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L'OPUNTIA COME MODULATORE DELLE PROTEINE CHE TRASPORTANO IL COLESTEROLO

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SCOPO DELLA RICERCA

In questo 9° numero presentiamo una ricerca condotta all'università di Lisbona che ha esaminato l'effetto dei composti fenolici presenti nel decotto di cladodi di Opuntia ficus-indica sulla regolazione dei trasportatori del colesterolo nelle cellule epatiche HepG2. Le cellule trattate hanno mostrato una diminuzione dell'abbondanza dei trasportatori NPC1L1, ABCA1, ABCG5 e ABCG8 rispetto al controllo, con una riduzione compresa tra il 13% e il 70%. L'analisi FTIR ha rivelato cambiamenti nella quantità di RNA, suggerendo che la diminuzione dei trasportatori del colesterolo potrebbe essere legata a modifiche nella sintesi proteica. Questi risultati suggeriscono un potenziale ruolo del decotto di cladodi di Opuntia ficus-indica nella modulazione dei livelli di colesterolo attraverso la regolazione dei trasportatori di membrana nelle cellule epatiche.

SCRITTO DA:

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TITOLO:

Le proteine trasportatrici del colesterolo nelle cellule HepG2 possono essere modulate da composti fenolici presenti nelle soluzioni acquose di Opuntia ficus-indica

INTRODUZIONE:

L'Opuntia ficus indica è una pianta che cresce spontanea nelle regioni aride del pianeta (Ennouri et al., 2006). I suoi cladodi sono utilizzati nella preparazione di alimenti e bevande con diverse funzioni bioattive riconosciute (Osunamartínez, Reyes-esparza, & Rodríguez-fragoso, 2014), tra cui per il trattamento dell'ipercolesterolemia (Galati, Tripodo,
Trovato, Aquino e Monforte, 2003). Questo effetto è stato dimostrato negli animali da laboratorio (Galati et al., 2003; Padilla-Camberos et al., 2015) e una riduzione della permeazione del colesterolo è stata osservata nei monostrati di Caco-2 (Ressaissi et al., 2017).
L'omeostasi del colesterolo è controllata principalmente dalla sintesi endogena, dall'assorbimento intestinale e dall'escrezione epatica. Uno squilibrio di questi processi può portare a elevate concentrazioni di colesterolo nel plasma, accumulo di colesterolo in diversi tessuti e aumento del rischio di malattie cardiovascolari aterosclerotiche (Park & Carr, 2013).
Il fegato svolge un ruolo centrale nell'omeostasi del colesterolo, regolando la sintesi de novo di una percentuale significativa di colesterolo nel corpo. Inoltre, produce e assorbe lipoproteine contenenti colesterolo, fondamentali per regolare le concentrazioni di colesterolo nel sangue, e converte il colesterolo in acidi biliari regolando quindi l'efficienza di assorbimento intestinale di lipidi e vitamine. Il fegato secerne nella bile sia il colesterolo non esterificato che gli acidi biliari derivati dal colesterolo, ed è quindi importante per lo smaltimento del colesterolo. Tutti questi percorsi nel fegato sono processi mediati dalle proteine (Jia, Betters e Yu, 2011).
Diversi studi hanno identificato la proteina Niemann-Pick C1 like 1 (NPC1L1) come trasportatore dell'assorbimento del colesterolo (Yu et al., 2006), localizzato nella membrana apicale degli enterociti e nella membrana canalicolare degli epatociti. Funziona come trasportatore di steroli per mediare l'assorbimento intestinale del colesterolo e controbilanciare l'escrezione epatobiliare del colesterolo (Jia et al., 2011). Esistono anche proteine ATP-binding cassette (ABC) come ABCG5 e ABCG8 che sono trasportatori di efflusso di colesterolo nelle cellule intestinali ed epatiche (Brown & Yu, 2009) e ABCA1, una proteina trasportatrice di colesterolo di membrana che media l'efflusso del colesterolo ai lipidi apolipoproteina povera (apoAI) (Lv et al., 2014).
La riduzione dell'escrezione del colesterolo attraverso l'inibizione dei trasportatori è un meccanismo attraverso il quale gli alimenti funzionali e i nutraceutici possono diminuire
livello di colesterolo plasmatico (Chen, Jiao e Ka, 2008).
È stato dimostrato che i composti fenolici presenti nella dieta agiscono sull'omeostasi del colesterolo (Davalos et al., 2006).

