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In vitro Protective Effect of Berdetox® on Fatty Acid Induced Steatosis in HepG2 Cells

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Abstract: There is a demand in the market of products for liver health, therefore, Plameca has developed a formula that contains berberine and curcumin. There is existing evidence for those ingredients on their beneficial effect in non-alcoholic fatty liver diseases (NAFLD). In addition, the formula contains other traditionally used active ingredients with health benefits for the liver. The objective of the present work was a primary evaluation of the effects of the dietary supplement syrup Berdetox® on the prevention and the reversion of mild steatosis in the in vitro HepG2 cell model. HepG2 cells were incubated with a mixture of oleic acid and palmitic acid at a respective proportion of 2:1 and a final concentration of 0.25 mM for 24 h to induce steatosis. Cells were exposed to the test syrup 2 h prior and during lipid overload or after removal of the lipid mixture. The intracellular lipid content and the membrane integrity were determined by Nile red and neutral red staining, respectively. The effect of the metabolites of the active components of the syrup was assessed indirectly using the same experimental approach after previous incubation of the syrup with human hepatocytes. The lipid mixture effectively induced steatosis in the HepG2 cells. The intracellular lipid content was reduced dose dependently after simultaneous exposure to test syrup with or without previous incubation with human hepatocytes. However, the reversive effect of the syrup was of the same magnitude than the treatment with incubation medium only. The cell membrane integrity remained unaffected after lipid overload and after the treatment with the test syrup or its excipients. Berdetox® demonstrated a preventive effect against steatosis in HepG2 cells as measured by the reduction of the intracellular lipid content in vitro at in vivo meaningful concentrations of its marker ingredients. The experimental conditions used in this work were based in the induction of mild steatosis as a response to cell overload with a mixture of oleic and palmitic acids at a proportion considered to mimic the reversible initial phase of liver disease. The human hepatic metabolites of the active components showed also equivalent efficacy. The reversive effect of the test ingredients could not be demonstrated because steatosis was reverted by endogenous cell reparation mechanisms.

Keywords: Berdetox®, Steatosis, HepG2, Berberine, Silymarin, Curcumin, Non-alcoholic Fatty Liver Diseases (NAFLD)

1. Introduction

Liver steatosis is the clinical manifestation of the chronic accumulation of triglycerides in the cytoplasm of hepatocytes. It is associated to the pathogenesis of alcoholic and non-alcoholic fatty liver diseases (AFLD and NAFLD, respectively), that are often considered as the early stage of severe pathologies such as fibrosis, steatohepatitis, liver cirrhosis and hepatocellular carcinoma [1, 2]. While alcohol intake constitutes the main risk to develop AFLD, the most important risk factors for NAFLD development are obesity, type-2 diabetes, insulin resistance and metabolic syndrome [2, 3].

Many potential induction factors of steatosis have been described whose persistence enhances the development of more severe stages of liver disease. These factors are often promoted by an unhealthy lifestyle and dietary habits and include: increased lipogenesis, reactive oxygen species and lipid peroxidation; damage of cell components (membranes, mitochondria, proteins and DNA); induction of proinflammatory cytokines, transcription factors and apoptosis; reduced mitochondrial content, oxidative phosphorylation and lipid oxidation as well as depletion of

ATP stores [4-8]. Moreover, steatosis can be produced as a primary toxic hepatic response to exposure of chemicals, drugs or their metabolites constituting a histological manifestation of the so-called drug induced liver injury (DILI). Some of the agents that can produce DILI-related steatosis as side effect include bisphenol A, valproic acid, cyclosporine, tamoxifen together with some antipsychotics [9-12].

At present, there are no approved drug therapies for the treatment of NAFLD. However, a number of plant-derived polyphenols have shown relevant efficacy either in vivo or in vitro, being HepG2 cells one of the most widely used and predictive model for in vitro assessment of liver toxicity [2, 7, 8, 13, 14]. Berdetox® (syrup) is a dietary supplement that is used to preserve the liver function when taken orally at a dose of 25 mL daily. It combines plant extracts and substances traditionally used for liver- and body-detoxification and contains three groups of polyphenol molecules that are considered as the markers for efficacy evaluation: berberine, silymarin and curcuminoids, the latter are formulated as a complex with β-cyclodextrin to enhance its usually low bioavailability. Berdetox® formulation additionally include choline, inositol and methionine in order to act in other liver detoxifying pathways like oxidative stress or phase II conjugation of xenobiotics (see Table 1).

Table 1. *Composition of test syrup and syrup excipients (test and reference items).*

Component	Test syrup		Syrup excipients
	%w/v	Concentration at 1% diluted Test Syrup	% w/v
Potassium sorbate	0.18	-	0.18
Stevia glycosides	0.01	-	0.01
Sucralose	0.02	-	0.02
Xanthan gum	0.15	-	0.15
Glycerine	4.50	-	4.50
Artichoke dry extract (2.5% cynarine)	2.56	-	-
Milk thistle dry extract (80% total silymarin)	1.25	100 ng/ml (200 nM)	-
Desmodium dry extract	0.12	-	-
Boldo dry extr5act (0.05% boldine)	0.12	-	-
Berberine chloride	0.0013	0.17 ng/ml (0.32 nM)	-
Methionine	1.50	-	-
Curcuminoid complex* (19% curcuminoids)	0.40	7.6 ng/ml (~20 nM)	-
Inositol	0.05	-	-
Citric acid anhydrous	0.04	-	0.04
Strawberry flavour	3.00	-	3.00
Purified water	87.40	-	91.00

*Curarti® curcuminoid complex contains a curcuminoid extract from Curcuma longa L. roots embedded in beta-cyclodextrin for enhanced bioavailability.

