Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 227 (2020) 117570

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Antioxidant effect of phenolic compounds (PC) at different concentrations in IEC-6 cells: A spectroscopic analysis

G. Barraza-Garza [a], J.A. Pérez-León [a], H. Castillo-Michel [b], L.A. de la Rosa [a], A. Martinez-Martinez [a], M. Cotte [b], E. Alvarez-Parrilla [a, *]

[a] Departamento de Ciencias Químico Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, PRONAF y Estocolmo s/n, 32310, Juárez, Mexico

[b] X-ray and Infrared Microspectroscopy Beamline ID21, European Synchrotron Radiation Facility, 71 avenue des Martyrs, 38000, Grenoble, France

ARTICLE INFO

Article history:
Received 7 June 2019
Received in revised form
24 September 2019
Accepted 25 September 2019
Available online 20 October 2019

Keywords:
Reactive oxygen species (ROS)
Flavonoids
IR spectroscopy
Immunofluorescence microscopy
Nrf2

ABSTRACT

Phenolic compounds (PC) have been proposed as natural antioxidant agents that protect cells against oxidative stress-related diseases. Nonetheless, their low bioavailability forecasts controversy about mechanisms on their in vivo scavenging activity against reactive oxygen species (ROS). It has been proposed that PC reduce directly ROS concentration. An alternative or complementary action of PC could be the activation of the cell's antioxidant pathway, involving the regulation of gene expression, like that initiated by the Nrf2 transcription factor. To date there is not enough experimental data to support or discard this possibility. In the present study, we evaluated the use of several PC to prevent peroxidation of macromolecules and to elicit the activation of the Nrf2 transcription factor in H_2O_2-stresed IEC-6 enterocytic cell line. Synchrotron microspectroscopy demonstrated that PC compounds protected proteins, lipids and nucleic acids against oxidation induced by H_2O_2. Immunofluorescence results showed that treatment with quercetin (Qc), catechin (Cat) and capsaicin (Cap) induced the translocation of Nrf2 into the nucleus, at the same level as did H_2O_2 treatment, thus mimicking the action of the endogenous cell response to peroxidation. Even though the detailed mechanism still needs to be elucidated, we demonstrated the activation of Nrf2 by PCs in response to oxidative stress.

1. Introduction

Phenolic compounds (PC) are natural antioxidant metabolites found in plant foods, known for their ability to scavenge reactive oxygen species (ROS) and thus protect the cells against oxidative stress-related diseases. However, due to their low bioavailability, there is controversy about how they can act as antioxidant compounds, thus the direct in vivo ROS-scavenging activity is in doubt [1,2]. It has been proposed that the antioxidant effect of PC may be related to an indirect interaction of the cells with ROS. In this indirect mechanism, PC seem to act as mild prooxidants that can activate a series of biochemical pathways of the cellular antioxidant system [3,4]. This mild prooxidant effect has been related to the capacity of PC to chelate metal ions and produce ROS, semiquinones and quinones which are potentially cytotoxic. Some prooxidative effects have been observed in insects that consume high levels of phenols, due to the presence of non-absorbed metal ions in their gastrointestinal tract. Data available on mammals gave different results, which make it difficult to infer what happens in these cases [5]. This mild prooxidant effect of PC can be beneficial because it can trigger the antioxidant defense mechanism, resulting in the protection of cells [5]. In a previous work by our research team, the antioxidant effects of six different PC against H_2O_2-generated oxidative stress were evaluated in isolated rat enterocytes, observing that catechin and capsaicin presented high antioxidant effect, while quercetin showed a mild prooxidant effect [6]. These results indicated that at, the same concentration, PC may present both antioxidant and prooxidant properties, however the mechanism involved in these differential properties is still unknown. It is proposed that PC may present a direct activity by neutralizing ROS (ROS-scavenging activity) or an indirect activity in which PC exert the activation of antioxidant pathways within the cell like the Nrf2-Keap1 pathway. The effect of different PC concentrations on their antioxidant or prooxidant activities is not well known. For instance, it has been reported that quercetin, eugenol and polyphenols from green tea extract exert antioxidant and prooxidant activities in cancer cells, depending on their concentration [7–9]. To gain

* Corresponding author.
E-mail address: ealvarez@uacj.mx (E. Alvarez-Parrilla).

https://doi.org/10.1016/j.saa.2019.117570
1386-1425/

information on the effect of PC concentration on their antioxidant properties, the aim of the current study was to evaluate the protective effect of different concentrations of catechin, capsaicin and quercetin on H_2O_2-induced oxidative stress, using an enterocyte-derived cell line (IEC-6) as model. To evaluate the antioxidant or prooxidant effects of PC and H_2O_2, we used Synchrotron radiation-based Fourier transform infrared microspectroscopy (FTIRM), which allowed us to observe the state of lipids, carbohydrates and proteins at single cell level [6,10], the data obtained were compared to standard spectrophotometric techniques such as conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS). Furthermore, we used immunofluorescence to analyze the activation of the Nrf-Keap-1 pathway in the same experimental conditions. This study reveals how different concentrations of PC affect their protective activity over IEC-6 cells, how this protective activity is exerted and how this is related to the PC structure.

2. Materials and methods

2.1. Chemicals and reagents

Catechin [Cat]. quercetin [Qc], capsaicin [Cap], chloroform, cyclohexane, thiobarbituric acid, trichloroacetic acid, tetramethoxypropane (TMP), sodium bicarbonate, H_2O_2, trypan blue, penicillin-streptomycin, hydrochloric acid and IEC-6 cell line (ATCC: CRL-1592) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol was purchased from Hycel (Jalisco, Mexico). Primary anti-Nrf2 antibody and secondary antibody Alexa Fluor 594 were purchased from Thermo-Fisher Scientific (Waltham, Massachusetts, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and trypsin-like enzyme Tryple Express™ were purchased from Gibco (Gaithersburg, Maryland, USA).

2.2. Cell culture

The IEC-6 enterocytic cell line derived from rat (*Rattus norvegicus*) small intestine was used in the present work. After arrival, cells were maintained at −80 °C until used. For the culture, cells were thawed at 37 °C for 1 min in a water bath. Subsequently the cells contained in the 1.5 mL vial were transferred to a T-25 culture flask, containing 3 mL of DMEM (10% de FBS y 1% de PEST) and incubated at 37 °C in a 5% CO_2 atmosphere. DMEM culture medium was changed every other day until cells reached between 85% and 90% of confluence. Before their use in any experiment, cell culture at optimal confluence were tripsinized with 1 mL of Tryple Express™ 1x for 10 min. Afterwards, 1 mL of DMEM medium was added to stop the enzyme activity, and the content of the flasks were transferred into 1.5 mL plastic conic tubes and centrifuged at 820 x g for 5 min. Supernatant was discarded and the pellet containing cells resuspended in 500 μL of DMEM medium. A sample of 50 μL of the cell suspension was collected and mixed (1:1) with 0.4%. trypan blue and used for cell counting and viability (Neubauer chamber, examined with an upright microscope at 20x objective, Leica CME Leica Miscrosystems, Wetzlar, Germany) [11,12].

