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Modified natural clinoptilolite with quercetin and quercetin dihydrate and the study of their anticancer activity

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ABSTRACT

Natural zeolite of the clinoptilolite type (CT, particle size up to 200 μm) and its modified forms with different content (0.06–5%) of pharmaceutically active compounds (type of flavonoids): quercetin (Q) and quercetin dihydrate (QD) have been investigated by thermal analyses TG, DTA and DTG, FTIR spectroscopy, fluorescence spectroscopy, X-ray powder diffractometry, determination of the surface areas and the pore volumes by low-temperature adsorption of nitrogen and atomic force microscopy. The analyses checked the presence of Q or QD in modified zeolitic products (CTQ and CTQD).

Natural zeolite of the clinoptilolite type and their modified forms CTQ and CTQD with low content (0.06% and 1%) of Q and QD have been used for the study of their anticancer activity. Carcinoma cell lines Jurkat, CEM, HeLa, MCF7, A549 and MDA were treated with various amounts of natural clinoptilolite and their modified forms CTQ and CTQD. The water content of the channel system influences the cytotoxicity of zeolite. The results of the study of pure CT thermally activated at two different temperatures 110 and 400 °C confirmed the better cytotoxicity of CT activated at 110 °C (CT110) with higher content of water in comparison with CT activated at 400 °C (CT400). Clinoptilolite modified with quercetin dihydrate (CTQD) has shown better cytotoxicity compared with clinoptilolite modified with quercetin (CTQ).

1. Introduction

Natural zeolite of the clinoptilolite type is a unique volcanic mineral, one of the world's most abundantly occurring and most abundantly used zeolitic minerals. At present it is widely used in many fields of agriculture, agrochemistry, industry and building industry, ecology [1–6], but also in other areas such as medicine and pharmacy [7–10].

Recently intensive research is focused on the biological activity of natural clinoptilolite based on its structure as well as ion exchange, sorption and catalytic properties. Well defined structures and catalytic activity make aluminosilicates attractive model systems for protein and enzyme mimetics [7]. The best known positive biological activity of natural clinoptilolite is its action as antidiarrheic drug [11]. Natural clinoptilolite can be effective as glucose adsorbent [7,12] and as immunostimulant [13]. Immunostimulative effect of zeolite caused local inflammation that resulted in activation of intestinal macrophages with consequent release of cytokines, and activation of T-cell immunological response [14]. Some other mechanism were reported saying that serotonin (5-hydroxytryptamine 5-HT) regulates the immune system and serotonergic receptors are found on immunocompetent cells, while proinflammatory cytokines are responsible for 5-HT release [13]. Ability of clinoptilolite to influence signaling pathways inducing immunity can also explain the clinoptilolite effect on wound healing [7]. On the other hand, clinoptilolite has been shown to induce a better cell growth in hybridoma cell cultures by reducing the ammonia in the medium [14].

Antitumor effect of clinoptilolite and its potential role as adjuvant in anticancer therapy is known in two basic forms, as liquid zeolite and powdered micronized zeolite. Clinoptilolite has antitumor effect in vitro due to its indirect action on the cell media and the consequent effects on cell viability and underlying activity involved in signaling pathways regulating cell division, survival/apoptosis and stress response. Zeolite as food supplement in low doses changes the serum ion composition. Due to its ionic charge, zeolite is attracted to cancer cells only. There is relative insensitivity of normal cells to clinoptilolite effect in comparison with tumor cells [7]. Clinoptilolite effect is due to a partial adsorption of

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Table 1
Content of Q and QD in the zeolitic samples and the amount of zeolite and solution of Q or QD used during of preparation of modified zeolitic samples.

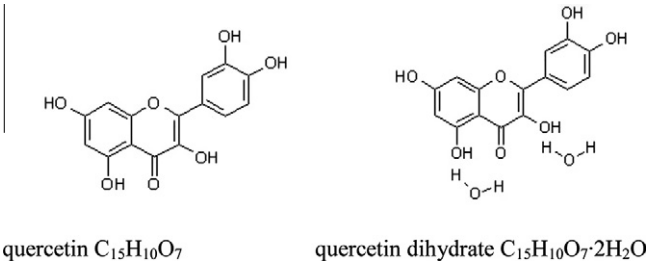
Samples	Average content of Q or QD	Precise content of Q or QD	Amount of used clinoptilolite during preparation (g CT)	Amount of used solution and its concentration during preparation
CT110Q	0.06% Q	0.06% Q	1.5	3 ml 1 mM Q
CT400Q	0.06% Q	0.06% Q	1.5	3 ml 1 mM Q
CT110QD	0.06% QD	0.067% QD	1.5	3 ml 1 mM QD
CT400QD	0.06% QD	0.067% QD	1.5	3 ml 1 mM QD
CT110Q	1% Q	0.8986% Q	1.5	4.5 ml 10 mM Q
CT400Q	1% Q	0.8986% Q	1.5	4.5 ml 10 mM Q
CT110QD	1% QD	1.0046% QD	1.5	4.5 ml 10 mM QD
CT400QD	1% QD	1.0046% QD	1.5	4.5 ml 10 mM QD
CT110Q	1.5% Q	1.49% Q	1	5 ml 10 mM Q
CT400Q	1.5% Q	1.49% Q	1	5 ml 10 mM Q
CT110QD	1.5% QD	1.66% QD	1	5 ml 10 mM QD
CT400QD	1.5% QD	1.66% QD	1	5 ml 10 mM QD
CT110Q	5% Q	4.79% Q	1.5	5 ml 0.05 M Q
CT400Q	5% Q	4.79% Q	1.5	5 ml 0.05 M Q
CT110QD	5% QD	5.34% QD	1.5	5 ml 0.05 M QD
CT400QD	5% QD	5.34% QD	1.5	5 ml 0.05 M QD
Physical mixture CT + Q low	0.06% Q	0.06% Q	1.1	0.000906 g Q
Physical mixture CT + Q high	5% Q	4.79% Q	1.1	0.05541 g Q

CT110: thermally activated natural zeolite of the clinoptilolite type at 110 °C.
CT400: thermally activated natural zeolite of the clinoptilolite type at 400 °C.

growth factors from serum in the medium. The results of the studies [7] partially explained clinoptilolite effect on serum, extracellular liquid and liquid in gastrointestinal tract.

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 of tumors size. Local application of clinoptilolite to skin cancers of dogs effectively reduced tumor formation and growth. Toxicology studies on mice and rats demonstrated that the treatment does not have negative effects. In vitro tissue culture studies showed that finely ground clinoptilolite inhibits protein kinase B (c-Akt), induces expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ tumor suppressor proteins, and blocks cell growth in several cancer cell lines [9].

