure during the 15 h treatment of virally infected culture media. This would be the most plausible explanation because a similar phenomenon is used in the method of viral concentration by capture on borosilicate glass powder although the particle size is much larger (100–200 µm) [10]. Furthermore, MZ adsorb essential minerals and amino acids from culture media [11]. Inhibition of viral proliferation by capture and/or adsorption of virions onto MZ crystalline microstructure requests further research (electron microscopy analysis, for instance).

Another possible mechanism of action of MZ onto viral particles is its ion exchange capability that could destabilise morphology of viral particles; namely as lipoprotein structure (viral envelope) is less resistant to environment than protein (viral capsid), this could explain why herpesviruses (enveloped) were more destabilised than adenoviruses (non-enveloped) by MZ. However, this theory is not completely accurate because the proliferation of enteroviruses (coxsackievirus B5 and echovirus 7), also non-enveloped virions were almost equally inhibited by MZ as those of herpesviruses (HSV 1). Thus, in such impoverished culture media the viral viability and infectivity is reduced. The exact mechanism of action of MZ based on the ion exchange property of their interaction with viral particles in an aqueous solution (culture media), needs further investigation, extensive biochemical analysis of media and virion changes.

The mechanisms of action of MZ upon different types of viruses are probably non-specific which makes it more interesting than conventional antiviral drug [12]. Such inactivation of viral particles by MZ would be extremely interesting for viruses that infect the digestive tract such as enteroviruses and adenoviruses, and because MZ can be orally administrated without toxicity [6] it could be used for therapeutic purposes. Beside that, MZ could be used as traditional natural antidiarrhoeal therapy such as clay and activated charcoal [12,13].

Herpesviruses are able to establish life-long latency after primary infection that can be reactivated, especially in immunocompromised transplant recipients and patients with AIDS. Generally, herpesvirus infections have been treated successfully with systemic administration of acyclovir [14]. However, drug resistance variants emerge after long-term treatment, which leads to treatment failures. This is why new efficient and inexpensive potential drugs such as MZ could be helpful to inhibit, if not eradicate, viral infections. Additionally, MZ could be administrated locally on skin as cream or gel in order to inhibit recurrent labial and genital herpesvirus infections that are often psychologically and physically very painful.

#### 4. Conclusion

Our preliminary results, indicate an antiviral property of clinoptilolite that open a possibility of therapeutic application of MZ either locally (skin) against herpesvirus infections or orally in cases of adenovirus or enterovirus infections. However, the inhibitory effect of viral proliferation was observed with high concentration of MZ (over 12 mg/ml) which makes the clinical applications and the dose-response effect difficult to establish. Fortunately, MZ could be used in purification of drinking water from different viral particles without concern of concentration of MZ for application.

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