2.3. PC treatment

For all the treatments, 24-well culture plates were used, inoculating 100,000 cells per well, to allow adherence, cells were grown overnight before applying the treatments into the plate. Three PC were used in this study: Cap, Cat and Qc at three different concentrations for the FTIRM, CD and TBARS experiments: 50, 100 and 250 μM, while only a 100 μM concentration was used for immunofluorescence microscopy analysis. Hydrogen peroxide (H_2O_2) was used to induce oxidative stress at a final concentration of 500 μM. Three different experiments were used for each polyphenol: (1) only PC was added to the medium, (2) PC added to the medium before H_2O_2 and (3) PC added to the medium after H_2O_2. Cells were exposed to H_2O_2 and PC for 30 min each. Control treatments and those treated with just one compound were first pre-incubated 30 min with DMEM and then washed with PBS (pH 7.4), centrifuged at 820 x *g* for 5 min and then treated with DMEM media added with the corresponding compound for 30 min. In the case of cells being treated with PC before or after H_2O_2 they were incubated with the first compound for 30 min with DMEM added media, washed, centrifuged and incubated with DMEM media added with the second compound for 30 min. After finishing the corresponding treatment, cells were centrifuged at 820 x *g* for 5 min and the supernatant was discarded. Finally, cells were washed twice with PBS (pH 7.4) and centrifuged at 820 x *g* for 5 min.

2.4. Fourier transform infrared microspectroscopy (FTIRM)

For FTIRM analyzes, cells obtained during the PC treatments were washed with PBS 1x and centrifuged at 820 x *g* for 5 min. After centrifugation the supernatant was discarded, and cells were fixed in paraformaldehyde 4% in PBS (PFA-PBS) for 20 min. Fixed cells were centrifuged for 10 min at 820 x *g*, supernatant discarded and the cells washed with deionized water, and finally resuspended in 200 μL of water [10]. Fixed cells were analyzed using the FTIRM end station in the line ID-21 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France using a Thermo Nicolet Continuum microscope (Thermo Scientific, Madison, WT, U.S.A.) coupled to a Thermo Nicolet Nexus FTIR spectrometer (Thermo Scientific, Madison, WT, U.S.A.). The IR microscope is equipped with a 32X objective, a motorized sample stage and a liquid nitrogen-cooled 50 μm mercury cadmium telluride detector. For observation, 20 μL of fixed cells (approximately 20000 cells) were left to dry in BaF_2 windows of 1 mm height. Spectra was recorded over the range of 4000 to 900 cm^{-1}, the spectral resolution was set to 6 cm^{-1} and 120 scans per spectrum were collected. Spectra of 30 individual cells were recorded for each treatment.

2.5. Infrared spectra analysis

Infrared spectra obtained by FTIRM were analyzed using Unscrambler X software (CAMO Software, Norway). Raw spectra were pre-processed first by a vector normalization and then by a second derivative using Savitsky-Golay of second polynomial order with 21 smoothing points [6,13,14]. Raw and second derivative analysis of all results are presented in supplementary files 1 and 2, respectively. Processed spectra were used for calculation of ratios (proportions) from IR signals at different spectral bands. Effects of oxidative stress in cells has been related to the displacement or reduction of some IR bands. The following bands were evaluated for distinct phenomena: For lipid oxidation: 1740 cm^{-1}/2960 cm^{-1}, lipid saturation: 2920 cm^{-1}/2960 cm^{-1} and lipid unsaturation: 3012 cm^{-1}/ 2960 cm^{-1}, protein aggregation: 1630 cm^{-1}/1650 cm^{-1} [15–19].

2.6. Spectrophotometric measures: determination of lipid peroxidation by conjugated dienes (CD) and determination of thiobarbituric acid reactive substances (TBARS)

Conjugated Dienes (CD) and thiobarbituric acid reactive substances (TBARS), which measure the presence of primary and secondary products of lipid peroxidation respectively, were measured as previously reported, using approximately 300,000 cells from the PC treatments, as described in our previous work [6]. For the CD analysis, PC treated, and control cells were lysed by sonication and then lipids extracted with chloroform. The extracted lipids were

then dissolved in cyclohexane, and immediately measured with a UV-spectrophotometer at 233 nm (Thermo Scientific, Madison, WT, USA) using cyclohexane as blank. The obtained absorbance was converted to conjugated dienes concentration using an extinction coefficient of 27000 $M^{-1}cm^{-1}$ and the results were expressed as conjugated dienes concentration (μM) per million cells. For TBARS analysis, PC treated, and control cells were lysed by sonication. Samples and calibration standards were mixed with TBA reagent (20% TCA, 0.5% TBA and 2.5 N HCl) and the mixture heated for 45 min in a boiling water bath. After cooling, the solution was centrifuged, and the absorbance of the supernatant was measured at 532 nm using the reaction mixture as a blank. Tetramethoxypropane (TMP, 1.25−20 μM) was used as standard and results were expressed as malonaldehyde (MDA) concentration (μM) per million of cells.

2.7. Immunofluorescence microscopy analysis

An immunofluorescence microscopy analysis was carried out to evaluate the activation of the Nrf2-Keap1 pathway. IEC-6 cells were grown in culture dishes as described in section 2.3. Then an initial population of ≈ 10,000 cells were inoculated into square glass slides (1 cm × 1 cm), allocated inside wells of a 24-wells culture plate, mixed with 1 mL of DMEM (10% de FBS y 1% de PEST) and left overnight to allow adherence. Subsequently cells were treated as described in the section 2.4, using a concentration of 100 μM of each PC [6]. After PC treatments, DMEM was removed from each well and the cells were rinsed three times with PBS 1x to remove excess of DMEM. Cells were fixed with a 4% PFA-PBS solution for 20 min and rinsed with PBS 1x twice. Subsequently, anti-Nrf2 primary antibody was added (50 μL 1:200) and incubated for 30 min. Incubation was stopped washing 3 times with PBS 1x. Then, the cells were incubated with the appropriate Alexa Fluor 594-conjugated secondary antibody-(50 μL1:400) for 30 min. At the end of the incubation, the slides were washed 3 times with PBS 1x. and counterstained in the dark for 15 min with DAPI, and washed three times with PBS 1x. Finally, slides were mounted using glycerol 70% and observed with a Leica DM2000 (Leica fluorescence microscope, Leica microsystem, Mexico) using the RGB, blue (DAPI) and fluorescent (fluorescein) filters with 20x and 40x targets (Wetzlar, Hesse, Germany) to acquire images of the treated cells.

2.8. Statistical analysis

All assays were carried out by triplicate and results are presented as averaged ± standard deviations (SD). CD and TBARS results were analyzed using a one-way analysis of variance (ANOVA). When ANOVA showed significant differences, a Tukey's post hoc test was applied. Statistical significance was regarded as $P<0.05$. In the case of IR spectra, proportions mentioned in section 2.6 were used. For immunofluorescence microscopy, a frequency chart with SD were used to compare results from the different treatments.

3. Results and discussion

In a previous study carried out by our research group, the effect of six different PC (Cap, Cat, Qc, gallic acid, caffeic acid and chlorogenic acid) against H_2O_2-induced oxidative stress in primary culture small intestine enterocyte was evaluated [6]. Results showed that some PC presented a protective effect when administered before or after the oxidative stimulus (H_2O_2). From the six PC analyzed Cap and Cat showed the best protective effect, while phenolic acids showed weaker protective activities. When administered at 100 μM in the absence of H_2O_2, all six PC behaved as mild pro-oxidants and this effect was more evident for Qc and phenolic acids. In order to describe the mechanism by which this PC present their protective effect, in the present study the effect of Cap, Cat and Qc at three different concentrations was evaluated, to determine if there is a dose-dependent effect of these PC against the H_2O_2-induced stress using a rat enterocyte cell line (IEC-6) as model.