Per spiegare l'effetto dei composti fenolici sull'assorbimento del colesterolo precedentemente riscontrato (Ressaissi et al., 2017), il livello delle proteine trasportatrici del colesterolo nelle linee cellulari HepG2 è stato studiato utilizzando l'analisi western blot e la spettroscopia FTIR. Poiché i livelli proteici nelle cellule sono regolati da una varietà di meccanismi complessi, non solo quelli legati all'espressione genica o al conteggio degli mRNA, abbiamo utilizzato un approccio per valutare l'abbondanza proteica basato sulla metodologia Western Blot utilizzando anticorpi specifici (Mahmood & Yang, 2012) contro trasportatori del colesterolo nelle linee cellulari HepG2. La spettroscopia infrarossa a trasformata di Fourier (FTIR) è stata ampiamente utilizzata per studiare i sistemi biologici. La spettroscopia FTIR è diventata un potente strumento per l'analisi dei componenti cellulari, come membrane (Mantsch & McElhaney, 1991), proteine (Fale & Chan, 2015) e acidi nucleici (Taillandie & Liquier, 1992). I cambiamenti spettrali delle cellule possono essere studiati utilizzando la spettroscopia FTIR, riflettendo il meccanismo d'azione delle molecole bioattive o la reazione delle cellule nei loro confronti (Fale & Chan, 2015).
L'obiettivo del presente lavoro era di studiare l'effetto dei composti fenolici del decotto di Opuntia ficus indica sulle proteine trasportatrici del colesterolo, spiegando l'effetto precedentemente notato sulla permeazione del colesterolo attraverso le cellule.

CONCLUSIONI:

I polifenoli del decotto di cladodi hanno ridotto il contenuto proteico totale nelle cellule umane, compresi i trasportatori di membrana del colesterolo NPC1L1, ABCG5/ABCG8 e ABCA1. Queste modifiche potrebbero essere correlate a una diminuzione della trascrizione dell'RNA (espressione proteica) quando le cellule erano sotto l'effetto dell'estratto, come osservato dall'analisi FTIR. L'effetto notato può spiegare la diminuzione del livello di colesterolo nel sangue descritta dalle persone che usano i cladodi negli alimenti o nelle bevande.

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Cholesterol transporter proteins in HepG2 cells can be modulated by phenolic compounds present in *Opuntia ficus-indica* aqueous solutions

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ABSTRACT

Increased blood cholesterol is a risk factor for atherosclerotic cardiovascular disease. This study tested the hypothesis that phenolic compounds can modulate the level of cholesterol transporters including Niemann-Pick C1-like 1 (NPC1L1) and ATP-binding cassette transporters in liver cells. HepG2 cells, used as a model of hepatocytes, showed a decrease in the abundance of cholesterol transporters comparatively to the control when treated with the *Opuntia ficus-indica*'s cladodes decoction. The decrease was between 13–70%, 25–60%, 9–60% and 23–60% for NPC1L1, ABCA1, ABCG5 and ABCG8 transporters, respectively, when using between 0.15 and 0.35 mg/mL of decoction in the culture medium. FTIR analysis showed changes in the amount of RNA, which may be the cause of the decrease in the level of several proteins. These *in vitro* results pave the way to a molecular explanation for the decoction of cladodes effect on cholesterol levels as it reduced the membrane cholesterol transporter proteins, NPC1L1, ABCG5/ABCG8 and ABCA1, in HepG2 cells.

1. Introduction

Opuntia ficus indica is a plant growing wild in dry regions of the planet (Ennouri et al., 2006). Its cladodes are used in food and beverage preparation with several proclaimed bioactive functions (Osuna-martínez, Reyes-esparza, & Rodríguez-fragoso, 2014), among which for the treatment of hypercholesterolemia (Galati, Tripodo, Trovato, Aquino, & Monforte, 2003). This effect has been demonstrated in laboratory animals (Galati et al., 2003; Padilla-Camberos et al., 2015), and cholesterol permeation reduction was observed in Caco-2 monolayers (Ressaissi et al., 2017).

Cholesterol homeostasis is controlled mainly by endogenous synthesis, intestinal absorption, and hepatic excretion. An imbalance of these processes may lead to high cholesterol concentrations in the plasma, cholesterol accumulation in different tissues, and increased risk of atherosclerotic cardiovascular diseases (Park & Carr, 2013).

The liver plays a central role in cholesterol homeostasis, by regulating the de novo synthesis of a significant proportion of cholesterol in the body. Also, it produces and takes up lipoprotein containing cholesterol, critical for regulating blood cholesterol concentrations, and converts cholesterol to bile acids therefore regulating intestinal absorption efficiencies of lipids and vitamins. Liver secretes both unesterified cholesterol and cholesterol-derived bile acids into bile, and thus is important for cholesterol disposal. All these pathways in the liver are protein-mediated processes (Jia, Betters, & Yu, 2011).

