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Hypocholesterolaemic and antioxidant properties of *Olea europaea* L. leaves from Chlef province, Algeria using *in vitro, in vivo* and *in silico* approaches

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ABSTRACT

Aqueous and ethanol extracts prepared from leaves of *Olea europaea* L. were evaluated for *in vitro* antioxidant and *in vivo* hypocholesterolemic effect. The result of administration of *O. europaea* leaf extracts on serum total cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) in hypercholesterolaemic mice was evaluated. In addition, rutin and luteolin, reported to occur naturally in *O. europaea* leaves, were docked against HMG-CoA reductase, the rate-limiting enzyme in cholesterol metabolism. Mice treated with both extracts showed reduced total cholesterol (246.6 and 163.4 mg/dl, for mice groups treated with respective extracts) and LDL (150.16 and 81.28 mg/dl, for mice groups treated with respective extracts) levels as compared to the hypercholesterolaemic group (total cholesterol 253.00 mg/dl and LDL 160.00 mg/dl). Mice treated with aqueous extract (200 mg/kg body weight) showed significantly reduced triglyceride and VLDL levels as compared to the group treated with atorvastatine. HDL level of mice administered with *O. europaea* aqueous extract was comparable to the atorvastatine-treated group. The ethanol extract of *O. europeae* leaves was a potent antioxidant (IC_{50} 69.15 mg/ml, % inhibition 54.98, 82.63 mg ascorbic acid equivalent/g extract, 7.53 mol of Fe^{2+}/g extract, and % inhibition 49.71, for the DPPH, β-carotene bleaching, total antioxidant capacity, FRAP, and ferric thiocyanate assays, respectively). Docking studies revealed that rutin showed higher binding affinity with HMG-CoA reductase as compared to luteolin. Data gathered from this study support the development of a prophylactic biomedicine from *O. europaea* leaves for the management of hypercholesterolemia and atherosclerosis.

1. Introduction

Well established links exist between hypercholesterolemia and the occurrence of cardiovascular complications (Hanning et al., 2016). Indeed, cholesterol is a versatile molecule, having multiple functions including cell membrane component, human brain constituents, precursor of steroid hormones, and regulator of the function of signalling molecules. Required cholesterol is mainly produced by the liver while the surplus from exogenous sources such as cholesterol rich food, increase the risk of hypercholesterolemia. In hypercholesterolemic patients, excess cholesterol is deposited in the arteries leading to narrowing of the arteries and restricted blood flow to the heart. Blockage of oxygen-rich blood to the heart can cause angina and lead to a heart attack (Hanning et al., 2016). Besides, hypercholesterolemia has been associated to cancer (Baek et al., 2017) and diabetes type 2 (Razi et al., 2017). Interestingly, patients are increasingly turning to plant-based complementary and alternative medicines to manage hypercholesterolemia (Littleton et al., 2012).

Olea europaea L., belonging to the Oleaceae family, is one of the oldest cultivated tree in the world and is a typical crop of the Mediterranean region (Scognamiglioa et al., 2012). Commonly known as olive tree, *O. europaea*, has been cultivated for centuries for its fruits and oil. Nowadays, *O. europaea* crops are found in different regions of the world and exist in different cultivars, some specific to certain regions (Di Donna et al., 2010). A strong body of scientific evidence highlight the role of *O. europaea* in the prevention and management of

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several diseases, including cardiovascular diseases.

The beneficial potential of *O. europaea* might be attributed to its secondary metabolites. Flavonoids, flavone glycosides, flavanones, iridoids, iridane glycosides, secoiridoids, secoiridoid glycosides, triterpenes, biophenols, benzoic acid derivatives, xylitol, sterols, isochromans, and sugars were isolated from *O. europaea* (Hashmi et al., 2015). Decoction, infusion, and essential oil of *O. europaea* have been employed in traditional medicine to relieve all sorts of diseases, namely urinary infections, gallstones, bronchial asthma, diarrhoea (Khan et al., 2007), tapeworm, high blood pressure, intestinal disease, renal lithiasis, constipation, liver pain, burns, rheumatism, diabetes, and gout (Hashmi et al., 2015).

The phenolic rich extract of *O. europaea* has been reported to induce apoptosis in colon cancer cell line (Zeriouh et al., 2017). Peptides from *O. europaea* seeds reduced oxidative stress in human cervical cancer cells (Hernández-Corroto et al., 2018). Treatment with aqueous extract of *O. europaea* leaves (400 mg/kg body weight) improved glucose control, antioxidant, and lipid profile of streptozotocin induced diabetic rats (Al-Attar and Alsalmi, 2017). The fruit pulp extract of *O. europaea* showed hepatoprotective effect in carbon-tetrachloride treated mice (Kang and Koppula, 2014).

