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Research Article

In vitro Screening of the Pancreatic Cholesterol Esterase Inhibitory Activity of Some Medicinal Plants Grown in Syria

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ABSTRACT

Hypercholesterolemia is implicated in atherosclerosis and associated cardiovascular diseases. Plants have increasingly become attractive alternatives to prevent or treat these conditions. The aim of this study was to evaluate the cholesterol esterase inhibitory activity of *Ecballium elaterium*, *Pinus brutia*, *Plantago lanceolata*, *Schinus molle*, and *Hedera helix in vitro*. Methanolic and water extracts of different parts of plants were prepared. Essential oils extracted from leaves and fruits of *Schinus molle* and fruit juice of *Ecballium elaterium* were also tested. Results have shown that percentage inhibition of pancreatic cholesterol esterase for fruit juice and dry aqueous extract from leaves of *Ecballium elaterium*, dry methanolic extract of *Hedera helix* leaves and *Pinus brutia* bark was 22.25±0.9%, 21.58±0.48, 17.96±0.60%, and 19.97±0.29%, respectively at concentration of 0.2mg/ml. These findings demonstrate that these plants seem to be potential inhibitors of cholesterol esterase which may result in reduced cholesterol absorption and consequently delay of the progression of micro- and macrovascular complications.

Keywords: Hypercholesterolemia, cholesterol esterase, inhibitory activity, medicinal plants.

INTRODUCTION

Hypercholesterolemia is the most important risk factor for atherosclerosis, which is the major cause of cardiovascular disease (CVD)[1]. Hypercholesterolemia is increasing day by day due to the ingestion of foods rich in saturated fats and cholesterol. It is more common in obese people. Familial hypercholesterolemia is a genetic disorder causes high levels of low density lipoprotein (LDL)[2]. Diagnosing and managing of hypercholesterolemia as a way to prevent CVD is a common approach for primary care. Therefore, many drugs have already been proved to be useful in lowering serum cholesterol levels, such as statins which block the enzyme HMG-CoA (5-hydroxy-3-methylglutaryl-coenzyme A) reductase that is required for the biosynthesis of cholesterol[3]. In addition, dietary and biliary cholesterol absorption inhibitors such as ezetimbe have been introduced as a new class of lipid-lowering drugs[4]. Dietary cholesterol is comprised of free and esterified cholesterol. The hydrolysis of cholesterol esters in the lumen of the small intestine is catalyzed by pancreatic cholesterol esterase, which liberates free cholesterol. Cholesterol esterase (CEase; EC 3.1.1.13) also called bile salt-stimulated lipase, carboxyl ester lipase and pancreatic lysophospholipase, is a glycoprotein that belongs to α/β hydrolase family of enzymes[5-7]. It is synthesized in the acinar cells of the pancreas and is stored in zymogen granules. CEase is released into the intestinal lumen in response to a fat-containing meal and constitutes 1–2% of total protein in pancreatic juice[6,8]. It has been found in a number of mammalian tissues such as pancreas, small intestine, liver, brain and placenta of numerous species also it has been found in milk. Cholesteryl esters, phospholipids, lysophospholipids, triglycerides and esters of lipid-soluble vitamins are among the substrates of this enzyme[9,10]. Several observations suggest that the role of CEase extends beyond that of simply hydrolyzing of dietary cholesterol. CEase may provide the transport function for delivery of cholesterol from micelles to enterocytes[11]. It is noteworthy the role of CEase in intestinal micelle formation where CEase participates with phospholipase A_2 in hydrolysis of lecithin to lysolecithin which is required for formation of intestinal micelles that efficiently deliver free cholesterol[12]. Pharmacological inhibition of this enzyme might lead to a reduction of cholesterol absorption and thereby a reduction in plasma cholesterol concentration. Interestingly, it has been reported that the ability to absorb cholesterol administered as cholesterol esters in cholesterol esterase gene-knockout mice was impaired[8]. More recently, phenoxyphenylcarbamate WAY-121,898, a synthetic novel inhibitor of cholesterol esterase, was shown to be effective inhibitors of cholesterol absorption in normal and cholesterol-fed rats and dogs. The effect was observed by both oral and parenteral administrations[13]. Medicinal plants constitute an important source of potential therapeutic agents for hypercholesterolemia with lower side effects that chemical drugs have such as myopathy and hepatotoxicity[14,15]. Thus in this study we investigated the inhibitory effect of *Plantago lanceolata*,

