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Characteristics of quercetin interactions with liposomal and vacuolar membranes

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ABSTRACT

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is claimed to exert many beneficial health effects. With application of ^{1}H NMR (nuclear magnetic resonance) and FTIR (Fourier-transform infrared) techniques, quercetin interaction with liposomes formed with dipalmitoylphosphatidylcholine (DPPC) was analyzed. Patch-clamp technique was employed to study quercetin effects at single channel level of vacuolar membranes in the liverwort *Conocephalum conicum*. Light and electron microscopy were applied to study quercetin effects on human negroid cervix carcinoma cells (HeLa). Enzymatic measurements along with DPPH (1,1-diphenyl-2-picrylhydrazyl) bioassay were performed to investigate the influence of quercetin on antioxidant enzymes and reactive oxygen species (ROS) production. The inclusion of quercetin to the membrane exerted pronounced ordering effect on the motional freedom of lipids in the head group region as manifested by broadening of the ^{1}H NMR spectral line representing the choline groups. FTIR analysis revealed quercetin incorporation into DPPC liposomes via hydrogen bonding between its own hydroxyl groups and lipid polar head groups in the C–O–P–O–C segment. Both, FTIR and NMR techniques indicated also quercetin spectral effects in the region corresponding to alkyl chains. Patch-clamp experiments showed that quercetin stabilizes tonoplast and promotes a close state of SV channels. Microscopic observations of HeLa cells revealed characteristic changes in ultrastructure and morphology of the examined cells in comparison to control cells. Pretreatment of HeLa cells with quercetin alleviated H_2O_2-induced cell injury by improving redox balance as indicated by the increase in glutathione content and SOD (superoxide dismutase) levels as well as by the decrease in ROS level. \In conclusion, the incorporation, distribution and the changes of biophysical properties of the membranes are very important for the effectiveness of phenolic compounds as antioxidant and anticancer factors.

1. Introduction

Plant-derived compounds are believed to exert a beneficial influence on human health and have been used as medicines. The interaction with membranes plays a crucial role in their biological activity. An understanding of the effects of flavonoids on membranes may help to better elucidate their mechanisms of action as anticancer as well as antioxidants. Quercetin belongs to flavonoids and constitutes an important component of people's daily diet. The common sources of quercetin are: onion, apples, tomatoes and red wine [1].

Apart from human health, flavonoids are crucial for plant growth and development. They protect plants from environmental stresses such as UV, heat, cold, pests and pathogens, and in general have

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPH• radical, 1,1-diphenyl-2-picrylhydrazyl radical; FTIR spectroscopy, Fourier-transform infrared spectroscopy; NMR spectroscopy, nuclear magnetic resonance spectroscopy; SV channels, slowly-activating vacuolar channels; WV, whole-vacuole

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significant role in plant resistance [2,3]. The antioxidant ability of flavonols (including quercetin) was also reported [4]. In addition to their stress protecting abilities, they are active compounds as attractants or feeding deterrents [5]. Flavonoids influence photosynthesis as catalysts of electron transport and are also involved in ion channel regulation and vacuolar transport [6–8]. They can modulate polar auxin transport [9] and stimulate nodulation and nitrogen fixation in plants [10,11]. Ca^{2+} transport and metabolism are also affected by flavonoids [12–14].

Quercetin (3,3′,4′,5,7-pentahydroxyflavone), the object of our study (Fig. 1), is the most widespread bioflavonoid in plants. It is found in a big amount in soybean [15]. It exhibits a broad spectrum of biological activities. One of the targets of this natural drug are cell membranes as well as membranous organelles [16]. Movileanu and coworkers studied the effect of quercetin on electrical properties of the planar lipid bilayers suggesting that the depth of flavonoid embedding is strongly pH-dependent [17]. It was found that quercetin can interact with human erythrocyte membranes and such interaction proceeds via reaction with membrane lipids and proteins [18]. Quercetin is able to interact and permeate lipid bilayer and such capacity is very important because there is correlation between antioxidant activity and ability to incorporate into membranes [19,20]. Once incorporated into membranes, quercetin changes biophysical parameters of the membrane for example its fluidity, cooperativity and the temperature of phase transition [21,22].

Electrophysiological effects of quercetin are widely documented in animal organisms. It was shown that in rat coronary artery rings examined by the patch-clamp technique in the whole-cell configuration, quercetin (>0.1 μM) increased the outward currents in the whole range of potentials, hyperpolarized cell membranes and increased the frequency of spontaneous transient outward currents carried by BKCa (large conductance Ca^{2+}-activated K^+) channels [23]. The authors of another report found that, quercetin regulated voltage-operated Ca^{2+} channels (VOCCs) in clonal rat pituitary GH_4C_1 cells. Quercetin potentiated Ca^{2+} entry (at 20 μg/ml) by 54.1%, induced a rapid and marked increase in both the transient (143.1%) and delayed (198.8%) Ca^{2+} currents, measured by the whole cell patch clamp technique [24]. Quercetin can regulate a number of ligand-gated ion channels including the nicotinic acetylcholine receptor, 5-HT receptor, glutamate kainite receptor and $GABA_A$ and $GABA_C$ receptors [25–27].

Flavonoids and their interaction with the membranes are frequently appearing subject of articles in recent years but only very few of them deal with quercetin influence on plant cells. Here quercetin was of interest to us because of a lack of published data concerning its effects on plant membrane current changes examined by patch-clamp technique. The interactions with membrane lipids and proteins could mediate certain biological effects of flavonoids, among them anticancer or antioxidant. Polyphenols could affect cell function by modifying plasma membrane structure and physical characteristics such as fluidity and electrical properties. For these reason, using patch-clamp technique and vacuoles with attendant slowly-activating vacuolar (SV) channels as the most convenient object, we decided to examine quercetin influence on the electro-parameters of the tonoplast in the liverwort *Conocephalum conicum*. Since the effect of quercetin on ion channels may depend on its interaction with lipid molecules we applied nuclear magnetic resonance (^{1}H NMR) technique. We examined possible membrane effects of quercetin on DPPC liposomes another model of membranes. Further, Fourier-transform infrared spectroscopic (FTIR) investigation was applied to address the problem of molecular interaction of quercetin with multilamellar DPPC membranes. Considering quercetin potential protective function against cancer and oxidative stress damage we subjected this flavonol to cell culture of human negroid cervix carcinoma cells in order to assess its effect on selected antioxidant enzymes, reactive oxygen species (ROS) production, morphology and ultrastructure of the cells.

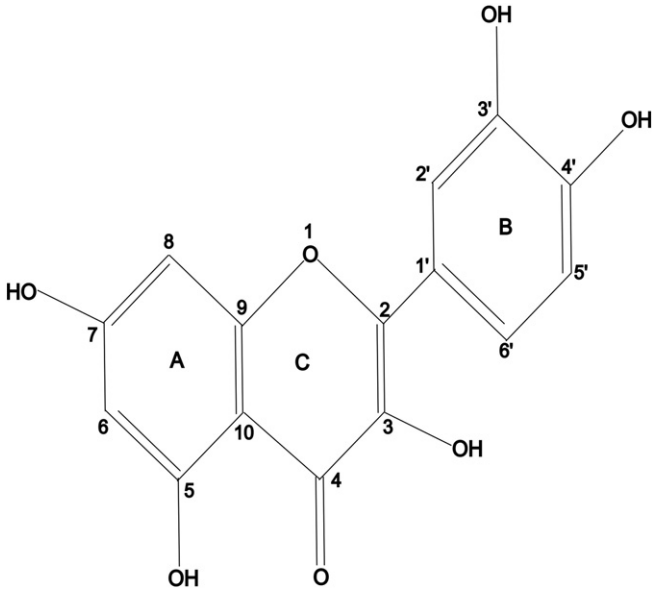

Fig. 1. Structure of quercetin.

2. Materials and methods

2.1. Chemicals

To dissolve quercetin (3,3′,4′,5,7-pentahydroxyflavone) (Sigma Chemical, USA) in the standard medium a stock solution (25 mg ml^{-1}) in DMSO (Merck, Germany) was applied. DMSO concentration in the test solution did not exceed 0.1%. Stock solutions were kept in darkness. The tested flavonol was used at different concentrations: 5 μg/ml; 10 μg/ml; 15 μg/ml; 25 μg/ml. For NMR and FTIR studies a stock solution of quercetin was dissolved in absolute ethanol (10 mg ml^{-1}). Deuterium oxide (D_2O) was purchased from ARMAR Chemicals Co. (Switzerland). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), morpholineethanesulfonic acid (MES) and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Sigma Chemical Co.