Several studies have identified Niemann-Pick C1 like 1 (NPC1L1) protein as a cholesterol uptake transporter (Yu et al., 2006), located in the apical membrane of enterocytes and the canalicular membrane of hepatocytes. It functions as a sterol transporter to mediate intestinal cholesterol absorption and counterbalances hepatobiliary cholesterol excretion (Jia et al., 2011). There are also ATP-binding cassette (ABC) proteins such as ABCG5 and ABCG8 which are cholesterol efflux transporters in intestinal and liver cells (Brown & Yu, 2009) and ABCA1, a membrane cholesterol-transporter protein mediating the efflux of cholesterol to lipid-poor apolipoprotein (apoAI) (Lv et al., 2014). Reduction of cholesterol excretion via transporters inhibition is a mechanism by which functional foods and nutraceuticals can decrease plasma cholesterol level (Chen, Jiao, & Ka, 2008).

Phenolic compounds present in the diet have been shown to act on cholesterol homeostasis (Davalos et al., 2006). To explain the effect of phenolic compounds on cholesterol absorption previously found (Ressaissi et al., 2017), the level of cholesterol transporter proteins in HepG2 cell lines was studied using western blot and FTIR spectroscopy analysis. As protein levels in cells are regulated by a variety of complex

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mechanisms, not only those related to gene expression or mRNA counts, we have used an approach to evaluate protein abundance based on western blot methodology using specific antibodies (Mahmood & Yang, 2012) against cholesterol transporters in HepG2 cell lines. Fourier-transform infrared (FTIR) spectroscopy has been widely used to investigate biological systems. FTIR spectroscopy has become a powerful tool for the analysis of cell components, such as membranes (Mantsch & McElhaney, 1991), proteins (Fale & Chan, 2015) and nucleic acids (Taillandie & Liquier, 1992). Spectral changes of cells can be studied using FTIR spectroscopy, reflecting the mechanism of action of bioactive molecules, or the reaction of cells towards them (Fale & Chan, 2015).

The objective of the present work was to investigate effect of phenolic compounds from *Opuntia ficus indica* decoction on cholesterol transporter proteins explaining the effect previously noticed on cholesterol permeation through cells.

2. Material and methods

2.1. Chemicals

Trypsin, Dulbecco's modified Eagle medium (DMEM), glutamine, Pen-Strep (penicillin and streptomycin mixture), Phosphate-Buffered Saline (PBS), Lysis buffer (Igepal 4%, DTT 1%, Urea 6 M), TBS (250 mM,1920 mM tris-Glycine, 20% methanol, 70% water), TBST (TBS + 1%Tween 20) and Fetal Bovine Serum (FBS) from Lonza (Verviers, Belgium) were bought from VWR International. Methanol was obtained from Merck (Darmstadt, Germany bought to VWR International). SDS-PAGE gels and and Western Blot Reagent Kit (Amersham ECL™ Prime™) were obtained from VWR International, ABCA1, ABCG5/G8 and NPC1L1 were obtained from Novus Biologicals and α-tubulin was obtained from Sigma-Aldrich.

The secondary antibody, Amersham ECL™ anti-rabbit IgG, horseradish peroxidase-linked whole antibody (from donkey) was from VWR International.

2.2. Plant extract preparation

Cladodes of *Opuntia ficus indica* (L.) Mill were harvested from La Manouba, Tunisia in November 2014, 36°48′42.4″N 10°05′16.9″E. Cladodes (10 g) were boiled with water (100 mL) for 20 min, filtered with Whatman paper n°1 and finally lyophilized using a Heto PowerDry LL3000. To separate fibers from the aqueous extract, Ethanol was added to the dried extract (32:1 v/w) and maintained for 24 h at 4 °C. The mixture was then centrifuged, and the supernatant was evaporated at 40 °C under vacuum in a rotary evaporator (with a yield of 0.6% from fresh cladodes).

2.3. Cell culture

HepG2, Human hepatocellular liver carcinoma cell lines (ATCC HB-8065) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine at 37 °C in an atmosphere with 5% CO_2 by seeding approx. 2×10^4 cells/cm^2. The medium was changed every 48–72 h, and cells were trypsinized before reaching confluence.

Cells were seeded in T25 flasks. After reaching confluence they were incubated for 24 h with different aqueous extract concentrations below the $IC_{50\%}$ (Ressaissi et al., 2017). The concentration used in this study were 0.15, 0.25 and 0.35 mg of extract/mL of medium which correspond to 26, 43, 60 mg of fresh cladodes/mL of medium. For the FTIR analysis the concentration of cladodes decoction used was equal to IC_{40} (0.5 mg/mL). A blank was also prepared by growing cells in medium without extract. Culture medium was removed, and cells were washed twice with cold PBS. Cells from each T25 were scrapped and transferred to a pre-weighed test tube. To each test tube lysis buffer was added in order to get the same weight of cells/mL (Silva et al., 2013).