However, it has been reported that the different environmental conditions of growth tend to determine the bioactivity of a cultivar and among different cultivars (Scognamiglioa et al., 2012). It was reported that the fruit extracts of seven different cultivars of *O. europaea* showed varying therapeutic activity (Dekdouk et al., 2015). In the present study, the biological properties extracts prepared from leaves of *O. europaea* collected from Chettia, from the Chlef province of Algeria was studied. The hypocholesterolaemic activity of *O. europaea* was evaluated *in vivo* using male Swiss albino mice. Furthermore, the antioxidant potential of *O. europaea* was investigated using several *in vitro* standard bio-assays. Besides, the interaction between two previously isolated bioactive phenolic compounds present in *O. europaea* and HMG-CoA reductase, a key enzyme in cholesterol metabolism was studied using *in silico* molecular docking.

In silico molecular docking approach is increasing being employed as a powerful tool to predict possible interaction between target molecule and proteins such as enzymes at the atomic level. In this way, docking allows the characterization and study the behaviour of bioactive molecules in the binding site of target enzymes as well as to elucidate fundamental biochemical processes. It has been mainly used in structure-based virtual screening for the identification of new bioactive compounds in drug discovery programs (Xuan-Yu et al., 2011). In the present study, molecular docking is used to highlight possible binding of active compounds such as phenolics from *O. europaea* and preferred orientation with HMG-CoA reductase to form a stable complex.

2. Materials and methods

2.1. Plant materials

Fresh leaves of *Olea europaea* were collected from the Chettia (Latitude: 36°10′11.66″N, Longitude: 1°13′41.63″E, Altitude = 94 m) Wilaya of CHLEF and identified by a local botanist. They were dried in the shade at room temperature and were then powdered.

2.2. Animals

In vivo study was carried out on male Swiss albino (NMRI) mice weighing between 25 and 30 g for hypocholesterolaemic activity. They were procured from the SAIDAL-Alger Research and Development Center (CRD) with appropriate ethical clearance granted. The animals were housed in polypropylene cages with a label holder containing the name of the batch, treatment applied, dates of the experiments and were exposed to light 12 h/24 h. The premises were equipped with a dynamic air conditioning with a temperature of 20–24 °C and a humidity level of 50 ± 10%. The animals had free access to water and standard food.

2.3. Preparation of extracts

100 g of dried *O. europaea* leaves were dissolved in 1 L of water and boiled for 15 min, to allow disruption of the cell, at 100 °C (Bennani-Kabchi et al., 2000). After cooling, the mixture was filtered through Wattman filter paper and the filtrate was concentrated using a rotary evaporator at a temperature of 55 °C. Ethanol extract was prepared by macerating 10 g of the leaves in a 50% aqueous-alcoholic solution for 72 h. The macerate was then filtered and concentrated using a rotary evaporator at a temperature of 48 °C (Peixoto et al., 2011; Cheurfa and Allem, 2016).

2.4. Determination of total phenolic and flavonoid content

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method as described by Wong et al. (2006) while the total flavonoids content (TFC) in the extracts was determined by spectrophotometry using the aluminium chloride method (Zhou et al., 2005). The results were expressed as gallic acid (mg GAEs/g extract) and quercetin acid equivalents (mg QEs/g extract) for the respective assays.

2.5. Evaluation of in vitro antioxidant activity

2.5.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Various concentrations of aqueous and ethanol extracts (250 µl) were added to 1 ml of 0.004% methanol solution of DPPH (Melucci et al., 2018; Hu et al., 2018). The mixture was stirred vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the solution was then measured at 517 nm. Butylated hydroxytoluene (BHT) was used as positive control. The percentage inhibition of DPPH was calculated using the following equation: % inhibition of DPPH = $\{(A_B - A_A)/A_B\} \times 100$; where A_B: Absorbance of DPPH solution without extract; A_A: Absorbance of DPPH solution in the presence of extract or standard. The results were expressed as percentage inhibition of DPPH and the IC_{50} (concentration corresponding to 50% inhibition of DPPH) was determined from a graphical curve of the absorbance of DPPH as a function of the concentration of the extract or BHT.

2.5.2. β-Carotene bleaching assay

1 ml of a solution of β-carotene in chloroform was introduced into a flask containing 40 µl of linoleic acid and 400 mg of Tween 40. After evaporation of chloroform, 100 ml of hydrogen peroxide was added with vigorous stirring. 2.5 ml of this solution were then transferred into tubes and 200 µl of extract or BHT was added. All the tubes were incubated in a water bath at 50 °C for 3 h and the absorbance of the solution was measured at 470 nm. The percentage of antioxidant activity was calculated according to the following equation (Anusuya et al., 2012): $AA\% = [1 - (A_0 - A_t)/(A_0^1 - A_t^1)] \times 100$; where A_0 et A_0^1: Absorbance of the extract or BHT and control (200 µl of absolute ethanol), respectively, measured at zero incubation time; A_t et A_t^1: Absorbance of the extract and control, respectively, measured after incubation.