The aim of the present work was to evaluate the in vitro protective effect of Berdetox® on fatty acid induced mild steatosis in HepG2 cells as a preliminary phase to the demonstration of its efficacy. To overcome one of the major drawbacks of HepG2 model predictivity, which is the downregulation of cytochrome P-450 (the main drug metabolizing enzyme family), the syrup was used with and without previous separate incubation with human hepatocytes in order to generate polyphenol hepatic metabolites.

2. Material and Methods

2.1. Test Syrup, HepG2 Cells and Chemicals

The whole commercial dietary supplement mixture and the mixture of its excipients only (syrup excipients) were prepared by Plameca S.A. (Pallejà, Barcelona, Spain). The composition of both solutions is shown in Table 1. Throughout this manuscript, syrup and syrup excipients are indistinctly named also as test and reference items, respectively. HepG2 human Hepatocellular carcinoma cell line was purchased from ATCC (Manassas, Virginia, USA). Cells were characterized by the supplier in terms of: morphology, growth properties, viability post-thawing, mycoplasma negative assays and assay for species confirmation through human DNA profile. Absence of human pathologic viruses was also determined. Once arrival and regularly during freeze-thaw cycles, the mycoplasma test MycoAlert® (Lonza, Basel, Switzerland) was run to ensure absence of mycoplasma contamination.

Analytical standards of: berberine, 98.5% purity; curcuminoid mixture, >98% purity (curcumin, demethoxycurcumin and bis-demethoxicurcumin) and milk thistle extract, namely silymarin (silibilin A, silibinin B, isosilibinin A, isosilibinin B, silicristin and silidianin) were purchased respectively from TCI Europe (Eschborn, Germany), United States Pharmacopeia (North Bethesda, Maryland, USA) and European Pharmacopeia (Strasbourg, France). Quercetin, 97.1% purity was purchased from Alfa Aesar (Tewksbury, Massachusetts, USA). All reagents including Nile red, neutral red, thiazolyl Blue tetrazolium bromide (MTT) and culture medium components were purchased from Merck Sigma-Aldrich (St. Louis, Missouri. USA) and Thermo-Fisher Scientific (Agawam, Massachusetts, USA), except where indicated otherwise. Solvents used for HPLC analysis were of liquid chromatography-mass spectrometry (LC-MS) grade. Human cryopreserved hepatocytes from 5 individual donors were purchased from Cytes Biotechnologies S.L. (Barcelona, Spain).

2.2. HepG2 Cell Culture Maintenance and Seeding

HepG2 cells were routinely grown in culture flasks in an incubator at 37°C under 5% CO_2 atmosphere and saturating humidity. The culture medium consisted of high glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 10% mL fetal bovine serum (FBS, heat inactivated, Lifetech Scientific. Shenzhen, China) and 2 mM L-glutamine. The medium was changed every 2 days and cells reaching 70-80% confluence were removed by trypsinization and sub-cultured. After trypsinization, cells were re-suspended in HepG2 culture medium and the cell density and viability were determined by the trypan blue staining method. The cells were seeded to 96-well plates (100 µL/well, 105 viable cells/well). The plates were incubated in the CO_2 incubator for 24 h to achieve cell adhesion.

2.3. Preliminary Cytotoxicity Assessment

MTT was dissolved in HepG2 culture medium without FBS freshly each day of experimentation protected from light until use.

After 24 h from HepG2 cell seeding, the culture media was replaced by incubation medium in the presence and absence of test syrup or syrup excipients (previously incubated with human hepatocytes, see below) and quercetin. The cells were incubated for 2h at 37°C in the CO_2 incubator. Afterwards, different oleic acid (OA, Alfa Aesar) + palmitic acid (PA, sodium salt, Acros organics, Geel. Belgium) mixtures were added to the wells without test or reference compounds which were incubated for additional 24 h. After incubation, the culture medium was removed and the cells washed twice with PBS. MTT working solution was added to the wells and the plates were incubated for 2 to 4 h at 37°C. For analysis, the medium containing MTT was removed, 100 µL of DMSO were added to each well and the plates were gently shaken for 5 minutes. The absorbance at 570 nm was determined in a microplate reader (Synergy H1, Biotek-Agilent. Santa Clara, California. USA). Conditions leading to absorbances lower that 70% of the non-treated cells were considered as cytotoxic as a widely accepted endpoint [15].

2.4. Incubation of Test Syrup and Its Excipients with Human Hepatocytes

Human cryopreserved hepatocytes from five separate individual donors were thawed according to the supplier´s recommendations. After thawing, the cells were pooled, counted and the viability assessed using the trypan blue exclusion method. The overall viability of hepatocytes after thawing was 91%. The hepatocytes were re-suspended in DMEM medium and the cell density was adjusted to 0.5 x 106 viable hepatocytes/mL.

Test syrup and excipients were incubated at 2% w/v concentration with the hepatocytes in suspension at 37°C for 30 minutes using low binding tubes. The reactions were started by the addition of test or reference items dissolved in dimethyl sulfoxide (DMSO, 1% final concentration) and were quenched by immersion of the tubes in ice and centrifugation at 16000 x g for 15 minutes at 4°C. Blank and control incubations were prepared without test or reference items and without cells, respectively.

After centrifugation, bovine serum albumin (BSA, fatty acid free), FBS, L-glutamine, streptomycin and penicillin were added to each pool to obtain the following respective concentrations: 1%, 10%, 100 µg/mL, 100 U/mL and 2 mM.

Samples corresponding to T=0 containing the same components, were prepared on ice and were immediately centrifuged at 16000 x g for 15 minutes. The specimens were stored at -80°C in aliquots until use and analysis.