3.1. Oxidative stress and antioxidant activity evaluated by infrared spectral analysis

Infrared spectroscopy was used to obtain information on the oxidation of proteins and lipids in IEC-6 cells treated with H_2O_2 and/or three concentrations of Cat, Cap and Qc, by comparison of the FTIR spectra of control cells versus spectra from PC-treated cells [6]. Two sections of the FTIRM spectra were analyzed. The first section, in the range of 1750-1470 cm^{-1}, known as the protein region, shows changes in aldehyde, amide I and II bands. The second section in the 3200-2800 cm^{-1} range, known as lipid region, shows changes in CH_2 and CH_3 alkyl chains and olefinic bond bands. Fig. 1 shows the FTIR absorbance spectra (1A) and second derivative spectra (1B) from control and H_2O_2-treated cells, to illustrate how raw and processed spectra data are seen. In order to evaluate the protective effect of PC, different FTIRM signal ratios related with lipid and protein oxidation, were analyzed: 1740 cm^{-1}/2960 cm^{-1} (lipid oxidation), 2920 cm^{-1}/2960 cm^{-1} (lipid saturation), 3012 cm^{-1}/2960 cm^{-1} (lipid unsaturation) and 1630 cm^{-1}/1650 cm^{-1} (protein aggregation). Fig. 2A shows the 1740 cm^{-1} / 2960 cm^{-1} ratio results, which allowed us to evaluate the effect of oxidative stress on lipid oxidation (MDA production) [15]. From the analysis of this figure, it is possible to observe a significant difference between the H_2O_2

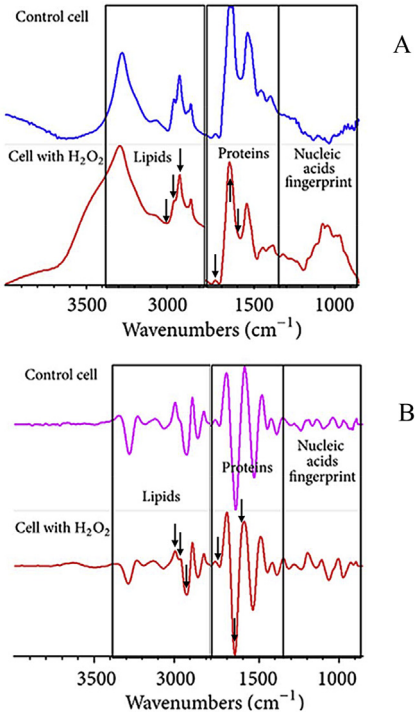

Fig. 1. FTIRM infrared spectra (A) and second derivative analysis (B). (A) control treatment (DMEM 1 h) upper spectra and H_2O_2-treated cells (DMEM 30 min + 30 min 500 μM H_2O_2 for 30 min) lower spectra. Spectra were obtained with a spectral resolution set to 6 cm^{-1}. (B) Average second derivative spectra using Savitsky-Golay with 21 smoothing points and a second polynomial order. (A) control treatment (DMEM 1 h) upper spectra and H_2O_2-treated cells (DMEM 30 min + 30 min 500 μM H_2O_2 for 30 min) lower spectra. Three major zones of interest were observed: Fingerprint and nucleic acid zone (1470-900 cm^{-1}), protein zone (1720-1470 cm^{-1}), and lipid zone (3200-2800 cm^{-1}). The last two zones were used in this work. Arrows indicate the main points of analysis at 1630 cm^{-1}, 1650 cm^{-1}, 1740 cm^{-1}, 2920 cm^{-1}, 2960 cm^{-1} and 3012 cm^{-1}.

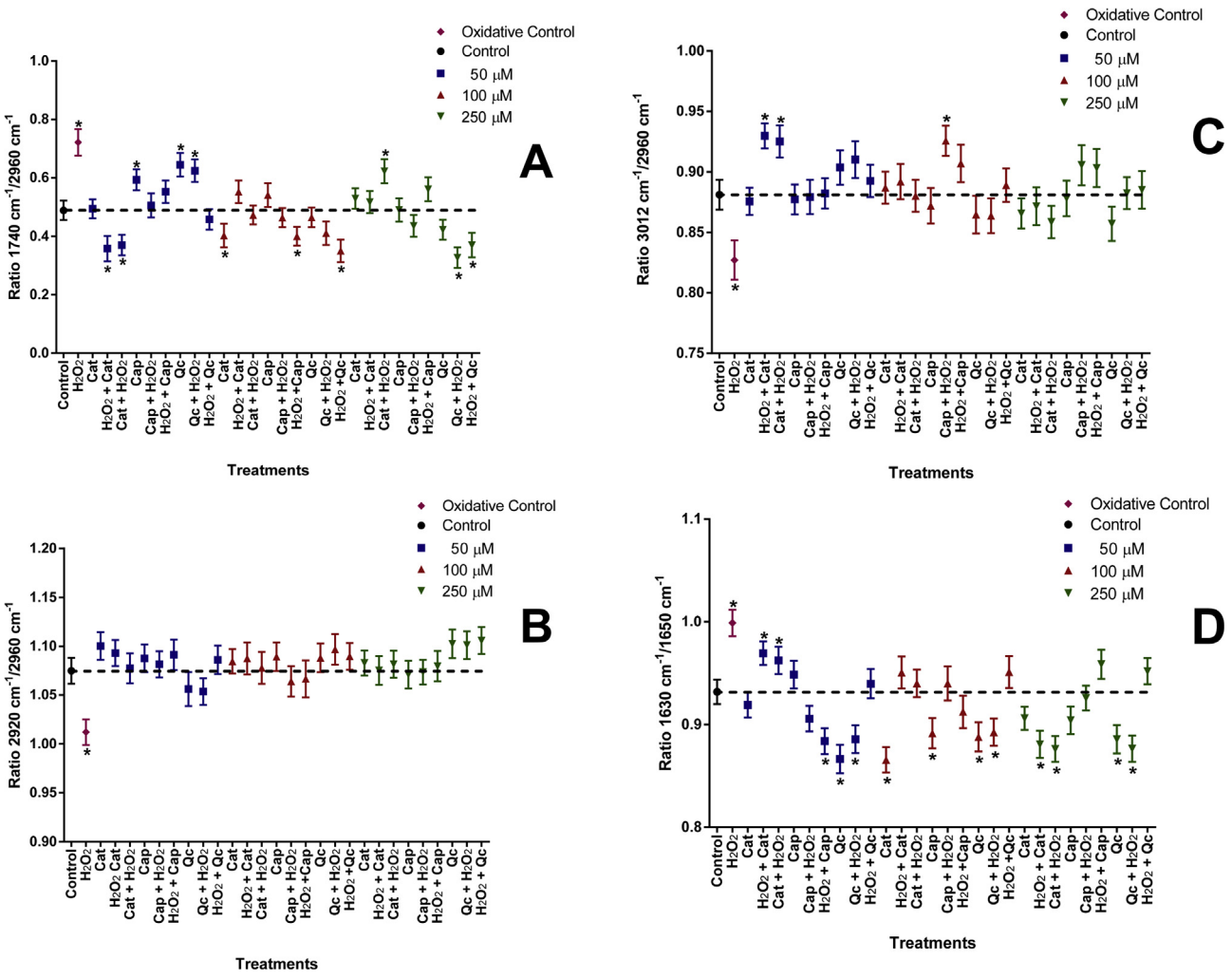

Fig. 2. Ratios between chemical bonds related to the lipid peroxidation observed with the FTIRM spectra data. (A) 1740 cm^{-1}/2960 cm^{-1} ratio assigned to lipid oxidation, (B) 2920 cm^{-1}/2960 cm^{-1} ratio assigned to lipid saturation, (C) 3012 cm^{-1}/2960 cm^{-1} ratio assigned to lipid insaturation and (D) 16ik30 cm^{-1}/1650 cm^{-1} ratio assigned to protein aggregation. The dotted line indicates the mean value of the control cells for each ratio, all ratios were compared to the control ratio in each case, while their significance was measured via Anova. * = Statistically differences respect to control treatment.