Latest results in vitro and in vivo research indicate that some dietetic supplements do indeed show anticancer activity. The strongest anticancer action has been demonstrated by antioxidants which modulate the activity of protein kinases. Polyphenols from plant extracts show the strongest anticancer activity [8,15–17]. Quercetin (Q) and quercetin dihydrate (QD) are naturally occurring flavonoid compounds found in most plant tissues [13,18–21]. Laboratory studies show they may have anti-inflammatory and antioxidant properties [18,19]. They are studied for apparent beneficial effects including cardiovascular protection, anti-cancer activity, anti-ulcer effects, antihistaminic activity, cataract prevention and antiviral activity [13,19–21]. The flavonoid aglycone constituents were found to be selectively adsorbed on the clinoptilolite surface. The antioxidant activity measurements performed for the Ginkgo biloba leaf extract solutions showed decreasing antioxidant activities due to adsorption. The decrease in antioxidant activity was attributed to the adsorption of phenolic constituents on the clinoptilolite surface [22]. The studies of quercetin adsorption on the surface of the medicinal carbonic adsorbent showed that quercetin does not loose its antioxidative activity. However, adsorption leads to instability of quercetin caused mainly by oxidation during storage [23].



quercetin $C_{15}H_{10}O_7$ quercetin dihydrate $C_{15}H_{10}O_7 \cdot 2H_2O$

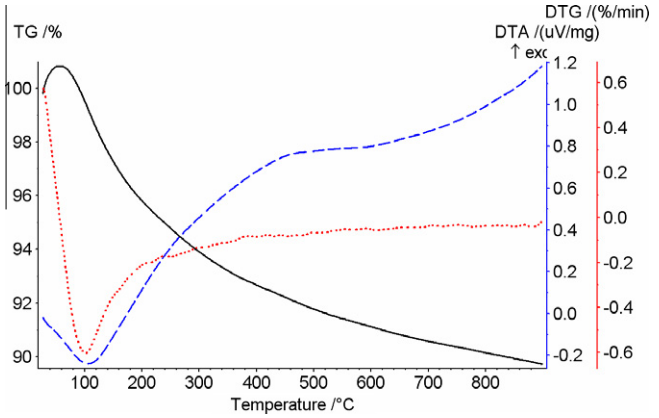


Fig. 1. TG (—), DTA (- - -) and DTG (···) curves of natural zeolite of the clinoptilolite type (CT).

In the previous studies concerning the possibility of application of natural zeolite of the clinoptilolite type from Nižný Hrabovec the attention was also focused on its use in the field of veterinary pharmacy. Natural zeolite of the clinoptilolite type was studied as an additive to animal feed in very small amounts (1–2%) [2,24,25], as a carrier of pharmaceuticals for veterinary pharmacy [26–29] and as a solid disinfectants [2,30].

The aim of our present study was the preparation of modified forms of natural zeolite of the clinoptilolite type with different content of pharmaceutically active compounds: quercetin and quercetin dihydrate and the study of their anticancer activity.

In this paper the attention is focused on altering modes of preparation of modified forms of natural zeolite of the clinoptilolite type with different quercetin or quercetin dihydrate content and characterization of the obtained zeolitic products by CHN analyses, thermal analysis (TG, DTA and DTG), IR spectroscopy, fluorescence

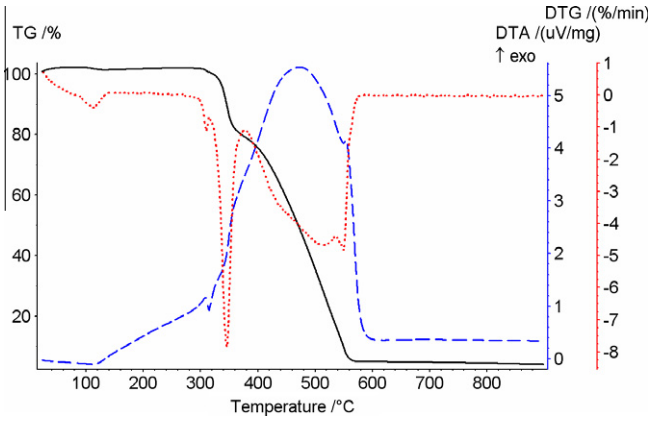


Fig. 2. TG (—), DTA (- - -) and DTG (···) curves of quercetin (Q).

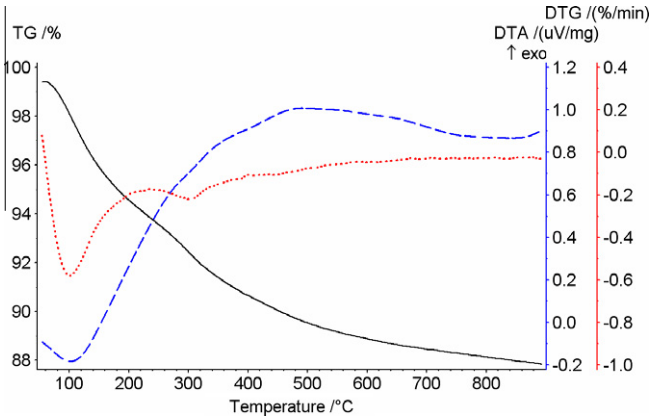


Fig. 3. TG (—), DTA (- - -) and DTG (···) curves of modified zeolitic product with 1% of quercetin CT110Q(1%).

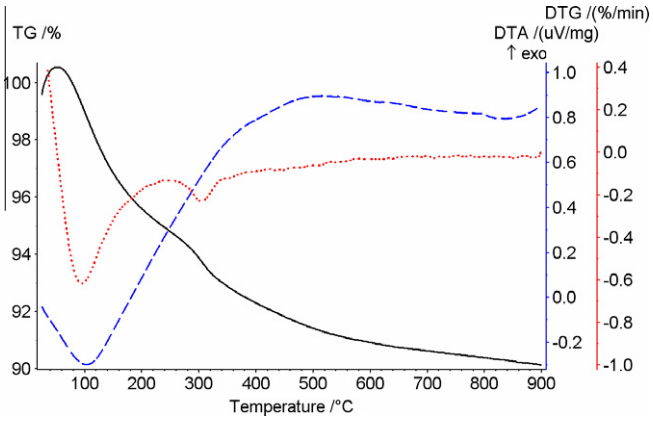


Fig. 4. TG (—), DTA (- - -) and DTG (···) curves of modified zeolitic product with 1% of quercetin dihydrate CT110QD(1%).