2.5.3. Total antioxidant capacity (phosphomolybdate assay)

This method is based on the reduction of Mo (VI) to Mo (V) in the presence of antioxidant compounds and the subsequent formation of a green phosphate/Mo (V) complex at an acidic pH (Sahu and Laloo, 2011). 0.3 ml of each extract was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction solution were incubated at 95 °C for 90 min and the absorbance of the solution was then measured spectrophotometrically at 695 nm. The total antioxidant capacity was expressed in milligrams of ascorbic acid equivalent per gram of extract.

2.5.4. FRAP (ferric reducing antioxidant power) assay

The FRAP method is based on the reduction of the ferric tripyridyltriazine complex to tripyridyltriazine at low pH. This causes the formation of blue coloured tripyridyltriazine-ferrous complex which can be measured at 593 nm. The FRAP reagent was prepared by mixing sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ in a ratio of 10:01:01 (v/v/v). 200 µl of each extract was added to 3 ml of FRAP reagent. After incubation in the dark at 37 °C for 30 min, the absorbance of the solution was measured at 593 nm against the blank (Benzie and Strain, 1996). Concentrations were calculated according to the calibration curve, which was obtained using $FeSO_4$ solutions. The results were expressed in moles of Fe (II) per gram of extract.

2.5.5. Ferric thiocyanate assay

This method is based on the oxidation of linoleic acid which generates the formation of peroxides which oxidizes Fe^{2+} to Fe^{3+}. These ions form a complex with thiocyanate which has a maximum absorbance at 500 nm (Gülçin et al., 2007). The various extracts (40 mg) and standard (40 mg vitamin C and gallic acid) were mixed with 4 ml of absolute ethanol, 4.1 ml of linoleic acid (2.52%), 8 ml of 0.02 M phosphate buffer (pH 7.0) and 3.9 ml distilled water. The mixed solution was incubated at 37 °C. At regular intervals during incubation, 0.1 ml of the mixture was diluted with 3.7 ml of ethanol, followed by the addition of 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous chloride in hydrochloric acid. The level of peroxidation was determined by reading the absorbance at 500 nm. This step was repeated every 24 h until the negative control reached its maximum absorption value. The percent inhibition of lipid peroxidation was calculated by the following equation: % inhibition of lipid peroxidation = $100 - [(A_{sample}/A_{control}) \times 100$; $A_{control}$: Absorbance of negative control; A_{sample}: Absorbance in the presence of the sample or standards.

2.6. In vivo hypocholesterolaemic activity

Hypercholesterolemia was induced in mice by a diet containing 1% cholesterol for 30 days. All mice had free access to food and *ad libitum* water during the experimentation period. The mice were divided into batches of 5. The treated groups simultaneously received extracts of the *Olea europaea* leaves orally at a dose of 200 mg/kg. The 200 mg dose was selected because it is lower than the lethal dose according to previous studies. 200 mg of extracts were dissolved in 10 ml of appropriate solvent and the mice were administered due to 10 ml/kg. The reference group received a dose of atorvastatin (10 mg/kg) orally while the control group received 0.5 ml of distilled water each morning. The control group received a normal diet (Cheurfa and Allem, 2015). After 30 days of experimentation, blood samples were taken from mice of all groups to measure the total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), very low density lipoproteins (VLDL) and high density lipoprotein (HDL), as described previously (Cheurfa and Allem, 2015).

2.7. Statistical analysis

Data presented in this study was analysed using XL Stat Pro 7.5 statistical software. The experiments were done in triplicate. Results were presented as mean values and standard deviation. ANOVA test was conducted to determine any significance differences. $P < 0.05$ was considered as statistically significant.

2.8. Docking calculations

Two bioactive compounds previously reported to be present in Olive leaves (rutin and luteolin) and control (cerivastatin) were selected for docking studies. The starting structure of the compounds, rutin, and luteolin were downloaded from zinc database (Irwin et al., 2012). The structures were optimized to their minimum energy structure using AM1 semi-emperical method implemented in gaussian09 software (Frisch et al., 2009). The crystal structure of the enzyme human HMG-CoA reductase was downloaded from Protein Databank RCSB PDB (pdb code:1HWJ), in which the enzyme was in complex with cerivastatin as inhibitor (Istvan et al., 2000). Docking calculations were performed using Autodock 4 software (Molinspiration Database). Water molecules and other co-crystalized molecules were removed from the protein. Polar hydrogens were added and the structure was neutralized using Kollman united atom charges. The dimensions of the grid box was 60 × 60 x 60 with 0.375 Å distance between points. Lamarckian genetic algorithm was used to run 100 run for the inhibitors rutin and luteolin and the control ligand cervastatin. The docked conformations were ranked and listed according to the binding free energy (ΔG). The intermolecular interactions with the active site were visualized using Discovery studio 5.0 visualizer. In addition, the obtained binding free energies were normalized by calculating the Z score according to the equation ($Z = (E - \sigma)/s$) (Olson, 1987) where E is the binding free energy of each ligand, σ is the mean of the binding free energies of a set of the best binding modes and s is the standard deviation.