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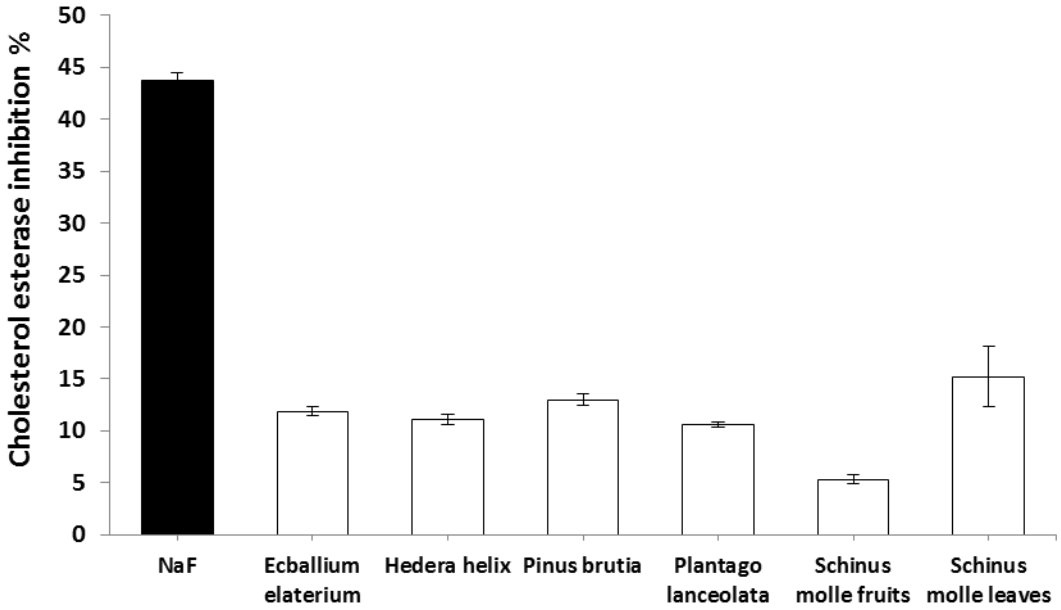

Figure 1: The effect of methanolic extracts on pancreatic cholesterol esterase. Results were expressed as mean values± S.E.M, N=6.

Table 1: List of selected plants grown in Syria for pancreatic cholesterol esterase inhibitory activity.

Plant name	Family	Used part
Ecballium elaterium	Cucurbitaceae	fruits juice leaves
Hedera helix	Araliaceae	leaves
Pinus brutia	Pinaceae	bark
Plantago lanceolata	Plantaginaceae	leaves
Schinus molle	Anacardiaceae	fruits leaves

Pinus brutia, *Hedera helix*, *Ecballium elaterium*, *Schinus molle* on pancreatic cholesterol esterase activities.

MATERIALS AND METHODS

Chemicals

Cholesterol esterase from porcine pancreas (CEase) was purchased from Sigma (USA). P-nitrophenyl butyrate (p-NPB) 99%was purchased from IT (USA), Triton X-100 (TX) was obtained from Rasayan Laboratory Sd fine chem limited (Mumbai). All other chemicals were commercially obtained and were of analytical grade.

Instruments

Spectrophotometric assays were done on V-650 UV-VIS Spectrophotometer (Jasco, Japan) with a photomultiplier tube detector. Incubation was performed using Bio TDB-120, BIOSAN, Dry block thermostat (Latvia). Ultrasonic instrument (POWERSONIC 405) was made by Hwashin Technology (Korea). Evaporation was done using Rotavapor R-215, Buchi (Switzerland).

Centrifuge: Heraeus Megafuge 2.0R (Germany).

Plant collection

Plants were collected from Aleppo, Syria in 2014 and were botanically identified by Dr. Abdel Aleem Bello (Plant Taxonomy, Faculty of Science, University of Aleppo). Fruits were harvested at the mature stage. The list of plants used in this study is given in Table 1.

Preparation of plant extracts

Samples of each plant were dried at room temperature and ground to fine powder, using a grinder. 10g of each plant part was extracted with100 ml methanol at 50±1°C, or with100 ml distilled water at 90±1°C for 60 min using ultrasonic bath (40KHz). The extracted suspension was centrifugally separated at 4000 rpm for 10 min. In this step we have got methanolic extract and it was stored in refrigerator at 4°C. For dry methanolic extract and dry aqueous extract the supernatant was evaporated in a rotary evaporator at 40±1°C. Then, they were stored in refrigerator. Fruits of *Ecballium elaterium* were squeezed manually, and their juice was collected in glass jars and then centrifuged at 4000 rpm for 10 min. It was then stored at –20 °C.