2.4. Cell and protein quantification

Cells collected with water were lyophilized and weighed then the lysis buffer was added in order to have the same concentration of cells. After disrupting the cells, the amount of protein was quantified using Bradford reagent. To 800 µL of water and 200 µL of Bradford, 2 µL of lysis buffer for the blank and 2 µL of extracted proteins from the samples were added. After 5 min the absorbances were read at 519 nm and the amount of protein was calculated using a calibration curve done with BSA.

2.5. Western Blot assays

The Western Blot assays were carried out as previously described with small modifications (Lai et al., 2015). The suspensions of cells previously prepared were disrupted by sonication in an ultrasound bath for 5 min. Samples were centrifuged for 20 min at 5000*g* and separated under reducing conditions in SDS-PAGE 4–12% gradient gels. Per lane the amount of protein loaded was contained in the same mass of cells (161.3 mg of cell /mL of lysis buffer). Gels ran according to the manufacturer's instructions, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) during 2 h and blocked with 5% non-fat milk in TBST buffer for 1 h. The membranes were incubated with primary antibodies overnight at 4 °C, according to the protein under investigation, and after that washed for four times with TBST for a total of 30 min, then the secondary antibodies conjugated with horseradish peroxidase was added for 1 h at room temperature and washed for four times with TBST. The blots were visualized with the western blotting detection reagents according to the manufacturer's instructions. The protein quantification in each band was performed using ImageJ densitometry analysis.

2.6. FTIR analysis

The FTIR analysis were carried out as previously described with small modifications (Chan & Fale, 2014). HepG2 were maintained in T25 cell culture flasks using DMEM medium supplemented with 10% FBS and 2 mM L-glutamine, in a 5% CO_2 environment at 37 °C. The cells were trypsinzed and harvested when they had reached ~80% confluence. The cell suspension was diluted in medium to reach a cell density of $\sim 1.2 \times 10^6$ cell/mL. The resulting cell suspension was seeded directly onto CaF_2 windows and incubated overnight. The cells were treated with a concentration of 0.5 mg/mL of extract (40% toxicity) for 24 h (Ressaissi et al., 2017). The spectra acquisitions were performed using Nicollet 6700 FT-IR apparatus from Thermo Electron Corporation®. All measurements were acquired with a spectral resolution of 4 cm^{-1} and a spectral range of 900–3000 cm^{-1}. FTIR spectra were water vapor subtracted and normalized to Amide II band. The OMINC software was used for acquisition and processing of spectra.

2.7. Data analysis

All the data were expressed as mean ± standard deviation of 3 replicates. Additional analysis of variance (ANOVA) was performed with $\alpha = 0.05$ using software SPSS, version 24.

3. Results and discussion

Decoction of cladodes was previously shown to contain piscidic and eucomic acids, and isorhamnetin derivatives, by LC-MS/MS (Ressaissi et al., 2016), Fig. 1. The bioavailability assays showed that the compounds were able to permeate the intestinal barrier and could reduce cholesterol permeation through intestinal cells (Ressaissi et al., 2017). One of the hypotheses that phenolic compounds can use to reduce

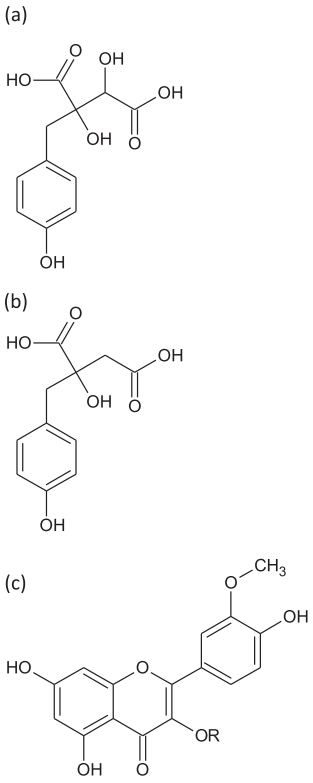

Fig. 1. Chemical structure of (a) piscidic acid; (b) eucomic acid; (c) isorhamnetin derivatives, R = glucosyl-rhamnosyl-rhamnoside, R = glucosyl-rhamnosyl-pentoside, R = glucosyl-pentoside, R = rutinoside.

cholesterol absorption is by modifying cholesterol-transporter proteins. Therefore, to study the effect of cladodes decoction on cholesterol permeation HepG2 cells were used. These express several cholesterol transporter proteins, such as NPC1L1 (Jia et al., 2011), ABCG5/ABCG8 (Wang et al., 2015) and ABCA1 (De Haan, Karasinska, Ruddle, & Hayden, 2014). To analyze the level of these proteins under the effect of cladodes decoction, western blot was used. FTIR spectroscopy was employed to verify if this extract produced modifications at RNA level were used.

