Hypolipidemic Activity of Extracts from *Eriobotrya japonica* and *Olea europaea*, Traditionally Used in the Greco-Arab Medicine in Maintaining Healthy Fat Levels in the Blood

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Abstract: Loquat (*Eriobotrya japonica)* and olive leaves (*Olea europaea*) were investigated for their safety, anti-oxidant, and anti-hypercholesterolemic properties. Therefore, a fixed combination of dry extracts of *Eriobotrya japonica* and *Olea europaea* was prepared into so called Cholevel tablets and tested for safety and efficacy *in vitro* and *in vivo* tests. No sign of toxicity (LDH-release) was observed in cultured human fibroblasts incubated with increasing concentrations of Cholevel. Similar results were seen in MTT assay in co-cultures of human hepatocytes and monocytes. In addition, a high level of safety was seen in rats with an LD_{50} of 17.3 g/kg. The extent of Lipid peroxidation in isolated human LDL fractions, rat liver homogenates, and ferrosulphate was substantial at low concentrations of Cholevel. Hypolipidemic properties were assessed in a double blinded- randomized clinical study carried out among 41 human volunteers with hyperlipidemia values. The volunteers were divided into three groups. They were asked to continue their usual diet and medications unchanged and were evaluated for efficacy and tolerability of Cholevel for 3 months. Group 1 included 12 of persons who were at a fixed dose of statins therapy without fully responding to their medications they consumed Cholevel tablets 1 x 3 daily. Group 2 included 20 volunteers who consumed only Cholevel tablets 1 x 3 daily. Group 3 (control group) included 9 volunteers who consumed placebo tablets 1 x 3 daily. Cholevel was well tolerated by all subjects and no side effects were reported. Cholesterol levels were significantly reduced in groups 1 and 2 by 24% and 14.3% after three months of Cholevel consumption, respectively. Parallel reductions in both LDL and triglycerides levels and increments in HDL levels were observed. Taken together, results demonstrate safety, tolerability and efficacy of Cholevel that seems to have dual inhibition on both the absorption and production of cholesterol.

Keywords: *Eriobotrya japonica*, *olea europaea*, cholevel, cholesterol, Greco-Arab medicine.

INTRODUCTION

Hypercholesterolemia is often associated with obesity, diabetes mellitus and hypertension, each and all contribute to elevated cardiovascular mortality [1]. There is a general consensus that these metabolic disorders share hyperinsulinemia and insulin resistance as a common link [2,3] leading to both micro- and macro-angiopathies [4]. During the last two decades, statins have been in clinical use as selective inhibitors of the key enzyme, Hydroxymethylglutaryl Coenzyme-A (HMG-CoA)-reductase that determines the rate of cholesterol synthesis in hepatocytes. In population studies, statins have been evidenced to reduce cholesterol levels by about 25% and mortality due to myocardial infarction by about 42% [5].

Atherogenesis is a multifactor process that includes oxidative modification of LDL which triggers pathological events through multiple pathways leading to atherosclerosis [6]. Research in recent years has been directed towards dietary antioxidants of plant-derived foods to normalize the augmented levels of cholesterol atherogenous fractions, mainly LDL, and of glucose in an attempt to reduce the cardiovascular risk [4,7,8].

Greco-Arab Herbal Medicines: Based on scientific and traditional knowledge, there is little doubt that the concept of Greco-Arab and Islamic herbal medicinal therapy has shown remarkable success in healing acute as well as chronic diseases. According to recent surveys, there are about 350-450 medicinal plants in the Eastern region of the Mediterranean and about 230 medicinal plants in coastal Mediterranean region in Egypt. These plants are used by healers for the treatment and preventions of almost all types of human diseases, such as cancer; skin, respiratory, digestive, liver dis-

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eases; diabetes; and others. These plants are sold or traded in market places in the Mediterranean region or internationally. In many cases plant extracts are prepared into a mixture [9,10]. Several plant species have been investigated and bioactive ingredients extracted to treat various human diseases. Plant parts used included leaves, flowers, stems, roots, seeds, and berries [9,10].