2.2. Liposomes preparation

Multilamellar liposomes were obtained by shaking [28,29]. The concentration of the lipid in TRIS buffer in D_2O (100 mM, pD 7.4) was 10^{-5} M (for FTIR) and in D_2O (pD 7.4) 3.2×10^{-2} M (for NMR). The concentration of quercetin was 1 mol% with respect to lipid. Dispersion of multilamellar liposomes of DPPC (73 mg/ml of chloroform) was prepared by mixing solutions of respective compounds by evaporation of solvent, first in a stream of nitrogen and subsequently by vacuum (3 h). DPPC samples were hydrated with TRIS buffer in D_2O or with pure D_2O, for FTIR and NMR respectively, by vigorous shaking at the temperature above the main phase transition of the lipid (41 °C) until optical homogeneity of the mixture was observed.

2.3. Plant material

Conocephalum conicum L. was collected together with a soil in its natural habitat, in a forest nearby Zwierzyniec (Poland). *Conocephalum* thalli were then grown in a greenhouse under highly moist conditions and at constant temperature (25 °C). The thalli were cut from the rest of the organism, detached from the soil and thoroughly rinsed with distilled water.

2.4. Isolation of vacuoles

The vacuoles of liverwort were isolated by a method described by Trębacz and Schönknecht [30]. The square-sliced fragments of the thallus were plasmolysed in a medium containing: 100 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES/TRIS, 500 mM sorbitol, pH 7.2. After 1 h incubation in the plasmolysing medium, the thallus fragments were cut with a sharp razor blade. Protoplasts were liberated through incised cell walls during a stepwise deplasmolysis. Reduction of the osmolality of the perfusion solution to 300 mOsm/kg caused rupturing of protoplasts and isolation of vacuoles.

2.5. Experimental solutions for patch-clamp

Bath and pipette solutions for electrophysiological recordings routinely contained (in mM): 100 KCl and 2 $CaCl_2$, and were adjusted to 300 mOsm with sorbitol. The solution facing the vacuolar site of a tonoplast was buffered with 15 mM MES and TRIS to pH of 5.85, while those facing the cytoplasmic site — with 15 mM HEPES and TRIS to pH 7.2. Pipette solutions were filtered through a filter (pore size, 0.2 μm) directly before use.

2.6. Patch-clamp experiments

Vacuolar ion currents were measured under voltage-clamp conditions on either whole vacuole (analogous to whole cell) or vacuole-out (vacuolar side of the tonoplast faces the bath) excised membrane patches [31]. The convention of current and voltage was according to Bertl and coworkers [32], i.e., the sign of voltage refers to the cytosolic side, and positive (outward) currents represent cation flux into the vacuole. Patch pipette *was* made from Kimax-51 glass capillaries (Kimble Products, Vineland, N.J., USA) by a two-step pulling protocol (electrode puller L/M-3P-A; List Medical, Darmstadt, Germany). The tip diameter of patch pipette after fire polishing (microforge MF 200-2, World Precision Instruments, USA) was 2 to 3 μm and about 1 μm for whole-vacuole and single-channel measurements, respectively. An Ag/AgCl reference electrode was connected to the bath solution via a 2% agar bridge filled with 100 mM KCl solution. Electrical measurements were carried out using an Heka EPC-9 amplifier (Heka Elektronik, Lambrecht, Germany). Currents were filtered at 10 kHz, digitized by an acquisition system (Patchmaster, Heka Elektronik, Germany) and stored on a hard drive of a PC. Measuring protocols were controlled by the software package. Each experiment was repeated at least three times. The data are presented as mean ± SD.

2.7. ^{1}H NMR measurements of DPPC liposomes

For the ^{1}H NMR spectroscopy, mixtures of phospholipids and quercetin were co-dissolved in chloroform/ethanol mixture (55:1/v:v) at the respective concentration. The solvents were evaporated under a stream of nitrogen and then by vacuum (overnight). Then samples were hydrated with D_2O, vigorously shaken (1 h) on a shaker at the temperature above the main phase transition of lipid (41 °C) in order to obtain optical homogeneity of the mixture. Then the lipid suspension in D_2O was sonicated to yield a homogenous lipid dispersion. Directly before measurements 4 mM praseodymium trichloride ($PrCl_3$) was added. ^{1}H NMR spectra were acquired on a Bruker Avance 300 NMR spectrometer using 5-mm probe with pulsed field gradient capabilities. The ^{1}H NMR parameters were as follow: spectral window 4496 Hz, digital resolution 0.274 Hz, pulse width 6.0 ms, acquisition and delay time were 1.82 s and 1 s, respectively, and acquisition temperature 330 K.

2.8. FTIR measurements

Infrared absorption spectra of flavonol alone, pure DPPC liposomes and liposomes with quercetin addition (DPPC) were recorded with the Fourier-transform infrared absorption spectrometer equipped with the attenuated total reflection set-up (ATR–FTIR) [33]. The samples were deposited on the ATR crystal element by evaporation from TRIS buffer in D_2O (pD 7.4). The spectra were then recorded with a Vector 33 spectrometer (Bruker, Germany). The internal reflection element was a ZnSe crystal (45° cut) yielding 10 internal reflections. Typically, 10 scans were collected, Fourier transformed and averaged for each measurement. Absorption spectra at a resolution of one data point every 2 cm^{-1} were obtained in the region between 4000 and 400 cm^{-1} using a clean crystal as the background. All experiments were done at 21 °C. The spectral analysis was performed with OPUS (Bruker, Germany) and Grams AI software from ThermoGalactic (USA). The instrument was purged with argon for 40 min before and continuously during measurements. The ATR crystals were cleaned with organic solvent (ethanol).

2.9. Cells and culture conditions

Human carcinoma cell line (HeLa B, ECACC No 85060701) cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS) (GIBCO BRL) (v/v) and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml) was used. Cells at density of 1×10^6 cells/ml were seeded in Falcon vessels and incubated at 37 °C in humidified atmosphere with 5% CO_2. The cultures were incubated with 5, 10, and 15 μg/ml of quercetin. The final concentration of DMSO in culture medium did not exceed 0.25%. To evoke oxidative shock (Os) cells were being incubated with hydrogen peroxide (2 mM) for 1 h. Then the cells were rinsed with phosphate buffer (pH 7.4) and incubated at 37 °C for 18 h. In other experimental variant the cells were incubated with quercetin and hydrogen peroxide for 2.5 h at 37 °C. Quercetin was added 1.5 h before the oxidative shock and was present during oxidative shock.

2.10. Cell viability analysis by neutral red (NR) uptake assay

NR cytotoxicity assay was based on the uptake and lysosomal accumulation of the supravital dye, neutral red. Dead or damaged cells do not take up the dye [34]. Cells were grown in 96-well multiplates (HeLa B) in 100 μl of culture medium with quercetin, hydrogen peroxide and with combination of quercetin and hydrogen peroxide for 6, 1.5 and 2.5 h respectively at the same doses as for culture of the cells in vitro for respective compounds. Subsequently, the medium was discarded and 0.4% NR (Sigma) solution medium was added to each well. The plate was being incubated for 3 h at 37 °C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, cells fixed with 1% calcium chloride ($CaCl_2$) in 4% paraformaldehyde, and thereafter the incorporated dye was solubilized using 1% acetic acetate in 50% ethanol solution (100 μl). The plates were being gently shaken for 20 min at room temperature and the extracted dye absorbance was measured spectrophotometrically at 550 nm using a microplate reader (Emax; Molecular Devices Corp., Menlo Park, CA). Three independent experiments were performed. The results were calculated as a percentage of control, arbitrarily set to 100%.

2.11. Light and transmission electron microscopy

The cells were being treated with quercetin at the concentration of 15 μg/ml and with 2 mM hydrogen peroxide. HeLa cells were gently scraped off flasks using a cell scraper. Then they were fixed in 4% glutaraldehyde in 100 mM cacodylate buffer for 2 h and in 1% osmium tetroxide for the next 2 h, all at 4 °C. The cells were dehydrated in series of alcohol and acetone and embedded in LR White resin. Semi-thin sections were cut with a glass knife on microtome RMC MT-XL (Tucson, AZ, USA), collected on glass slides and stained with 1% toluidine blue. The samples were examined under NIKON E800 microscope. Ultrathin sections were cut with a diamond knife on microtome RMC MT-XL (Tucson, AZ, USA), collected on copper grids and contrasted with the

use of uranyl acetate and Reynold's liquid. For each experimental variant at least 100 cells were examined. The samples were observed under LEO–Zeiss 912 AB electron microscope (Oberkohen, Germany).