3. Results and discussion

3.1. Total phenolic and flavonoid contents

Olea europaea is largely distributed all around the Mediterranean basin and represent significant genotypic and phenotypic variations resulting from centuries of natural selection (Bouarroudj et al., 2016; Abdessemed et al., 2015). In the present study, we attempted to investigate into the biological activities of *O. europaea* leaves collected from Chettia, Algeria. Firstly, the total phenolic and flavonoid contents of the ethanol and aqueous extracts of *O. europaea* leaves were assessed using standard *in vitro* methods. The ethanol extract showed highest TPC (24.58 mg GAE/g) and TFC (1.06 mg QE/g) as compared to the aqueous extract (1.40 mg GAE/g and 0.98 mg QE/g, for TPC and TFC, respectively) as shown in Table 1. The current findings tend to provide evidence that ethanol is a more suitable in extracting phenolic compounds from *O. europaea* leaves compared to water. Likewise, the methanol (98%) extract of *O. europaea* leaves collected from Tunisia, Malta, and Montenegro (205.17, 149.23, and 127.18 mg GAE/g, for respective regions) showed high phenolic content (Stanković et al., 2017).

3.2. In vivo cholesterol lowering activity

The serum lipid profile was determined by estimating the levels of LDL, VLDL, triglyceride, and HDL. LDL, the main target of atherosclerosis reduction therapy, is the predominant cholesterol-carrying lipoprotein, and is considered to be the main atherogenic lipoprotein (Orozco-Beltran et al., 2017). Besides, cholesterol rich diet has been reported to decrease fatty acid oxidation, thereby increasing triglyceride level. Unlike, the clear and exact relationship of LDL in the pathogenesis of cardiovascular complications, the role of triglyceride remains unclear. However, a growing body of epidemiological evidences have demonstrated the implication of triglycerides in the formation of atherosclerosis and pathogenesis of cardiovascular complications

Table 1
Total phenolic and flavonoid contents of *O. europaea* extracts.

Extract	TPC (mg GAE/g)	TFC (mg QE/g)
Aqueous	1.40 ± 0.037	0.98 ± 0.005
Ethanol	24.58 ± 0.027	1.06 ± 0.031

Gallic acid (mg GAEs/g extract) and quercetin acid equivalents (mg QEs/g extract).

Table 2
Effects of *O. europaea* extracts on lipid parameters.

Treatment	TC (mg/dl)	TG (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)
Aqueous extract (200 mg/kg)	246.60 ± 17.38	111.20 ± 21.77	150.16 ± 44.18	22.24 ± 4.35*	62.00 ± 5.14
Ethanol extract (200 mg/kg)	163.40 ± 67.77	164.60 ± 35.01	87.28 ± 44.89*	30.92 ± 20.30	45.20 ± 14.00
Atorvastatine (10 mg/kg)	130.60 ± 4.18	121.80 ± 42.65	43.44 ± 9.35	24.36 ± 8.53	62.80 ± 3.49
Hypercholesterolemic	253.00 ± 31.60**	200.00 ± 12.32**	160.00 ± 31.60**	40.00 ± 2.46**	31.60 ± 1.74**
Normal	116.00 ± 0.89	146.40 ± 10.86	38.52 ± 8.13	29.28 ± 2.17	48.20 ± 6.40

*$p < 0.05$ significantly different compared to hypercholesterolemic group.
**$p < 0.05$ significantly different compared to normal group. Total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), very low density lipoproteins (VLDL) and high density lipoprotein (HDL).

Table 3
Antioxidant activity of *O. europaea* extracts.

	DPPH (IC_{50} mg/ml)	β-carotene bleaching (%)	Total antioxidant capacity (mg EAA/g extract)	FRAP (moles of Fe^{2+}/g extract)	Ferric thiocyanate (% inhibition)
Aqueous extract	92.04 ± 0.049[c]	50.87 ± 0.09[c]	75.75 ± 0.06[c]	04.01 ± 0.01[b]	45.95 ± 0.00[d]
Ethanol extract	69.15 ± 0.063[b]	54.98 ± 0.03[b]	82.63 ± 0.02[b]	07.53 ± 0.06[a]	49.71 ± 0.21[c]
BHT	06.95 ± 0.002[a]	96.19 ± 0.19[a]	401.01 ± 1.24[a]	/	/
Gallic acid	/	/	/	/	59.28 ± 0.00[a]
Vitamin C	/	/	/	/	52.80 ± 0.00[b]

Values with the same letter are not significantly different ($p > 0.05$). Control: BHT, gallic acid, and vitamin C.