2.5. HepG2 Incubation Medium

The HepG2 incubation medium consisted of culture medium containing 1% BSA and 1% DMSO. The incubation medium was prepared in the presence or absence of quercetin (positive control, 20 µM), test item or reference item (both at 0, 0.25, 0.5, and 1% w/v).

For the test and reference items incubated with human hepatocytes, the supernatants from these incubations were directly diluted with incubation medium to give target 0, 0.25, 0.5, and 1% concentrations.

2.6. Medium for HepG2 Lipid Overload

The medium containing fatty acids was prepared based on the procedure described by Papas A. et al with modifications [16] Briefly, PBS (Corning, New York, Massachusetts. USA) containing 4% BSA was warmed at 37°C and PBS alone was warmed at 70°C. A solution of PA was prepared in PBS at 70°C until complete dissolution. Once dissolved, the PA solution was mixed with the same volume of 4% BSA in PBS and the solution was maintained at 37°C. Afterwards, OA was added to obtain a molar proportion of 2:1 (OA: PA; total fatty acid concentration of 2.5 mM). The solution was prepared freshly each day of experimentation.

2.7. Experimental Procedure for HepG2 Incubations and Fatty Acid Overload

After 24 h from HepG2 cell seeding, the culture media in each well was replaced by 100 µL of incubation medium containing quercetin or the test and reference Items, the two latter with and without previous incubation with human hepatocytes. The plates were then incubated for 2 h. Non-treated cells were incubated with incubation medium only. After the 2-h of incubation period, 10 µL of the lipid overload solution were added to each well and the culture plates were incubated for 24 h at 37°C in the CO_2 incubator (final fatty acid concentration and proportion: 0.25 mM and 2:1 OA to PA ratio). Ten microliters of incubation medium alone were added to the non-treated cell group.

In another set of experiments intended to assess the reversive effect of the test syrup, the cells were exposed to incubation medium containing the lipid overload solution for 24 h in the absence of test and reference items. After lipid overload, the medium was replaced by 100 µL of incubation

medium containing quercetin or the test syrup or the excipients (both previously incubated with human hepatocytes). Incubation medium alone was used for the non-treated cells group and for the recovery group that was treated with fatty acid overload medium. The plates were incubated for an additional 24 h period at 37°C.

2.8. Nile Red and Neutral Red Staining

A solution of 0.5 mg/mL of Nile red was prepared in acetone and was stored at -20°C. Working solutions of 325 ng/mL Nile red were prepared immediately before use by dilution in PBS in tubes protected from light.

After exposure of HepG2 cells to the test and reference items or positive control and to the lipid overload mixture, the cells were washed twice with PBS. After washing, 100 µL of Nile red working solution were added to the wells and the plates were incubated at 37°C for 15 minutes protected from light. After incubation the wells were washed twice with PBS. For analysis, 100 µL PBS were added to the wells and the fluorescence determined in a microplate reader with excitation and emission wavelengths at 488 nm and 550 nm, respectively.

Neutral red 0.33% stock solution was diluted freshly to 50 µg/mL with HepG2 culture medium without FBS, at room temperature and protected from light. For neutral red staining, the cells were also washed twice with PBS and the methodology described by G. Repetto et al was followed [17]. The plates were analysed in a microplate absorbance reader at 540 nm. HepG2 cells treated with 100 µL 0.1% w/v sodium dodecyl sulphate (SDS, lysis buffer) per well were used as positive toxicity controls for neutral red determinations.

After all measurements, the plates were centrifuged at 300 x g for 10 minutes, the medium removed from the wells and the plates stored at -80°C until total protein determination. The cell pellets were treated with 0.1% SDS and the micro-BCA protein assay kit (Thermo Fisher) was used to this purpose following the manufacturer's instructions.

2.9. Calculations and Statistics

Fluorescence (Nile red) or absorbance values (neutral red) were normalized by the protein content. The results were calculated per each plate using the respective non-treated and 100% overload groups for comparison purposes. Results were expressed as mean ± SEM values of percentage vs control non-treated cells for the number of replicates used in each case (from 8 to 24). The differences among groups were analysed by means of one-way ANOVA. Tests for comparison (Dunnett's test) between control and treated groups were run, if necessary. The level of significance was set at $p < 0.05$. Graphpad prism software was used for statistical analyses (Graphpad Holdings. San Diego, California. USA).

2.10. LC-MS Analysis of Hepatocyte Incubates to Determine the Fraction Metabolized of the Test Item Markers

To assess the fraction metabolized of the marker/active ingredients: curcuminoids, berberine and silymarin, the hepatocyte incubates were analysed by liquid chromatography coupled to mass spectrometry (LC-MS, Acquity UPLC coupled to SQD Mass Detector, Waters. Mildford. Massachusetts. USA). The content of each active ingredient in the samples corresponding to 30 minutes' hepatocyte incubation were compared to T=0 samples. A separate LC-MS methodology was developed for each respective active ingredient (see Table 2 for LC-MS conditions).

Table 2. *LC-MS conditions and percentage metabolized of berberine, curcuminoids and silymarin by humans hepatocytes.*

Analytes	MS Voltage		SIR m/z	Mobile Phase		Column Fow Rate Temp.	Percentage Metabolized
	ESI (+)	Cone		Sovent A	Sovent B		
Berberine	2500 v	25 v	336.1	0.1% FA in H_2O	0.1% FA in AcN	Column 1 0.6 ml/min 50°C	0.0
Curcumin	200 v	25 v	369.1				79.4
Denethoxycurcumin			339.1				68.9
Bis-demethoxycurcumin			309.2				0.0
Silicristin	3500 v	35 v	481.5	10 mM Ammonium Acetate + 0.1% FA	Methanol	Column 2 0.25 ml/min 30°C	12.2
Silidianin							10.7
Silibinin A							17.1
Silibinin B							18.0
Isosilibinin A							17.9
Isosilibinin B							1.8

LC Columns were, 1: Acquity BEH C18 (50 x 2.1 mm, 1.7 µm, Waters) for berberine (isocratic 20%solvent B) and curcuminoids (40% solvent B) and 2: Luna C18 (2) (100 x 4.6 mm, 3 µm, Phenomenex) for silymarin (isocratic 51% solvent B). ESI (+): Electrospray Ionization voltage (positive ion mode); SIR: Single Ion Recording; FA: Formic Acid.