2.12. Enzymatic measurements

Medium was removed from flasks, and monolayer cells were rinsed with PBS, scraped using cell scrapers, and resuspended in cold PBS. Following centrifugation for 10 min 8000 ×*g*, at 4 °C, the supernatant was discarded, cells were resuspended in cold PBS and homogenized in an ice-chilled motor-driven Potter S homogenizer (Sartorius, Germany). The homogenates were then centrifuged for 10 min at 10,000 ×*g*, at 4 °C, and an aliquot of the supernatant was used for the respective enzyme assay. Catalase (CAT) activity was determined by following decrease of absorbance during the decomposition of H_2O_2 [35]. Glutathione reductase (GR) activity was determined by the method described by Carlberg and Mannervik [36]. Superoxide dismutase (SOD) activity was assayed by its ability to inhibit the auto-oxidation of adrenaline determined by the increase in the absorbance at 480 nm at 30 °C, as previously described [37] and expressed as U/mg of proteins. The measurement of the level of superoxide anion radicals (SARs) was done according to method previously described [38]. For GSH level measurements the cells were homogenized in 5% sulfosalicylic acid and the content of GSH was measured according to Anderson [39].

2.13. DPPH• free radical scavenging test

Free radical scavenging activity of quercetin was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) assay [40]. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH• (Sigma) to the yellow colored diphenyl-picrylhydrazine. Briefly, 100 μl of DPPH• solution (0.2 mg/ml in methanol) was added to 100 μl of quercetin at concentrations 5; 10; 15 μg/ml. Trolox (Sigma) at increasing concentrations (1–50 μg/ml) was used as a reference for free radical scavenging activity. After 10 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower the absorbance, the higher the free radical scavenging activity of quercetin. The activity of quercetin was determined by comparing its absorbance with that of a blank solution (reagents without quercetin) and standard. The capability to scavenge DPPH• radical was calculated by the following formula:

$$\text{DPPH}\cdot\ \text{scavenging effect}(\%) = [(\text{Xcontrol} - \text{Xquercetin}/\text{Xcontrol}) \times 100] \quad \text{where :}$$

Xcontrol is the absorbance of the control and Xquercetin is the absorbance in the presence of quercetin.

3. Results

3.1. ^{1}H NMR investigations of DPPC liposomes

In Fig. 2 the ^{1}H NMR spectra of DPPC liposomes and DPPC liposomes with addition of 1 mol% quercetin are shown. Several bands can be recognized in the spectra, corresponding to major molecular features of the membranes: the CH_3 and CH_2 groups of the hydrophobic regions of the membrane as well as the bands of choline groups from polar head region of the membrane. To the liposome suspension $PrCl_3$ was added. Addition of this chemical compound effects in the split of the ^{1}H NMR band corresponding to the $-N^+(CH_3)_3$ group owing to the pseudocontact shifts produced by shift reagents from the group of lanthanides (e.g. Pr^{3+}) [41–43]. The resonance maximum shift towards lower ppm values corresponds to the inner liposome surface whereas shift towards higher ppm values corresponds to the lipid molecules forming the outer leaflet of the liposome membranes. The ratio of the areas under the signal assigned to the outer layer to that assigned to the inner layer (I_{out}/I_{in}, outer to inner) is proportional to the number of choline heads in the outer and inner layers. It is known that the number of lipid molecules in the outer layer is greater than that in the inner layer. Therefore for unilamellar liposomes the ratio I_{out}/I_{in} is greater than 1.

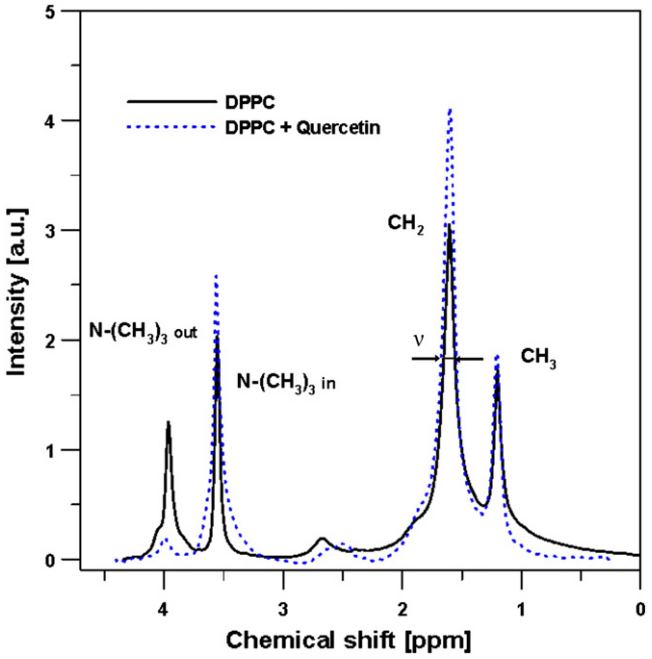

Fig. 2. ^{1}H NMR spectra of liposomes formed with pure DPPC and DPPC with quercetin addition in 1 mol%. $PrCl_3$ was added to the samples before measurement. The following parameters were determined: the full width at a half height (ν) and the I_{out}/I_{in} ratio.

Addition of quercetin caused the change in the full width at a half height (ν) of the ^{1}H NMR bands. The slight decrease in the case of CH_3 group (2%) was observed whereas for the CH_2 groups a pronounced decrease by 40% was noted. This result indicates fluidizing effect of quercetin with respect to the hydrophobic core (alkyl chain region) of the membrane. On the other hand, pronounced ordering effect on the motional freedom of lipids was observed in the head group region as manifested by an increase in the ν value of the bands representing the choline groups. The presence of quercetin caused the increase in ν by 33% (inner leaflet of membrane). Moreover, presence of quercetin changed dramatically the I_{out}/I_{in} ratio, from 0.086 in pure DPPC to 0.62 in the liposomes with addition of the examined flavonol. Both ratios indicate formation of multilamellar liposomes but in the case of DPPC liposomes with quercetin, liposomes formed were composed of more layers in comparison to pure lipids.

3.2. FTIR investigations of DPPC liposomes

Fig. 3 presents the infrared absorption spectra of DPPC membranes, pure and the one modified with quercetin. The polar head groups vibrations are represented by three main spectral bands: the antisymmetric stretching of the PO_2^- groups (1243 cm^{-1}), symmetric PO_2^- stretching (1087 cm^{-1}) partially overlapped with the band representing the C–O–P–O–C stretching modes (1054 cm^{-1}) and the band representing the antisymmetric N^+-CH_3 stretching vibrations (971 cm^{-1}). The relatively333,333 strong band centered at 1735 cm^{-1} corresponds to the stretching vibrations of the ester carbonyl groups. The scissoring vibrations of the CH_2 groups are represented by the bond at 1467 cm^{-1} while the relatively weak band at 1380 cm^{-1} represents the umbrella deformation vibrations of the CH_3 groups of alkyl chains. The principial band between 2800 and 3000 cm^{-1} represents the C–H stretching modes with the maxima of peaks at 2849 cm^{-1} and at 2916 cm^{-1}, corresponding to the symmetric and antisymmetric stretching in the CH_2 groups of alkyl chains, respectively, with minor

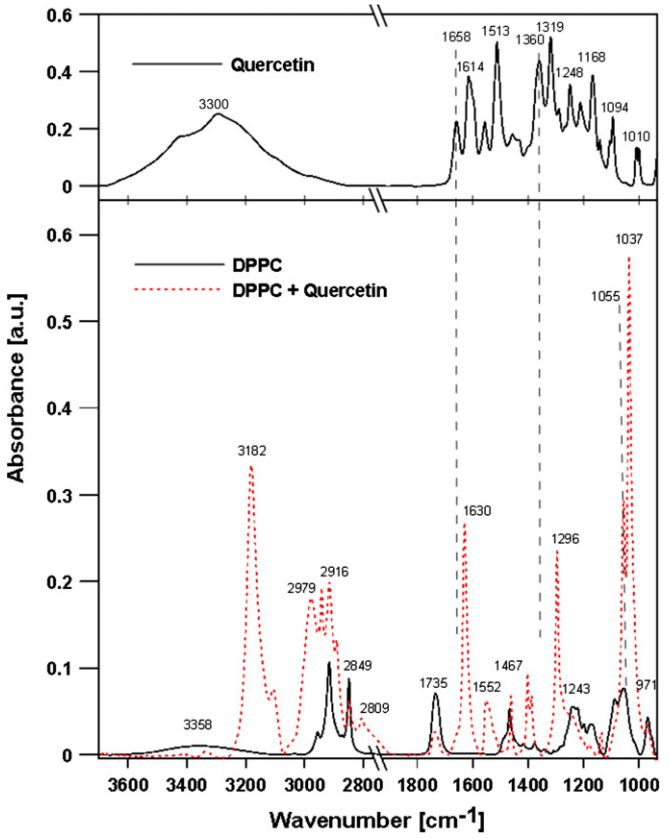

Fig. 3. Infrared absorption spectra of liposomes made of pure DPPC (continuous line), liposomes with addition of quercetin (dashed line) and pure quercetin (upper part of the graph). The spectra were normalized by dividing by the surface beneath the band centered at 971 cm^{-1}, corresponding to the stretching vibration of the choline group (ν_a, N^+-CH_3).