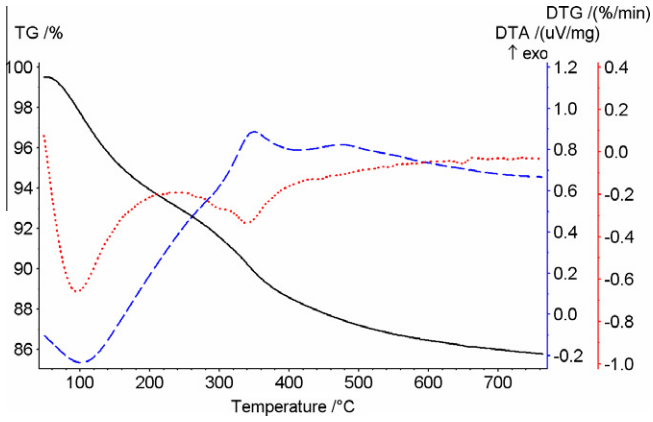


Fig. 5. TG (—), DTA (- - -) and DTG (···) curves of modified zeolitic product with 5% of quercetin CT400Q(5%).

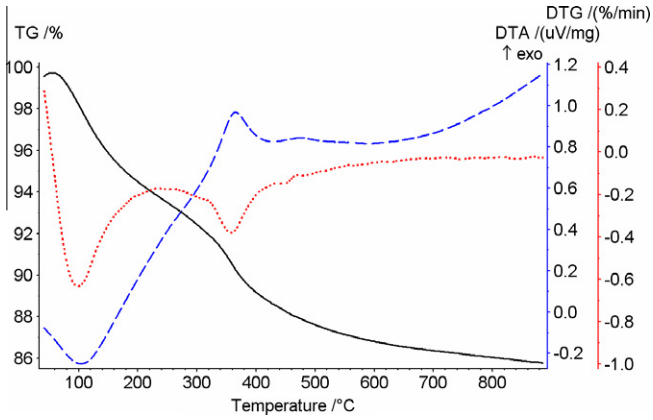


Fig. 6. TG (—), DTA (- - -) and DTG (···) curves of modified zeolitic product with 5% of quercetin CT400QD(5%).

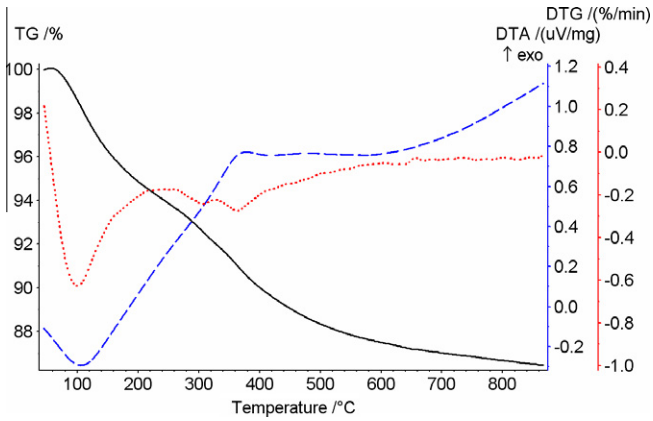


Fig. 7. TG (—), DTA (- - -) and DTG (···) curves of natural zeolite of modified zeolitic product with 5% of quercetin CT110Q(5%).

spectroscopy, X-ray powder diffractometry, analysis of the surface areas and the pore volumes by low-temperature adsorption of nitrogen and atomic force microscopy. The cytotoxic effect of natural zeolite of the clinoptilolite type (CT110 and CT400) and its modified forms with low content (0.06% and 1%) of Q and QD were studied on cancer cell lines Jurkat (human T cell leukemia cells), HeLa (cervical carcinoma cells), CEM (human lymphoid cells), MCF7 (breast cancer cells), A549 (carcinomic human alveolar basal epithelial cells) and MDA (human breast adenocarcinoma cells).

2. Experimental

2.1. Chemicals and materials

In our study we have used natural zeolite of the clinoptilolite type (fine-grained, the particle size up to 200 μm, content of clinoptilolite 84%) from the East Slovakian deposit in Nižný Hrabovec. Natural clinotilolite was thermally activated at two different tem-

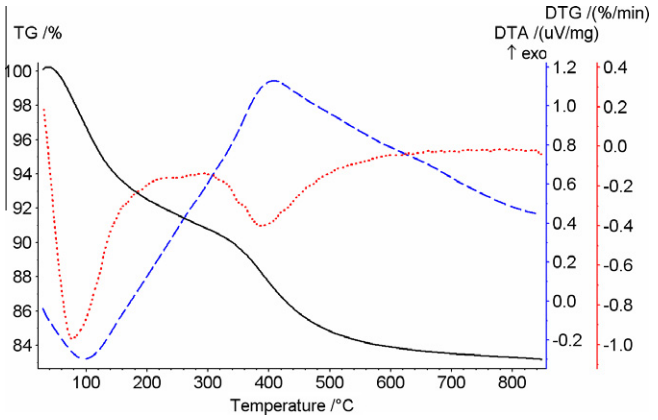


Fig. 8. TG (—), DTA (- - -) and DTG (···) curves of physical mixture of natural clinoptilolite (CT110) and 5% of quercetin (Q).

Table 2
Characteristic bands on IR spectra of natural zeolite of the clinoptilolite type.

ν (cm^{-1})	Vibrations
3620	Asymmetric stretching vibration of H_2O
3440	Symmetric stretching vibration of H_2O
1632	Bending vibration of H_2O
1207, 1055	Asymmetric stretching vibrations of tetrahedra
796, 720	Symmetric stretching vibrations of tetrahedra
466	O–T–O bending vibration

Table 3
Characteristic bands on IR spectra of quercetin.

ν (cm^{-1})	Vibration
3367	O–H stretching vibration
1670	C=O stretching vibration
1609, 1511	Aromatic C=C stretching vibrations
1363–1160	C–C, C–O skeleton vibrations
1090–599	Out-of-plane bending vibrations of aromatic C–H groups

peratures: at 110 °C for 2 h, denoted as CT110 and at 400 °C for 1 h, denoted as CT400. For cytotoxicity tests a part of natural clinotilolite was irradiated by germicidal lamp (special type of lamp which produces ultraviolet light).

Flavonoids – quercetin (Q) and quercetin dihydrate (QD) and other chemicals were of p.a. purity, quercetin from Lachema Brno, Chemapol, quercetin dihydrate from Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, Pécs, Hungary. Purity of QD was checked by TLC (thin layer chromatography) and GC (gas chromatography) methods. The compounds have been used without further purification. Absolute ethanol for UV spectroscopy was obtained from Merck, Germany. Immediately before measurements of fluorescence, Q and QD were dissolved in ethanol (c = 1 mM) and kept in the dark.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was from Sigma–Aldrich Chemie (Steinheim, Germany).