For the analysis of berberine and silymarin, the hepatocyte incubates were diluted with LC mobile phase. For the analysis of curcuminoids, a solid phase extraction step was conducted for each sample using Strata-XL cartridges (30 mg; 1 mL, Phenomenex, California. USA) The eluates (1 mL acetonitrile) were evaporated to dryness and the residue was dissolved with 100 µL of LC mobile phase. All the samples were centrifuged at 6000 x g for 15 minutes and the supernatants injected to the LC-MS system.

3. Results

3.1. Preliminary Cytotoxicity Assessment

The cytotoxicity of different concentrations of test syrup,

syrup excipients, quercetin and fatty acid mixtures was determined by the MTT method (a marker of mitochondrial damage) in order to select the final working conditions for the study. The results shown in Table 3 indicate that the test syrup and the excipients could be cytotoxic at concentrations of 2% and 1.5%, while quercetin was non-cytotoxic from 5 to 20 µM. Likewise no cytotoxicity was found neither in any of the compositions of the fatty acid mixtures used for lipid overload (OA:PA ratios of 2:1 and 3:1 both at 0.5 and 0.25 mM).

Table 3. *Preliminary assessment of cytotoxicity by the MTT method.*

Treatment	MTT Assay
Non-treated cells	100 ± 3.1
0.1% SDS (Positive Cytotoxicity Control)	0 ± 0.1
OA:PA (2:1) 0.25 mM	95 ± 3.6
OA:PA (3:1) 0.25 mM	96 ± 3.2
OA:PA (2:1) 0.5 mM	85 ± 2.6
OA:PA (3:1) 0.5 mM	96 ± 2.4
Hep. Incubate Test Syrup 2%	64 ± 1.8
Hep. Incubate Test Syrup 1.5%	67 ± 2.2
Hep. Incubate Test Syrup 1%	74 ± 1.9
Hep. Incubate Test Syrup 0.5%	83 ± 3.1
Hep. Incubate Test Excipients 2%	57 ± 2.1
Hep. Incubate Test Excipients 1.5%	64 ± 2.1
Quercetin 5 µM	74 ± 5.3
Quercetin 10 µM	85 ± 1.7
Quercetin 20 µM	70 ± 4.6

SDS: Sodium Dodecyl Sulfate; OA: Oleic Acid; PA: Palmitic Acid.

3.2. Preventive Effect of Berdetox® on Fatty Acid Induced Steatosis

The preventive effect of test syrup was studied by incubating it with HepG2 cells for 2 h followed by the addition of the fatty acid overload solution. Test syrup treatment was performed under two conditions, with and without previous incubation with human hepatocytes (conditions 1 and 2, respectively). For fatty acid overload, HepG2 cells were incubated for 24 h in the simultaneous presence of fatty acid mixture and test item. Syrup excipients (see Table 2 for the composition of test and reference items) were included in the experiments, together with 20 µM quercetin as positive control and incubation medium only for the non-treated control group.

Figure 1 shows the results of the determination of intracellular fatty acid accumulation by the Nile red staining method in condition 1. HepG2 cells were incubated with test syrup and quercetin (panel A) or syrup excipients (panel B) for 2 h. Afterwards the fatty acid overload solution was added and the cells were incubated for 24 h. Results are expressed as mean ± SEM of Nile red fluorescence normalized by cell protein relative to control lipid overload cells (n=24 for non-treated and lipid overload groups; n=8 for the treatment groups). The treatment with test syrup showed decreased Nile red derived fluorescence indicating reduction of steatosis (Figure 1, panel A). Syrup at 1% concentration showed a 37% reduction of the reference value in the lipid overload group (statistically significative decrease). A dose-dependence was observed in the preventive effect of test syrup at 0.5% concentration (13% reduction of the reference value) and no effect was found at 0.25% concentration. Syrup excipients at all the concentration levels showed steatosis levels not statistically different to those in the lipid overload group (Figure 1, panel B).

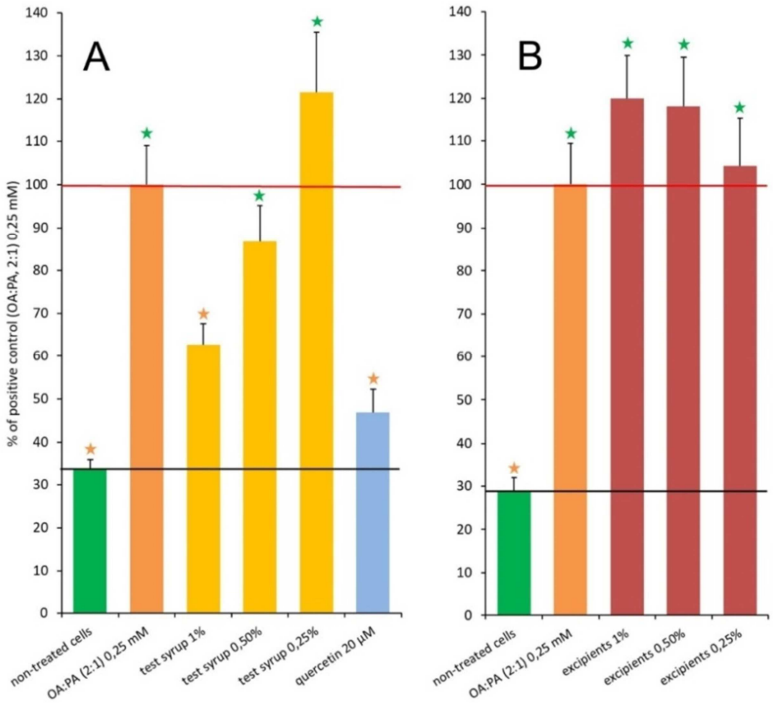

Figure 1. *Preventive effect of test syrup (A) and syrup excipients (B) on fatty acid induced steatosis in HepG2 cells.*