contribution from the symmetric and antisymmetric stretching vibrations in CH_3 groups at 2873 cm^{-1} and 2955 cm^{-1}, respectively. The broad band centered at 3358 cm^{-1} represents O–H stretching in water molecules associated with the membranes via hydrogen bonding. The samples were prepared in D_2O in order to avoid the contribution from bulk water in the spectral region corresponding to the O–H stretching of quercetin, which is the subject of interest. The results of our measurements show that preparation of samples in D_2O and short-time incubation does not result in pronounced hydrogen substitution neither in quercetin nor in DPPC. Deposition of the samples on ZnSe crystal, via partial evaporation, removes most of bulk water but we observed small band in the region between 2300 and 2600 cm^{-1}, corresponding to D_2O (not shown). Incorporation of quercetin resulted in dramatic changes in the infrared absorption spectra, indicating the localization of the flavonol with respect to the membrane and a type of interaction with lipids. The band at 3182 cm^{-1} is particularly sharp, intensive and appears in the relatively low frequency region. Owing to this fact we have attributed this particular band to the O–H stretching vibration involved in hydrogen bonding. Due to the fact that this band appears in the broad region of C–H and O–H stretching vibrations of quercetin and enhanced oscillator strength we deem that this band can represent the stretching vibrations of the O–H groups in quercetin involved in hydrogen bonding.

The spectral shift of the band at 1360 cm^{-1}, representing the C–O stretching vibrations in quercetin, towards lower wavenumbers (1296 cm^{-1}) after incorporation into membranes supports the concept on involvement of polar groups of the flavonoid (hydroxyl groups) in water binding in the membrane environment. The appearance of additional band at 2809 cm^{-1} can be interpreted as associated with the small fraction of alkyl chains with considerably reduced motional freedom. Alternatively, overall broadening of the entire C–H stretching band may represent penetration of water into the hydrophobic core of the membrane combined with formation of weak hydrogen bonds between water molecules and C–H groups of alkyl chains.

Spectral shift of the band centered at 1658 cm^{-1}, assigned to the C═O stretching, towards lower frequencies (to 1630 cm^{-1}) is observed. It can therefore be concluded that quercetin binds to the polar zone of the membrane via hydrogen bonding between oxygen groups of lipid and the keto groups of the flavonoid. The relatively weak band at 1552 cm^{-1} can be assigned to the –C═C-skeletal vibrations at the membrane-bound quercetin. The most pronounced spectral effect accompanying quercetin presence in the membranes can be observed in the spectral region corresponding to the C–O–P–O–C vibrations: shift towards lower frequencies accompanied with considerable increase in the oscillator strength. Such effect represents, most probably, hydrogen bonding of polar heads of DPPC with quercetin and indicates the localization of an additive.

3.3. Electro-parameters of the tonoplast in the liverwort C. conicum under quercetin influence

Fig. 4 depicts typical SV currents in symmetrical solutions (bath/pipette) of 100 mM KCl, 2 mM $CaCl_2$ in whole-vacuole (WV) configuration before (control) and after supplementation of bath with 10 μg/ml quercetin. Since quercetin was dissolved in DMSO, the second control with DMSO (final concentration 0.04%) was also done (n = 3, data not shown). There was no significant difference in SV currents under control- and DMSO-condition.

As seen in Fig. 4, the significant action of quercetin was manifested as a current drop (and an immediate increase in membrane resistance). To resolve the quercetin-induced current decrease at a single channel level, we were addressing the flavonol solution to membrane patches. Fig. 5 presents the original traces from one out of six similar experiments, where vacuole-attached mode was succeeded by vacuole-out configuration. Characteristic kinetics of SV currents with clear single channel openings at negative potentials and time-dependent activity at the beginning of positive potential clamping was always observed in the absence of quercetin (Fig. 5A). To avoid uncertainty in determination of single channel openings at positive potentials, only ion currents at negative voltages were chosen for the calculation of single channel conductance (Fig. 5D). The single channel conductance did not differ significantly between control (35 ± 7 pS; n = 6) and quercetin (35 ± 3 pS; n = 4). This points to the fact that quercetin decreases open probability or stabilizes a close state of SV channels. Since

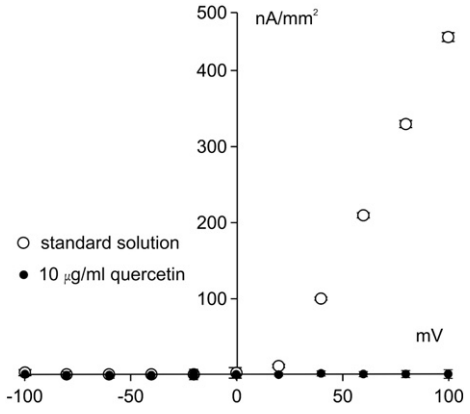

Fig. 4. Whole-vacuole current density/voltage dependencies of SV channels in *Conocephalum conicum* vacuoles: standard solution = control (open circles), quercetin-treated (filled triangle). Pipette and bath solutions consisted of 100 mM KCl and 2 mM $CaCl_2$; the former was buffered with 15 mM MES/TRIS to pH 5.85, while the latter with 15 mM HEPES/TRIS to pH 7.2. Pulse voltages between −100 and +100 mV were applied in 20 mV steps. The data are presented as mean ± SD for number of repetition (n) = 3.