Table 4
Fluorescence maxima λ_{max} (nm) or fluorescence intensity of studied samples in the synchronous fluorescence spectra SFS ($\Delta\lambda$ = 70).

Studied samples	λ_{max} (nm)	F
Q	440	257
QD	440	187
CT	472	16
CTQ	450	39
CTQD	449	38

Table 5
Fluorescence maxima of Q and QD fluorophores in the excitation/emission and synchronous spectra.

Fluorophore (sample)	$\lambda_{ex}/\lambda_{em}$ (nm)	F	$\lambda_{ex}/\Delta\lambda$ (nm)	F
Q	440/520	268	441/70	388
QD	440/520	192	437/80	252

2.1.1. Material preparation

Four series of modified forms with different amount of quercetin (Q) or quercetin dihydrate (QD) were prepared. The average content of Q or QD is:

0.06%: content of Q = 0.06%, content of QD = 0.067%,
1%: content of Q = 0.8986%, content of QD = 1.0046%,
1.5%: content of Q = 1.4888%, content of QD = 1.66%,
5%: content of Q = 4.79%, content of QD = 5.34%.

Modified forms of natural clinoptilolite with different content of quercetin or quercetin dihydrate (Table 1) were prepared:

Thermally activated natural clinoptilolite (CT110 or CT400) was mixed with quercetin or quercetin dihydrate solution of different concentrations: 0.1 mmol dm^{-3} (0.1 mM), 10 mmol dm^{-3} (10 mM) and 0.05 mol dm^{-3} (0.05 M). Solutions of Q or QD were prepared with ethyl alcohol. The heterogeneous mixture of natural clinoptilolite and pharmaceutical active compound (Q or QD) were

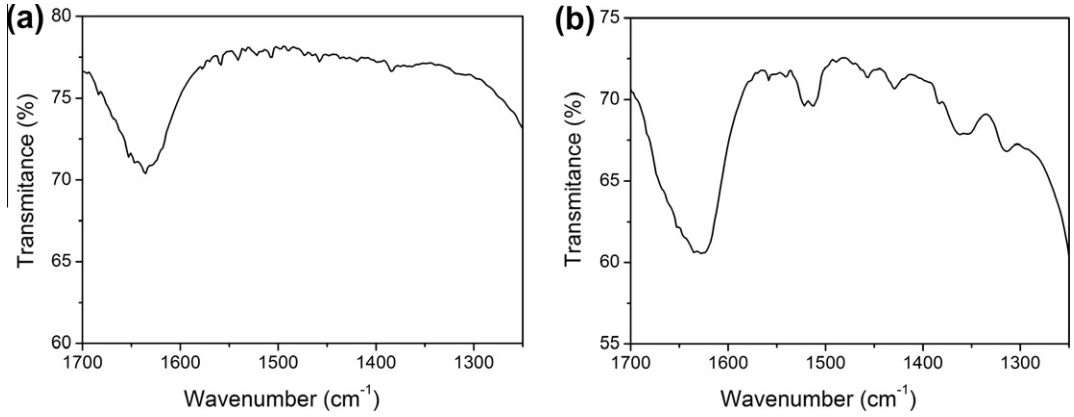


Fig. 9. IR spectrum of: (a) modified zeolitic product CT110Q(5%), (b) physical mixture of natural clinoptilolite (CT110) and 5% of quercetin (Q).

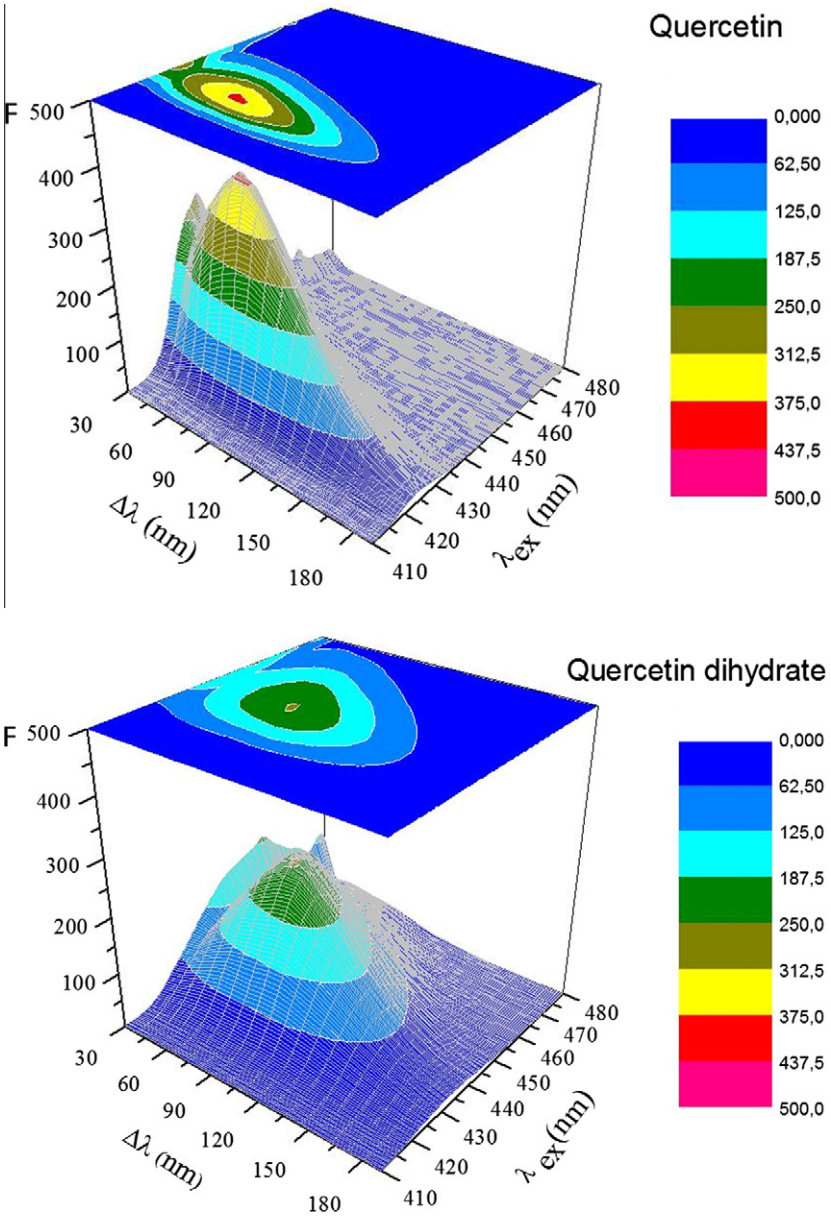


Fig. 10. Three dimensional synchronous fingerprint of Q and QD. Circular pattern of SFF is result of fluorescence, which is characteristic fingerprint of Q and QD. In the center of Q fingerprint is fluorescence maximum F_{max} = 388 with spot height at ($\Delta\lambda$ = 70/λ_{ex} = 441) nm. In the center of QD fingerprint is fluorescence maximum F_{max} = 252 with spot height at ($\Delta\lambda$ = 80/λ_{ex} = 437) nm.

three times stirring (15–20 min) and then dried at room temperature in a small desiccator over silica gel (the samples of modified forms were in open dark container). The products were denoted simple as CTQ and CTQD or CT110Q, CT400Q, CT110QD, CT400QD and in parentheses with content of Q or QD, e.i. CT110Q(1.5%).