Olive and Loquat: Recent data have evidenced antiatherogenic and antioxidant activities of extracts from leaves from both olive [12] and loquat [13].

The safety of both herbs used in the present study is documented by their use in traditional Greco-Arab medicine through centuries [11,14]. The leaves of loquat are well known and safe household remedy especially in the Far [13] and Middle East [15]. Extracts from these leaves have been reported to exhibit a significant hypoglycaemic effect both in Italy [16-18] and Pakistan [19]. They were also reported to have antiviral [18], antitumour effects [20] and to exhibit cytotoxic effects against tumour cell lines but not against normal cells [21]. Moreover, they have been evidenced as potent natural antioxidants [13,22]. In all these scientific publications, no evidence of any adverse effects of loquat leaf extracts has been indicated. On the contrary, liver protective effects of seed's extracts have been evidenced in animals [23]. As for safety of olive leaf extracts, it has been widely documented both in Europe [4, 12, 24-27] and in the Middle East [15] and their anti-atherogenic, antioxidant and antidiabetic properties have been evidenced both in Germany [12] and France [4].

The present study aimed at investigating the safety and efficacy of a fixed mixture of both olive and loquat leaves. Safety studies were carried out in animals and *in vitro* whereas therapeutic efficacy was evaluated in human volunteers. The study was undertaken in accord with legal and ethical requirements as well as current scientific standards as indicated by European Good Clinical Practice Guidelines and the declaration of Helsinki.

MATERIAL AND METHODS

Preparation of Cholevel: The leaves of loquat and olive were collected from the Galilee region, dried under shade and powdered to a fine grade as extracts by Antaki Ltd.-laboratories, Kfar Cana, Israel. Cholevel (310 mg/tablet) were prepared at Karmat Micro Encapsulation laboratories, Kibbutz Ramot Menashe, Israel. Each tablet contained 98mg loquat, 56mg olive, 7 mg Vitamin C, 2 mg Vitamin E, and 147 mg Tricalcium phosphate (TCP).

Safety Studies

Cell Culture: The effects of Cholevel on cell viability was assessed in a cell culture system using cells from the mouse fibroblast cell line (3T3), the human hepatoplastoma cell line HepG2, and from the human monocyte cell line THP1. HepG2 cell line retains differentiated parenchymal functions of normal hepatocytes, including the expression of P450 isoenzymes, thus permitting long-term studies to be performed. The cells from both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with a high glucose content (4.5 g/L) supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 µg/mL streptomycin. Cells were maintained in humidified atmosphere with 5% CO_2 at 37°C. The medium of cells from both cell lines was changed twice a week. At 70-80% confluence, cells were trypsinized and seeded in 96-well plates in cell density of 1.5 x 10^4 HepG2 cells and 5 x 10^3 THP1 cells. Twenty four hours after cell seeding cells were exposed to various concentrations of the plant extracts in fresh serum-free medium.

MTT Assay: The tetrazolium dye, MTT, is widely used to assess the viability and/or the metabolic state of the cells [28,29]. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the red formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Twenty four hours after cell seeding, cells were incubated with varying concentrations Cholevel for 24 hours at 37°C. Following the removal of the media from each well, cells were washed in phosphate buffered saline. The cells were then incubated in serum free DMEM to which MTT (0.5 mg/mL) was added to each well (100 µL), and incubated for a further four hours. Then the medium was removed and the cells were incubated for 15 minutes with 100µL of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 570 nm in an ELISA-reader. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Lactate Dehydrogenase: In the Lactate dehydrogenase (LDH) assay the leakage of the cytoplasm located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium is indicative of cell membrane damage [28,29].

For the LDH assay, 1.5 x 10^4 HepG2 cells and 5 x 10^3 THP1 were seeded per well of 96-microtiter plates. Twenty-four hour after cell seeding, cells were exposed to varying concentrations of the Cholevel. After 24h of treatment, the supernatants were collected from each well. Cell monolayers were then treated with a cell lysis solution for 30 minutes at room temperature to lyse. The cells and the lysate were collected. LDH activity was measured in both the supernatants and the cell lysate fractions by using CytoTox 96 a nonradioactive cytotoxicity assay kit (Promega, WI, USA) in accordance with the manufacturer's instruction. The intensity of the color is proportional to LDH activity. The absorbance is determined at 490 nm with 96-well plate ELISA reader. The percent of LDH release from the cells was determined using the formula: LDH release = (Absorbance of the supernatant)/ (absorbance of the supernatant and cell lysate)*100.

