Antihyperlipidemic effect of *Rhamnus alaternus* L. leaves methanolic extract in Triton WR-1339-induced hyperlipidemic rats and human HepG2 cells

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Abstract

Rhamnus alaternus L. is a plant used in traditional medicine in Mediterranean countries. Here, we aimed to characterize its phenolic compounds and explore its anti-hyperlipidemic activity *in vivo* and relevant cell models. The study was conducted on crude methanolic extract (CME) from leaves of *R. alaternus*. The extract was fractionated by liquid-liquid extraction and its molecular profile characterized by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI-MS^2). Effects on circulating lipids were evaluated in Triton WR-1339-induced hyperlipidemic rats. Effects on intracellular lipid accumulation were evaluated by Oil Red O staining in human hepatoma HepG2 cells and differentiating 3T3-L1 adipocytes. Effects on the expression of key genes related to fatty acid metabolism were tested by real-time qRT-PCR. The HPLC/ESI-MS^2 profile showed the presence in the CME of fifteen compounds of which eleven were identified. Oral administration of the CME markedly decreased blood levels of cholesterol and triacylglycerols in Triton WR-1339-injected rats. In HepG2 cells, exposure to the CME dose-dependently decreased intracellular lipids and up-regulated the mRNA expression of carnitine palmitoyltransferase 1 involved in fatty acid oxidation. In the 3T3-L1 model, CME favored preadipocyte proliferation and adipogenesis, suggesting positive effects on adipose tissue expandability. Our results suggests novel uses of *R.alaternus* L. in herbal medicine by showing that its leaves are rich in flavonoids and flavonoid derivatives with an anti-hyperlipidemic effect *in vivo* and in hepatic cells. Anti-hyperlipidemic effects of *R.alaternus* L. extracts had not been previously described.

Keywords: *Rhamnus alaternus* L., Rhamnaceae, flavonoids, Triton WR-1339, HepG2, hyperlipidemia, fatty acid oxidation

Introduction

Hyperlipidemia is a main risk factor for the development and progression of cardiovascular diseases. It is characterized by elevated levels of one or more plasma lipids: triacylglycerols, cholesterol, cholesterol esters, phospholipids, LDL cholesterol, VLDL cholesterol, free fatty acids and apolipoprotein B, as well as by reduced levels of HDL cholesterol **(Amit et al., 2011; Harikumar et al., 2013; Shattat, 2015)**. Many endogenous and exogenous factors can influence the prevalence of hyperlipidemia such asethnic group, genetic background, life style including dietary habits and regular exercise, poorly controlled diabetes, high alcohol consumption and stress **(Rohilla et al., 2012; Ruixing et al., 2006)** Hyperlipidemia can cause severe complications, among them: atherosclerosis, coronary artery disease, myocardial infarction, angina pectoris and ischemic stroke or cerebrovascular accident **Rohilla et al., 2012; Verma, 2017)**. Currently, the major classes of drugs used in the treatment of hyperlipidemia include statins, nicotinic acid derivatives, fibrates, bile acid binding resins and cholesterol absorption inhibitors **(Mahamuni et al., 2012; Shattat, 2015)**.

The use of traditional medicine has a long history in disease prevention and treatment. Many individual countries and international organizations are developing policies to promote the safe and effective use of traditional medicine **(Organization, 2014)**. Plants with hypolipidemic or anti-hyperlipidemic properties are increasingly being studied for their potential usefulness in the management of cardiovascular health. Knowledge on these plants can serve as the basis for the development of novel lipid lowering pharmaceuticals and nutraceuticals/functional foods **(Dhaliya et al., 2013; Thompson and Ernst, 2003**).

Rhamnus alaternus L. (Rhamnaceae) is a small tree that is distributed along the Mediterranean coasts. It grows in the countries of North Africa (Algeria, Morocco and Tunisia) **(Bas et al., 2009)**. In Algeria, it grows in forests and rocks. It's located principally in

the North, where it is known as ‘‘Imlilesse, M’lila, Soitfaïr or Safir” **(Ait Youssef, 2006)**. It has traditionally been used as a digestive, diuretic, laxative, hypotensive **(Boukef, 2001)**, and for the treatment of hepatic and dermatological complications **(Ait Youssef, 2006)**. In human cells, extracts of *R. alaternus* leaves modulate the expression levels of genes implicated in both DNA repair and oxidative defense systems **(Ammar et al., 2007a)**. Other studies have demonstrated that *R. alaternus* extracts possess potent antioxidant, free radical scavenging, antimutagenic and antigenotoxic activities **(Ammar et al., 2009; Ammar et al., 2008)**, as well as antiproliferative and pro-apoptotic effects in human cancer cell lines **(Ammar et al., 2007b; Ammar et al., 2011)**

To our knowledge, no studies have addressed the antihyperlipidemic properties of *R. alaternus*. Therefore, the aim of the present study was to evaluate the effect of methanolic extract from *R. alaternu* sleaves on blood lipid parameters in Triton WR-1339-induced hyperlipidemic rats, and its effect on intracellular lipid accumulation in two different cell line types: human hepatoma cell line HepG2 and mouse preadipocytes 3T3-L1. ~~A~~ The characterization of the phenolic compounds ~~composition~~ of *R. alaternus* CME and its fractions was carried out by HPLC/ESI-MS^2.