treatment (oxidative control), control treatment and PC treatments. H_2O_2 treatment showed the highest ratio value, followed by Qc (free and Qc + H_2O_2) and Cap at low concentration (50 μM), and 250 μM Cat + H_2O_2. These results seem to indicate that the oxidative control and treatments with lower concentrations of Cap and Qc induced a higher production of MDA, a by-product of lipid peroxidation characterized by the presence of an aldehyde group (1740 cm^{-1}). Similar results (increased MDA production) were observed for 250 μM Cat treatment before H_2O_2-induced oxidative stress, even though to a lesser degree than the oxidative control (H_2O_2), suggesting a low protective effect of Cat at this concentration. There is a series of treatments, mainly at 100 and 250 μM PC, with or without H_2O_2, without a statistical difference when compared to the control cells, which indicates that these treatments generated the same amount of MDA as the control. Interestingly, there was a third group of treatments which showed lower MDA formation (lower 1740 cm^{-1}/2960 cm^{-1} ratio) than the control cells. These treatments were 50 μM Cat and 250 μM Qc before or after H_2O_2. The data shown in Fig. 1 seems to indicate that the production of MDA may be triggered by low Cap and Qc concentrations (50 μM), in agreement with our previous work that showed higher production of MDA in Qc treated cells when compared to control cells [6]. Interestingly, Qc showed a protective effect (lower MDA production) at the highest concentration (250 μM). Similar results have been reported in murine sperm cells [20] and PC12 cells [21] under oxidative stress, where higher Qc concentrations (100–500 μM) inhibited MDA production better than lower concentrations (<100 μM). The effects of Cat and Cap in the production of MDA were less dependent of their concentration, in agreement with previous results, which showed concentration-independent effects of Cap in an animal model [23] and of Cat in Caco-2 cells [24]. Interestingly, Cat which, similar to QC, has a catechol group at ring B, but lacks the presence of the double bond and oxo group in ring C and therefore, has a less rigid structure than QC, didn't show a prooxidant activity against parasites unless its concentration was high enough to be considered toxic to the organism [22]. The two hydroxyl groups in an ortho position in Cap seem to favor its scavenging activity when used against parasites or *in vitro*, showing lower oxidative stress [22,25]. These results suggest a Structure-Activity Relationship (SAR) between the structure of the PC and their protective effect against H_2O_2-induced oxidative stress.

The 2920 cm^{-1}/2960 cm^{-1} ratio evaluates the stability of lipids, associated to the proportion between primary and secondary

carbons present in the lipid structure [6,15]. The 2920 cm^{-1}/2960 cm^{-1} ratio results are shown in Fig. 2B, where it is possible to observe that the oxidative control (H_2O_2 treatment) exhibited a lower ratio than the control, indicating a significant effect on lipid saturation related to the loss of integrity in the lipid skeleton and the formation of new shorter aliphatic chains. No significative difference was observed between any PC treatment and the control, independently of the PC concentration. These results may indicate that the three studied PC can protect against the effects of H_2O_2 on lipid saturation and integrity, and that this effect is neither dose nor structure dependent. The unsaturated lipids are targets for ROS. Thus, the third evaluated ratio, 3012 cm^{-1}/2960 cm^{-1} (Fig. 2C), describes the effect of peroxidation on the decrease of unsaturated lipids inside the cell [15]. In agreement with previous results [6], H_2O_2-treated cells showed the lowest 3012 cm^{-1}/2960 cm^{-1} ratio, indicating the highest oxidation of unsaturated lipids. Treatments with 50 μM Cat before and after H_2O_2 and 100 μM Cap before H_2O_2 reduced the unsaturated lipid peroxidation when compared to the control, indicating that these PC decrease the H_2O_2-induced basal oxidation of unsaturated lipids in the interior of the cells. Similar results have been reported when the effect of Cat was evaluated against ethanol-induced lipid peroxidation in rat livers [26]. The rest of the treatments presented a 3012 cm^{-1}/2960 cm^{-1} ratio without significant differences with control cells, indicating that, they avoided H_2O_2-induced damage to unsaturated lipids.

Fig. 2D shows the 1630 cm^{-1}/1650 cm^{-1} ratio, related to protein aggregation caused by protein oxidation as a product of oxidative stress [15]. In agreement with previous results [6], H_2O_2-treated cells showed the highest 1630 cm^{-1}/1650 cm^{-1} ratio, indicating the highest protein aggregation due to oxidative stress. The treatments of Cat + H_2O_2 and H_2O_2 + Cat at 50 μM showed intermediate values between control and H_2O_2-treated cells, indicating that Cat at low concentration showed smaller protective effects against protein aggregation. Interestingly, several treatments reduced protein aggregation compared to basal levels in control cells: all Qc concentrations, used alone or before H_2O_2; 50 μM Cap before or after H_2O_2; 100 μM Cap before H_2O_2; 100 μM Cat without H_2O_2; and 250 μM Cat with or without H_2O_2. Maurya et al., observed that Cat at low concentrations (20 μM) showed a higher protein aggregation in erythrocyte hemoglobin under oxidative stress, probably due to a prooxidant effect [27]. It has been reported that Qc presented a protective effect against protein aggregation at concentrations above 20 μM, however, its protective mechanism has not been described [28].

To evaluate the effect of the PC treatments on the relation between lipids and proteins, the 2920 cm^{-1}/1650 cm^{-1} ratio was used to compare the CH_2 from lipid skeletons with those of amide I bonds from protein (data not shown). Results showed that aside from the treatments H_2O_2 + Cap and H_2O_2 + Qc at 50 μM, no significant differences were observed between all other treatments, including H_2O_2, and control, meaning that the relation between lipids and proteins was not affected by H_2O_2-induced oxidative stress. The differences observed between the control and the H_2O_2+Cap and H_2O_2 + Qc 50 μM treatments can indicate a better protection of lipids by a low concentration of Cap and Qc after the addition of H_2O_2, which is in agreement with TBARS results.

In summary we have seen that protection against lipid peroxidation by PC is dose dependent. Higher concentrations tend to exert better protective effect, except for Cat + H_2O_2 at 250 μM. Interestingly, at low concentration (50 μM) PC seem to exert a prooxidant effect against lipids. The presence of the three PC protected the lipid skeleton and lipid unsaturation against H_2O_2-induced damage, regardless of its concentration. Finally, protein aggregation mediated by H_2O_2-induced oxidative stress was suppressed by all PC, except low Cat concentration, in agreement with previously reported results [27].