3.1. Effect of polyphenol on total protein amount and α-Tubulin expression

The method used to study the effect of cladodes decoction on cholesterol transporter proteins was electrophoresis, more precisely western blot with the respective use of antibodies. One of the main problems of this technique is the standardization of the protein content to allocate to the gel. Usually, the standardization is carried out using the same protein content and after then using α-tubulin or actin as an internal standard (Li & Shen, 2013).

The effect of cladodes' decoction from *Opuntia ficus-indica* in the cell growth was analyzed on the cell protein and α-tubulin content to be sure that polyphenols do not affect either the protein amount in the cell or α-tubulin expression.

It can be seen in Fig. 2, that the effect of extract concentration on cell total protein shows a positive correlation with % of total protein levels decrease. The highest concentration of decoction used, 0.35 mg/mL, a value lower than the IC_{50} (0.84 mg/mL) for HepG2 cells (Ressaissi et al., 2017) produced 60% decrease in total amount of cell protein. These results denote that the cells were probably under stress. Several stress conditions are known to produce a decrease in total protein content of the cells (Spriggs, Bushell, & Willis, 2010), although no reports were found for this type of effect on total protein content produced by phenolic compounds.

Other approaches to standardize the applications of proteins on SDS-Gels for western blot, use constitutive proteins, such as α-tubulin, as internal standards. Therefore the effect of the extract on α-tubulin amount was also investigated. Again, the application in the lane for electrophoresis gel was carried out according to the same dry mass of cells. The results in Fig. 3, reveal again that the increase in the extract concentration in the cell culture medium originated a decrease in α-tubulin expression inside the cells with more than 40% decrease for the highest concentration. Other researchers found that quercetin can affect the expression of actin in concentration-dependent manner (Böhl, Czupalla, Tokalov, Hoflack, & Gutzeit, 2005)

Cellular proliferation and differentiation during development is controlled by many regulatory processes. These include the selective induction, expression and regulation of the activity of genes which are likely to encode for DNA binding proteins or proteins that interact with them and control the transcription of several other genes (Kessel & Gruss, 1990). The obtained results showed that the phenolic compounds downregulate the total protein expression and downregulate α-Tubulin (Fig. 3) these can be due to the interference in the transcription factors, for instance. Nevertheless, as these and previous studies indicated that phenolic compounds could change cell protein content (Böhl et al., 2005; Falé et al., 2012) the total protein content cannot be used as standardization measure for western blotting analysis and even actin cannot be used as a standard.

3.2. Effect of phenolic compounds on cholesterol transporters

The effect of phenolic compounds on cholesterol transporter proteins was analyzed by western blotting. HepG2 cells were treated with different concentrations of polyphenols from cladodes decoction, 0.15–0.35 mg/mL, during 24 h, to understand the effect of cladodes decoction on cholesterol transporter proteins in the liver. The abundance of proteins NPC1L1, ABCA1, ABCG5 and ABCG8 were analyzed with specific antibodies in Western blot, Fig. 4, the bands intensities were quantified using the ImageJ program and the % of decrease was determined relatively to the same protein in the control. A reduction in the band intensity can be seen, depending on the decoction concentration (Fig. 4).

It can be seen in Fig. 4, that the polyphenols treatment of HepG2 cells decreased the abundance of all the cholesterol transporter proteins under investigation. In these experiments a dose-dependent effect was observed. In Fig. 4a western blot showing the effect of 3 extract concentrations on the cholesterol transporter proteins relatively to the cells control is shown. In Fig. 4b the quantification of these changes is represented. It indicates a decrease in NPC1L1 of 12.9 and 70.7% with 0.15 and 0.35 mg/mL of cladodes decoction, respectively. In addition to NPC1L1, the expression of ABC transporters was studied and all of them showed a decrease in the expression ranging from 8.8 and 62.9%, in a dose-dependent manner.

Other polyphenols have been shown to inhibit NPC1L1 expression, such as curcumin (Feng, Ohlsson, & Duan, 2010), quercetin and luteolin (Nekohashi et al., 2014). The decoction of cladodes contains isorhamnetin derivatives, piscidic and eucomic acids (Ressaissi et al., 2016). Isorhamnetin was also shown to decrease NPC1L1 amount in cells (Chua, 2014), however to authors knowledge there are no reports on the effect of piscidic acid or eucomic acid, the phenolic compounds present in the cladodes extract under investigation.