Results

The extraction protocol applied resulted in an extraction yield of 18.3 of *R. alaternus* leave powder. The total phenolic and flavonoid contents of CME were, respectively, 155.98 mg ± 4.63 GAE/g of extract and 74.63 mg ± 1.37 QE/g of extract. These contents indicate that *R. alaternus* leaves CME extract is rich in phenolic compounds, in particular flavonoids.

Base Peak Chromatograms of CME and its different fractions are shown in Fig. 1S. Fifteen different peaks were found in total. Peak attribution was performed by comparing their mass spectra with those found in the literature ~~references~~ **(Boussahel et al., 2015;**

Cuoco et al., 2014; Pikulski and Brodbelt, 2003; Segueni et al., 2016). Eleven of the peaks were identified, while four remained unidentified. Peak attributions are listed in Table 1.

The chromatographic profile of CME (Fig. 1S, CME) showed the presence of two major compounds: 1 and 4 (kaempferol hexoside and rhamnetin hexoside). After fractionation, these two compounds were present in three of the four fractions (CF, BF and RMF fractions), but were absent in the EAF fraction. All other peaks were found at least in one of the fractions, but not in the CME (Fig. 1S and Table 1). These results suggest that CME has high concentration of compounds 1 and 4, and low concentrations of the other compounds. On the other hand, the single fractions are less concentrated in compounds 1 and 4, and this allows the other compounds to be detected by injecting more concentrated solutions. The use of solvents with different polarity is therefore particularly useful, since a full characterization of the extract can be obtained by the comparison of the chromatograms of all fractions.

Chloroform fraction (Fig. 1S, CF) gave the richest profile of phenolic compounds, since eleven compounds (#1, 4, 5, 6, 7, 8, 10, 11, 13, 14, 15) of the fifteen characterized were present (Table 1). Of these eleven compounds, four (#8, 11, 14, 15) were exclusively present in this fraction (Table 1). The ethyl acetate fraction (Fig. 1S, EAF) showed the presence of 6 compounds, of which two (#9, 12) were present only in this fraction (Table 1). The two other fractions: butanol fraction and residual methanol fraction (BF and RMF) had similar chromatograms, with some compounds in common (Fig. 1S and Table 1).

Antihyperlipidemic properties of *R. alaternus* leaves CME were evaluated in the model of Triton WR-1339–induced hyperleptinemia in rats. As show in Fig.1, 24 h after Triton injection serum triacylglycerol and total cholesterol levels were markedly increased (by 700% and 285%, respectively) in the hyperlipidemic control group compared to the normal

control group, indicating that hyperlipidemia was readily induced by the protocol applied. Oral treatment with 200 mg/kg and 400 mg/kg CME immediately after Triton injection resulted in much lower triacylglycerol (Figure 2A) and total cholesterol (Figure 2B) blood levels in the CME-treated groups compared to the hyperlipidemic control group. The hypolipidemic effects were stronger at the 200 mg/kg dose than the 400 mg/kg dose. Specifically, serum triacylglycerol levels were decreased by 70% and 42%, and serum total cholesterol by 60% and 40%, in the groups treated with 200 mg/kg and 400 mg/kg, respectively, relative to the hyperlipidemic control group.

We used the HepG2 hepatocyte cell model to get further insight into the metabolic basis of the antihyperlipidemic effect of *R. alaternus* leaves CME. At concentrations up to 500 μg/mL, CME lacked cytotoxicity in HepG2 cells, as assessed by the LDH release assay (data not shown). We tested the impact of 24h exposure of the cells to varying concentrations of CME on intracellular lipid content, using Oil Red O (ORO) staining. Exposure to the CME potently and dose-dependently decreased intracellular lipids in HepG2 cells (Fig. 2A). The decrease was already significant ($P<0.05$) at 10 μg/mL concentration (40% decrease), reaching 60% and 70% decrease at concentrations of 500 μg/mL and 750 μg/mL, respectively. Decreased lipid content was evident from the ORO stained cells photomicrographs (Fig. 2C). Exposure to CME did not affect crystal violet staining of the cultures (Fig. 2B), suggesting lack of cytotoxicity, in accordance with the results of the LDH release assay.

Reduced intracellular lipids in CME-exposed HepG2 cells could result from diverse mechanisms, such as decreased lipogenesis, increased lipid oxidation or both. To further investigate in the mechanism(s) involved, we examined the effect of exposure to varying concentrations of *R. alaternus* leaves CME (from 1 to 500 μg/mL) on the mRNA expression levels of key genes related to fatty acid metabolism. As a control, we used HepG2 cells