3.2. Evaluation of lipid oxidation by conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS)

To compare the FTIRM results, with standard spectrophotometric biochemical assays, primary and secondary lipid oxidation products were measured by CD and TBARS techniques respectively. Fig. 3A shows the results of CD from cells treated with Cap, Cat and Qc at three concentrations (50, 100 and 250 μM) in the presence and absence of H_2O_2. These results showed that cells treated with H_2O_2 alone produced the highest CD content with an average of 4.72 μM of CD / million cells, while control cells showed the lowest CD content with an average of 2.20 mM of CD / million cells (Fig. 3A). Interestingly, all IEC-6 treated cells showed the same trend on their CD values than primary cultures of rat enterocyte cells [6], but with lower values, probably because IEC-6 cells suffered less stress during manipulation.

Data in Fig. 3A, shows that all PC treatments presented CD values similar to those of the control, in agreement with results obtained with primary enterocyte cells [6]. When the effect of the PC concentration was evaluated, a non-statistical dose dependent increase on CD values was observed for all treatments (PC alone, before or after the addition of H_2O_2) as the concentration increased from 50 to 250 μM.

When evaluating the effect of PC on the generation of MDA, through TBARS (Fig. 3B), it was possible to observe that as in the case of CD, H_2O_2-stressed cells showed the highest MDA content, followed by PC treatments and finally positive control, which showed the lowest MDA content. MDA content of IEC-6 cells showed lower values compared to primary cultures of rat enterocyte cells [6], indicating once again that IEC-6 cells suffered less stress during manipulation.

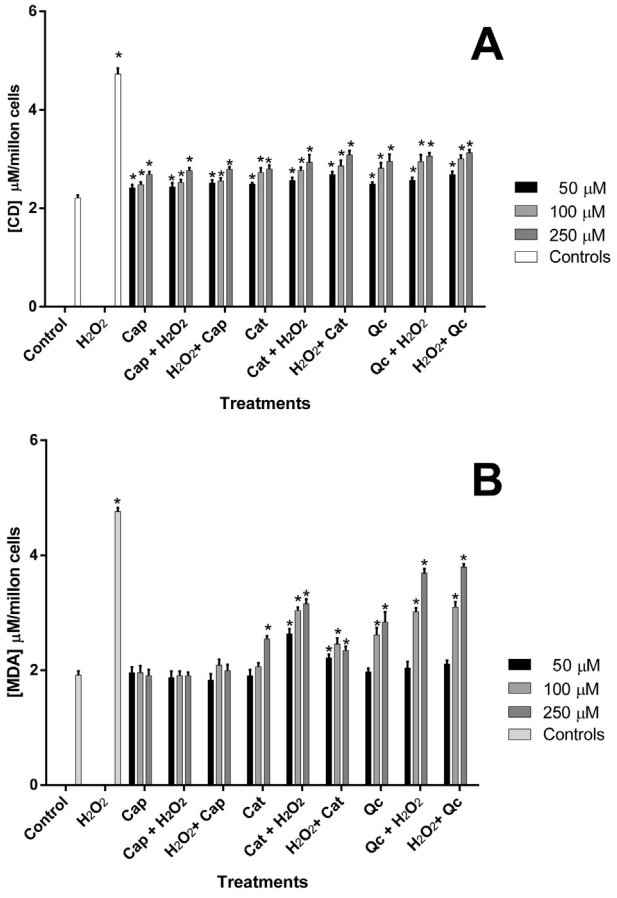

Fig. 3. Spectrophotometric assays to assert lipid peroxidation on IEC-6 treated cells. (A) CD assay. (B) TBARS assay Values presented are averages + standard deviations of 6 individual measurements. * = Statistically differences respect to control treatment.

