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Virucidal effect of Crithmum maritimum L. extracts on ECHO 9 virus

Wirusobójcze działanie ekstraktów *Crithmum maritimum* L. na wirusa ECHO 9

The development of medical science, especially virology and pharmacology allowed to obtain a huge progress in the treatment of various viral infections. However, the use of antiviral drugs, no matter how potent or effective they are, inevitably leads to the emergence and selection of resistant strains of viruses, which are a huge problem in antiviral therapy [18, 19, 20]. Another matter of great concern is the high cost and toxicity of administered antiviral drugs [23]. Those factors forces scientists all over the world to seek new substances with virucidal activity and potential application in antiviral therapy.

Plants are a rich source of chemical compounds possessing various biological activities, including antiviral activity, and have shown significant antiviral activity even against viral strains resistant to conventional antiviral agents [1, 17].

The first step in the evaluation of this activity is screening of the virucidal activity of plant extracts. If the extract is found to be effective, further investigation, including determination of virucidal activity of isolated pure compounds can be performed in order to seek out the compound or compounds responsible for the activity of the extract [1, 11, 17].

In this study, the cytotoxicity and virucidal activity of sea fennel (*Crithmum maritimum* L, *Apiaceae*) extracts were examined.

MATERIAL AND METHODS

Plant material. The plant material – blossoming, aerial parts of *Crithmum maritimum* L., *Apaiceae* – was collected on the island of Rhodes. After identification, the plant material was dried at 35ºC and ground.

Extraction. The extracts were obtained with the use of laboratory water bath (LW, WSL, Poland). Samples of 10g were extracted under reflux condenser for 30 min with 100 ml of selected extractant – methanol or mixture of methanol and water (1:1 *v/v*). After the extraction, the solvent was removed under pressure and the samples for further examination were prepared by dissolving of selected dry extract in solvent or a mixture of solvents used for previous extraction to the concentration of 50 mg/ml.

Cell cultures. In all *in vitro* experiments GMK (Green Monkey Kidney) cell culture obtained from BIOMED Serum and Vaccine Production Plant Ltd in Lublin (Poland) was used.

The culture media (minimum essential medium Eagle's 1959) was supplemented with 10% of calf serum (BIOMED), 100 U/ml of penicillin (Polfa-Tarchomin, Poland) and 100 µg/ml of streptomycin (Polfa-Tarchomin, Poland). Cell cultures were incubated at 37ºC in the atmosphere containing 5% of CO_2.

Cytotoxicity assay. For cytotoxicity assays, portions of 100 µl of GMK cell culture were seeded into 96-well plates (NUNC) at a density of 2×10^4 cells per well. After incubation for 24 h at 37°C the medium was removed from the culture and different concentrations of tested extracts in culture media supplemented with 2% calf serum were added, while the dilution medium without the sample was used as the control. Final concentrations of tested *Crithmum maritimum* extracts were as follows: 0.025; 0.05; 0.1; 0.25; 0.5; 1; 2; 5 mg/ml. The cells subjected to plant extracts and control cultures were incubated at 37ºC in the atmosphere containing 5% CO_2 for 24–72 h

Cytotoxicity of the tested extracts was estimated with the use of MTT method, described by Takenouchi and Munekata [24]. MTT method is a colorimetric toxicity test, based on transformation of yellow, soluble tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple-blue insoluble formasane. This process occurs naturally in mitochondria of living cells. After dissolution in an organic solvent, formasane is quantified spectrophotometrically at two wavelengths – 540 and 620 nm in a multiwell scanning spectrophotometer (96-well plastic plates, Organon Teknika).

On the basis of the results, cytotoxic concentration (CC_{50}), which is the amount of the tested substance that caused 50% decrease of cell activity compared to control culture, was determined.