exposed for 24h to 1 μM all-trans retinoic acid (ATRA), since effects of ATRA on the expression of lipid metabolism related genes in HepG2 cells are well known **(Amengual et al., 2012; Roder and Schweizer, 2007; Roder et al., 2007)**. Exposure to CME did not affect gene expression of lipogenesis-related enzymes (FASN, SCD1) or lipogenic transcription factors (SREBP1, PPARG and LXRA) but resulted in a potent and dose-dependent induction of CPT1-L, encoding the rate-limiting enzyme in hepatic mitochondrial fatty acid oxidation **(Figure 4)**. CPT1-L induction was already evident at 10 μg/mL concentration and CPT1-L mRNA levels almost doubled in the cells exposed to 500 μg/mL CME. Induction of CPT1-L was unrelated to increases in the mRNA expression levels of PPARA, which remained unchanged in the CME-exposed HepG2 cells. Exposure to 1 μM ATRA induced both lipogenic (SREBP1,FASN, SCD1) and fatty acid oxidation (CPT1-L) related genes in the HepG2 cells, as expected **(Amengual et al., 2012; Roder and Schweizer, 2007),** and decreased PPARG gene expression levels in these cells.

Effects of *R. alaternus* leaves CME on adipose cells were assessed using the 3T3-L1 model. 3T3-L1 is apreadipocyte cell line of fibroblasts cells that are committed to differentiate into lipid-laden adipocytes upon stimulation with an adipogenic hormonal cocktail. In this process, first growth-arrested (post-confluent) 3T3-L1 preadipocytes synchronously reenter the cell cycle and undergo several rounds of mitosis (termed mitotic clonal expansion) followed by terminal differentiation **(Tang et al., 2003)**.

First, we tested the impact of CME on the proliferation/viability of 3T3-L1 preadipocytes, using the XTT assay. Cells grown in basal medium exposed to 20 to 1000 μg CME/mL displayed significantly increased XTT cleavage compared to control cells ($P<0.05$), suggesting an effect of CME increasing preadipocyte proliferation (Fig.4).

Next, to test the impact of CME on adipogenesis, 3T3-L1 cells were stimulated to differentiate into adipocytes in the presence of varying concentrations of CME, from day 0. Lipid accumulation at day 8 was visualized and quantified by ORO staining. As a control, we used cells exposed to 1 μM ATRA, which is known to inhibit adipogenesis when added to the cells at early stages of the adipogenic differentiation process **(Schwarz et al., 1997)**. CME exposure significantly affected lipid accumulation according to the ANOVA analysis, with a dose-dependent increase at the 100 ans 500 μg/mL doses relative to the lower CME doses (Fig.5A). Increased adipogenesis in the cultures exposed to 500 μg/mL CME was also apparent relative to the control cultures (31% increase, Fig. 5A; see also the microphotographs of ORO stained cultures in Fig. 5C). Crystal violet staining was unaffected at low CME doses and slightly but significantly increased (by up to 13%, $P<0.05$) in the cultures differentiated in the presence of 100 and 500 μg/mL (Fig.5B). The latter result suggests that exposure to high CME doses enhances mitotic clonal expansion of preadipocytes, and is in good concordance with the results of the XTT assay (Fig. 4). As expected, the presence of ATRA from the beginning of the differentiation process markedly inhibited adipogenesis (Fig. 5A-5C).

Finally, we tested if exposure to the CME can affect triacylglycerol content of cells at a late stage in the adipogenic differentiation program. To this end, differentiating 3T3-L1 cells were treated with CME (10 to 750 μg/mL) from day 6 to day 8 after initiation of differentiation. The results showed no significant differences in intracellular lipid content as assessed by ORO staining between control and late CME-exposed cultures at CME doses up to 500 μg/mL(Fig.5D). A slight (13%) reduction in ORO staining was noticed in the cultures exposed to the highest dose tested of 750 μg CME/mL(Fig.5D), which could reflect in part reduced cell viability, since crystal violet staining tended to be reduced in the cultures

exposed to this high dose (Fig.5E). Late exposure to ATRA for 48h did not affect lipid accumulation in the adipocytes, as expected **(Mercader et al., 2007)**.

Discussion

R. alaternus L. is a medicinal plant used in folk medicine in countries of North Africa (Algeria, Morocco, and Tunisia). Results in this work are first to reveal an anti-hyperlipidemic activity of methanolic extract of *R. alaternus* leaves, which is demonstrated *in vivo* and in an hepatocyte cell model. We attribute this activity to phenolic compounds and in particular flavonoids that, according to our HPLC-MS investigations~~analyses~~ and in agreement with previously reported analysis **(Boussahel et al., 2013; Moussi et al., 2015)**, are abundant in *R. alaternus* leaves.

In recent years, the composition of phenolic compounds of *R.alaternus* L. has been the subject of several studies. Many compounds have been identified particularly in leaves and bark. These studies revealed the presence of different compounds: : anthraquinone, flavone heterosides, coumarins, tannins, glycosylated and non-glycosylated flavonoids, phenolic acids and anthraquinine aglycones **(Ammar et al., 2009; Ammar et al., 2008; Ben Ammar et al., 2005; Boussahel et al., 2013; Boussahel et al., 2015; Izhaki et al., 2002; Moussi et al., 2015; Stocker et al., 2004)**.