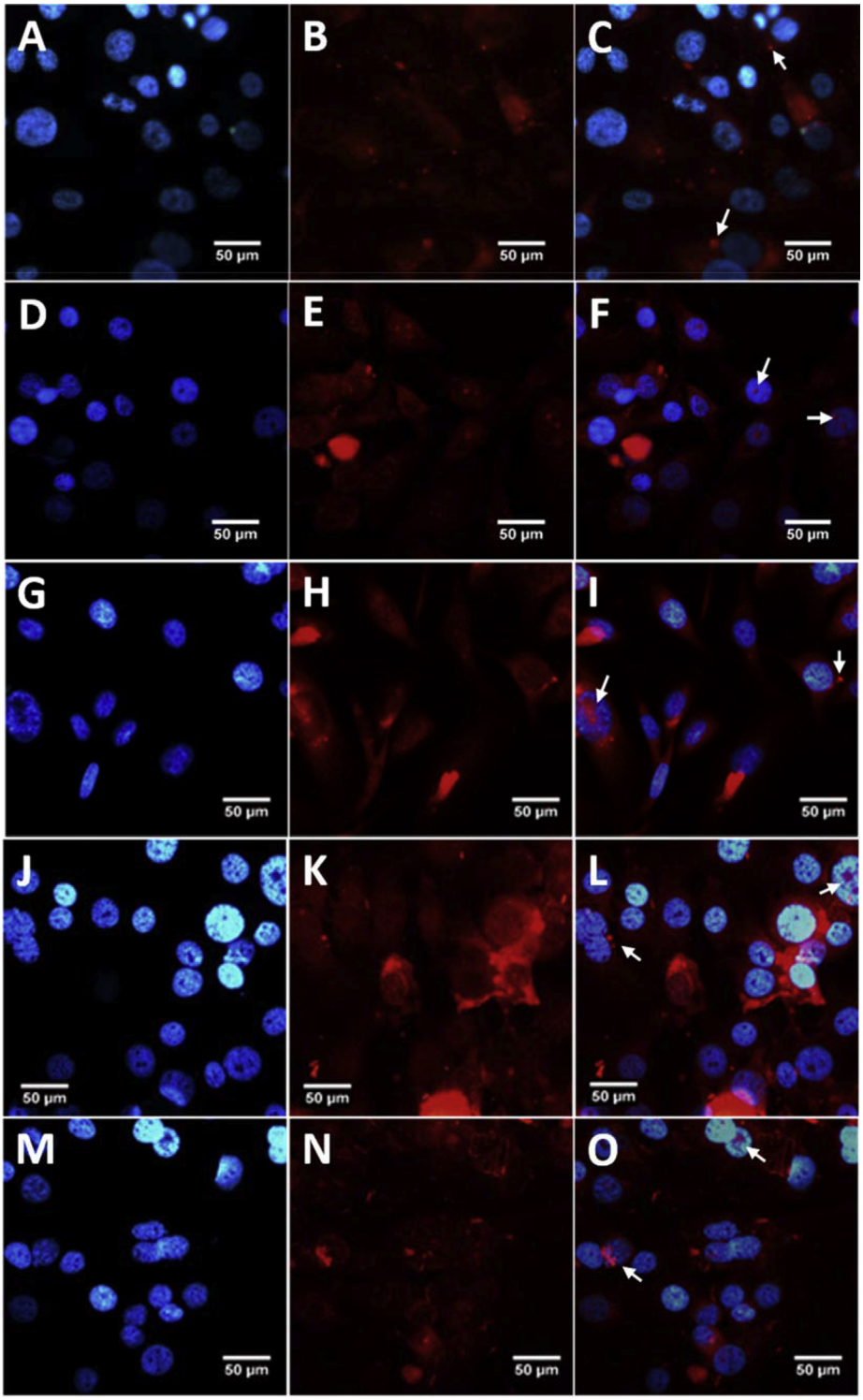

Fig. 4. Immunofluorescence of transcription factor Nrf2 in IEC6 cells. Images A to C, are of cells without any treatment (A) Nuclei with DAPI counterstaining. (B) Location of Nrf2. (C) Co-localization of labels from (A) and (B). Images D to F, are of cells treated with H2O2 (500 μM) (D) Nuclei with DAPI counterstaining. (E) Location of Nrf2. (F) Co-localization of labels from (D) and (E). G to I Qc 100 μM (G) Nuclei with DAPI counterstaining. (H) Location of Nrf2. (I) Co-localization of labels from (G) and (H). J-L, treatment with Qc (100 μM) administered prior to H_2O_2 (500 μM) treatment. (J) Nuclei with DAPI, (K) Anti-Nrf2 fluorescence, (L) Overlap of fluorescence signals from (J) and (K). M – O, treatment with Qc (100 μM) administered after H_2O_2 (100 μM); (M) DAPI nuclei counterstaining (H) Anti-Nrf2 fluorescence, (I) Overlap of fluorescence signals, DAPI stain and anti Nrf2 fluorescent labelling co-localize. White arrows in the merger images indicates some points in which Nrf2 is detected.

Fig. 3B shows the effect of the PC concentration on the production of MDA. It is possible to observe a dose-dependent increase on MDA concentration as Qc concentration increases, being this effect higher in the presence of H_2O_2. Similar results have been observed in liver cells [29] and in erythrocytes [30] treated with Qc at concentrations greater than 100 μM. This increase in MDA values shows that Qc presents higher prooxidant effect as its concentration in the cell increases [30] and that its protective effect against H_2O_2 is better at lower concentrations, since at higher concentrations, it might present a prooxidant effect. In the case of Cat, a significant dose-dependent increase in MDA concentration was observed for Cat alone and administered before the H_2O_2. This type of behavior

indicates that Cat has a slight prooxidant effect at concentrations higher than 100 μM and that it is more effective to rescue cells from an oxidative stress-state than to prevent it. This effect has been previously reported when Cat was administered to endothelial cells [31] and HepG2 cells [32]. Finally, in the case of the Cap treatment, no significant variations in the concentration of MDA were observed after increasing Cap concentration, which indicates that Cap was the best protector against the formation of lipid oxidation products. Similar effect of Cap on the generation of MDA was observed in murine liver cells at concentrations below 100 μM [33].

In summary, all PC, at all concentrations analyzed in the present study, showed no significant effects on CD content when administered by themselves and a complete protection against the H_2O_2-induced CD increment, which means that the protective effect is similar among different PC used. The TBARS assay showed that Cap presented the best protective effect. These results are in agreement with those reported with the FTIRM, in which, independently of the Cap concentration, the production of MDA was lower when compared with the other two PC, indicating that this PC shows a better protective effect against H_2O_2-induced lipid peroxidation.

3.3. Immunofluorescence for PC treatments

The immunofluorescence experiments were carried out to evaluate if the Nrf2-Keap-1 pathway was activated by the interaction of PC (alone or in the presence of H_2O_2) with IEC-6 cells. PC can exert an antioxidant effect on the cells through two mechanisms: a direct or an indirect effect. In the case of a direct effect, the PC exerts its antioxidant activity through neutralization or stabilization of ROS [34]. While in an indirect effect, the PC do not interact directly with the ROS, but activate different biochemical pathways that increase the cell's antioxidant defenses, such as the Nrf2-Keap1 pathway which is common in all cell types. The exact mechanism by which a PC activates the indirect mechanism is still not well known [35], but it has been suggested that a mild prooxidant effect from PC within the cell can trigger it [34]. The binding of the Nrf2-Keap1 complex allows the ubiquitination of Nrf2, for its subsequent proteolysis, thus maintaining the levels of Nrf2 low and hindering its translocation to the nucleus. Inactivation of Keap1 by cysteine oxidation allows Nrf2 to translocate to the nucleus where it acts as a transcription factor for the Antioxidant Response Element (ARE) triggering the transcription of Phase II and antioxidant enzymes [36].

In order to test the hypothesis of activation of Nrf2 translocation by PC, IEC-6 cells were incubated with 100 μM solutions of Qc, Cap and Cat, in the presence or absence of H_2O_2. Fig. 4 (A–I) shows the translocation of Nrf2 to the nucleus in the presence of Qc. Similar results were reported for Cap and Cat. Fig. 4 A–C are control cells, in which Nrf2 is located in the cytoplasmic area which indicates that without an oxidative stimuli Nrf2 is not translocated to the nucleus. Also, in Fig. 4B we can see how the lack of translocation allowed us to see the nucleus of the cells without the need for DAPI staining. Fig. 4 D–F correspond to the oxidative control cells, in which 500 μM H_2O_2 was added. Fig. 4E and F shows that Nrf2 has been translocated to the cell nucleus, due to the oxidative stimulus. This translocation is necessary to activate the antioxidant and cell-protective response. Fig. 4 G–O show the effect of 100 μM Qc treatments, in which loss of integrity in some nucleus can be observed through DAPI staining (Fig. 4G, J and 4M) when compared with the control cells (4A-C). Fig. 4H, K and 4N also show Nrf2 translocation to the nucleus in a similar pattern to the one observed for H_2O_2-treated cells (4D-F). Finally, the merged Fig. 4I, L and 4O shown that most of Nrf2 has been translocated to the nuclei, which indicates a robust activation of the Nrf2-Keap1 pathway. Similar results were observed for 100 μM Cat and Cap treatments (data not shown). Similar effects on the translocation of Nrf2 have been reported for Qc (25 μM) in neurons [37], Cat (50 μM) in human mammary epithelium cells [34,38], Cap

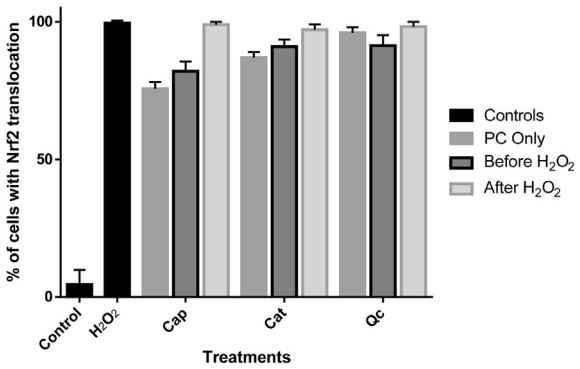

Fig. 5. Percentage of cells with translocation of Nrf2 to the nucleus. The negative and positive controls are shown in black. Meanwhile PC treatments are shown for every combination of treatments used: PC Only, PC before the addition of H_2O_2 and after addition of H_2O_2. Every treatment shows its own SD.

(200 μM) in HepG2 cells [39], and for other phytochemicals such as isothiocyanates [40], resveratrol [41], curcumin [35] and others [35,37] in different cell systems.