Drugs often used for treating hypercholesterolemia by inhibition of cholesterol biosynthesis like statins, have also been studied on their effect on cholesterol transporter proteins. It has been shown that pravastatin decreased the levels of NPC1L1 and ABCG5/G8 in 70% and 80% comparative to the control, respectively (Kawase, Hata, Takagi, & Iwaki, 2015). In this study similar results were obtained for cladodes decoction using a concentration of 0.35 mg /mL, suggesting a comparable effect to pravastatin. In addition, in the present work we demonstrate that the level of ABCA1 decreases under the effect of the

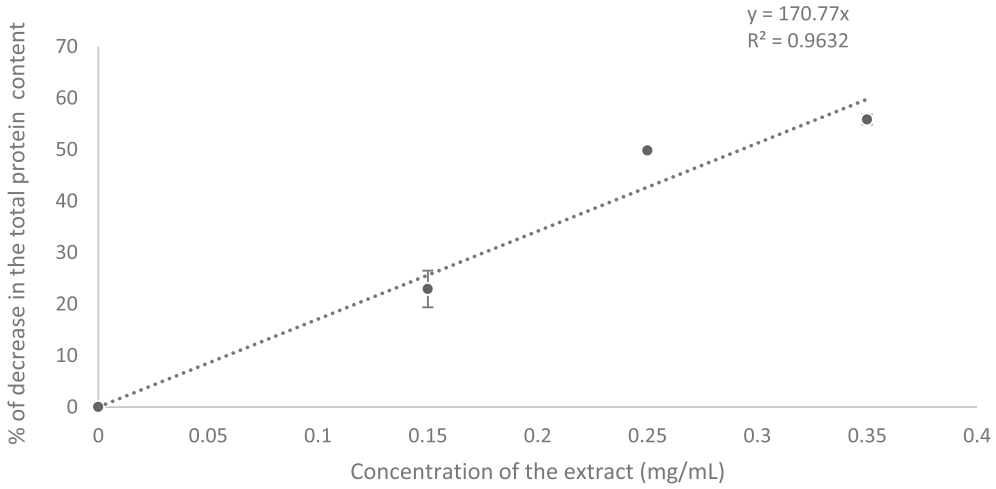

Fig. 2. Effect of Cladodes extract on the amount of protein using the same weight of cells.

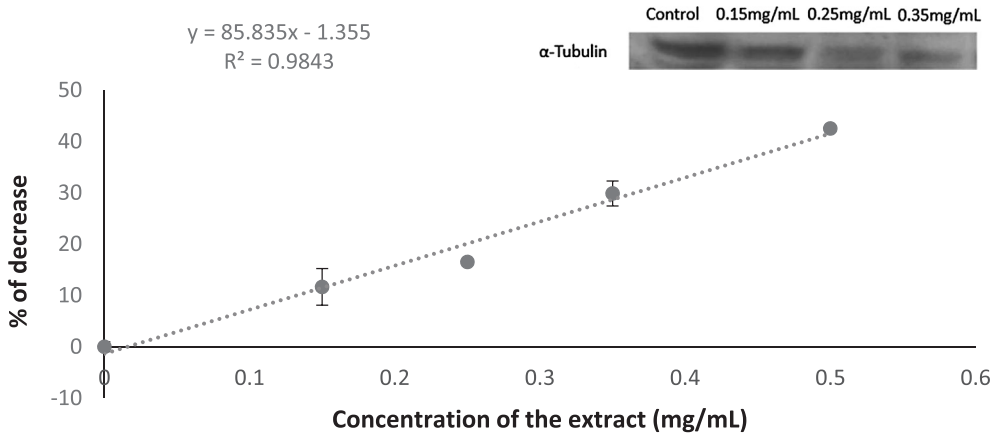

Fig. 3. Effect of polyphenol on α-Tubulin expression in HepG2 cell lines using the same weight of cells for different concentration of extract.

extract. Analogous results were found for atorvastatin and simvastatin, suggesting that these lipid-lowering drugs downregulate ABCA1 and ABCG1 expression by limiting the availability of oxysterol ligands to Liver X Receptors "LXRs" (Genvigir et al., 2010; Wong, Quinn, Gelissen, Jessup, & Brown, 2008), whereas pravastatin did not change the ABCA1 mRNA and protein level in HepG2 (Ando et al., 2004).

The changes in the abundance of several cholesterol transporters in the liver can be related to the mechanism of action or the mode by which the compounds interact with the cells when they are treated with phenolic compounds. To better understand the underlying mechanism of lipid lowering drugs on cholesterol transporters, investigators examined the mRNA expression of LXRα, LXRβ, which are two isoforms of LXRs that stimulate reverse cholesterol transport 'RCT' by regulating cholesterol transporters expression (Baranowski, 2008). Changes in LXRα and LXRβ mRNA levels after treatment with pravastatin were observed (Ando et al., 2004; Kawase et al., 2015). Indeed, it has been shown that statins, as HMG-CoA reductase inhibitors, cause the suppression of LXRs activity induced by the decrease of mevalonic acid