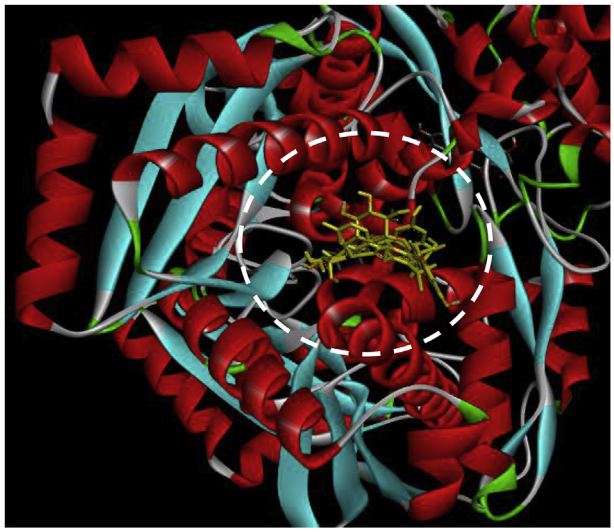

Fig. 1. The docked compounds, rutin, luteolin and cerivastatin at the active site of HMG-CoA enzyme.

(Arbel et al., 2016). Although, VLDL represents an unused target for risk prediction and risk reduction intervention, high concentration of VLDL in the blood confer an increased risk of developing atherosclerosis formation. A study conducted on a cohort consisting of 30 378 participants, showed that elevated VLDL level was significantly related to the increased risk of developing coronary heart disease (Ren et al., 2010). On the other hand, HDL level is inversely correlated to the occurrence of cardiovascular complications. HDL, involved in the uptake and transport of cholesterol to the liver through reverse cholesterol transport process, is an ideal therapeutic target to reduce the risk of developing atherosclerosis. Indeed, substantial epidemiological and clinical studies have shown that low level of HDL increased the risk of atherosclerosis formation (Baskaran et al., 2015). Thus, it can be argued that the balance of lipoproteins is a key factor to determine the formation and progression of atherosclerosis plaque and associated complications. These facts sustain the renewed interest for the discovery of lipid modulation agents as important therapeutic target for cardiovascular risk reduction.

In the present study, the effect of administration of aqueous and ethanol extracts of *O. europeae* leaves on the lipid profile of mice fed with cholesterol-rich diet was evaluated. The serum TC, triglyceride, LDL, VLDL, and HDL levels of mice treated with 200 mg of extract or atorvastatine per kg body weight are presented in Table 2. Mice groups treated with both aqueous and ethanol extracts showed reduced total cholesterol (246.6 and 163.4 mg/dl, for mice groups treated with respective extracts) and LDL (150.16 and 81.28 mg/dl, for mice groups treated with respective extracts) levels as compared to the hypercholesterolemic group (253.00 and 160.00 mg/dl for TC and LDL, respectively). Mice treated with aqueous extract (200 mg/kg body weight) of *O. europeae* leaves showed significantly reduced triglyceride and VLDL levels compared to the group treated with atorvastatine (Table 2). HDL level of mice administered with *O. europaea* aqueous extract and atorvastatine was comparable and higher than mice from other groups. A significant increase in HDL level was observed in mice administered with *O. europaea* aqueous extract. Data collected from this study demonstrate the modulating effect of *O. europaea* leaves on lipid profiles.

The therapeutic utility of *O. europaea* as a hypocholesterolaemic agent has been indicated in traditional medicine (Shen et al., 2014). The hypocholesterolaemic effect of oleuropein and its aglycone, found in abundance in *O. europaea* leaves, was attributed to their ability to lower serum TC, TG, and LDL levels in Wistar rats fed with cholesterol-rich diets (Jemai et al., 2008). Hydroxytyrosol, recovered from *O. europaea* leaves, was also found to decrease serum TC, TG, and LDL in Wistar rats fed with cholesterol-rich diets (Jemai et al., 2008). Rutin was identified as one of the major flavonol occurring in *O. europaea* leaves (Abaza et al., 2015). A study conducted on hypercholesterolemic rats, showed that rutin (100 mg/kg) significantly reduced the total cholesterol and LDL (Ziaee et al., 2009). Luteolin, also identified from *O. europaea* leaves (Kontogianni et al., 2013), was found to reduce cholesterol absorption in human epithelial colorectal adenocarcinoma cells by inhibiting NPC1L1 cholesterol transporter (Nekohashi et al., 2014).