★: Statistically significative differences with the lipid overload group
★: Statistically significative differences with the non-treated group; P< 0.05.

Figure 2 shows the results obtained for experiments under condition 2, in which the test and reference items were incubated with human hepatocytes prior to HepG2 experiments. HepG2 cells were exposed to the hepatocyte supernatants or quercetin for 2 h. Afterwards the fatty acid overload solution was added and the cells were incubated for 24 h. Under these experimental conditions, test syrup at the three concentrations tested induced 29% to 26% reduction of Nile Red fluorescence from the lipid overload group (Figure 2, panel A). The effect was dose-dependent, but differences were statistically significative only at the 1% syrup concentration. Syrup excipients showed fluorescence values not statistically different from the lipid overload group (Figure 2, panel B).

The positive control quercetin showed statistically significant reduction of intracellular lipid accumulation in both sets of experiments (53% and 36% as compared to steatosis cells, respectively (Figure 2, panel A), thus validating the experiments.

Table 4. *Assessment of membrane integrity (neutral red staining) in HepG2 cells. Effect of treatment with test and reference items, fatty acid mixture and positive or reference controls.*

Treatment	Neutral red	
	Preventive assay	Reversive assay
Non-treated cells	100.0 ± 7.2	100.0 ± 2.2
OA:PA (2:1) 0.25mM	100.8 ± 6.4	92.4 ± 2.7
0.1% SDS (Positive Cytotoxicity Control)	6.2 ± 0.3	6.9 ± 0.1
Test syrup 1%	92.6 ± 10.1	101.8 ± 1.2
Test syrup 0.50%	89.7 ± 10.4	102.2 ± 2.4
Test syrup 0.25%	88.0 ± 7.1	103.7 ± 2.4
Excipients 1%	101.6 ± 8.4	90.3 ± 3.5
Excipients 0.50%	91.7 ± 7.2	103.1 ± 2.8
Excipients 0.25%	114.6 ± 8.6	102.4 ± 2.7
Quercetin 20 µM (Reference Control)	106.7 ± 7.2	101.9 ± 1.4

SDS: Sodium Dodecyl Sulfate; OA: Oleic Acid; PA: Palmitic Acid
Results are expressed as mean ± SEM (n=8) absorbance relative to untreated cells (treated with incubation media). 70% was considered as the cytotoxicity threshold.

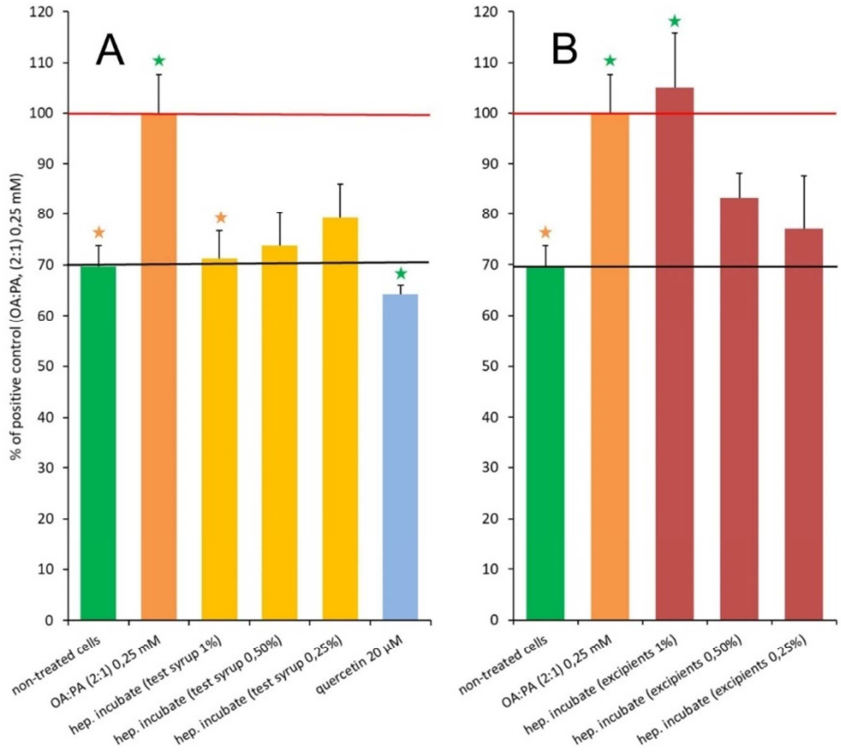

Figure 2. *Preventive effect of test syrup (A) and syrup excipients (B) on fatty acid induced steatosis in HepG2 cells after incubation with human hepatocytes.*

Results are expressed as mean ± SEM of Nile red fluorescence normalized by cell protein relative to control lipid overload cells (n=24 for non-treated and the lipid overload groups; n=8 for the treatment groups).
★: Statistically significative differences with the lipid overload group.
★: Statistically significative differences with the non-treated group; P< 0.05.