Fig. 5 shows the percentage of Nrf2 translocation to the nucleus, considering cells exposed to H_2O_2 as the positive control (100% translocation of Nrf2 to the nucleus), and DMEM-treated cells as the negative control (4.5% translocation). All PC treatments produced a clear translocation of Nrf2 to the nucleus, with Cap showing the lowest Nrf2 translocation percentage with 76% and H_2O_2 + Cap the highest with 99%. These results show that the exposition to IEC-6 cells to Cap, Cat and Qc exerted a clear translocation of Nrf2 to the nucleus.

From the FTIRM, CD, TBARS and immunofluorescence results, we can conclude that Cat, Cap and Qc induced significant antioxidant effects in IEC-6 cells than can be mediated through the translocation of Nrf2 and activation of cellular antioxidant response although a direct antioxidant effect cannot be excluded, especially in lipid peroxidation.

4. Conclusion and perspective

After analyzing the FTIRM spectra, and standard spectrophotometric biochemical assays for lipid oxidation (CD and TBARS) results, it was concluded that, treatments with low concentration (50 μM), presented a mild oxidative stress effect, being this effect higher for Qc. Higher concentrations (100 and 250 μM) were more effective against H_2O_2-induced oxidative stress. Thus, higher PC concentrations showed a better antioxidant effect. Immunofluorescence studies in IEC-6 cells showed that all treatments with 100 μM PC produced a translocation of Nrf2 to the nucleus, suggesting the activation of the Nrf2-Keap1 pathway. While these results expand our knowledge about how PC concentration can affect its antioxidant effect within enterocytes, there is still doubts about how the Nrf2-Keap1 pathway is activated and whether the concentration of PC affects the quantity of phase II enzymes that are produced by the cell. Finally, if there is an indirect antioxidant effect of the PC, the question arises whether this is the only mechanism, or there is also a direct scavenging activity at the same time.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The financial support provided by the Consejo Nacional de Ciencia y Tecnología, CONACYT, Mexico (CB-2011-01-167932 and

CB-2011-01-167164) and by ESRF (LS-2445 proposal) is greatly acknowledged. G. B.-G. wishes to thank CONACYT for the PhD scholarship granted. We also acknowledge the support of Dr. Fernando Plenge Tellechea for lending laboratory installations at Universidad Autonoma de Ciudad Juarez. There is no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.117570.

References

[1] D. Vauzour, A. Rodriguez-Mateos, G. Corona, M.J. Oruna-Concha, J. Spencer, Polyphenols and human health: prevention of disease and mechanisms of action, Nutrients 2 (11) (2010) 1106–1131, https://doi.org/10.3390/nu2111106.

[2] D. Vauzour, Dietary polyphenols as modulators of brain functions: biological actions and molecular mechanisms underpinning their beneficial effects, Oxid Med Cell Longev 16 (2012) 2012, https://doi.org/10.1155/2012/914273. Article ID 914273.

[3] J. Lü, P. Lin, Q. Yao, C. Chen, Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems, J. Cell Mol. Med. 14 (4) (2010) 840–860, https://doi.org/10.1111/j.1582-4934.2009.00897.x.

[4] A. Tan, I. Konczak, I. Ramzan, D. Sze, Antioxidant and cytoprotective activities of native Australian fruit polyphenols, Food Res. Int. 44 (7) (2011) 2034–2040, https://doi.org/10.1016/j.foodres.2010.10.023.

[5] B. Halliwell, Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch. Biochem. Biophys. 476 (2008) 107–112, https://doi.org/10.1016/j.abb.2008.01.028.

[6] G. Barraza-Garza, H. Castillo-Michel, L. de la Rosa, A. Martinez-Martinez, J.A. Pérez-León, M. Cotte, E. Alvarez-Parrilla, Infrared spectroscopy as a tool to study the antioxidant activity of polyphenolic compounds in isolated rat enterocytes, Oxid Med Cell Longev (2016) 10, https://doi.org/10.1155/2016/9245150. Article ID 9245150.

[7] S. Forrester, J. Lambert, The role of antioxidant versus pro-oxidant effects of green tea polyphenols in cancer prevention", Mol. Nutr. Food Res. 55 (2011) 844–854, https://doi.org/10.1002/mnfr.201000641.

[8] D. Pereira, G. Gadelha, M. Castro, D. Pergentino, The dual antioxidant/prooxidant effect of eugenol and its action in cancer development and treatment", Nutrients 9 (2017) 15, https://doi.org/10.3390/nu9121367.

[9] E. Schmalhausen, E. Zhlobek, I. Shalova, O. Firuzi, L. Saso, V. Muronetz, Antioxidant and prooxidant effects of quercetin on glyceraldehyde-3-phosphate dehydrogenase", Food Chem. Toxicol. 45 (10) (2007) 1988–1993, https://doi.org/10.1016/j.fct.2007.04.015.

[10] A. Vargas-Caraveo, H. Castillo-Michel, G. Mejia-Carmona, D. Pérez-Ishiwara, M. Cotte, A. Martínez-Martínez, Preliminary studies of the effects of psychological stress on circulating lymphocytes analyzed by synchrotron radiation based-Fourier transform infrared microspectroscopy, Spectrochim. Acta A. Mol. Biomol. Spectrosc 128 (2014) 141–146, https://doi.org/10.1016/j.saa.2014.02.148.

[11] P. Chougule, G. Herlenius, N. Hernandez, P. Patil, B. Xu, S. Sumitran-Holgersson, Isolation and characterization of human primary enterocytes from small intestine using a novel method, Scand. J. Gastroenterol. 47 (11) (2012) 1334–1343, https://doi.org/10.3109/00365521.2012.708940, 2012.

[12] A. Nik, P. Carlsson, Separation of intact intestinal epithelium from mesenchyme, Biotechniques 55 (1) (2013) 42–44, https://doi.org/10.2144/000114055.

[13] G. Bellisola, C. Sorio, Infrared spectroscopy and microscopy in cancer research and diagnosis, American J. Cancer Res. 2 (1) (2012) 1–21, https://doi.org/10.1038/nprot.2010.133.

[14] F. Martin, J. Kelly, V. Llabjani, P. Martin-Hirsch, I. Patel, J. Trevisan, N. Fullwood, M. Walsh, Distinguishing cell types or populations based on the computational analysis of their infrared spectra, Nat. Protoc. 5 (11) (2010) 1748–1760, https://doi.org/10.1038/nprot.2010.133.

[15] N. Benseny Cases, O. Klementieva, M. Cotte, I. Ferrer, J. Cladera, Microspectroscopy (μFTIR) reveals Co-localization of lipid oxidation and amyloid plaques in human alzheimer, Dis. Brains. Anal. Chem. 86 (24) (2014) 12047–12054, https://doi.org/10.1021/ac502667b.

[16] J. Chwiej, J. Dulinska, K. Janeczko, P. Dumas, D. Eichert, J. Dudala, Z. Setkowicz, Synchrotron FTIR micro-spectroscopy study of the rat hippocampal formation after pilocarpine-evoked seizures, J. Chem. Neuroanat. 40 (2010) 140–147, https://doi.org/10.1016/j.jchemneu.2010.03.008.

[17] C. Petibois, B. Drogat, A. Bikfalvi, G. Deleris, M. Moenner, Histological mapping of biochemical changes in solid tumors by FT-IR spectral imaging, FEBS Lett. 581 (28) (2007) 5469–5474, https://doi.org/10.1016/j.febslet.2007.10.052.