3.3. In vitro antioxidant activity

The literature tend to report the increased production of superoxide in cardiac cells of hypercholesterolemic animals, supporting the association hypercholesterolaemia and cardiac oxidative stress (Csonka et al., 2016). The modulation of oxidative stress in hypercholesterolaemia can be achieved by developing cholesterol-lowering agents

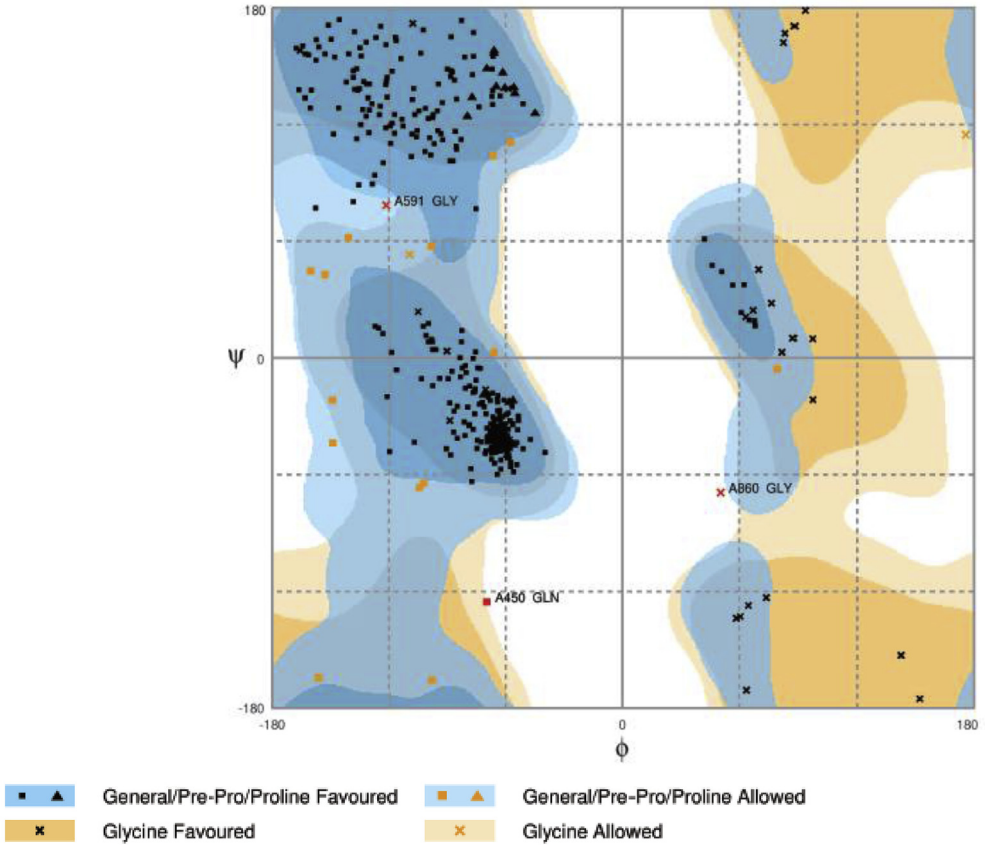

Fig. 2. Ramachandran plot of HMG-CoA enzyme.

possessing antioxidant properties. Antioxidants confer therapeutic action by transferring a hydrogen atom or an electron to the reactive species, making them stable and less reactive (Apak et al., 2016). Thus *in vitro* antioxidant assays probe the antioxidant capacity of molecules by measuring hydrogen atom transfer or electron transfer. In the present study, the antioxidant properties of *O. europaea* leaves extracts were evaluated based on different mechanisms of protection against oxidative damage. The phosphomolybdenum assay, based on the reduction of Mo (VI) to Mo (V), is a simple method to measure the antioxidant potential of plant metabolites (Ramdane et al., 2017). As shown in Table 3, the ethanol extract (82.63 mg AAE/g extract) of *O. europaea* leaves showed higher antioxidant capacity compared to the aqueous extract (75.75 mg AAE/g extract). The ethanol extract (IC_{50} 69.15 mg/ml) of *O. europaea* leaves showed significantly greater ($P < 0.05$) DPPH radical scavenging activity as compared to the aqueous extract (IC_{50} 92.04 mg/ml). Nonetheless, the positive control BHT (IC_{50} 6.95 mg/ml) was a more potent scavenger of DPPH compared to the studied extract. The DPPH radical, widely used to evaluate the radical scavenging activity of antioxidants, is reduced to DPPH-H by hydrogen transfer (Boudier et al., 2012). In a system composed of β-carotene and linoleic acid, β-carotene undergoes rapid discoloration in the absence of an antioxidant due to the oxidation of linoleic acid (Jeshvaghani et al., 2015). The ethanol extract (54.98%) of *O. europaea* leaves significantly ($P < 0.05$) prevented bleaching as compared to the aqueous extract (50.87%), but both were less potent than BHT (96.19%). The FRAP assay is an electron transfer based assay that measures the reduction of Fe^{3+} to an intensely blue Fe^{2+} complex by an antioxidant agent (Zhong and Shahidi, 2015). The ethanol extract (7.53 mol of Fe^{2+}/g extract) of *O. europaea* leaves was found to be significantly superior ($P < 0.05$) compared to the aqueous extract (4.01 mol of Fe^{2+}/g extract). A series of free radical-mediated chain reaction occur during lipid peroxidation and the ferric thiocyanate method measures the amount of peroxide produced during the initial stage of oxidation (Gülçin et al., 2010). Similar to the other antioxidant assays, percentage inhibition of lipid peroxidation was significantly higher in the presence of the ethanol extract (49.71%) of *O. europaea* leaves. Interestingly, rutin previously identified in *O. europaea* leaves, was reported to attenuate hepatotoxicity induced by oxidative stress in high cholesterol diet fed rats (AlSharari et al., 2016). Additionally, scientific evidence gathered from a previous study, suggested that luteolin might confer protective effects against the progression of diabetes-induced cardiac dysfunction by down regulating oxidative stress. Oleuropein and hydroxytyrosol were reported to induce antioxidant properties *in vivo* (Jemai et al., 2008).