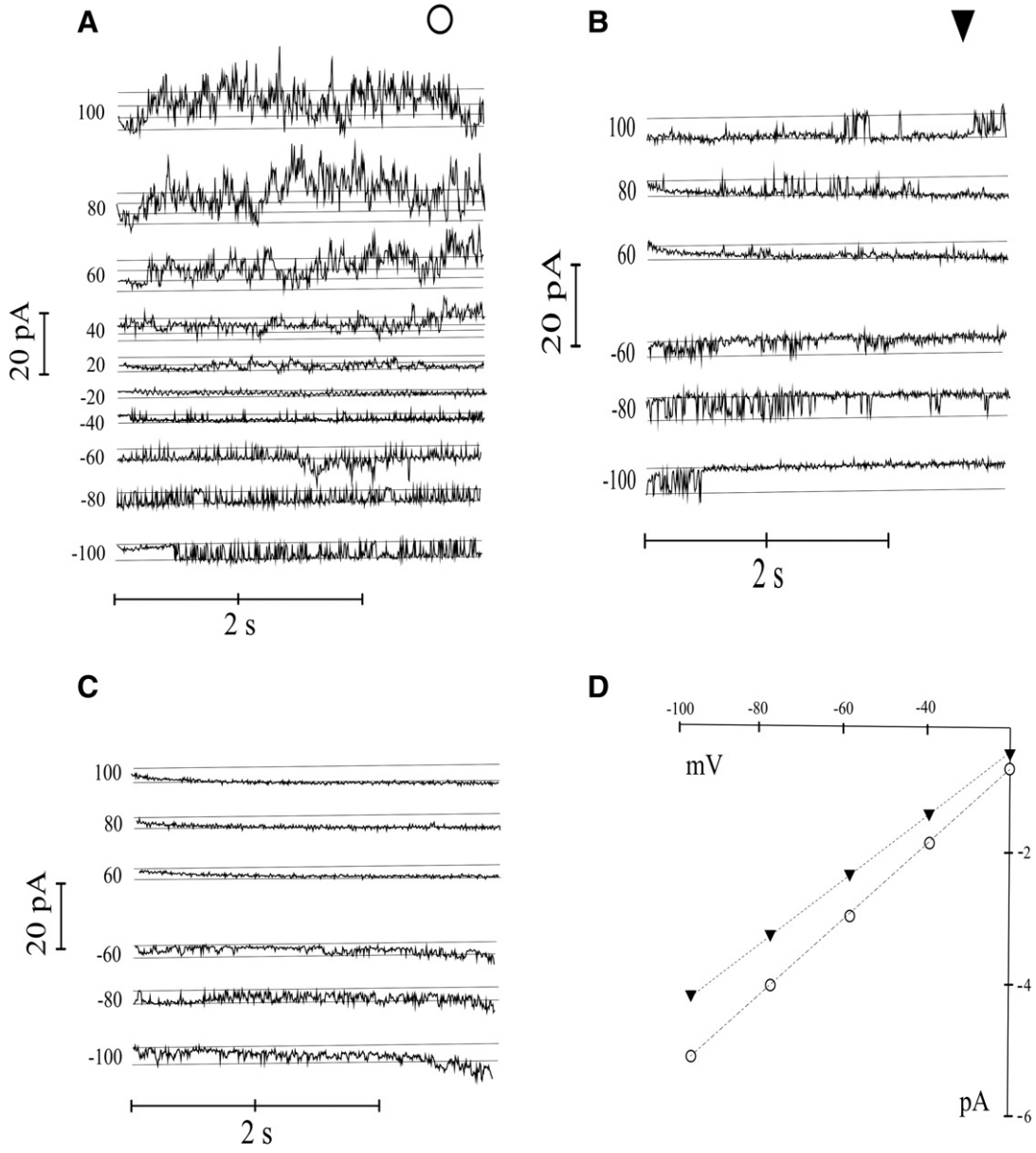

Fig. 5. The exemplary of traces of ion currents flowing through SV channels in *Conocephalum conicum* vacuoles in a vacuole-attached mode (A) or in vacuole-out configuration (B,C, D): (A) without quercetin in the pipette and bath solution; (B) 4 min after replacement of the standard bath solution with one supplemented with 10 μg/ml quercetin; (C) 6 min in after the replacement; numbers on the left denote command potentials in mV. Bath solution contained: 100 mM KCl, 2 mM $CaCl_2$, 15 mM Mes/TRIS (pH 5.85); pipette — 15 mM HEPES instead of MES (pH 7.2). (D) Current/voltage dependency depicting a single SV channel conductance (linear slope) under respective conditions: ○ — as in (A); ▼ — as in (B).

perfusion with the flavonoid made SV channels close (Fig. 5B, C), it is in good accordance with WV records. The higher the concentration of quercetin, the faster the SV inhibition (Fig. 6). With 5 μg/ml there was none or little effect within 20 min (a longer exposure was not examined). 10 and 15 μg/ml caused a complete SV-closure after 7.0 ± 0.8 and 3.8 ± 0.6 min, respectively. Quercetin at 25 μg/ml inhibited SV channels already during quercetin perfusion — SV were silent already during the very first record after bath solution exchange. If 10 μg/ml quercetin was added prior to seal formation either to the bath solution or to the pipette solution, there was no chance to record SV channel activity (n = 3).

3.4. Antioxidant enzymes in HeLa cells

Activities of selected antioxidant enzymes in HeLa cells are shown in Table 1. In the cells treated with hydrogen peroxide alone an increase in catalase (CAT) activity was observed.

Catalase (CAT) activity was lowered in cells exposed to quercetin and oxidative shock simultaneously in all the concentrations studied. The enzyme activity was statistically lowered by about 21% at the concentrations of 5 and 10 μg/ml. Superoxide dismutase (SOD) activity was increased by 59% and 56% with regard to oxidative shocked cells, at the quercetin concentration of 5 and 10 μg/ml. In the cells exposed to hydrogen peroxide conditions, activity of glutathione reductase (GR) was higher by about 73% in comparison to control cells. Statistically significant decrease in enzyme activity was observed in comparison to cells incubated with H_2O_2. The biggest decrease (by above 60%) in GR activity was for quercetin at the concentration of 5 μg/ml (Table 1).

3.5. Glutathione and radicals' level in HeLa cells

Glutathione (GSH) level in HeLa cells is shown in Table 1. The measurements revealed that the level of GSH increased in cells treated with H_2O_2 alone. In cells treated with quercetin at the concentration of 10

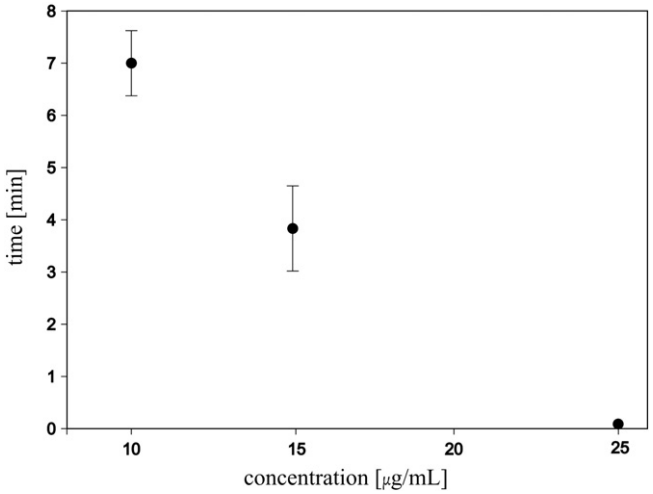

Fig. 6. Time/flavonoid concentration dependence; time after which there was a complete SV-closure noted for the respective concentration of quercetin. 0 means the moment, when the whole standard solution from an experimental chamber was replaced by the solution containing quercetin.

and 15 μg/ml and then exposed to H_2O_2 an increase of GSH level was noticed by 40 and 95%, respectively. Cells treated with H_2O_2 demonstrated a big increase in superoxide anion radical (SAR) level. More than 6-fold higher level of SAR was observed in H_2O_2 treated HeLa cells. When cells where preincubated with quercetin statistically significant, dependent on the quercetin concentration decrease in SAR level was noted. The biggest decline in the level of superoxide anion radicals (by 51%) was noted in the cells treated with flavonol at the concentration of 15 μg/ml in relation to control (Table 1).

The antioxidant activity of quercetin was additionally determined spectrophotometrically by the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging assay. The radical scavenging activity of quercetin at the doses of 5, 10 and 15 μg/ml was expressed as percentage of reduction of DPPH. As shown in Table 2, scavenging activity against DPPH• radical was 2.8-fold higher than the values obtained for Trolox, a synthetic vitamin E, as a reference compound. The effect of quercetin was concentration-dependent. The highest scavenging activity was noted for flavonol at the dose of 15 μg/ml.

3.6. Morphology of HeLa cells under the influence of hydrogen peroxide and quercetin with hydrogen peroxide

To demonstrate the effect of hydrogen peroxide and quercetin with hydrogen peroxide on cells' morphology the cells were observed under light and transmission electron microscopy. At the beginning control cells were examined. Microscopic observation of control cells revealed the presence of organelles of typical appearance. There were observed many control cells stained with toluidine blue. They had typical organization of nuclei with evenly distributed chromatin in which hetero- and euchromatin were easily distinguishable. The cells had several nucleoli (Fig. 7A). The control cells observed under electron microscope revealed normal subcellular organization (Fig. 7B–D). The endoplasmic reticulum (ER) had well preserved cisternae with many ribosomes abutted at the surfaces. The nuclei had homogenous karyoplasms and well discernible membrane. The uniformly shaped mitochondria had well-developed cristae. NR cytotoxicity assay has shown that viability of the examined cells after 18 h of incubation was 100%. Exposure of the HeLa cells to hydrogen peroxide caused decrease in viability of the cells (47.4 ± 7.95). In some cells cytoplasm had spongy appearance and the cells were swollen with many vacuoles inside (arrowheads) whereas in other cells cytoplasm and the whole cells were denser and condensed (arrows) (Fig. 7E). The observation of these cells under electron microscope has revealed typical changes: cell shrinkage, chromatin condensations, especially in marginal region of nuclei and later of whole nuclei and membrane blebbing (Fig. 7F–G). Increased frequency of vacuoles was readily observable. Some of the big vacuoles contained electron densities. In cytoplasm autophagy occurred (Fig. 7F–H). The cells treated with quercetin and then by hydrogen peroxide revealed irregular chromatin condensation, swollen cytoplasm (arrowheads) as well as dense, granular cytoplasm with condensed nuclei showing marginal heterochromatization (arrows) (Fig. 7I). There were also observed cells with unchanged morphology. NR cytotoxicity assay has shown that viability of the examined cells in this experimental variant achieved 41.63 ± 11.01. Quercetin alone did decrease the viability of the cells to a small extent (89.14 ± 8.41). Electron microscopy observation has supported the presence of the cells with swollen cytoplasm, ER scattered all over cytoplasm and mitochondrial matrix swelling (Fig. 7L, J) as well as the cells exhibiting continuing blebbing and apoptotic body formation (Fig. 7J–K). In the pool of the

Table 2
Free radical scavenging activity of quercetin in DPPH test.

Quercetin (μg/ml)	5	10	15
% of reduction as compared to control (0% of reduction)	12.62 ± 0.86*	25.08 ± 0.85*	38.54 ± 1.03*
Reduction value, which corresponds to the following Trolox concentration	4.45 ± 0.31	8.93 ± 0.31	13.75 ± 0.37

Values are means ± SD of three independent experiments. The absorbance of the solutions was measured spectrophotometrically at 515 nm. Trolox was used as a reference for free radical scavenging activity.
n = 3.
* $p < 0.05$.

Table 1
CAT, SOD, GR activities and superoxide anion radical (SAR) and GSH level in HeLa cells incubated with quercetin and treated with oxidative shock.