For comparison two physical mixtures were prepared: CT110 + Q with low content of Q (0.06%) and CT110 + Q with high content of Q (5%).

2.1.2. Cell lines and culture

The following human cancer cell lines were used for this study: Jurkat (human T cell leukemia cells), HeLa (cervical carcinoma cells), CEM (human lymphoid cells), MCF7 (breast cancer cells), A549 (carcinomic human alveolar basal epithelial cells) and MDA (human breast adenocarcinoma cells). All cell lines used were kindly provided by Dr. Hajduch (Olomouc, Czech Republic). Jurkat, HeLa and CEM cells were cultured in RPMI 1640 medium. MCF7, A549 and MDA were cultured in growth medium consisting of high glucose DMEM medium. Both media were with Glutamax-supplemented with 10% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all from Invitrogen, USA), in a humidified air atmosphere of 5% CO_2 at 37 °C. Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

2.2. Methods

2.2.1. Analytical methods

CHN elemental analyses were performed on a Perkin Elmer 2400 Elemental Analyzer. Thermal analyses (TG, DTA and DTG) were carried out at temperature up to 800 °C in air on a NETZSCH STA 409 PC/PG under the conditions: sample weight 20 mg, heating rate 10 °C/min, Al_2O_3 crucible.

Infrared spectra were obtained with KBr disc technique in the range 400–4000 cm^{-1} using AVATAR 330 FT-IR Thermo Nicolet IR spectrometer.

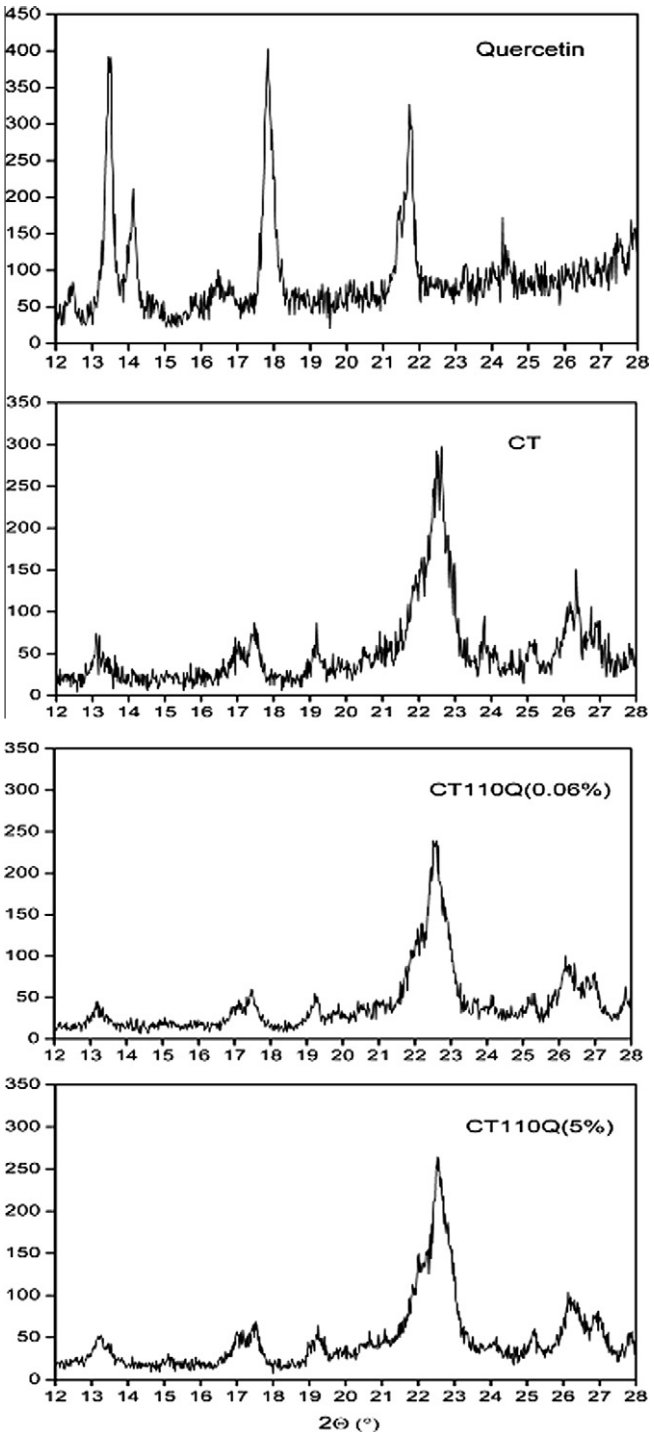


Fig. 11. The X-ray diffraction patterns of quercetin Q, CT110, CT110Q(0.06%) and CT110Q(5%).

The fluorescence spectra were run on a Perkin–Elmer Model LS 55 Luminescence spectrophotometer in range of wavelengths (400–600 nm) by single excitation, emission, synchronous spectra and synchronous fluorescence fingerprint (SFF). The excitation and emission maxima of Q and QD in ethanol were investigated by single excitation and emission spectra. For scanning of synchronous spectra, the wavelength difference between the excitation and emission monochromators was adjust to $\Delta\lambda = 70$ nm. The synchronous technique SFF consists of varying simultaneously both excitation and emission wavelengths while keeping a constant wavelength interval $\Delta\lambda$ between them, number of synchronous spectra scan

Table 6
The surface areas (S_{BET}) and pore volumes of the CT and studied zeolitic samples with different content of Q and QD.

Sample	Average content of Q or QD (%)	S_{BET} (m^2/g)	Pore volume (cm^3/g)
CT	–	27.3205	0.0431
CT110Q	1	21.3204	0.0372
CT110QD	1	20.9963	0.0383
CT110Q	1.5	19.3507	0.0324
CT110QD	1.5	18.9767	0.0303
CTQ110	5	15.7248	0.0261
CTQD110	5	13.7802	0.0232

20 with increment 10 (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200).