Table 4 shows the results of membrane integrity assessment by the neutral red uptake method. These determinations were performed for the set of experiments corresponding to condition 2. HepG2 cells were incubated for 2 h with or without test and reference Items or quercetin. For fatty acid overload HepG2 cells were incubated for 24 h or incubation medium alone. 24-h treatment with 0.1% SDS served as positive cytotoxicity control. The test syrup and the excipients showed absorbance values well above 70% of the non-treated group. Non-significative differences were found

from the non-treated HepG2 cells, indicating no damaging effects on cell membrane in both groups. The lipid overload group and the cells treated with quercetin showed neutral red uptake not statistically different from the non-treated cells. The positive toxicity control (0.1% SDS) showed a 95.8% reduction of absorbance as compared to non-treated cells thus validating the experiments.

3.3. Reversive Effect of Berdetox® on Fatty Acid Induced Steatosis in HepG2 Cells

The reversion of steatosis by the test syrup was studied using test item pre-treated with human hepatocytes (condition 2) as described in materials and methods.

The results in Figure 3 indicate that fatty acid induced HepG2 steatosis reverts after withdrawal of the lipid overload solution from the cell monolayers, either in the presence of test and reference Items or in their absence (Figure 3, panels A and B, respectively). No differences were found between the test syrup and the excipients groups, but a tendency to enhance reversion of steatosis by 20 µM quercetin was observed (Figure 3, panel A).

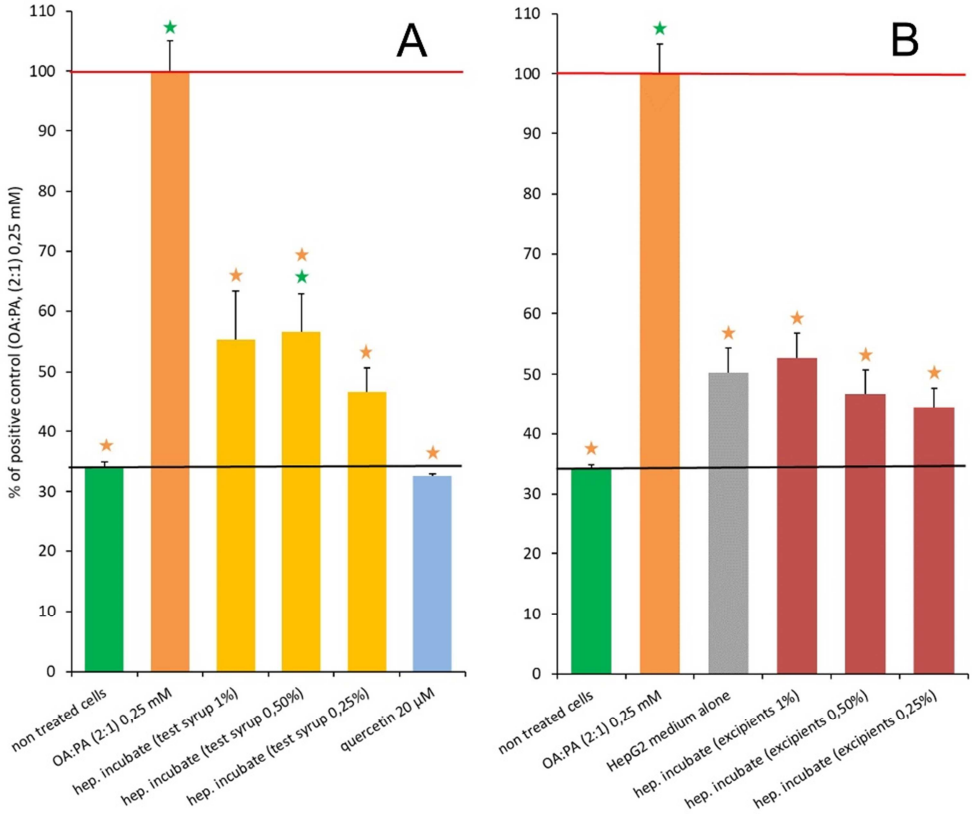

Figure 3. *Reversive effect of test syrup (A) and syrup excipients (B) on fatty acid induced steatosis in HepG2 cells after incubation with human hepatocytes.*

★: Statistically significative differences with the lipid overload group
★: Statistically significative differences with the non-treated group; P< 0.05.

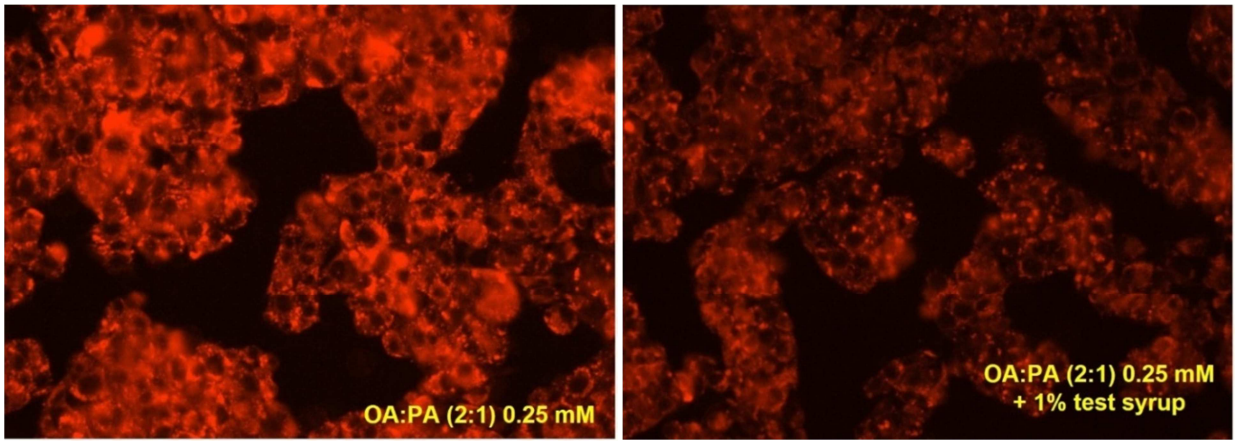

Figure 4. *Fluorescence microscopy pictures of 24 hours lipid accumulation in HepG2 cells by Nile red staining.*