3.4. Docking calculations

It is well known that docking calculations provides essential information about the binding conformation of the inhibitors at the active site and is the best tool for ranking inhibitors based on their binding free energy. Flavonoids, namely, rutin and luteolin, have been previously identified and isolated from the leaves of *O. europaea* (De Laurentis et al., 1998; Mylonaki et al., 2008). In this study, docking calculations were used to explore the binding free energy of rutin, luteolin and reference inhibitor cerivastatin which was crystalized at the active site of HMG-CoA reductase, an enzyme involved in the production of cholesterol. Fig. 1 shows the docked compounds at the active site of the enzyme. Overlapping of the three compounds at the active site sustained that the chosen site was ideal for docking.

Naturally, the tertiary structure of the enzyme is composed of four subunits in which the active site is shared between two neighbouring subunits. In order to investigate the secondary structure features of the protein, Ramachandran plot (Lovell et al., 2002) is sketched and shown in Fig. 2. Obviously, the most favoured secondary structures are beta

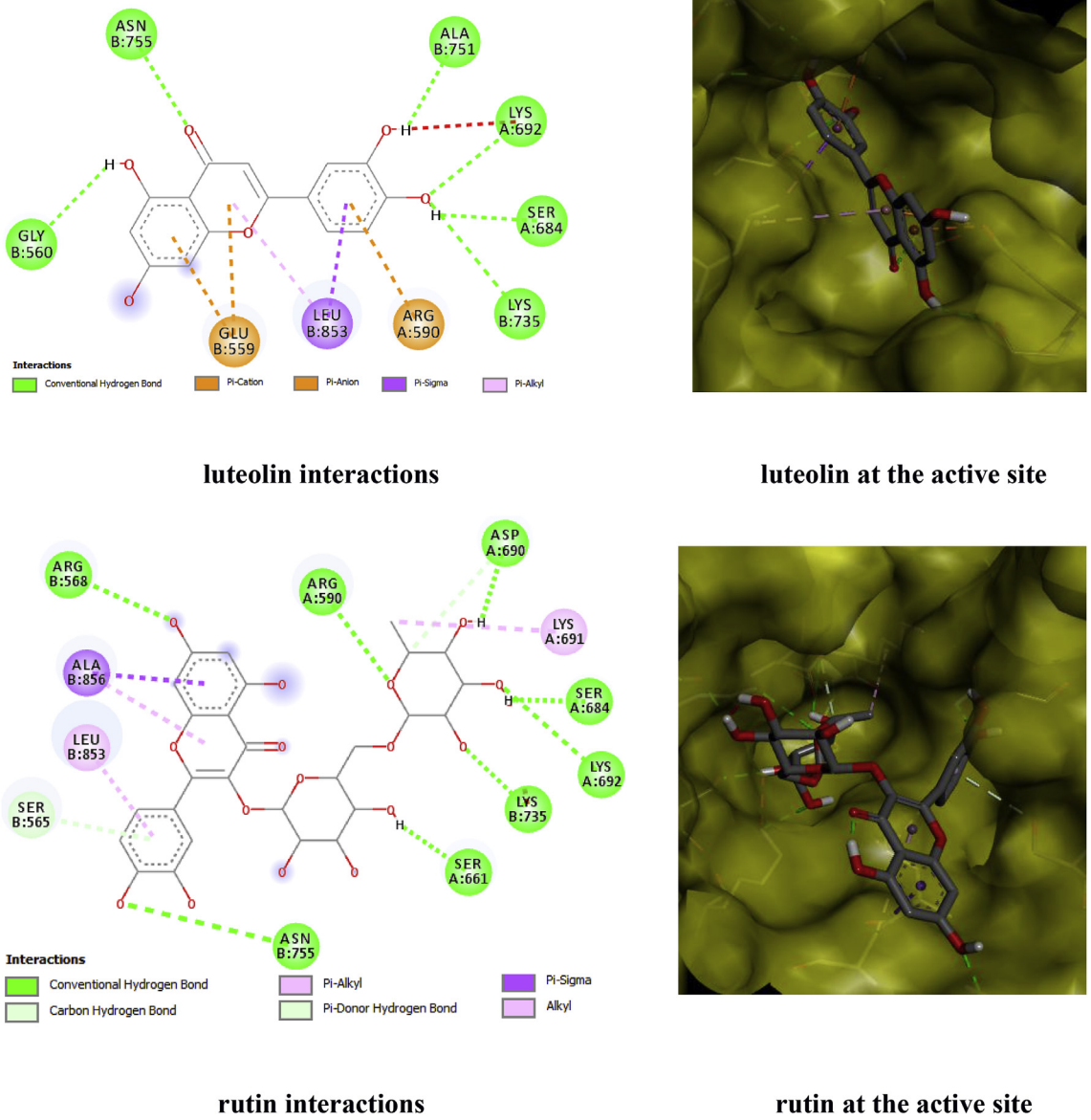

Fig. 3. The docked compounds, rutin and luteolin at the active site of HMG-CoA enzyme and their interactions.

strands located at Quadrant II and right-handed alpha helices located at Quadrant III. The docking results are summarized in Fig. 3, in which the best docked conformation for the two phenolic compounds is shown. The binding free energy, Z score, predicted inhibition constant and distances between the inhibitor and residues at the active site (Table 4). As shown in Table 4 the Z score of the docked ligands are negative and higher than the value of the control which means and confirm the strong interaction between these ligands and the protein. Fig. 3 presents the best docked conformation for rutin and luteolin. Different interactions were formed between the phenolic compounds, rutin, and luteolin and the active site as shown in Fig. 3. It was observed that hydrogen bonding was more common. It was noted that both phenolic compounds showed higher binding affinity with the enzyme compared to the control compound, cerivastatin. Indeed, rutin showed highest binding affinity (−9.5 kcal/mol) although it formed less hydrogen bonds at the active site compared to luteolin (−8.0 kcal/mol).