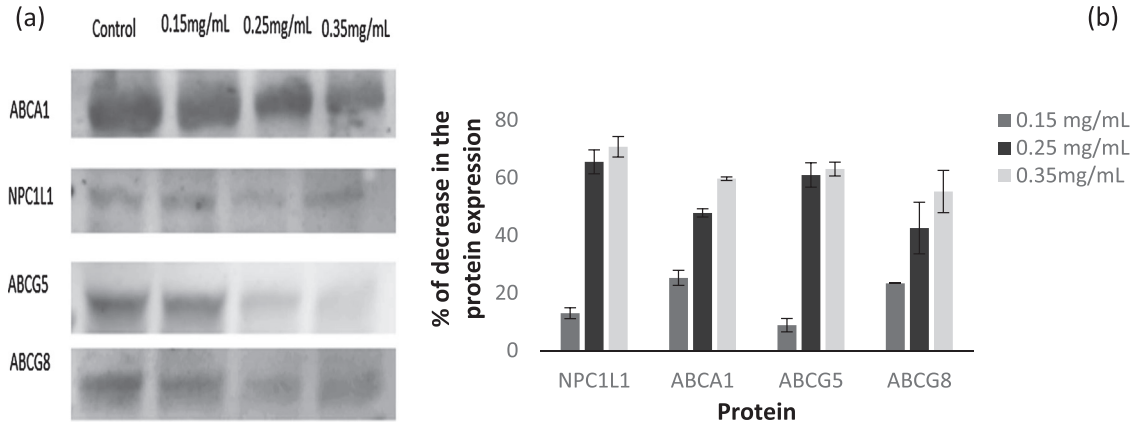

Fig. 4. The effect of phenolic compounds, decoction on the amount of different cholesterol transporters in HepG2 cells using the same weight of cells (161.3 mg of cell /mL of lysis buffer). (a) Bands intensities for several proteins obtained by Western Blot, (b) % of decrease in the protein expression under the effect of cladodes.

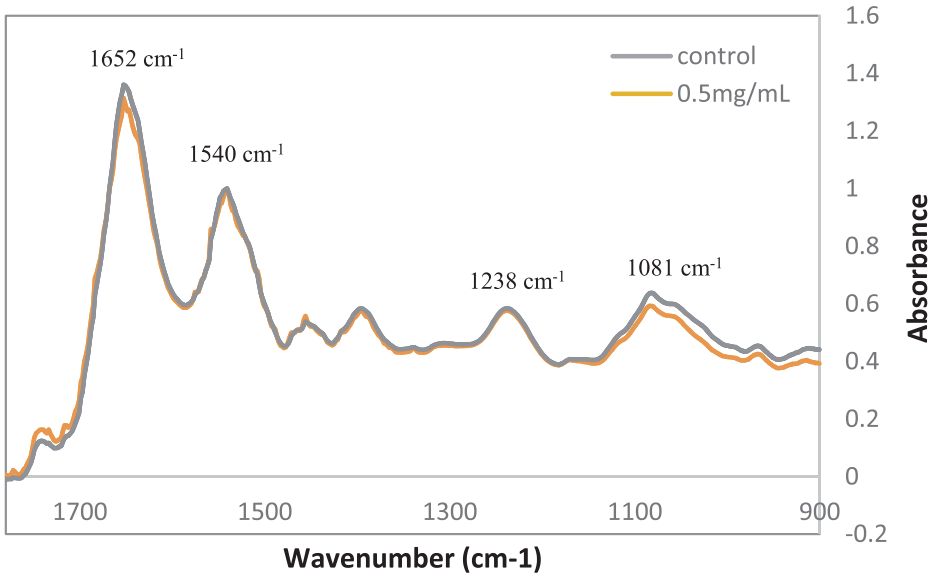

Fig. 5. FTIR spectra between 1800 and 900 cm^{-1} of HepG2 cells and HepG2 cells exposed to 0.5 mg/mL of cladodes extract.

(Ando et al., 2004). However, since cladodes decoction can also inhibit HMG-CoA reductase (Ressaissi et al., 2017), its effect on ABC transporters and NPC1L1 may be associated to changes in the availability of oxysterols ligands and consequently in LXRs activity in the same way as statins.

3.3. DNA/RNA and protein analysis by FTIR

To confirm that the effects noticed on the cholesterol proteins transporters could be due to changes at DNA/RNA level, FTIR analysis was carried out in HepG2 cells treated with cladodes decoction, comparatively to the untreated cells. Cells were analyzed under the spectral rage of 1700–900 cm^{-1}, the region of interest to analyze the proteins and DNA/RNA changes is shown in Fig. 5.