The wavelength scan speed of both monochromators was 1200 nm/min. Setting of instrument's excitation slit was 10 nm and emission slit was 15 nm. Data processing was managed by the Winlab software package of Perkin Elmer. A quartz cuvette (1 cm) with a volume of 3.0 ml was used for all fluorescence measurements at room temperature.

X-ray powder diffraction patterns were recorded with reflecting technique. A Philips X'pert diffractometer with $\lambda = CuK_\alpha$ radiation and an aluminum same holder without internal standard was used.

The analyses of surface areas and the pore volumes of the zeolitic samples were realized by GEMINI 2360 (Micrometrics, USA). The specific surface area was determined by low-temperature adsorption of nitrogen. Before measurements the samples were heated for 2 h at 105 °C.

Topographic images of Q and QD layers deposited on the glass slides were carried out by atomic force microscope (ICON, Bruker, USA) in tapping mode with silicon tips (MikroMasch, NSC35 series) with radius of curvature ~10 nm. Quercetine and quercetine dihydrate layers were prepared by pipetting a droplet of 50 μl of Q or QD (0.025 mol l^{-1}) in ethanol on the glass slides, which were dried at room temperature.

2.2.2. Biological application – cytotoxicity assay

The cytotoxic effect of CT110 and CT400 was studied by using colorimetric microculture assay with the MTT endpoint [31]. The method is based on conversion of a tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in cells to insoluble formazan. The conversion depends on mitochondrial dehydrogenases of living cells. Briefly, 8×10^4 cells were plated per well in 96 well polystyrene microplates (Sarstedt, Germany) in the culture medium containing the tested CT110 and CT400 at the doses of 5, 10 and 20 mg. After 72 h of incubation, 10 μl of MTT (5 mg/ml) were added in each well. After additional 4 h, during which insoluble formazan was produced, 100 μl of 10% sodium dodecylsulphate were added in each well and another 12 h were allowed for the formazan to be dissolved. The absorbance was measured at 540 nm using the automated MRX microplate reader (Dynatech Laboratories, UK). The absorbance of the control wells was taken as 100% and the results were expressed as a percentage of the control.

3. Results and discussion

Natural zeolite of the clinoptilolite type (CT, fine grained, the particle size up to 200 μm) from the East Slovakian deposit in Nižný Hrabovec have been used for preparation of modified zeolitic products with different content (on average: 0.06%, 1%, 1.5% and 5%) of quercetin (CTQ) or quercetin dihydrate (CTQD). Natural clinoptilolite (CT) as well as their modified forms (CTQ, CTQD) with low content of quercetin or quercetin dihydrate (0.06% and 1%)

were used for the study of their anticancer activity. The zeolitic products with higher content of Q or QD were prepared only for better characterization of these modified forms.

Natural clinoptilolite is as a slowly releasing carrier of pharmaceutical active compounds: quercetin or quercetin dihydrate.

Before preparation natural clinoptilolite was thermally activated at two different temperatures: at 110 °C (CT110) and at 400 °C (CT400). The results of the first biological tests of anticancer activity of natural clinoptilolite showed higher anticancer activity of CT110 in comparison with CT400. Natural clinoptilolite thermally activated at the temperature 110 °C contains a higher content of water. It can contribute to its higher biological activity.

Modified zeolitic forms with quercetin or quercetin dihydrate were analyzed by the CHN analyses, thermal analyses – TG, DTG, DTA, FTIR spectroscopy, fluorescence spectroscopy, X-ray powder diffractometry, determination of the surface areas and the pore volumes by low-temperature adsorption of nitrogen and atomic force microscopy.

CHN analyses were carried out only for comparison of content of carbon in starting zeolitic samples CT and modified zeolitic samples CTQ and CTQD. In all cases the content of carbon was higher in modified samples due to the presence of Q and QD.

3.1. Thermal analysis

The methods of thermal analysis significantly contributed to the characterization of sorption processes of quercetin and quercetin dihydrate by natural clinoptilolite. The results of thermal analyses clearly show different properties of the natural clinoptilolite (Fig. 1), pure quercetin (Fig. 2) and modified forms CTQ or CTQD with different content of Q or QD (Figs. 3–7). The modified zeolitic products containing Q or QD have different TG, DTG and DTA curve in comparison with starting zeolite CT. Fig. 8 shows the physical mixture of CT110 and 5% of Q for comparison with modified sample CT110Q(5%) containing 5% of Q (Fig. 7).

By thermal activation (by heating) of CT to 110 °C about 13% (1/8) of the total amount of water disappears. This comes from the most remote sites which thus can be replaced by for instance Q or QD molecules. By thermal activation to 400 °C about 67% (2/3) of the total water is released which gives space to more hidden molecules. However after thermal activation the zeolitic samples were cooled in a closed small desiccator and during this cooling process a small part of water from air atmosphere can be again resorbed.

In temperature range from 260 to 550 °C on TG curve (Fig. 1) is the continuous weight loss slope, typical for a true zeolite. Anomalies from the smooth descent TG indicate that even a small addition of 0.06% Q, surprisingly, is observed.

3.2. IR spectroscopy

Infrared spectroscopy was used to characterize the modified zeolitic products containing quercetin (CTQ) or quercetin dihydrate (CTQD) in comparison with starting natural zeolite of the clinoptilolite type (CT), quercetin (Q) and quercetin dihydrate (QD). Characteristic absorption bands of natural zeolite of the clinoptilolite type are in Table 2. The results are in a good agreement with literature [32]. Characteristic absorption bands of quercetin are in Table 3.

No significant changes were observed on the IR spectra of original natural zeolite CT and its modified zeolitic product CTQ and CTQD containing different amount of Q or QD. A very small change appeared in the region from 1300 to 1550 cm^{-1}.

IR spectra of modified zeolitic product with 5% of quercetin CT110Q(5%) and physical mixture of natural clinoptilolite (CT110) and 5% of quercetin (Q) in the range from 1700 to 1250 cm^{-1} are depicted in Fig. 9. On the IR spectra of the modified zeolitic product CT110Q(5%) the absorption bands corresponding to the quercetin appeared only in the in the range from 1300 to 1550 cm^{-1} (Fig. 9a) with very low intensity in comparison with physical mixture (Fig. 9b) of natural zeolite (CT110) and 5% of quercetin (Q).