Essential oil extraction

The essential oil was isolated by hydrodistillation using a Clevenger type apparatus. 50g of *Schinus molle* leaves or fruits was transferred into a distillation flask and 250 ml of distilled water was added. The extraction process was carried out for 5 hours. The obtained essential oil was dried over anhydrous sodium sulphate and stored at -20°C in dark brown bottle until analysis.

Cholesterol esterase enzyme inhibitory activity

The pancreatic cholesterol esterase inhibition was measured spectrophotometrically at 25±0.5°C using the method of Pietsch and Gutschow with some modifications[6,16]. Lyophilized CEase was dissolved in 1 ml of 100 mM sodium phosphate buffer at pH 7.0, separated into 200 µl aliquots and stored at −80°C. Prior to use, an aliquot was thawed and diluted to 5 µg/ml with the same buffer. Reaction volume of 1ml, containing 20 µl of various inhibitors (10 mg/ml in acetonitrile) were pre-incubated with 500 µl of Triton X-100(5%w/w), 20 µl of p-nitrophenyl butyrate (p-NPB) (0.05 M in acetonitrile), 40 µl of acetonitrile (2%) in 400 µl of assay buffer (100 mM sodium phosphate, 100 mM NaCl, pH 7.0) at 25 °C and thoroughly mixed for 5 min. Reaction was initiated by adding 20 µl of CEase(5µg/ml). After

Table 2: Percentage yield of plant extract.

Sample	Part	Percentage yield (w/w %)	
		Dry aqueous extract	Dry methanolic extract
Ecballium elaterium	Leaves	19.4	5.5
Hedera helix	Leaves	19.2	10.6
Pinus brutia	Bark	29.8	32.2
Plantago lanceolata	Leaves	30.4	18.2
Schinus molle	Fruits	26.8	28.2
	Leaves	22.4	16.6

incubation for 15 min, liberated p-nitrophenoxide was determined by measuring the absorbance at 405 nm. Uninhibited enzyme activity was determined by adding acetonitrile instead of the inhibitor solution. Sodium fluoride (NaF) was used as positive control[17]. Percentage of inhibition was calculated using the formula (A-B)/A×100, where A and B are the absorbances resulting from enzymatic hydrolysis without and with inhibitor, respectively. Six sets of data were collected for each sample.

Statistical Analysis

Values were expressed as mean±standard error of the mean (S.E.M) of six independent experiments. The data were analyzed by Student's t-test to study the CEase inhibitory of the extracts. $P< 0.05$ was considered to be statistically significant.

RESULTS

Extraction yield

As shown in table 2 the percentage of extraction yield for methanolic extract was ranged from 5.5 to 32.2% (w/w). The yield of *S. molle* oil was found to be 4.0% (w/w) fruits and 0.75% (w/w) leaves in relation to the dry weight. plant extracts12 plant extracts were evaluated for their cholesterol esterase inhibition activity as well as juice of *Ecballium elaterium* fruit and essential oil from leaves and fruits of *S. molle.* The percentage of inhibition of cholesterol esterase by plant extracts at concentration of 0.2 mg/ml is presented in Table 3. Among these plants, *Ecballium elaterium*, *Pinus brutia*, and *Hedera helix* were the most effective pancreatic cholesterol esterase inhibitors with values 17-22 %, whereas *Schinus molle* fruits, whether it is a dry aqueous or dry methanolic was the least potent inhibitor among the tested extracts. Other extracts showed moderate inhibitory activity (8-15%). Comparably, NaF used as positive control markedly inhibited the cholesterol esterase about (43.70±0.78) %. The inhibitory effect of methanolic extracts on cholesterol esterase is shown in Figure 1. Their inhibitory activity was ranged from 5.31 to 15.21%. Fruit juice of *Ecballium elaterium* markedly inhibited pancreatic cholesterol esterase activity by 22.25±0.9%, whereas fruits and leaves oil of *Schinus molle* inhibited this enzyme activity by 8.41±0.68, and 9.04±0.51%.