4. Conclusion

This study showed that *O. europaea* could be considered as an effective alternative natural remedy for the management of hypercholesterolaemia, consequently reducing the risk of atherosclerosis. Data collected showed that extracts of *O. europaea* leaves effectively reduced the levels of TC, LDL, VLDL, and TG and increased the level of HDL *in vivo*. Besides, *O. europaea* leaves extracts, particularly the ethanol extract, showed potent antioxidant activities in different oxidant mechanisms. *In silico* molecular docking showed that both phenolic compounds interacted with the active site of HMG-CoA rutin showed higher binding affinity compared to luteolin and cerivastatin. These findings advocate the need for further investigation on the mechanism of action of *O. europaea* and the identified phenolic compounds in the inhibition of atherosclerosis. Besides, data gathered support the development of a prophylactic agent from *O. europaea* leaves for the management of hypercholesterolaemia and atherosclerosis.

Table 4
Binding energy (ΔG), inhibition constant, interaction sites, and distances between residues at the active site of HMG-CoA and chosen ligands.

Ligand	ΔG (kcal/mol)	Z score	Interaction site	Distances (Å)
Rutin	**−9.5 (1.35 μM)**	−1.26	Lys A:692, Ser A:684, Arg A:590, Lys B:735, Leu B:853, Glu B:559, Gly B:560, Asn B:755, Ala B:751	Lig – Lys A:692 (HB)* (3.18) Lig – Ser A:684 (HB) (2.27) Lig – Arg A:590 (4.14) Lig – Lys B:735 (HB) (3.19) Lig – Leu B:853 (3.89) Lig – Glu B:559 (3.75) Lig – Gly B:560 (HB) (2.33) Lig – Asn B:755 (HB) (3.22) Lig – Ala B:751 (HB) (2.26)
Luteolin	**−8.0 (1.83 μM)**	−1.05	Arg A:590, Asp A:690, Lys A:691, Ser A:684, Lys A:692, Ser A:661, Lys B:725, Asn B:755, Ser B:565, Leu B:853, Ala B:856, Arg B:568	Lig – Arg A:590 (HB) (3.32) Lig – Asp A:690 (HB) (2.93) Lig – Lys A:691 (2.45) Lig – Ser A:684 (HB) (2.09) Lig – Lys A:692 (HB) (2.80) Lig – Ser A:661 (HB) (1.89) Lig – Lys B:725 (HB) (3.06) Lig – Asn B:755 (HB) (2.96) Lig – Ser B:565 (3.74) Lig – Leu B:853 (4.95) Lig – Ala B:856 (3.76) Lig – Arg B:568 (HB) (2.87)
(control)	**−7.4**	0.04		

(HB)* = Hydrogen bond.

Transparency document

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