Antioxidant marker (unit)	Control	Os	Q5/Os	Q10/Os	Q15/Os
CAT (U/mg)	125 ± 25	147 ± 34.1***	115 ± 33.4****	116 ± 15.6****	121 ± 8.17***
SOD (U/mg)	430 ± 92	382 ± 41	608 ± 37*	596 ± 42***	492 ± 35*
GR (U/mg)	0.015 ± 0.002	0.026 ± 0.002*	0.01 ± 0.001*	0.023 ± 0.001*	0.021 ± 0.009*
SAR	0.001 ± 0.0007	0.0073 ± 0.0017****	0.0046 ± 0.002***	0.0036 ± 0.001***	0.0036 ± 0.0007***
GSH (mmol/mg)	1.6 ± 0.31	2.37 ± 0.48*	2.92 ± 0.43	3.31 ± 0.63**	4.61 ± 0.75**

Values are means ± SD of three independent experiments. The relative level of SAR was measured spectrophotometrically at 560 nm. Control cells; Os — hydrogen peroxide treated cells; Q/Os — cells preincubated with quercetin and then shocked.
n = 3.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.005$.
**** $p < 0.0001$.

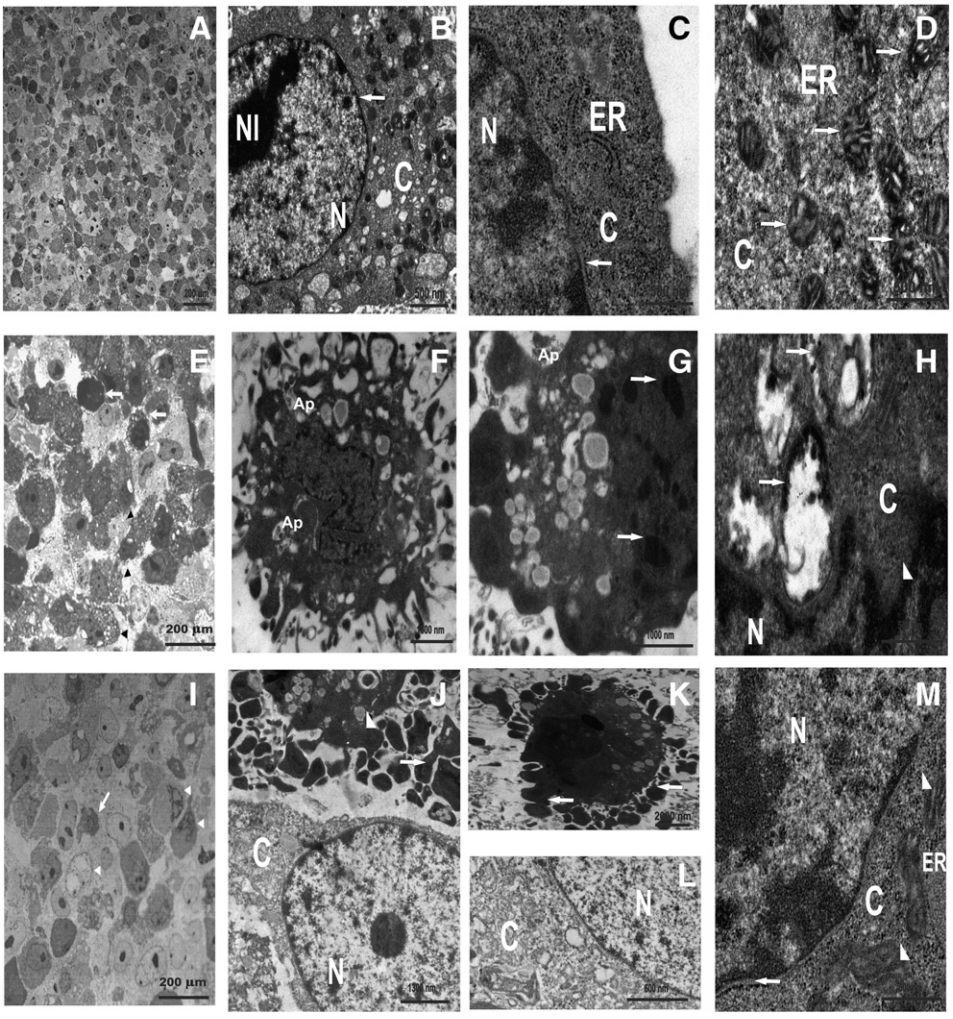

Fig. 7. The changes in morphology of the human negroid cervix carcinoma cell line — HeLa under the influence of oxidative shock and quercetin. (A–D) control cells; (A) control cells stained with toluidine blue; (B) control cells with typical subcellular organization: nucleus — N with well discernible nucleolus — Nl and nuclear membrane — arrow, cytoplasm with many organelles; (C) portion of cell with cytoplasm — C and fragment of nucleus — N. An arrow indicates nuclear envelope, in cytoplasm well preserved cisternae of endoplasmic reticulum with many ribosomes abutted at the surfaces are visible; (D) fragment of cytoplasm — C with uniformly shaped mitochondria — arrows; (E–H) the cells treated with hydrogen peroxide; E) shocked cells observed in semithin section; (F) cell treated with H_2O_2 demonstrates morphological features of early apoptosis: cell shrinkage, chromatin condensation — marginal heterochromatization and integrity of plasma membrane; (G) human cervical cancer cell showing traits of intermediate apoptosis: cell shrinkage, chromatin condensations — arrows and membrane blebbing; (H) fragment of shocked cell with characteristic vacuoles with some electron densities inside, nucleus does show marginal heterochromatization; (I) cells pretreated with quercetin and then shocked observed under light microscope; (J) one fragment of cell shows cell in late apoptosis: nuclear collapse — arrowhead, continuing blebbing and apoptotic body formation — arrow; the second fragment of cell with swollen cytoplasm; (K) cell showing cell shrinkage, chromatin condensations and membrane blebbing — arrows; (L) cell showing distinct swelling of cytoplasm — C with swollen mitochondria, ER scattered all over cytoplasm; (M) fragment of cell with normal structure: nucleus — N, nuclear membrane — arrow, cytoplasm — C without abnormal changes, well recognizable mitochondria — arrowheads and ER cisternae — ER.

cells there were also observable cells that demonstrated normal structure of the nucleus and cytoplasm without abnormal changes with well recognizable mitochondria and prominent endoplasmic reticulum cisternae (Fig. 7M).

4. Discussion

4.1. Dualistic behavior of quercetin in DPPC liposomes

The partitioning of anti-cancer dietary factors, such flavonoids, into membranes influences their physical properties [1,21]. Ability to interact with membranes is strictly coupled with flavonoid's non-polar properties [1]. Quercetin, the subject of this study contains five hydroxyl groups responsible for its polarity and weak acidic properties. On the other hand quercetin consists of two aromatic rings A and B linked by oxygen-containing heterocyclic ring (Fig. 1). This part shows high affinity towards hydrophobic environment. In the present paper, the interaction of quercetin with liposomal membranes (DPPC) was studied by ^{1}H NMR technique. The model membrane made of dipalmitoylphosphatidylcholine (DPPC) is ideally suited for flavonoid-membrane interaction because of its T_m value of 314 K [44]. ^{1}H NMR technique was proved to be a good tool for the investigation of the dynamic and structural properties of the membranes.

The analysis of the full width at a half height (ν) of a certain maximum in spectra recorded from liposomes with flavonol addition indicated that quercetin is localized within a lipid bilayer. The effect of the narrowing of spectral peaks which is directly related to liberations of the segmental movement of lipid molecules was very strong in the case of CH_2 groups of alkyl chains. This is an indication of the fluidizing effect of quercetin to the hydrophobic part the membrane. Proton-resonance in the choline group of pure membranes and membranes with addition of quercetin was compared. The inclusion of quercetin