In the present study, phenolic compounds from leaves of *R.alaternus* L. were extracted using methanol. This organic solvent has an ability to inhibit polyphenol oxidase and is often used for the extraction **(Yao et al., 2004)**. The CME was then fractionated by liquid-liquid extraction in separatory funnel into four fractions using different organic solvent increasing polarities: chloroform, ethyl acetate and butanol to obtain chloroform fraction (CF), ethyl acetate fraction (EAF), butanol fraction (BF) and residual methanol fraction (RMF). HPLC-ESI-MS/MS analyses of CME and fraction showed that the majority of the phenolic

compounds identified in our study have already been reported by the previously cited studies; nevertheless, three compounds are newly identified: pilosinhexoside (#6), pilosin (#11), and rhamnocitrin (#15) (Table 1).

R. alaternus leaves CME counteracted the development of hypertriglyceridemia and hypercholesterolemia in Triton WR-1339-injected rats, a model of drug-induced hyperlipidemia that is widely used for screening chemical or natural hypolipidemic products **(Schurr et al., 1972)**. Triton WR-1339 (Tyloxapol) blocks the uptake of lipoprotein from the blood circulation by extra hepatic tissues and stimulates hepatic cholesterol biosynthesis by increasing 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase activity, resulting in an increase in the level of circulating lipoproteins and hyperlipidemia **(Goldfarb, 1978; Schurr et al., 1972)**. Hyperlipidemia induced by triton WR-1339 is effective for 72h after application of the drug. After this period, there was a decrease in cholesterol values to levels similar to baseline values, and therefore may not reflect the action of a lipid-lowering product **(Bertges et al., 2011)**.

Hypertriglyceridemia is a possible risk factor for the development of ischaemic heart **(Andersen, 1991)**. Blood triacylglycerol- and cholesterol-lowering effects are therefore considered beneficial. The characterization of the composition of CME shows that the extract is rich in flavonoids, especially kaempferol hexosides and rhamnetin hexosides. In animal models of hyperlipidemia (drug-induced, diet-induced and genetic), flavonoids or flavonoid-rich extracts have been reported to lower serum lipids through a variety of mechanisms. These include: down-regulating the production of intestinal-associated lipoprotein apoB48 **(Ma et al., 2015)**; inhibiting the activity of hepatic HMG-CoA reductase **(Khamis et al., 2017; Kuang et al., 2017)**; inhibiting hepatic lipogenesis through suppressed expression of SREBP1 and fatty acid synthase **(Bao et al., 2016; Hoang et al., 2015; Kuang et al., 2017; Zang et**

al., 2015); and stimulating hepatic fatty acid oxidation **(Chang et al., 2011; Mulvihill et al., 2011; Noh et al., 2013)**.

The liver plays a central role in lipid metabolism in the whole body and in determining blood lipid levels. HepG2 is a human hepatoma cell line widely used as a hepatocyte cell model in metabolic studies. *R. alaternus* leaves CME decreased the intracellular lipid content of HepG2 cells in a dose-dependent manner, likely due to enhanced fatty acid oxidation in the face of unchanged lipogenesis, since gene expression of CPT1-L was induced while that of lipogenic genes (SREBP1, PPARG, FASN, LXRA and SCD1) remained unchanged following exposure of the cells to the extract. CPT1-L catalyzes the translocation of long-chain fatty acids from cytosol into mitochondrial matrix, the first and rate limiting step in β-oxidation **(Zammit, 2008)**. To be noted, in ATRA-treated HepG2 cells, in which both lipogenic and fatty acid oxidation genes are induced, the intracellular lipid content is not affected (this work and) **(Amengual et al., 2012)**

Increased expression of CPT1-L in liver or HepG2 cells through the action of plant extracts or phenolic compounds has been reported for *Euchresta horsfieldii* Benn. **(Kim et al., 2011)**, walnuts **(Shimoda et al., 2009)**, *Ginkgo biloba* **(Wei et al., 2014)**, genistein **(Kim et al., 2004)** and nobiletin **(Mulvihill et al., 2011)**. In most **(Kim et al., 2011** ; **Kim et al., 2004** ; **Shimoda et al., 2009)** though not all **(Mulvihill et al., 2011)** of these studies, CPT1-L induction could be related to the induction/activation of PPARα, a main transcriptional controller of enzymes in hepatic fatty acid metabolism **(Dubuquoy et al., 2008)**. In our study, effects of *R. alaternus* CME in HepG2 cells were independent of changes in gene expression of PPARα.

Although further *in vivo* studies are required to better establish the mechanism(s) of the antihyperlipidemic effects of *R. alaternus* leaves CME, our studies in the HepG2 model

suggest that enhancement of hepatic fatty acid oxidation might be involved. Therefore, *R. alaternus* is revealed in this work of potential interest not only in relation to the management of blood lipids and cardiovascular risk, but also in relation to the prevention of non-alcoholic fatty liver disease, due to its modulatory activity on hepatocyte lipid metabolism.