[18] W. André, C. Sandt, P. Dumas, P. Djian, G. Hoffner, "Structure of inclusions of Huntington's disease brain revealed by synchrotron infrared microspectroscopy: polymorphism and relevance to cytotoxicity", Anal. Chem. 85 (7) (2013) 3765–3773, https://doi.org/10.1021/ac400038b.

[19] A.D. Meade, C. Clarke, H.J. Byrne, F.M. Lyng, Fourier transform infrared microspectroscopy and multivariate methods for radiobiological dosimetry, Radiant Res 173 (2) (2010) 225–237, https://doi.org/10.1667/RR1836.1.

[20] F.B. Abdallah, H. Fetoui, N. Zribi, F. Fakfakh, L. Ammar-Keskes, Antioxidant supplementations in vitro improve rat sperm parameters and enhance antioxidant enzyme activities against dimethoate-induced sperm damages, Andrologia 44 (2012) 272–279, https://doi.org/10.1111/j.1439-0272.2011.01177.x.

[21] D. Bao, J. Wang, X. Pang, H. Liu, Protective effect of quercetin against oxidative stress-induced cytotoxicity in rat pheochromocytoma (PC-12) cells", Molecules 22 (7) (2017) 1122, https://doi.org/10.3390/molecules22071122.

[22] J. Baldim, B. Gonçalves, O. da Silva Domingos, M. Gomes, I. Santana, R. Dias, T. Branquinho, J. Ghilardi, D. Aparecida, The correlation between chemical structures and antioxidant, prooxidant, and antitrypanosomatid properties of flavonoids", Oxid Med Cell Longev 12 (2017) https://doi.org/10.1155/2017/3789856.

[23] A.F. El-kott, M.M. Bin-Meferij, Suppressive effects of capsaicin against N-nitrosomethyurea induced tumorigenesis in rats, Biomed. Pharmacother. 98 (2018) 673–679, https://doi.org/10.1016/j.biopha.2017.12.036.

[24] J. Intra, S.M. Kuo, Physiological levels of tea catechins increase celular lipid antioxidant activity of vitamin C and vitamin E in human intestinal Caco-2 cells, Chem. Biol. Interact. 169 (2007) 91–99, https://doi.org/10.1016/j.cbi.2007.05.007.

[25] M. Reyes-Escogido, E. Gonzalez-Mondragon, E. Vazquez-Tzompantzi, Chemical and pharmacological aspects of capsaicin, Molecules 16 (2) (2011) 1253–1270, https://doi.org/10.3390/molecules16021253.

[26] J. Ostrowska, W. Luczaj, I. Kasacka, A. Ronzanski, E. Skrzydlewska, Green tea protects against ethanols-induced lipid peroxidation in rat organs, Alcohol 32 (2004) 25–32, https://doi.org/10.1016/j.alcohol.2003.11.001.

[27] P.K. Maurya, P. Kumar, P. Chandra, Biomarkers of oxidative stress in erythrocytes as a function of human age, World J. Methodol. 5 (4) (2015) 216–222, https://doi.org/10.5662/wjm.v5.i4.216.

[28] S. Hasanbašić, A. Jahić, S. Berbić, M. Tušek Žnidarič, E. Žerovnik, Inhibition of protein aggregation by several antioxidants", Oxid Med Cell Longev 2018 (2018) 12, https://doi.org/10.1155/2018/8613209. Article ID 8613209.

[29] C.M. Liu, Y.L. Zheng, J. Lu, Z.F. Zhang, S.H. Fan, D.M. Wu, J.Q. Ma, Quercetin protects rat liver against lead-induced oxidative stress and apoptosis, Environ. Toxicol. Pharmacol. 29 (2) (2010) 158–166, https://doi.org/10.1016/j.etap.2009.12.006.

[30] A. Jamshidzadeh, A.R. Mehrabadi, Protective effect of quercetin on oxidative stress in glucose-6-phosphate dehydrogenase-deficient erythrocytes *in vitro*, Iran. J. Pharm. Res. (IJPR) 9 (2) (2010) 169–175.

[31] T.T. Yang, M. Koo, Inhibitory effect of Chinese green tea on endothelial cell-induced LDL oxidation", Atherosclerosis 148 (1) (2000) 67–73, https://doi.org/10.1016/S0021-9150(99)00239-7.

[32] L. Chen, X. Yang, H. Jiao, B. Zhao, Tea catechins protect against lead-induced cytotoxicity, lipid peroxidation, and membrane fluidity in HepG2 cells, Toxicol. Sci. 69 (1) (2002) 149–156, https://doi.org/10.1093/toxsci/69.1.149.

[33] R. Gangabhagirathi, R. Joshi, Antioxidant activity of capsaicin on radiation-induced oxidation of murine hepatic mitochondrial membrane preparation", Res. Rep. Biochem. 2015 (5) (2015) 163–171, https://doi.org/10.2147/RRBC.S84270.

[34] T. Nguyen, P. Nioi, C.B. Pickett, The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress", J. Biol. Chem. 284 (20) (2009) 13291–13295, https://doi.org/10.1074/jbc.R900010200.

[35] H. Erlank, A. Elmann, R. Kohen, J. Kanner, "Polyphenols activate Nrf2 in astrocytes via H2O2, semiquinones, and quinones", Free Radic. Biol. Med. 51 (12) (2011) 2319–2327, https://doi.org/10.1016/j.freeradbiomed.2011.09.033.

[36] B. Zou, G. Xiao, Y. Xu, J. Wu, Y. Yu, M. Fu, Persimmon vinegar polyphenols protect against hydrogen peroxide-induced cellular oxidative stress via Nrf2 signalling pathway", Food Chem. 255 (2018) 23–30, https://doi.org/10.1016/j.foodchem.2018.02.028.

[37] F. Arredondo, C. Echeverry, J.A. Abin-Carriquiry, F. Blasina, K. Antúnez, D.P. Jones, Y.M. Go, Y.L. Liang, F. Dajas, After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult, Free Radic. Biol. Med. 49 (5) (2010) 738–747, https://doi.org/10.1016/j.freeradbiomed.2010.05.020.

[38] H.K. Na, Y.J. Surh, Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG", Food Chem. Toxicol. 46 (4) (2008) 1271–1278, https://doi.org/10.1016/j.fct.2007.10.006.

[39] E.J. Joung, M.H. Li, H.G. Lee, N. Somparn, Y.S. Jung, H.K. Na, S.H. Kim, Y.N. Cha, Y.J. Surh, Capsaicin induces heme oxygenase-1 expression in HepG2 cells via activation of PI3K-Nrf2 signaling: NAD(P)H:quinone oxidoreductase as a potential target", Antioxidants Redox Signal. 9 (12) (2007) 2087–2098.

[40] C. Xu, X. Yuan, Z. Pan, G. Shen, J.H. Kim, S. Yu, T.O. Khor, W. Li, J. Ma, A.N. Kong, Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2, Mol. Cancer Ther. 5 (8) (2006) 1918–1926, https://doi.org/10.1158/1535-7163.MCT-05-0497.

[41] T.C. Hsieh, X. Lu, Z. Wang, J.M. Wu, Induction of quinone reductase NQO1 by resveratrol in human K562 cells involves the antioxidant response element ARE and is accompanied by nuclear translocation of transcription factor Nrf2, Med. Chem. 2 (3) (2006) 275–285, https://doi.org/10.2174/157340606776930709.