into membranes decreased the motional freedom of polar head groups. Moreover, quercetin increased the number of liposome layers in comparison to pure liposomes. The effects shown above point out to the polar head region as the site of localization of the examined flavonol. Quercetin can mediate intermediate interactions between polar groups of neighboring layers. Thus, results from NMR technique showed that quercetin incorporates into membrane and causes biphasic effect on the fluidity. Present in the polar head group zone quercetin influences the alkyl chains conformation by increasing their fluidity. Localization of quercetin in the lipid head group region results in increase of the average area occupied statistically by a lipid molecule and therefore can weaken van der Waals interactions between alkyl chains. In our earlier work on quercetin DPPC membranes [22], in work of Saija [45] as well as in work of Sinha [44] the shift of the phase transition temperature towards lower values combined with the broadening effect of temperature profiles was observed. Such decrease in the lipid gel–liquid crystalline transition temperature (T_m) coupled with broadening of transitions peaks indicates flavonol incorporation into the membrane and supports location at the polar–non-polar interface [46]. In another report it was concluded that flavonoids preferably located in the polar interface region of the bilayer can fluidize membrane [47]. Similarly, Cieślik-Boczula and coworkers found that quercetin at the level of hydrocarbon chains increases the number of gauche conformers [48]. Van Dijk and coworkers [49] suggested that quercetin is localized at the boundary between the polar and hydrophobic region or inside the hydrophobic region of the bilayer and hence interacts with the hydrocarbon chains of lipids. Scheidt and coauthors, using pulse field gradient NMR spectroscopy, showed that distribution of flavonoids within membranes could be quite broad. The most affected were the proton signals associated with the lipid glycerol backbone and few upper groups of lipid alkyl chains [50]. Similarly, Siarheyeva and coauthors have shown that the highest concentration of quercetin is between the phosphate of the lipid head group and the upper segments of the lipid hydrocarbon chain by using ^{1}H NOESY MAS NMR method [51]. In our previous work on DPPC membranes, in which EPR technique was applied, we observed quercetin localization in the polar head region. Quercetin changed the ordered structure of lipid membranes to less compact at the temperature below the temperature of the phase transition and oppositely above this temperature, quercetin behaved in different way and made the spin label penetration more difficult [52]. Similarly, in our present study, we observed pronounced ordering effect of quercetin in the head group region. The findings from the current study correspond to the results reported by Tsuchiya and coworkers [21]. Quercetin showed biphasic effect on the tumor cell model membranes consisting of 20 mol% cholesterol and 80 mol% phosphatidylcholine rigidifying or fluidizing it. Rigidifying effect of quercetin was observed by Tsuchiya and coworkers who applied florescence polarization measurements on membranes made of unsaturated phospholipids and cholesterol [53].

4.2. Molecular interaction of quercetin and DPPC liposomes

In order to address the problem of molecular nature of quercetin–lipid bilayer interaction we applied FTIR spectroscopic investigation. Fig. 3 shows the IR absorption spectra of the DPPC membrane and DPPC-membrane with quercetin addition in different spectral regions. The most pronounced spectral effect is observed in the spectral region corresponding to the C–O–P–O–C (1054 cm^{-1}) vibration. The finding from this investigation shows that quercetin incorporates into DPPC via hydrogen bonding with the polar heads of DPPC. In line with our data Sinha and coauthors [44] showed that quercetin is located at the lipid/water interfacial region. Using molecular dynamic simulation they indicated phenolic-OH groups in quercetin as major participants in the formation of hydrogen bonds with the polar head of the lipid bilayer. Oteiza and coworkers drew similar conclusions. They examined bilayers composed of brain phosphatidylcholine and phosphatidylserine and suggested that interaction of flavonoids probably occurs through hydrogen bond formation between the hydroxyl group of flavonoids and the polar head groups of the membrane lipids [20]. OH groups as the crucial factor for the interaction of flavonoids at the water–lipid interface were also suggested in the work of Erlejman and coworkers [54]. The findings from the current study strictly correspond to our previous studies where FTIR spectroscopy was applied. We found a wide distribution of genistein and apigenin, flavonoids having three hydroxyl groups, within the DPPC membranes with a maximum in the upper region of the membrane [28,29]. One should remember that quercetin insertion into membranes is strongly pH-dependent [17]. In acidic medium this flavonol is completely liposoluble whereas in alkaline media reaction site of the flavonoid is restricted to the hydrophilic domain of the membrane due to deprotonation process [55]. At physiological pH, quercetin is translocated in the polar part of lipid membrane. Thus, the findings from the current study are in agreement with other reports [20,44,48,54] reporting localization of quercetin and other polyphenols in the polar region of the membrane. As a consequence of such interaction, radical scavenging activity of flavonoids can arise [43,55,56] together with protection against lipid peroxidation [57].

Another effect (observed in Fig. 2) concerning the band at 1360 cm^{-1} in quercetin-doped DPPC liposomes supports the concept of the involvement of quercetin's hydroxyl groups in water binding in the membrane environment. It is very likely that water bridges link additionally the polar groups of DPPC, including the phosphate group, with the hydroxyl groups of quercetin. Similarly Sinha and co-authors, who applied 2D NOESY technique, found that quercetin interacts with the hydrophilic head group of DPPC model membrane involving OH groups. These groups are present at positions: 3′, 4′ of ring B and position 7 of ring A [44]. The existence of quercetin linkage via hydrogen bonds between the keto groups of a flavonoid and oxygen groups of lipid cannot be excluded too. The proposed assignments are in accordance with other literature data [43,48]. Quercetin intercalates also into aliphatic chain zone as indicated by FTIR data. The 2809 cm^{-1} band was assigned by us above to the small fraction of alkyl chains with considerably reduced mobility. On the other hand the shift is relatively large and one would expect rather line broadening in such a case. It is worth mentioning that this particular band has been also observed in other lipid–flavonoid systems, such as DPPC–apigenin, the sharp band at 2808 cm^{-1} [29]. In both cases this particular band is not observed in the spectra of the pure components but it is always observed in the mixture of DPPC and a flavonoid. Due to this fact we find it highly probable that this band represents molecular interactions between the components. An alternative explanation for the appearance of this band in the two-component lipid–flavonoid system could be a slight shift of the frequency of the C–H stretching vibrations towards lower values, owing to weak hydrogen bonding to OH or other groups [58,59]. Both interpretations corroborate with the concept on interaction of a flavonoid with the hydrophobic core of the lipid membrane. ^{1}H NMR data support also such location of apigenin within the DPPC membranes. Quercetin interacts with lipid bilayer that is coupled to its flat structure [47]. Murota and coworkers have shown that quercetin has strong affinity for the liposomal membrane. The authors have also shown that membrane partitioning of flavonoids correlated with their uptake and transport in Caco-2 cells [60]. Pampel and co-authors, using the pulse field gradient NMR (PFG NMR) [61], supported that natural compounds such as flavonoids, show dynamic penetration of different regions of the membrane. Our data showed that quercetin has the highest affinity to the polar head group region and below the head group. Our earlier study, on red blood human membranes, with application of the electron spin resonance technique, indicated quercetin influence on polar region of the bilayer [18]. In line with our data, Scheidt and coauthors found a broad distribution of flavonoids along the membrane with a maximum in the lipid/water interface. Using the high-resolution magic angle spinning NMR spectroscopy and monounsaturated (POPC) model membranes this group has indicated at lipid glycerol backbone and few upper groups of acyl chains as the most affected region, in terms of flavonoid's distribution [50].

4.3. SV channel activity under the influence of quercetin

The most abundant (expressed in most plant tissues) and also the best characterized vacuolar channels are slowly-activating vacuolar (SV) channels [62]. The currents passing through them are evoked by positive voltages in the presence of high $[Ca^{2+}]_{cyt}$ and are outward-directed (cations enter the vacuole). Recognized for their characteristic time-dependence (hence the name "slow vacuolar"), Ca^{2+}-activation and cation-selectivity, SV channels are present in all tested plants except *Characean algae* [63]. Although *C. conicum* belongs to the oldest terrestrial organisms, SV existence has been electrophysiologically proved for it [30,63]. In the present work we aimed at checking how endogenous plant substance — quercetin influences plant vacuole at single channel level by the patch clamp technique. We found that quercetin caused SV channels' closure. Degree of SV inhibition was correlated with the dose of quercetin. Dose-dependent decrease in SV channel activity in vacuoles from mesophyll cells of *Arabidopsis thaliana* was observed for other type of flavonoid—naringenin [64]. Similar experiments performed on vacuoles from carrot root have shown that naringenin addition to the bath solution strongly reduced the currents. It is known from our previous study that quercetin exerts strong influence on electrical membrane properties in *C. conicum* [12,65]. We suggested that quercetin can affect the H^+ pump engaged in the second stage of repolarization during an action potential (AP) by maintaining high membrane potential of the plasma membrane. The effect of flavonoids on membranes of isolated vacuoles may be associated with the changes in lipid fluidity and in this way affect ion channels. As it was shown in the study of Nurminski and co-authors [66], dihydroquercetin affected active and passive transport systems of the vacuolar membranes possibly, as it was concluded, via its combined effect on the sulfhydryl groups of proteins and the lipid component of the membrane. Quercetin indeed strongly affects the lipid membrane as it was shown in the current investigation with application of FTIR and NMR techniques. Quercetin after incorporation into membranes alters biophysical properties of the lipid membrane. Alteration of biophysical properties of the lipid bilayer, mainly its fluidity changes, can be an important factor in transport regulation by proteins. For instance it was shown that activity of membrane transport proteins (like P-gp, ABCB1) can be modulated by the physical state of the surrounding lipids [67,68]. Direct acting on proteins cannot be excluded as well. As it was shown by Conseil and co-workers [69] quercetin can bind to NBD2 (nucleotide binding domain) of P-glycoprotein, to ATP binding site and also to the vicinal hydrophobic protein region interacting with substrate. This partially explains mechanism of P-gp modulation by quercetin. Quercetin has big influence on electric properties of membranes in animals that is widely documented [23–27] but scarce data concerning this problem in plants inclined us to employ patch clamp technique to examine plant vacuoles using the liverwort *C. conicum* – a well elaborated model plant – to resolve quercetin effects in more detail, i.e. at single channel level. From the present study it can be summarized that quercetin stabilizes tonoplast (reduces leak currents) and promotes a close state of SV channels. On the basis of current results and other reports, we can state that quercetin interacts directly or indirectly with the cell membrane, in which channel proteins are located.