Exposure to *R. alaternus* CME favored to some extent preadipocyte proliferation, clonal expansion and adipogenesis in the 3T3-L1 model. These effects could reflect the activity of phenolic compounds in the extract. Rhamnetin suppresses 3T3-L1 adipogenesis **(Ji et al., 2012)**, but other flavonoids, particularly certain kaempferol and quercetin derivatives, stimulate adipogenesis in this model **(Matsuda et al., 2011)**. Similar to *R. alaternus* leaves CME, other flavonoid rich plant extracts were shown to display hypolipidemic (and hypoglycemic) activity *in vivo* in rats and pro-adipogenic activity in 3T3-L1 cells **(Sharma et al., 2008)**. White adipose tissue is the natural place for healthy storage of excess energy in the form of lipid droplets. It plays an important role in the homeostasis of blood lipids and glucose and prevents lipotoxic fat deposition in ectopic places **(Virtue and Vidal-Puig, 2010)**. If translatable to the *in vivo* setting, a positive effect of *R. alaternus* CME on (subcutaneous) adipogenesis may contribute to increased adipose tissue expandability and prevent the deposit of fats in ectopic sites under conditions of positive energy balance. Such effect may be good in the context of the metabolic syndrome. Indeed, in the metabolic syndrome and diabetes, hyperlipidemia is associated with lipid accumulation in non-adipose tissues such as the liver and the heart, which precedes organ dysfunction **(Schaffer, 2016)**.

In conclusion, the present study apparently validates the folk medicinal use of *R. alaternus* and provides the first evidence that it may be a good natural lipid-lowering agent by reducing lipid plasma levels. Our results also demonstrate that methanolic extract of *R. alaternus*can reduce intracellular lipids in HepG2 cells and increase fatty acid oxidation capacity in these cells. The extract has also a positive effect on adipogenesis in 3T3-L1 cells,

suggesting it may promote healthy lipid storage in adipose tissue. These effects are likely to be due to the action of flavonoids and flavonoid derivatives present in the extract.

Materials and methods

Plant material

R. alaternus L. were collected from Sidiayad, Bejaia (Algeria) (GPS coordinates: 36°36'07.72 North, 4°42'52.71 East). Authentication of the plant was carried out by a botanist at the Gouraya National Park (Bejaia, Algérie). Leaves were cleaned, air dried and ground, and the particles obtained were sieved to obtain a homogeneous powder (Ø = 250µm).

Preparation of crude methanolic extract

Fifty grams of *R. alaternus* leaves powder were extracted with 500 mL of methanol for 7 h at room temperature with continuously stirring. The supernatant was filtered. The same operation was repeated with the pellet overnight to accomplish an exhaustive extraction. Both supernatants were mixed and defatted several times with petroleum etherin a separatory funnel, and methanol was evaporated to obtain crude methanolic extract (CME). CME was dissolved in saline solution for the *in vivo* study. For cell culture studies, traces of methanol in CME were completely removed by exposing it to a pressure of nitrogen gas and then the extract was dissolved in DMEM (Dulbecco's Modified Eagle Medium), filtered on 45µm and 22µm diameter filters and stocked at -20°C until treatment of cells.

Determination of total polyphenols content

CME total polyphenols content was determined by the Folin–Ciocalteu method **(Wolfe et al., 2003)**. 250 µL of a known dilution of CME were added to 1.25 mL of Folin-Ciocalteu reagent (0.1 N in distilled water) and allowed to react for 5 min. Then, 1 mL of 7.5% sodium carbonate solution was added. The mixture was homogenized, incubated for 90

min and absorbance of blue colored mixture was read at 740nm against a blank (Biochrom WPA Lightwave II UV/Visible). Gallic acid was used in a standard curve and the concentration of total phenolic compounds is expressed as milligrams of gallic acid equivalents per g of extract (mg GAE/g). All measurements were done in triplicate.

Determination of total flavonoids content

CME total flavonoids content was determined according to the protocol that uses aluminum chloride ($AlCl_3$) as reagent **(Quettier-Deleu et al., 2000)**. 1 mL of a known dilution of CME was added to 1 mL of a solution of aluminum chloride (2%).The mixture was homogenized and absorbance was read after 15 min at 410 nm against a blank. Quercetin was used in standard curve and the concentration of flavonoid compounds is expressed as milligrams of quercetin equivalents per g of extract (mg QE/g). All measurements were done in triplicate.

Fractionation of the CME

The CME was dissolved in distilled water and extracted successively with different organic solvents: chloroform, ethyl acetate and butanol. The solvents were evaporated to give chloroform fraction (CF), ethyl acetate fraction (EAF), butanol fraction (BF) and residual methanol fraction (RMF).

HPLC/ESI-MS^2 analysis

Solutions of the extracts were prepared from the dried extracts in bidistilled water. Optimal concentrations were chosen to ensure high peak intensities and resolutions in the chromatograms. The following concentrations were used: CME 100ppm, CF 1000ppm, EAF 100ppm, BF 70ppm, and RMF 600ppm.