Virucidal activity. For the virucidal activity assessment of *Critchmum maritimum L.* extracts ECHO 9 virus (ATCC VR-1050) from American Type Culture Collection, belonging to *Picornaviridae*, was used. ECHO 9 virus was propagated in GMK cell culture. Virus stock was stored at - 70°C until use. The titre of the virus was $5x10^5$ $TCID_{50}$ ml^{-1}. The suspension of the virus was mixed (1:1 *v/v*) with the extracts diluted in media without serum, and the final concentration of the extracts were 0.5; 1 and 2 mg/ml (methanolic extract) and 1, 2, 5 mg/ml (methanol/H_2O extract). The concentrations of extractants used in this study were non-toxic to cell cultures and viruses. The mixtures were incubated at 37°C for 1 h and then the virus was titrated in the GMK cell culture. Virus diluted in the culture media without tested extracts was used as a control. After 24 h incubation at 37°C cytopathic effect (CPE) was assessed using Reed-Muench method (21). The analysis was performed in triplicates.

RESULTS

The influence of *Crithmum maritimum* L. extracts on the viability of GMK cells is shown in Table 1. Investigated methanolic and methanol/H_2O *Crithmum maritimum* L. extracts, after 72h incubation, were non-toxic for GMK cells at the concentrations 0.5–2 mg/ml and 1–5 mg/ml, respectively. The results of antiviral assays are shown in Table 2.

The virucidal effect was observed for both tested extracts at the concentrations non-toxic to cells. The methanolic extract at the concentration of 2 mg/ml decreased ECHO 9 replication by 1.37 log, and methanol/H_2O extract at the concentrations of 2 and 5 mg/ml by 1.14log.

Table 1. The influence of *Crithmum maritimum* L. on the viability of GMK cells. The results are estimated as the percentage of viability of GMK cells to the control cell culture

Extracts [mg ml^{-1}]	methanol			methanol/ H_2O (1:1 v/v)		
	exposition time (h)			exposition time (h)		
	24	48	72	24	48	72
	%	%	%	%	%	%
0 (control)	100.0	100.0	100.0	100.0	100.0	100.0
0.025	100.0	100.0	90.5	96.7	95.6	91.8
0.05	97.7	99.5	100.0	94.7	94.0	98.5
0.1	96.7	94.6	100.0	95.0	97.9	100.0
0.25	98.5	97.2	100.0	94.4	92.2	97.0
0.5	96.0	98.3	100.0	95.1	93.8	92.7
1	97.3	95.2	100.0	94.8	87.2	96.4
2	97.0	96.3	100.0	92.7	89.4	87.9
5	34.2	9.7	14.7	79.5	72.8	92.7

Table 2. Virucidal activity of the extracts from *Crithmum maritimum* L against ECHO 9 virus

ECHO 9 virus [$TCID_{50}$ ml^{-1}]*		
Extracts [mg ml^{-1}]	methanol	methanol/ H_2O (1:1 v/v)
0 (control)	3.37 ± 0.13	4.14± 0.19
0. 5	3.31 ± 0.51	-
1	3.71 ± 0.23	3.6 ± 0.15
2	2.0 ± 0.44	3.0 ± 0.39
5	-	3.0 ± 0.13

*The virus titers are shown in log

DISCUSSION

Many plant extracts and isolated compounds, belonging to various classes of compounds, were found to possess antiviral activity. Among different classes of plant constituents, antiviral properties were documented for furocoumarins and furanochromones, polysaccharides, alkaloids (b-carbolines, furanoquinolines, camptothecin, atropine, caffeine, castanospermine), terpenoids (sesquiterpenes, triterpenoids – moronic acid, ursolic acid, maslinic acid), lignans (podophyllotoxin and related lignans such as the peltatins, dibenzocyclooctadiene lignans such as schizarin B and taiwanschirin D) and lectins (isolated from *Canavalia ensiformis*, *Lens culinaris*, *Phaseolus vulgaris*, *Triticum vulgaris*), but the most promising class are phenolic compounds (caffeic acid, tannins, proanthocyanidins, phenylpropanoid glycosides); however, none of those compounds is potent and safe enough to be used in official treatment. For some compounds the mechanism of their antiviral action was discovered (inhibition of the formation of viral DNA or RNA, inhibition of the activity of viral reproduction), but further studies are required. The discovery of mechanism of antiviral action of plant constituents may lead to the development and synthesis of new antiviral drugs [8, 11, 13, 17].