A band at 1517 cm^{-1} corresponds to the frequency of Amide II vibration (Movasaghi, Rehman, & Rehman, 2008), which results from the combination out-of-phase of the N—H in plane bend and the C—N stretching vibration with smaller contributions from the C—O in plane bend and the C—C and N—C stretching vibrations (Barth, 2007). Bands located between 1638 and 1694 cm^{-1} (Movasaghi et al., 2008) represent the Amide I vibrations, characterized mainly from the C═O stretching with minor contributions from the out-of-phase C—N stretching, the C—C—N deformation and the N—H in-plane bend (Barth, 2007; Movasaghi et al., 2008). Absorptions between 1300 and 900 cm^{-1} were mainly attributed to phosphate associated with nucleic acids, DNA and RNA. The absorption bands at 1238 cm^{-1} and 1080 cm^{-1} are characteristic of asymmetric and symmetric phosphodiester vibrations of nucleic acids, respectively (Di Giambattista et al., 2009).

The results shown in Fig. 5 indicate that there are differences between the spectrum of control cells and the cells treated with cladodes decoction. To withdraw quantitative information from these results ratios between amideI/amideII, DNA/AmideII, RNA/AmideII, ν_{asym} PO_2^-/ν_{sym} PO_2^- and RNA/DNA were calculated using the absorbance of the main peaks in these regions, Table 1.

The difference between control and treated cells was seen to be significantly different at 95% confidence level for all ratios, except Amide I/Amide II. Both Amide I and Amide II bands arise from proteins and the fact that they are not significantly different means that the FTIR analysis is not distorted by baseline shifts.

The intensity of the ν_{sym} PO_2^-, whose band is located at 1081 cm^{-1}, is reported to be relate to the cell DNA content (Benedetti et al., 1986). The study of the ratio of bands at 1081 cm^{-1} and 1540 cm^{-1} is often used to illustrate the change of the cell's DNA /protein content (Mostaço-Guidolin, Murakami, Batistuti, Nomizo, & Bachmann, 2010). The treated cells with the cladodes extract showed a decrease in this ratio of DNA/protein, changing from 0.246 ± 0.003 in the control to 0.224 ± 0.001 in treated cells. A decrease in the ratio RNA/protein as well was obtained, from 0.093 ± 0.001 for the control to 0.077 ± 0.001 in treated cells, thus leading to a decrease in DNA content which is due to the mechanism of action which inhibits DNA replication (Taillandie & Liquier, 1992). In fact, it has been shown that the decrease in the 1081 and 1238 cm^{-1} region suggests that the amount of nucleic acid or triphosphate compounds in the cells may be lower relative to the protein content (Fale & Chan, 2015). The decrease in the ratio RNA/protein, together with the decrease in the ratio RNA/DNA can be related to a lower RNA content in cladodes treated cells suggesting a lower transcription level compared to the control (Marguerat & Bähler, 2012). Lower transcription level causes lower protein expression, which agrees with our results showing that cells treated with cladodes present a lower amount of protein per mg of cells and supports the hypothesis that the inhibition of protein expression in human cells occurs by cladodes, which was seen for cholesterol transporters.

Phenolic compounds from cladodes decoction caused changes in the nucleic acid region of cells, seen by FTIR spectroscopy, which may be related to the observed changes in protein expression.

4. Conclusion

Polyphenols from cladodes decoction reduced total protein content in human cells, including cholesterol membrane transporters NPC1L1, ABCG5/ABCG8 and ABCA1. These modifications may be related to a decrease in RNA transcription (protein expression) when the cells were under the effect of the extract, as seen by FTIR analysis. The noticed effect can explain the decrease in cholesterol blood level described by people using cladodes in food or beverages.

Authors participation

Asma Ressaissi: execute the experimental tests (PhD work).

Nebil Attia: supervisor from Tunisia, read all the work and made suggestions.

Rita Pacheco: supervised the cell work.

Table 1
Calculated ratios from the FTIR spectra for HepG2 cells as a control and under the effect of cladodes decoction.

Ratio (cm^{-1}/cm^{-1})	Ratio Attributions	Control	Cladodes decoction (0.5 mg/mL)
1652/1540	AmideI/AmideII[a]	1.347 ± 0.012	1.338 ± 0.016
1081/1540	DNA/AmideII[a]	0.246 ± 0.003	0.224 ± 0.001*
1120/1540	RNA/Amide II[a]	0.093 ± 0.001	0.077 ± 0.001*
1238/1081	$v_{asym}\ PO_2^-/v_{sym}\ PO_2^-$[b]	0.798 ± 0.022	0.841 ± 0.012*
1120/1081	RNA/DNA[a]	0.376 ± 0.007	0.344 ± 0.002*

Attributions according to:
[a] Mostaço-Guidolin et al. (2010).
[b] Duygu et al. (2012).
* Statistically different from the control ($\alpha = 0.05$).

Pedro Falé: supervised the FTIR studies.

Maria Luisa Serralheiro: supervisor from Portugal, designed the study, discussed all the results and supervised the western blot techniques.

Ethics statement

There are no ethic concerns in this work as no animals and or humans' studies were conducted during all the stages of the laboratory process.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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