Determination of LD_{50}

Thirty-six male Sprague Dawley rats (average weight: 148 ± 16g) were divided into 4 groups. Large single doses of Cholevel were placed directly into the stomach of each group and observed for 14 days to determine the LD_{50}. Animal study approval was given from the Faculty of medical school - Technion - Haifa in 1999.

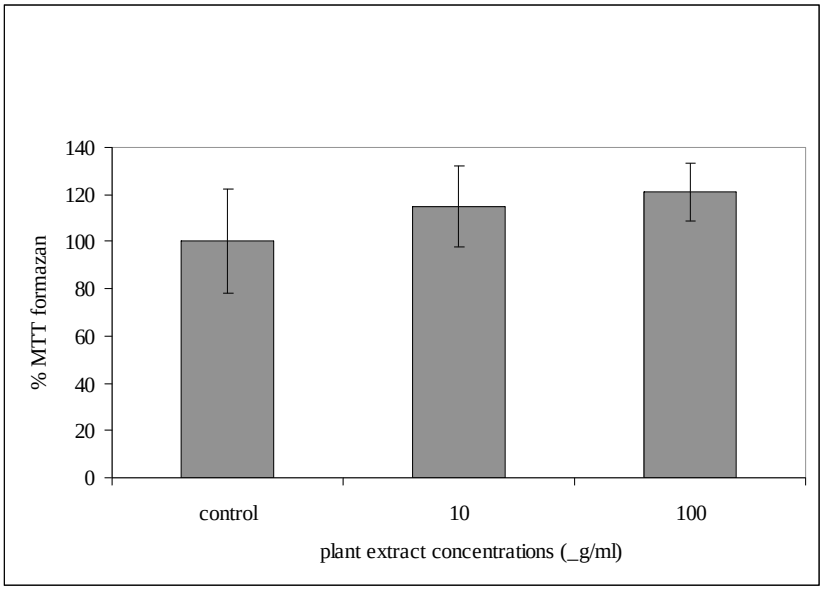


Fig. (1). MTT Assay in co-culture of HepG2 & THP1 cells after an overnight incubation with 10 μg and 100 μg Cholevel/mL. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells. Values given represent the mean ± standard deviations of three independent experiments carried out in triplicates.

Oxidative Stress

Rat liver homogenates and human blood LDL fractions were used to assess antioxidant effects of Cholevel.

Oxidative stress leads to generation of reactive oxygen species (ROS) which play an important pathogenetic role in different disease-states. Lipid peroxidation has damaging effects on liver cell membrane. The extent of lipid peroxidation was measured using a technique based on the thiobarbituric acid reactive substances (TBARS) assay that detects malondialdehyde (MDA), an end product of peroxidative decomposition of polyionic fatty acids in *in-vitro* systems. To accurately quantify TBARS in the analytical procedure, the protein was precipitated before the addition of thiobarbituric acid to the reaction, while the antioxidant butylated hydroxytoluene was added before the heating of samples.

Rat Liver Homogenates: Rat liver homogenates were incubated with 100 μM $FeSO_4$ as ROS generating system [30] and with various concentrations of the product.

Human Blood LDL Oxidation: Blood samples were collected, into vacutainer tubes containing EDTA (1 g/L blood), from normocholesterolemic adult volunteers, and centrifuged at 4°C (833 x *g* for 10 min) to isolate plasma. The LDL fraction was isolated by ultracentrifugation through a KBr discontinuous gradient and collected as the fraction floating at a density of 1.019–1.063 kg/L (1, 2). A rapid filtration through disposable desalting columns was needed to remove the EDTA, and LDL protein was measured by the method of Lowry *et al.*, [31] on the same day using bovine serum albumin as the standard. Filtered LDL was immediately used for oxidation experiments.