For plants belonging to the *Apiaceae,* antiviral activity was proven both for DNA and RNA viruses. What is more, this activity was observed for extracts, as well as for isolated compounds. It was discovered that plants from *Apiaceae* possess antiviral activity against HBV [6, 10, 27] and Coxsackie B viruses [5]. The investigation of biological activity of saikosaponins from *Bupleurum sp*. revealed their inhibitory effect on replication of HBV, Herpes Simplex type 1 and 2, influenza virus and HIV, through a direct influence on replication process [2, 3]. Karagöz et al. found that

different extracts from *Sanicula europea* L. possess antiviral activity against Human parainfluenza type 2 [12].

According to some reports plants extracts demonstrate antiviral activity against DNA rather than RNA viruses [4, 26].

Our experiment was intended to assess the virucidal properties of different extracts from the aerial parts of *Crithmum maritimum* L. Previous phytochemical studies and biological assays performed on *Crithmum maritimum* L. allowed to discover the main constituents and pharmacological properties, including antifungal [14], antioxidant [16, 22], antibacterial and anti-inflammatory properties [7] of this herb. What is more, essential oil obtained from *Crithmum maritimum* L. possesses antifungal [15] and antibacterial activity against Gram-positive and Gram-negative bacteria [9, 14, 22, 25].

CONCLUSIONS

In the presented study we have established a safe concentration of extracts in order to avoid extract-induced cell damage in following antiviral test. A slight virucidal activity of *Crithmum maritimum* L. was observed for both extract, but further studies need to be performed in order to find compounds responsible for this action and to seek out the possible mechanism of action. What is more, antiviral activity against other viruses has to be evaluated. As far as we know, this is the first report on cytotoxicity and virucidal activity of *Crithmum maritimum* L.

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SUMMARY

The investigation of cytotoxic activity of methanolic extracts from the aerial parts of *Crithmum maritimum* L (sea fennel) was carried out on GMK cell cultures. The investigated extracts were diluted in culture media containing 2% of calf serum to the concentration of 0.025; 0.05; 0.1; 0.25; 0.5; 1; 2; 5 mg/ml. The cultures were incubated at 37ºC in the atmosphere with 5% of CO_2 for 24–72 h. Cytotoxicity was determined using the colorimetric MTT (tetrazolium) method. After estimation of highest non-toxic concentration of extracts, the virucidal activity against ECHO 9 was assessed. Doses for methanolic and methanol/H_2O extracts non-toxic to GMK were established at the concentrations of 2 mg/ml and 5 mg/ml, respectively. A slight virucidal effect was observed for both extracts. The methanolic extract decreased ECHO 9 replication by 1.37 log, and methanol/H_2O extract by 1.14log.

STRESZCZENIE

Ocenę aktywności cytotoksycznej ekstraktów przeprowadzono wobec komórek linii GMK. Badane substancje rozcieńczano w płynie hodowlanym z dodatkiem 2% surowicy cielęcej do końcowego stężenia 0,025; 0,05; 0,1; 0,25; 0,5; 1; 2; 5 mg/ml. Komórki poddane działaniu ekstraktów inkubowano w 37ºC w obecności 5% CO_2 przez 24–72 h. Cytotoksyczność substancji oceniano przy użyciu testu formazanowego MTT. Po określeniu najwyższego nietoksycznego stężenia substancji oceniano ich aktywność wirusobójczą wobec wirusa ECHO 9. Ustalono następujące nietoksyczne dawki: metanolowego ekstraktu (100%) 2 mg/ml i metanolowo-wodnego ekstraktu (50% metanol) 5 mg/ml. Niewielkie wirusobójcze działanie wykazały obydwa ekstrakty. Ekstrakt metanolowy spowodował obniżenie poziomu replikacji wirusa o 1,37 log w stosunku do kontroli, natomiast ekstrakt metanolowo-wodny (2 mg/ml i 5 mg/ml) o 1,14 log.