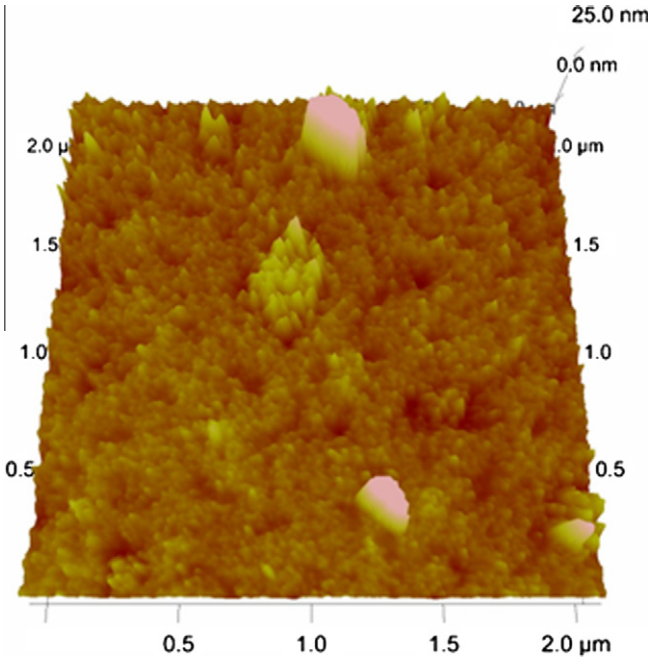


Fig. 12. Three-dimensional topographic image shows the surface of quercetine.

3.3. Fluorescence spectroscopy

Synchronous fluorescence spectra (SFS) measured at constant difference setting wavelengths ($\Delta\lambda = 70$ nm) between excitation and emission monochromators showed graphically, that molecules Q and QD interacts with zeolite (Table 4).

The results showed, that SFS fluorescence maxima of both fluorophores are placed at the same wavelength $\lambda_{max} = 440$ (nm). The lower fluorescence had QD ($F = 187$) in comparison with Q ($F = 257$). Quenching of QD fluorophore fluorescence causes the presence of two water molecules.

The modified zeolite with both fluorophores Q and QD significantly decreased fluorescence. The presence of zeolite caused the fluorescence maxima hypsochromic shift of CTQ modification about 22 nm and CTQD about 23 nm in the comparison with natural zeolite CT. Natural zeolite CT was the least fluorescent. Zeolite is not autofluorescent, but water is a very weak fluorophore (Table 5).

Molecules of Q and QD fluorophores were characterized by simple excitation and emission spectra (Table 5) and by three dimensional method of fluorescence fingerprint SFF (Fig. 10), which is used for comparison of spectra and reveals differences between spectra. Contour map is a planar projection, which is formed by connection of points with the same intensity of fluorescence. The result is a characteristic selective fingerprint, which identifies exactly the investigated material. Fluorescence intensity is spectral quantity characterized analogically as spot height on the maps. The fluorescence maxima in the centers of fluorophores Q (441/70, $F = 388$) and QD (437/80, $F = 252$) were identified by SFF method and revealed differences between them (Table 5), which are not visible at the first sight (Fig. 10).Excitation of Q or QD is placed at violet area of UV/VIS spectra (380–455 nm), while emission of these substances is in blue green area (495–575 nm).

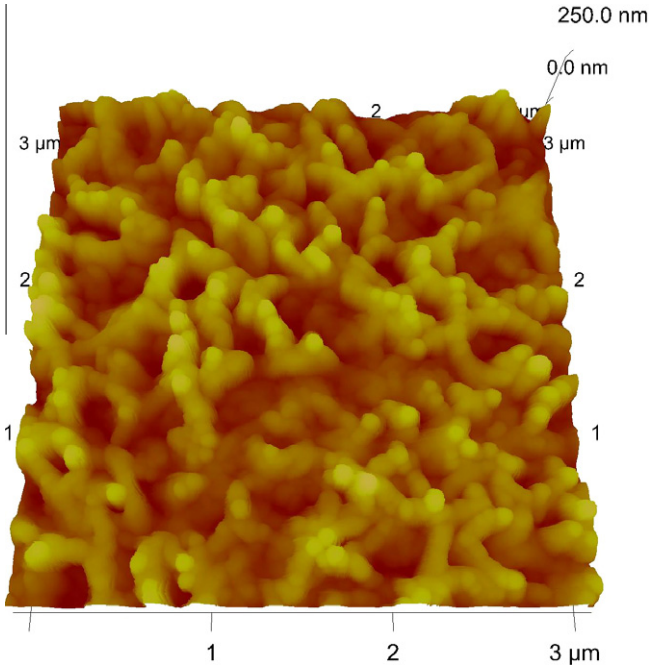


Fig. 13. Three-dimensional topographic image shows the surface of quercetine dihydrate.

3.4. X-ray diffraction analysis

The diagrams of the binary reaction products as well as the physical mixtures show one crystalline phase only i.e. the zeolite matrix. The detection limit of a minority part is around 4–5% which is the maximum content of Q or QD in those preparations.

Fig. 11 shows X-ray powder diffraction patterns of quercetin (Q), natural zeolite of the clinoptilolite type (CT110) and its modified products CT110Q(0.06% Q) containing 0.06% of quercetin and CT110Q(5% Q) containing 5% of quercetin. Due to this X-ray reflecting technique is not a decisive method for an interpretation in molecular terms. However, these data show that the zeolite behaves as a true zeolite i.e. no change in the cell dimensions is seen in spite of introductions of water and of Q or QD molecules.

Table 7
Effect of zeolite CT on the viability (%) of different cancer cell lines.

Zeolite CT110 and CT400 added to cancer cell lines		Viability of cancer cell lines in (%)					
	Zeolite CT	Jurkat	HeLa	CEM	MCF7	A549	MDA
		5 mg of modified zeolite added to cancer cell lines					
Non-UV irradiated zeolite	CT110	0	4.7	0	14.1	13.3	11.2
	CT400	0	6.9	0	33.4	32.9	28.0
UV irradiated zeolite	CT110	0	13.7	0	32.5	14.4	15.2
	CT400	0	14.2	0	26.5	21.5	35.4
		10 mg of modified zeolite added to cancer cell lines					
Non-UV irradiated zeolite	CT110	0	0	0	6.1	6.1	7.6
	CT400	0	0	0	22.1	22.9	31.7
UV irradiated zeolite	CT110	0	0	0	8.6	5.4	9.5
	CT400	0	0	0	19.6	19.0	38.3
		20 mg of modified zeolite added to cancer cell lines					
Non-UV irradiated zeolite	CT110	0	0	0	2.3	0.2	1.1
	CT400	0	0	0	13.3	9.5	27.9
UV irradiated zeolite	CT110	0	0	0	0.7	0.3	2.4
	CT400	0	0	0	12.2	9.6	23.9

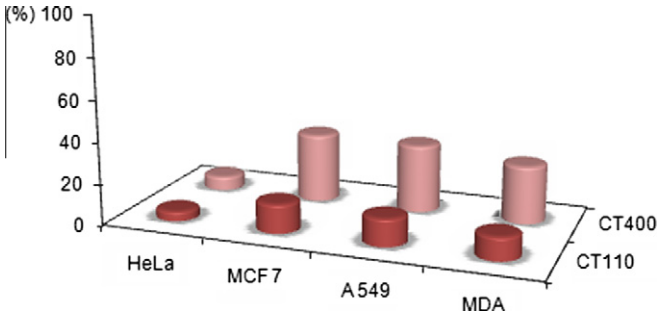


Fig. 14. Diagram for comparison of the viability of selected tumor cell lines after the addition of unirradiated natural zeolite (5 mg) thermally activated at two different temperatures: 110 °C (CT110) and 400 °C (CT400).