Likewise, the treatment with test syrup, syrup excipients and quercetin showed no differences in the cell membrane integrity as compared to non-treated cells as demonstrated by neutral red staining (Table 4). While the treatment with lipid overload solution alone showed a slight but non-toxic decrease (about 8% from the non-treated cell group) that was considered as non-relevant. Figure 4 shows representative images of fluorescence microscopy of HepG2 cultures corresponding to steatosis status and to HepG2 cells after preventive treatment with 1% test syrup. The upper panel corresponds to cells treated with fatty acid mixture: oleic acid (OA) + palmitic acid (PA), ratio 2:1, total concentration 0.25 mM for 24 h. and the lower panel Corresponds to the cells exposed to 1% test syrup for 2 h followed by addition of fatty acid mixture and incubation for 24 h. Magnification ×100.

4. Discussion

NAFLD is thought to affect about 25% of the general population and represents a chronic disease that can potentially undergo more severe and life-threatening pathologies if the causing mechanisms are exacerbated. The first histopathological manifestation of NAFLD is steatosis and involves the accumulation of triglycerides in the cytoplasm of hepatocytes. OA and PA are the main dietary fatty acids and consequently, they account for most of the fatty acid content in liver cell triglycerides. In steatosis condition they also accumulate in endolysosomes [12]. While OA accumulation is associated to low cytotoxicity and to reversibility of steatosis, the increase in the content of PA constitutes a health risk by eliciting pro-apoptotic and challenging cell toxic responses. Several dietary supplements and traditional medicine herb mixtures have been proven to ameliorate NAFLD symptoms in vivo, especially steatosis [18]. The most active chemical group of components in these treatments are plant derived polyphenols.

HepG2 cells are considered as the main in vitro model to study steatosis because most of the cellular and molecular mechanisms of induction and progress of steatosis have been shown to occur in this cell model, which is widely used for liver function investigations.

The objective of the present work was to primarily assess the preventive and the reversive effect of a dietary supplement syrup (Berdetox®) on steatosis in HepG2 cells. The test syrup is a mixture of different components considered to be beneficial for liver function, among them, three groups of polyphenols that constitute the basis of its efficacy: berberine, curcuminoids and silymarin (Table 1). Steatosis was effectively induced by incubation of HepG2 cells with a mixture of OA and PA at a proportion of 2:1, respectively, with a total final concentration of 0.25 mM. Other fatty acid ratios and concentrations were tested in preliminary optimization experiments such as 3:1 and 0.5 mM, that showed less reproducibility and more cytotoxicity when tested by the MTT assay (Table 3). Likewise, 24 h appeared as the optimal incubation time to obtain the best balance between maximum lipid overload and adequate dose-response of amelioration of steatosis tested by different concentrations of quercetin. According to data from Gómez-Lechon and others [1, 4], the 2:1 (OA:PA) proportion is associated to a reversible benign apoptotic model of low toxicity that shows very mild to negligible pro-apoptotic responses such slight Caspase 3 and 9 activation and almost no induction of proinflammatory cytokines such as tumour necrosis factor alpha (TNFα) and mitochondrial disfunction. Furthermore, high OA content can inhibit PA induced oxidative stress not only in HepG2 cells but also in other tissues such as pancreatic β-cells or cardiac myocytes [19, 20].

The working concentrations of the test syrup were selected from a preliminary cytotoxicity test using the MTT assay (Table 3). Taking into account this data and some published results on the pharmacokinetics of silymarin, curcuminoids and berberine, 1% was selected as the highest test syrup concentration in the present work that corresponded to approximately 100 ng/mL, 7.6 ng/mL and 0.17 ng/mL of total silymarin, curcuminoids and berberine, respectively.

The test syrup was able to decrease the intracellular lipid content in HepG2-treated cells if incubated 2 h prior to the addition of the fatty mixture and with maintained exposure during the 24 h lipid overload period. This decrease was of a magnitude up to 37% at the maximum syrup concentration tested (1%) and, in practice, returned the cells to a non-steatosis status. The cytoplasmic lipid decrease was determined by the Nile red staining method and was comparable to the one found by other authors using similar lipidic overload conditions, but different cell models such as rat hepatocytes and anti-steatosis agents such as resveratrol, quercetin and dietary polyphenol mixtures [4, 5, 8, 9, 14, 20]. The excipients of the test syrup alone showed no effect in the reduction of the lipid content. Quercetin, that was used as positive control and showed a 53% decrease of intracellular triglycerides in agreement with other published data [7, 8, 20].

The integrity of cell membrane was assessed in parallel to the content of cytoplasmic lipids. The neutral red staining method was used for this purpose. The incorporation of this dye in the lysosomes is dependent on the maintenance of the cellular pH gradient which is disrupted by alterations in cell surface that are often induced by toxic or pathologic stimuli [16, 21]. Neither the exposure to the lipid overload mixture, nor the treatment with test syrup, its excipients or the positive control quercetin modified the degree of neutral red staining, in agreement with the benign steatosis HepG2 model used in the present work and the efficacy of the test syrup. However, the aggressive disruption of cell membranes induced by the lysis solution consisting of 0.1% SDS reduced neutral red staining by more than 95% as compared to non-treated cells, and thus validated the experiments.