DISSCUSSION

It has been firmly established that hypercholesterolemia has directly proportionate relationship with atherosclerosis and ischaemic heart disease[18]. Therefore, the inhibition of enzymes that control cholesterol absorption and transportation is considered as a good target for reducing blood cholesterol. One of these enzymes is cholesterol esterase which plays an important role in the regulation of cholesterol metabolism by extending cholesterol intestinal absorption and transportation to enterocytes. In this study, some medicinal plants grown in Syria were selected to investigate their potential as an antihypercholesterolemic agent by testing their inhibitory effect against cholesterol esterase. Methanol and water extracts of different parts of plants were tested. Extracts of *Pinus brutia*, *Ecballium elaterium*, and *Hedera helix* exhibited cholesterol esterase inhibition activity ranging from 17.9 to 22.25%. Results indicated that dry aqueous, and dry methanolic extract were more effective than methanolic extract in inhibition of cholesterol esterase, this may be due to the highest concentration of bioactive compounds in dry extracts, which might have a beneficial impact on this enzyme. Published research reported that supplementation with *Pinus pinaster* bark extract rich in polyphenols has favourable effects on risk factors for coronary artery disease, i.e., reducing total cholesterol and LDL-cholesterol levels and increasing high-density lipoprotein (HDL)-cholesterol levels, resulting in a better atherosclerotic index[19]. In another randomized, double blind, placebo controlled study significantly lowered LDL and increased HDL was observed after administration of PYCNOGENOL®, French Maritime Pine Bark, in 155 menopausal woman during a treatment period of 6 months[20]. Evidence from *in vitro* study reveals that polyphenols, gallic acid, catechin and epicatechin, significantly inhibit cholesterol esterase in a concentration-dependent manner[21]. On the other hand, animal studies demonstrate that proanthocyanidins reduce the plasma levels of atherogenic LDL-cholesterol but increase antiatherogenic HDL-cholesterol[22]. It is noteworthy that the highest amount of total procyanidins, were found in *Pinus nigra* and *Pinus brutia* among different Pinus species. Also, *Pinus brutia* bark is rich in polyphenols[23,24]. From this point of view, it can be inferred that the pancreatic cholesterol esterase inhibitory activity of pinus extracts may be partly due to the effect of procyanidins and polyphenols. These previous reports studied different species of the genus *Pinus* and up now there are no studies on the effect of *Pinus brutia* on cholesterol. *Hedera helix* has been reported to contain saponin as an active component[25]. In general, the effect of saponins, natural or synthetic, in lowering the levels of cholesterols has been demonstrated in a number of studies. Morehouse *et al*. observed that the synthetic saponins pamaqueside and tiqueside inhibit intestinal cholesterol absorption in rabbits resulting in decreased plasma and hepatic cholesterol levels[26]. It is interesting to note that alfalfa saponins are responsible for its effect in

Table 3: Effect of plant extracts on the inhibition of cholesterol esterase.

Sample	Part	Percentage inhibition of pancreatic cholesterol esterase%	
		Dry aqueous extract	Dry methanolic extract
Ecballium elaterium	Leaves	21.58±0.48*	17.69±0.94*
Hedera helix	Leaves	16.24±0.64*	17.96±0.60*
Pinus brutia	Bark	14.78±0.28*	19.97±0.29*
Plantago lanceolata	Leaves	14.56±0.61*	16.72±0.36*
Schinus molle	Fruits	10.50±0.33*	8.98±0.21*
	Leaves	15.47±0.66*	13.45±0.36*

Results are expressed as mean values±S.E.M. (standard error of the mean), N=6*, Significantly different from – control ($p<0.05$)

reducing cholesterol absorption[27]. In addition, saponins from *Panax quinquefolium* reduced the level of lipid peroxidation products subsequently reduce the risk of atherosclerosis[28]. Thus, it is reasonable to assume that the effect of *Hedera helix* on CEase might also be owing to the presence of saponins. Until now, there are no studies about the hypocholesterolemic effects of *Ecballium elaterium*, but our results indicate that both fruit juice and leaves of Ecballium have inhibitory effect on cholesterol esterase. According to our *in vitro* findings it can be suggested cholesterol lowering effect of these plant extracts through inhibition of cholesterol esterase activity, and the use of them may be a feasible therapeutic strategy for prevention and treatment of patients with hypercholesterolemia. We suggest an orientation about the extraction and the purification of principal ingredients which might be possess good hypocholesterolemic activity from these plants. More studies *in vivo* are needed to elucidate hypocholesterolemic activity of these extracts and confirm their mechanisms of action for the purpose of application in hypercholesterolemia prevention and treatment.

CONCLUSION

Our results clearly indicate that *Ecballium elaterium*, *Pinus brutia* and *Hedera helix* extract demonstrate good inhibitory activity against pancreatic cholesterol esterase. They might be useful in clinical field and since being plant extracts, they may exhibit lower incidence of side effects.

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