Clinical Investigations

Selection of Volunteers and Clinical Protocol: Human volunteers were selected on the basis of routine visits to their general physician in five clinics in Galilee if they were motivated to take herbal supplements to maintain a healthy fat level in the blood. Forty one human volunteers were chosen carefully on the base of close hyperlipidemaia values and divided into three groups

Group 1: Included 12 volunteers who were at a fixed dose of statins therapy but without therapeutically efficiency. Volunteers continued their usual diet and medications unchanged and consumed Cholevel tablets 1 x 3 daily for 3 months.

Group 2: Included 20 volunteers who consumed only Cholevel tablets 1 x 3 daily for 3 months.

Group 3: Included 9 volunteers who consumed placebo tablets 1 x 3 daily for 3 months. This group served as control.

After a thorough review of Cholevel-components, they were asked to continue their daily activities and food habits unchanged. An informed consent was obtained from each subject who was given a free of charge box containing 90 tablets of Cholevel.

At baseline, fasting cholesterol levels were estimated for every subject who was scheduled for the next visit on 4 weeks and was asked to return the Cholevel box so that returned tablets could be counted as the only measure of patient compliance with the protocol. This was repeated during each of the 3 consequent visits when fasting cholesterol levels were estimated and careful investigations of well being and of any adverse effect were undertaken.

Statistics

The Wilcoxon signed-rank test was used. Comparisons between groups were performed by the Wilcoxon rank-sum test. A 0.05 level of significance was set. Data obtained were expressed as mean ± standard error of mean (SEM).

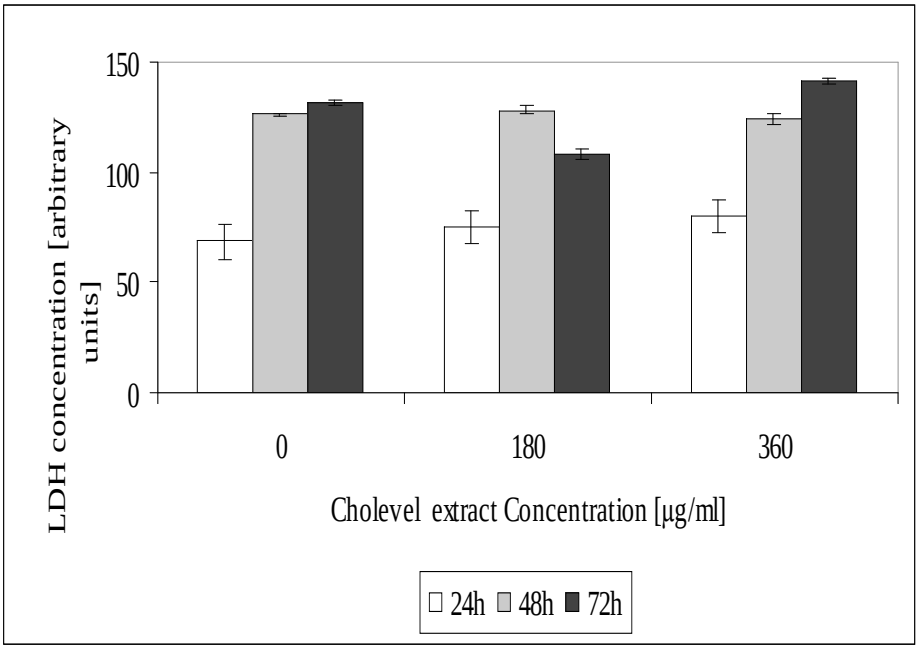


Fig. (2). LDH-release in arbitrary units from cultured human fibroblasts at baseline (0) and during incubating the fibroblasts with 180 and 360 µg Cholevel/ml for 24, 48, and 72 hours. Values given represent the mean ± standard deviations of three independent experiments carried out in triplicates.

RESULTS

Safety of Cholevel

LD50 in Rats: An extremely high dose of the Cholevel (17.3 g/kg) was necessary to obtain the LD_{50} value in rats.