HPLC/ESI-MS^2 ~~analyses~~ characterization of CME and fractions were carried out using an already set up method (**Mattonai et al., 2016**[E1]). The analyses ~~-~~were performed using a

1200 Infinity HPLC system, coupled with a 6530 Infinity Q-ToF mass spectrometry detector by a Jet Stream ESI interface (Agilent Technologies). Separations were performed on Ascentis Express RPAmide column (100 mm × 2.1 mm), particle size 2.7 µm (Supelco), with an Ascentis Express RP-Amide guard column (5 mm × 2.1 mm), particle size 2.7 µm (Supelco). An electrospray ionization source (ESI) interface was used and operated in negative ion mode. The ion source operated at a nebulizer gas pressure of 35 psig, with a capillary voltage of 4.5kV. Nitrogen (purity > 98%) was used both as drying gas (10mL/min, 350°C) and as a sheathing gas (11mL/min, 375°C), CID voltage 20 V. High-resolution MS and MS^2 acquisition range was set from 100 to 1000 m/z. Injection volume was 3 µL. The mobile phase was composed of water (A) and acetonitrile (B), both with 0.3% (v/v) formic acid. Mobile phase flow rate was 0.4mL/min, and column oven temperature was 40 °C. The following gradient was used for all experiments: 0–3.75 min at 100% (A); 3.75–19.50 min from 100% to 89% (A); 19.50–27.75 min from 89% to 79% (A); 27.75–44.25 min from 79% to 60% (A); 44.25–50.25 min from 60% to 39% (A); 50.25–51 min from 39% to 0% (A); 51.00–52.50 at 0% (A). Re-equilibration time was 12 min. Data were processed with Mass Hunter Qualitative Analysis software (version B.04.00).

Animals and housing conditions

~~Twenty-four~~Twenty-four adult albino Wistar rats of both sexes, weighing between 190-240gwere obtained from Pasteur institute (Algers, Algeria). Animal maintenance, handling and experimental procedureswere conducted in accordance with internationally accepted standard guidelines for use of laboratory animals (directive N° 2010/63/EU of 22 September 2010 on the protection of animals used for scientific purpose) Animals were maintained in standard laboratory conditions of temperature (25 ± 2 °C), relative humidity of 60–70%, with 12-hour light-dark cycle. Animals were fed with standard diet and water *ad libitum*, and acclimatized for a period of one week before the experiment.

Triton WR-1339induced hyperlipidemia experiment

Overnight fasted rats were randomly divided into 4 groups (n=6/group). Rats in group I (NCG: normal control group) received an intraperitoneal (i.p.) injection of normal saline, followed by oral gavage with normal saline. Rats in group II (HCG: hyperlipidemic control group) were i.p. injected Triton WR-1339 (Tyloxapol, Sigma–Aldrich, USA) at a dose of 250 mg/kg dissolved in normal saline and oral gavaged with normal saline. Rats in groups III and IV (CME 200 and CME 400) were also i.p. injected Triton WR-1339 at the same dose (250 mg/kg), and were oral gavaged with CME dissolved in normal saline at a dose of 200 mg/kg and 400 mg/kg, respectively. In the following period of study (24 h), animals had access to water only. At the end of this time period, blood was collected and centrifuged at 3000 rpm for 10 min and serum was used for lipid analysis.

Biochemical analysis

Serum total cholesterol and triacylglycerol levels were determined by enzymatic methods using cholesterol oxidase and lipoprotein lipase, respectively. These assays were done using standard diagnostic test kits (Spinreact, Girona, Spain).

Cell culture and differentiation

Human hepatoma cell line HepG2 and mouse embryonic preadipocyte cell line 3T3-L1 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA).

HepG2 cells were cultured in DMEM (DMEM 30-2002, from ATCC) containing 4mM glutamine antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin) (Sigma, St. Louis, MO, USA) and 10% fetal bovine serum (FBS) (Invitrogen, Life Technologies, Carlsbad, CA, USA), at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was renewed every two days.

3T3-L1 cells were routinely cultured in DMEM containing 1% penicillin-streptomycin (P/S) and 10% newborn calf serum (basal medium), at 37ºC and 5% CO_2. For differentiation into adipocytes, the cells were allowed to reach confluence and two days later (defined as day 0 of differentiation) the basal medium was replaced by differentiation medium consisting of DMEM supplemented with 10% FBS, 1% P/S, 5 µg insulin/mL, 0.25 mM 3-isobutyl-1-methylxanthine and 0.25 µM dexamethasone. After 2 days, differentiation medium was replaced by maintenance medium (DMEM containing 1% PS, 10% FBS and 5 µg insulin/mL). Cells were cultured until day 8, with replacement of the maintenance medium every 2 days.

For both cell lines, sub-culturing and cell count were performed by standard procedures (trypsinization and Trypan blue assay, respectively). The cells were treated with *R. alaternus* leaves CME or vehicle (DMEM) for time periods and doses as indicated.

Cytotoxicity and cell proliferation/viability assays

Cytotoxicity of *R. alaternus*leaves CME in HepG2 cells was evaluated by the lactate dehydrogenase (LDH) release assay, using a commercially available kit (Cytotoxicity Detection Kit (LDH), Roche Applied Science, Penzberg, Germany). For this, cells were seeded in 12-well plates at a density of 1×10^5, incubated for 48 h, then exposed to different concentrations of CME (10 to 500 µg/mL, f.c.) and further incubated for another 24 h, after which LDH activity in the culture medium was assessed following the manufacturer's instructions.