4.4. Antioxidant activity of quercetin against HeLa cells-challenged by hydrogen-peroxide

Excessive production of reactive oxygen species (ROS) leads to aging, inflammation, cancer, atherosclerosis and other diseases [70]. Oxidative stress appears to be the common apoptotic mediator [71–73]. Because the action modes of flavonoids, among them quercetin, are partly based on an anti-oxidative mechanism we decided to examine quercetin effect on chosen antioxidant enzymes and radical's level in the cultured human negroid cells. In this report we found that treatment of HeLa cells with 2 mM H_2O_2, a precursor of other ROS, caused significant increase in activities of GR, CAT, the content of GSH and the level of superoxide anion radicals. Similarly, Mansour and co-authors found that addition of H_2O_2 in the culture medium of HeLa cells resulted in an increase of the catalase, the enzyme that converts hydrogen peroxide into oxygen and water and this way enabling the cells to adapt to additional amount of H_2O_2 [74]. In this study the observation with application of light and electron microscopy has revealed the presence of the cells with apoptotic features. In agreement with our results are data obtained by other authors [71,72,75] who found the correlation between high level of ROS and apoptosis induction among human cervical, neuronal and endothelial cells. Natural antioxidants, like quercetin, can inhibit production of ROS or increase cellular antioxidant defenses thus can prevent apoptosis and exert protecting abilities against the damaging effects of oxygen radicals [71,76]. The ability of this flavonol to protect the enzymatic antioxidant system can be explained by the following mechanism: (1) this compound itself can scavenge free radicals (2) prevent the loss of antioxidants by ROS and (3) can also up regulate endogenous antioxidant defenses. In vitro studies revealed that flavonoids may have considerable antioxidant activities in a wide range of chemical oxidation systems [77–79]. Quercetin, a flavonol (Fig. 1) containing the 3′–4′- dihydroxy structure in the B ring (catechol B ring), is known to possess a high antioxidant ability by scavenging free radicals [76]. In agreement are our data obtained in DPPH decoloration test. Quercetin revealed very high scavenging activity in comparison to Trolox. Our results are also supported by those presented by Burda and Oleszek [80]. With the use of DPPH test they found that quercetin had high ability to scavenge radicals. Similarly Psotova and coworkers, basing on the same model, found quercetin to be the most effective scavenger [81]. In our experiments we observed protective abilities of quercetin on HeLa cells in terms of cellular antioxidant defenses. We showed that pretreatment of HeLa cells with quercetin alleviated H_2O_2-induced cell injury by improving redox balance as indicated by the increase in glutathione content and SOD levels as well as by the decrease in ROS level in comparison to H_2O_2-challenged cells. Our results are in agreement with other reports. Quercetin pretreatment protected NG108-15 cells from oxidative stress injury and hydrogen peroxide-induced apoptosis by improving redox imbalance of the cells [75]. Quercetin was also shown to reduce H_2O_2-induced apoptotic DNA damage in HUVEC cells [71]. Chen and co-workers have found that quercetin inhibited ROS-dependent apoptosis in rat glioma C6 cells [82]. Similarly, our data, not-included into this report, showed that quercetin pretreatment alleviated hydrogen-peroxide induced apoptosis of HeLa cells (data not shown). It is known that quercetin is potent in reducing the level of ROS and had high antioxidant activity [83]. One should remember that antioxidative activity of flavonoids per se does not confer protective effect. Scavenging of ROS acts cooperatively with other mechanisms. Alleviation of oxidative stress in a cell culture model can also occur by increasing intracellular GSH content [75], the effect we also found in the current study. Although the physiological benefits of flavonoids have been largely ascribed to their antioxidant properties, flavonoids may also protect cells by other mechanisms. The protective properties of flavonoids, among them quercetin, can also be accounted for by incorporation and stabilization of plasma membrane [78,84]. Our data from the current study give evidence for quercetin incorporation into membranes and altering their properties.

4.5. Quercetin influence on HeLa cells morphology

Microscopic observations of HeLa cells revealed characteristic changes in ultrastructure and morphology of the examined cells in comparison to control cells. Hydrogen peroxide at high concentration is toxic, causes oxidative shock and as aftermath affects cell viability [85]. Indeed, in our study, hydrogen peroxide treatment of the cells resulted in a decrease of cell viability by more than 50%. Oxidative stress, as a result of H_2O_2 exposure, appears to be a common mediator of apoptosis, as

it was mentioned previously [71,72]. Evaluation of the cells under microscopes has shown the presence of the cells with apoptotic traits such as: cell shrinkage, chromatin condensation, membrane blebbing and apoptotic body formation. Our observations are in agreement with the findings of other authors who observed an increase in apoptosis of endothelial, fibroblast and the cells of blood vessel smooth muscles in response to hydrogen peroxide treatment [37,86,87]. The same effect was reported when H_2O_2 was applied in vitro to neurons from NG108-15 cell line [75]. Exposure of HeLa cells to hydrogen peroxide induced also the appearance of the cell with necrotic modality (swollen cytoplasm, many vacuoles, irregular chromatin condensation). Oxidative stress-induced vacuolization of cytoplasm together with shrinkage of cells were observed by Psotova and coworkers in isolated rat heart cardiac myocytes [81]. Pro-apoptotic and pro-necrotic effects of H_2O_2 are dependent on its concentration and time of action. Low concentration ($\leq$0.5 mM) is not cytotoxic. With increasing concentration this effect increases and the cells are dying via apoptosis and later via necrosis [87]. Similar effects were observed by group of Chen and co-workers. Below 0.3 mM hydrogen peroxide did not affect cell viability of human fibroblasts (F65) whereas at higher doses strong inhibition of DNA replication occurred [88]. We, similarly as Ulug and co-workers [89], found that quercetin alone exhibited very slight toxic effect on HeLa cells under similar dose and time of incubation. Since H_2O_2 at 2 mM concentration significantly inhibited cell viability of HeLa cells, while quercetin had negligible effect on cell viability, therefore we initially hypothesized that quercetin would protect cells from H_2O_2 effect. Surprisingly NR assay has shown that pretreatment with quercetin further exacerbated cell viability reduction. The result may suggest that quercetin sensitized the cells to hydrogen peroxide exposure in a slight way. On the other hand, we found that pretreatment of cells with examined flavonol improved cell redox balance as was found by the increase in GSH, SOD levels and decrease in ROS level. Additionally, with usage of transmission electron and light microscopy techniques we still observed the cells with apoptotic features and/or dying in some other way but simultaneously there were observed cells showing normal morphology and ultrastructure. Our investigations of HeLa cells with usage of double staining (Hoechst dye and propidium iodide) to quantify the number of apoptotic and necrotic cells have revealed that quercetin itself causes little apoptosis whereas when used in combination with hydrogen-peroxide suppresses apoptosis induced by H_2O_2 (data not shown).

4.6. Conclusions

Because the interaction with membranes plays crucial role in flavonoids' biological activity, in this report we further disclose the mechanism of quercetin action on the membranes. Incorporation and alteration of membrane properties are crucial for transport regulation of proteins. Furthermore, very important for the effectiveness of phenolic compounds as antioxidant and anticancer factors is their incorporation, distribution and orientation in the membrane bilayer. It has been shown that quercetin significantly increased the accumulation of daunomycin and vinblastine in human pancreatic adenocarcinoma cell line (Panc-1). It was postulated that this flavonol inhibited MRP-1 (multidrug resistance associated protein-1) mediated drug transport by binding with MRP1, as well as by modulation of GSH concentrations [90]. Apoptotic and toxic effects of quercetin were investigated by transferring quercetin into the cells by carriers like liposomes [91]. The changes in the activity of integral proteins and in consequence the influence on transport and other membrane-related processes were also found for the human erythrocyte membrane [18]. According to obtained data and results of other authors we can conclude that quercetin exerts its beneficial effect on human health and has been used as medicine partly via its intercalation into membranes.

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