In Vitro Cytotoxicity Assessments: MTT and LDH assays were carried out with fibroblast cell line (3T3) and co-cultures of human hepatoplastoma cell line HepG2 and cells from the human monocyte cell line THP1. HepG2 cell line retains differentiated parenchymal functions of normal hepatocytes, including the expression of P450 isoenzymes thus permitting long-term studies to be performed.

MTT Test: The metabolic activity can be evaluated by measuring the activity of a mitochondrial enzyme succinate dehydrogenase using the MTT test. This test is widely used in the *in vitro* evaluation of the toxicity of plant extracts. We applied the MTT test to evaluate the safety of Cholevel. Cells were exposed to 10 µg Cholevel/mL and 100 µg Cholevel/mL of culture medium for 24h. No sign of any negative effects were observed after treatment with the two concentrations (Fig. **1**).

LDH-Release Test: Membrane integrity can be evaluated by measuring lactate dehydrogenase activity. Lactate dehydrogenase, an enzyme located in the cytoplasm, catalyses the conversion of lactate and pyruvate. When lactate dehydrogenase is found within the media on the cells, there are two possible causes: The first is cellular death and the second is a 'leak' in a cell membrane. When cells are disrupted, the lactate dehydrogenase activity is elevated. Results obtained indicate no significant changes of the LDH levels in the culture medium after exposure to Cholevel at concentration up to 360 µg/ml (Fig. **2**).

Efficacy of Cholevel

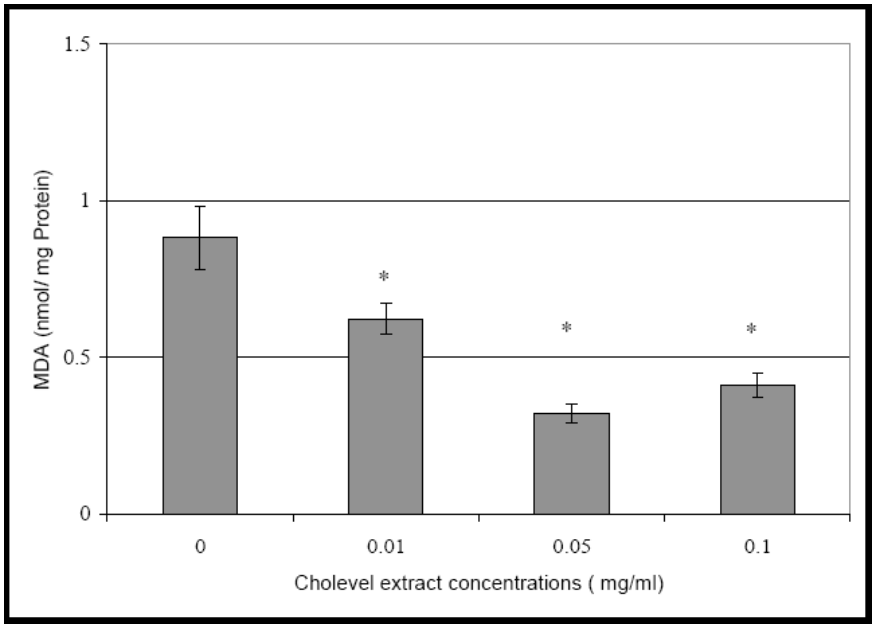
Lipid Peroxidation: Lipid peroxidation induced by incubating rat liver homogenates with ferrosulphate is expressed in Fig. (**3A**) as the extent of MDA production. The addition of very low dose of the Cholevel (10 µg/ml) to the medium significantly reduced MDA-release from 0.88 ±0.10 to 0.62 ± 0.05 nm/mg protein ($p < 0.01$). Higher concentrations of the Cholevel (50 µg/ml) further reduced MDA release to 0.32 ±0.03 nm/mg protein ($p < 0.001$). No further antioxidative effects were seen by increasing Cholevel concentration up to 100 µg/ml (Fig. **3A**).

LDL Oxidation: *In vitro* inhibition of human LDL oxidation by Cholevel in the presence of ferrosulphate is expressed in Fig. (**3B**) as the extent of MDA formation. Cholevel at low concentrations of 50, 100 and 400 µg/ml significantly inhibited the LDL oxidation and reduced the levels of MDA from 21 ± 3 to 15 ± 2, 12 ± 1.7 and 8 ± 1.5 nmole/mg protein, respectively.