Effects of *R. alaternus* leaves CME on 3T3-L1 preadipocyte proliferation and viability were determined by a colorimetric, XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate)-based assay, using Cell Proliferation Kit II (XTT) (Roche Applied Science). In brief, 3T3-L1 cells in basal medium

were seeded in a 96-well cell culture plate (5×10^3 cells per well) and treated with CME (1 to 1000 µg/mL, f.c.). After 48h, 50µl of XTT labeling mixture were added to each well, followed by incubation for 4h at 37°C. The absorbance of orange formazan dye, which is produced from XTT in viable cells, was then measured at 492 nm with reference wavelength at 690 nm. The percentage of cell viability was calculated, using the absorbance value in control cells not exposed to CME as 100%.

Analysis of intracellular lipids

HepG2 cells were seeded in a 24-well cell culture plate (3×10^4 cells per well). Four days later, confluent cellswere treated with different concentrations of *R. alaternus* leaves CME (1 to 750 µg/mL, f.c.) and further incubated for 24 h before staining of intracellular neutral lipids (triacylglycerols, steryl esters) with Oil Red O (ORO).

3T3-L1 preadipocyteswere seeded in 24-well cell culture plate at a density of $1x10^4$ cells per well. Two experimental designs were used. In the first design, 3T3-L1 cells were treated with varying concentrations of CME during the entire differentiation period (from day 0 until day 8, with refreshment every two days), and were stained with ORO on day 8. In the second design, the cells were treated with CME at a late stage of adipogenic differentiation (day 6), and stained with ORO 48 h later (on day 8). Differentiation was induced as described above.

For ORO staining, the cell culture medium was removed and the cells washed twice with PBS, fixed for 10 min and then for 1 h with 10% formalin, washed twice with Milli-Q water and then with 60% isopropanol for 5 min, and allowed to dry at room temperature. A volume of 0.5 mL of ORO working solution was then added to each well and incubated for 10 min, after which the solution was removed and the cells were washed 4 times with water. Images were acquired under a microscope (Axiovert 100, Zeiss, Germany). For

quantification, ORO was eluted from the cells using 1 mL 100% isopropanol and quantified with a spectrophotometer at 500 nm using 100% isopropanol as blank. After ORO elution, the cells were stained with 0.1% crystal violet solution (50 mg crystal violet powder in 5 mL ethanol/45 mLMilli-Q water) for 20 min and washed 3 times with Milli-Q water. The crystal violet dye was then eluted with 10% acetic acid solution and the absorbance was measured at 590 nm. Crystal violet binds to proteins and DNA and it was used as an indicator of the number of non-detached, viable, cells **(Feoktistova et al., 2016)**.

RNA extraction and gene expression analysis

Confluent HepG2 cells were treated with CME (1 to 500 µg/mL, f.c.) and further incubated for 24 h before isolation of total cellular RNA, using E.Z.N.A total RNA Kit I (Omega Bio-Tek, USA) according to the manufacturer's instructions. Total RNA was mixed with an equal volume of 70% ethanol and treated on-column with DNAse. High quality RNA was eluted in DEPC-treated water and further purified by standard ethanol/sodium acetate precipitation. Quantity and quality of isolated RNA were assessed using a Nano-Drop 1000 spectrophotometer (Thermo-Fisher Scientific, USA) and its integrity confirmed using 1% agarose gel electrophoresis stained with SYBR Safe (Life Technologies, USA).

Real-time qRT-PCRwas used to analyze the mRNA expression levels of genes of interest: sterol regulatory element binding transcription factor 1 (SREBP1), nuclear receptor subfamily 1 group H member 3 (NR1H3, also known as LXRA), fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD1), carnitine palmitoyltransferase 1A (CPT1-L, liver isoform), acyl-CoA oxidase 1 (ACOX1), peroxisome proliferator activated receptor alpha (PPARA) and peroxisome proliferator activated receptor gamma (PPARG). For retrotranscription, 6 µL of total RNA (0.25 µg) was denatured at 65°C for 10 min and then incubated with MuLV reverse transcriptase (50 U/µL), 10× RT buffer, 25 mM $MgCl_2$, dNTP mixture (2.5 mM

each), 50 µM random hexamer, RNase inhibitor (20 U/µL) and RNase free water (15min at 20°C; 30min at 42°C and 5min at 95°C) in an Applied Biosystems 2720 Thermal Cycler. For each PCR reaction, Power SYBR Green Master mix (Life Technologies), 250 nM of forward and reverse primers, and 4 ng cDNA equivalents were used. Primers were obtained from Sigma (Madrid, Spain) and are detailed in Table 1S. qPCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems), with the following profile: Step I (cycling step): 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min, for 40 cycles; Step II (Melt Curve step): 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The relative expression of each mRNA was calculated according to Pfaffl **(Pfaffl, 2001)**, using β-actin as reference gene. Melting curves for each PCR reaction were generated to ensure the purity of amplified product.

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was assessed by Mann-Whitney U test or one-way ANOVA followed by Bonferroni post-hoc multiple comparison analysis. The differences were considered as significant when $P<0.05$. The analyses were performed with SPSS 19.0 for windows (Chicago, IL, USA).