One of the main issues regarding the predictivity of the HepG2 cell model is the strong downregulation of the characteristic hepatocyte drug metabolism enzyme pattern. To overcome this situation, some authors have included breakdown products of polyphenols in the HepG2

incubations [8, 20]. However, the metabolites used for these authors are products of enzymatic reactions catalysed by the gut microflora but do not correspond to the more common metabolism pathways located mostly in the liver, that drive drug clearance and often induce the formation of either active or toxic metabolic derivatives. In the present work, the test syrup or its excipients were also tested after incubation with human hepatocytes, in order to generate phase I or phase II metabolites of the marker polyphenol substrates. As shown in Table 2, berberine was almost not metabolized by the human hepatocytes, in contrast to previously published data suggesting that berberine can undergo oxidation and demethylation in vivo together with further metabolite glucuronidation [22]. The source of these discrepancies is not clear but could be related to the short hepatocyte incubation time used in the present study, that was selected to maintain a mixture of parent compounds and metabolites. On the contrary, curcuminoids in the syrup showed more than overall 50% metabolic transformation after hepatocyte incubation (79% curcumin and 69% demethoxycurcumin, Table 2). This was in agreement with the results of some authors that describe reduction, glucuronidation and sulphation as the main pathways of liver metabolism of curcuminoids, leading also to active metabolites [23, 24]. Mono- and di-hydroxylation together with glucuronidation and sulphation have been also described for silymarin components [25, 26]. In the present study, silymarin syrup constituents were effectively metabolized by human hepatocytes and the metabolic fraction accounted from 1.8% (isosilibinin B) to 18% (silibinin A and isolisibinin A). When the HepG2 cells were exposed to the hepatocyte extracts containing polyphenols together with its liver metabolites, the mixtures were also capable of reducing OA:PA induced steatosis in the preventive condition. In this set of experiments, the non-treated cell group showed less difference in the intracellular lipid content as compared to the overload group (see Figure 2, panels A and B). This was attributed to a lower efficiency in the lipid loading process. Even though, the Nile red staining was reduced by 29% to 26%, meaning that a remarkable fraction of the formed metabolites can be considered as active against lipid-induced steatosis. By contrast to phase I metabolites, phase II metabolites often do not show pharmacologic activity due to the severe structural changes induced by conjugation with endogenous macromolecules. The potential formation of phase II metabolites during human hepatocyte incubation could explain the relatively lower reduction of steatosis as compared with the incubations with their parent compounds (condition 1 in the present work). However, in vivo phase II metabolites revert to the original substrates after hydrolysis in the gut. In these cases, entero-hepatic recirculation of the aglycones is a common feature for biliary excreted compounds that actually increase the efficacy of the treatments. This could be the case of curcuminoids and silymarin, for which biliary excretion constitutes a main elimination pathway [24, 25]. The actual concentrations of the marker polyphenols of Berdetox® at ≤ 1% used in the present work, were in the same range of those described in plasma in pharmacokinetic studies in humans (silymarin and berberine) and rats (curcuminoids) after single or chronic dosage regimens by the oral route [27-29]. This fact increased the predictivity of the experimental method used, and added promising efficacy value to the study.

The reversive (curative) effect of the test syrup was also studied. In this case, the lipid overload of HepG2 cells was induced for 24 h in the absence of syrup. After overload, the lipid mixture was removed and the cells were incubated with the test syrup or its excipients. The results after Nile red staining showed restoration of the intracellular lipid content to the normal status, even in the non-treated group incubated with medium only. This means that HepG2 cells returns to the non-steatosis status by removing the lipid mixture essentially by means of endogenous repair mechanisms. This is coherent with the results of Stellavato et al, that found similar results using an equivalent experimental approach [13]. However, a group treated with culture medium only was not included in these assays and the beneficial effects were essentially attributed to the mixture of polyphenols formulated in the presence of vitamin E, phosphatidylcholine among others. The recovery of HepG2 cells after removal of the challenging lipid mixture probably relies on the normalization of some key molecular mechanisms involved in NAFLD progression, such as suppression of steroid regulatory element binding protein P (SREBP1), induction of enzymes involved in the oxidation of fatty acids such as carnitine palmitoyl transferase 1 (CPT1), induction of superoxide dismutase 2 (SOD2) and restoration of survival factors and receptors such as AKT kinase or peroxisome proliferator-activated receptors (PPARs).

5. Conclusion

In conclusion, steatosis was effectively induced by incubation of HepG2 cells with a 0.25 mM mixture of PA and OA (2:1) for 24 h. This fatty acid proportion is associated to a reversible liver disease model compatible with chronic benign steatosis. The test syrup was able to restore the intracellular lipid content in HepG2 when these were treated in a preventive manner. The cytoplasmic lipid reduction was syrup dose-dependent, while its excipients alone did not show any effect. The efficacy of the test syrup was maintained when it was used after incubation with human hepatocytes, meaning that phase I or phase II metabolites of syrup components (essentially the polyphenols berberine, curcuminoids and silymarin) retain most of the activity of the parent compounds.

The reversive steatosis effect of the test syrup could not be assessed because HepG2 steatosis was restored by endogenous mechanisms after removal of the lipid overload mixture.

Finally, the integrity of cell membrane was measured by the neutral red staining method. The HepG2 cells did not show membrane alterations either after exposure to the lipid overload mixture, or after treatment with test syrup, its

excipients, or the positive control quercetin.

The initial results obtained for Berdetox® in the present work are encouraging and point out the need to further research to be conducted, especially on the mechanisms involved in steatosis down-regulation in vitro by the measurement of key liver function biomarkers and in vivo by assessing the bioavailability of Berdetox® marker polyphenol bioavailability of the current formulation.

Disclosure

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