3.5. Determination of surface areas and pore volumes

The results of the study of the surface areas changes of natural zeolite of the clinoptilolite type and its modified forms with Q and QD are in a good agreement with the results of the pore volumes (Table 6). After the comparison of the results of the starting CT and the samples after sorption of Q or QD it was found that surface area and pore volumes decreased due to their adsorption. The surface areas and pore volumes of the starting sample are relatively low because before measurements all samples were heated only at 105 °C for 2 h. Higher temperatures in order to remove water could not be applied since the desorption of Q or QD could already occur. The samples were measured only for comparison of the changes.

3.6. Atomic force microscopy

The results of atomic force microscopy showed difference between the surface of Q and QD, caused by the presence of water molecules in QD (Figs. 12 and 13).

The thin layer of Q consisted of larger globules approximately 50 nm large, each containing 4–5 smaller units. The size of the smaller units was 3–5 nm in height and 10–13 nm in width (Fig. 12).

The quercetin dihydrate in ethanol solution formed considerably smaller globules, about 10 nm in diameter, arranged in primary chains 50–70 nm long. The chains were interconnected into a three-dimensional arrangement (Fig. 13). This tertiary arrangement is probably due to the presence of the water molecules in the quercetine dihydrate.

3.7. Cytotoxicity

The effect of tested compounds on cell proliferation in vitro was studied on several human cell lines Jurkat, HeLa, CEM, MCF7, A549 and MDA cells. The sensitivity of cancer cells to tested compounds differed among cell types.

MTT assay showed that pure zeolite CT100 with a higher water content heated to 100 °C had greater antiproliferative effects in comparison with zeolite CT400 with lower water content heated to 400 °C (Table 7). There were no significant differences between UV irradiated or not UV irradiated zeolite. Different cytotoxicity of CT100 and CT400 is illustrated at Fig. 14. The growth of HeLa, MCF7, A549 and MDA tumor cell lines were lower after application of CT110 compared with CT400. The effect of CT100 and CT400 was the same on Jurkat and CEM cell lines.

Incorporation of pharmaceutically active compounds (type of flavonoids): quercetin (Q) and quercetin dihydrate (QD) with antiproliferative effect had no synergic effect of the zeolite cytotoxicity

Table 8
Anticancer activity of modified zeolite CT400Q and CT400QD added to different cancer cell lines.

Content of Q or QD (%)	Modified zeolite CT400	Viability of cancer cell lines in (%)					
		Jurkat	HeLa	CEM	MCF7	A549	MDA
		5 mg of modified zeolite added to cancer cell lines					
0.06	CTQ	100	17.1	20.0	26.6	65.8	100
	CTQD	90.3	80.6	80.0	9.1	32.0	100
1	CTQ	100	100	100	100	100	100
	CTQD	100	100	100	100	100	100
		10 mg of modified zeolite added to cancer cell lines					
0.06	CTQ	28.2	0	13.3	0	23.5	100
	CTQD	8.1	11.3	15.4	18.4	16.5	59.4
1	CTQ	100	100	100	61.9	41.7	100
	CTQD	100	100	75.1	100	84.3	100
		20 mg of modified zeolite added to cancer cell lines					
0.06	CTQ	0	0	0	0	0	0
	CTQD	0	0	5.5	0	0	0
1	CTQ	64.5	22.3	29.0	0	0	0
	CTQD	11.1	14.0	27.0	0	0	0

but the protective effect of cancer cells. The modified zeolite CTQD had greater antiproliferative effects than modified zeolite CTQ (Table 8). The tumor cell lines studied after application of modified zeolite CTQ or CTQD had lower antiproliferative activity in comparison with the natural zeolite of the clinoptilolite type CT in our experimental conditions.

We have studied the influence of cytotoxic compounds Q as well as QD with two different content 0.06% and 1% incorporated into zeolite. Modified zeolite with the content 0.06% of Q or QD were more cytotoxic compared with modified zeolite with the content 1%. The growing concentration (5, 10, 20 mg) of pure and modified zeolite CT added to cancer cell lines increased cytotoxicity (Tables 7 and 8).

The increased cytotoxic effect of tested compounds is probably associated with water content present in QD and CT. Zeolites have high adsorptive capacity for water. However water may be the potential source of promoting apoptosis.

According to the results shown in the Tables 7 and 8 and Fig. 14 is evident that the application of CT activated at different temperatures (CT110, CT400) as well as its modified forms (CT110Q, CT110QD, CT400Q, CT400QD) showed different effects after application on different tumor cell lines. Further information is however necessary to know for more detailed explanation of cytotoxic activity of modified zeolitic forms CTQ and CTQD.

4. Conclusion

Natural zeolite of the clinoptilolite type is a slowly releasing carrier of pharmaceutically active compounds (type of flavonoids). Pharmaceutically active compounds: quercetin (Q) or quercetin dihydrate (QD) in solutions was incorporated into zeolite. The character of the interaction of Q in the zeolite is registered by TA techniques, as well as by determination of surface areas and pore volumes. The water content of the channel system influences the cytotoxicity of zeolite. A certain linear relationship is found between water content and expected active substance no matter the level of the preheated zeolite.

From a clinical view we observed that pure zeolite CT showed the most antiproliferative effects. Pure CT thermally activated at 110 °C (CT110) showed better cytotoxicity in comparison with CT thermally activated at 400 °C (CT400). Better effect of cytotoxicity came from Q or QD preparations made from 110 °C thermally activated zeolites than from 400 °C ones. The most probable reason is found in the mechanism of stackingwater molecules in the matrix channels. Clinoptilolite modified with quercetin dihydrate (CTQD) has shown better cytotoxicity compared to clinoptilolite modified with quercetin (CTQ).

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