Supporting information

Sequences of the primers used for qPCR analysis are presented in Table 1S. Base Peak Chromatograms of *R. alaternus* leaves CME and its different fractions are shown in Fig. 1S.

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Conflict of interest

The authors declare that there is no conflict of interest.

Legends for figures

Fig. 1 Effect of *R. alaternus* L. crude methanolic extract (CME) on serum triacylglycerol (A) and total cholesterol (B) levels in hyperlipidemic rats. Overnight fasted Wistar rats were treated with CME (200 mg/kg and 400 mg/kg) dissolved in saline by oral gavage and blood was collected 24h after. To induce hyperlipidemia, rats received an intraperitoneal (i.p.) injection of Triton WR-1339 (250 mg/kg) dissolved in saline just before CME treatment. Serum lipid levels were determined by enzymatic methods. Data are mean ± SEM of 6 animals per group (3 male/3 female). Significant differences between means were tested by one-way ANOVA followed by Bonferroni post-hoc multiple comparison analysis. Values not sharing a common letter were statistically different ($P<0.05$). NCG: normal control group (i.p. injected and gavaged saline); HCG: hyperlipidemic control group (i.p. injected Triton and gavaged saline).

Fig. 2 Effect of *R. alaternus* L. crude methanolic extract (CME) on intracellular lipid accumulation in HepG2 cells. Confluent HepG2 cells were exposed to the indicated concentrations of CME for 24 h. Intracellular neutral lipids were stained and quantified with Oil Red O (ORO; A and C) and proteins and DNA with crystal violet (B). Control cells received the vehicle (DMEM). Data are means ± SEM (n=7/8 per condition) and are expressed relative to the mean values of control cells, which were set to 100. Significant differences between means were tested by one-way ANOVA followed by Bonferroni post-hoc multiple comparison analysis. Values not sharing a common letter were statistically different ($P<0.05$).In C, representative photomicrographs of ORO stained HepG2 cells exposed to the indicated concentrations of CME are shown (magnification x 320).

Fig. 3 Effect of *R. alaternus* L. crude methanolic extract (CME) on gene expression related to fatty acid metabolism in HepG2 cells. Confluent HepG2 cells were exposed to the indicated concentrations of CME or 1µM all-trans retinoic acid (ATRA) for 24 h. Control cells received the corresponding vehicles DMEM. mRNA levels were analyzed by real-time qRT-PCR. Data are means ± SEM (n=5/6 per condition) and are expressed relative to the mean values of control cells, which were set to 100. Significant differences between control and CME treatments were tested by one-way ANOVA followed by Bonferroni post-hoc multiple comparison analysis. Values not sharing a common letter were statistically different ($P<0.05$). The statistical significance of ATRA effects versus control was assessed by the Mann-Whitney U test (*, $P<0.05$). Genes analyzed were, from A to H: CPT1-L, carnitine palmitoyltransferase 1A; ACOX1, acyl-CoA oxidase 1;PPARA, peroxisome proliferator activated receptor alpha; PPARG, peroxisome proliferator activated receptor gamma; SREBP1, sterol regulatory element binding transcription factor 1; LXRA, nuclear receptor subfamily 1 group H member 3-NR1H3-, also known as LXRA; FASN, fatty acid synthase; and SCD1, stearoyl-CoA desaturase.

Fig. 4 Effect of *R. alaternus* L. crude methanolic extract (CME) on 3T3-L1 preadipocyte proliferation/viability. Preconfluent 3T3-L1 preadipocytes in basal growth medium were exposed to the indicated concentrations of CME. Control cells received the vehicle (DMEM). After 48h, cell proliferation/viability was determined by a colorimetric XTT-based assay. Data are mean ± SEM (n=8 per condition) and are expressed relative to the mean value of control cells, which was set to 100. Significant differences between means were tested by one-way ANOVA followed by Bonferroni post-hoc multiple comparison analysis. Values not sharing a common letter were statistically different ($P<0.05$).

Fig. 5 Effect of *R. alaternus* L. crude methanolic extract (CME) on intracellular lipid accumulation in3T3-L1 adipocytes. 3T3-L1 preadipocytes were exposed to the indicated concentrations of CME or 1µM all-trans retinoic acid (ATRA) during the entire differentiation period (from day 0 until day 8; A, B and C) or at a late stage of adipogenic differentiation (from day 6 to day 8; D and E). Control cells received the corresponding vehicles DMEM.Intracellular neutral lipids were stained and quantified with Oil Red O (ORO; A, C and D) and proteins and DNA with crystal violet (B and E) at day 8. Data are means ± SEM (n=3/4 per condition) and are expressed relative to the mean values of control cells, which were set to 100. Significant differences between control and CME treatments were tested by one-way ANOVA followed by Bonferroni post-hoc multiple comparison

analysis. Values not sharing a common letter were statistically different ($P<0.05$). The statistical significance of ATRA effects versus control was assessed by the Mann-Whitney U test (*, $P<0.05$). In C, representative photomicrographs of ORO stained 3T3-L1 adipocytes exposed to the indicated concentrations of CME throughout differentiation are shown (magnification x 100).