Clinical Studies: Forty one human volunteers were recruited into the study, 12 of them were already on a fixed dose of statins therapy. All 41 subjects were evaluated for efficacy and tolerability during all scheduled visits as patient compliance with the protocol was excellent. Cholevel was well tolerated by all 32 subjects (groups 1+2) and no minor or major adverse effect was noted by any subject. They all tolerated Cholevel with other medications they were on especially those who were on statins therapy (group 1). In two of the 32 subjects, cholesterol levels were not elevated at baseline and did not substantially change during the 3 months of Cholevel consumption.

Group1 (Cholevel + Statins): the cholesterol levels were significantly reduced within the first month of Cholevel con-

(a)



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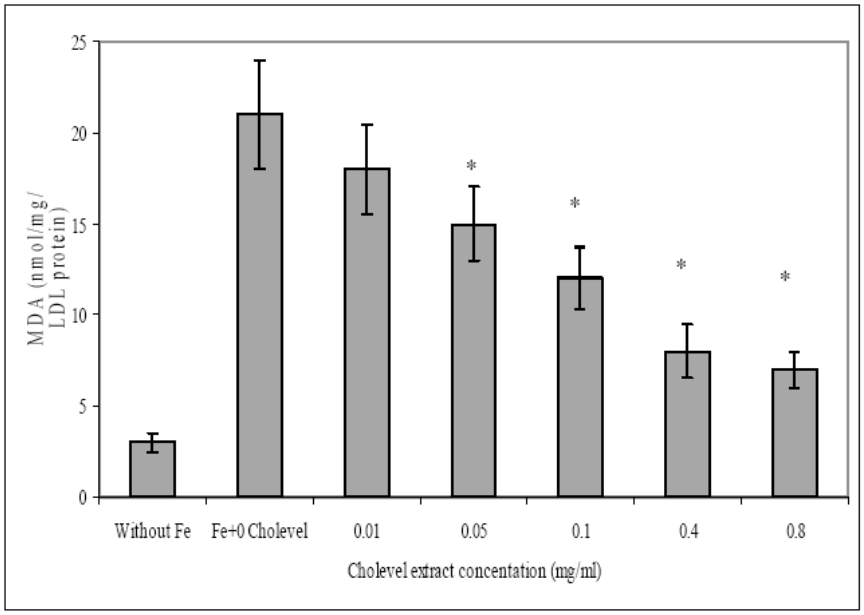

Fig. (3). Effects of different concentrations (10, 50, and 100 μg/ml) of Cholevel upon malondialdehyde (MDA) release that reflects the extent of lipid peroxidation in rat liver cells incubated with 100 μM ferrosulphate (**A**) and *in vitro* inhibition of human LDL oxidation by Cholevel in the presence of ferrosulphate (**B**). Values given represent the mean ± standard deviations of three independent experiments carried out in triplicates.

sumption by 13% and by 18.3% and 24.2% in the two other months of treatment respectively (Table **1**). In group 1, cholesterol levels were 287.6 ± 17.9 mg% at baseline and decreased to 249.5 ± 13, 229.4 ± 14 and 217.7 ± 11 mg% at 1, 2 and3 months of Cholevel intake. These reductions are highly significant. As shown in Table **1**, LDL levels decreased from 169.83 ± 7.17 mg% at baseline to 136.5 ± 7.35 mg% at 3 months, whereas, HDL levels increased from 40.08 ± 7.03 mg% to 48.83 ± 5.56 mg% at 3 months. These changes were accompanied by significant reductions in the levels of triglycerides after the study 3 months from 258 ± 30.56 mg% at baseline to 206.42 ± 16.23 mg% at 3 months (Table **1** and Table **4**).

Group 2 (Cholevel Only): cholesterol levels were 282.2 ± 13.8 mg% at baseline and decreased to 268.3 ± 14, 254.6 ± 12.8 and 241.7 ± 10.6 mg% at 1, 2 and 3 months of Cholevel intake (Table **2**). As shown in Table **2**, LDL levels decreased from 175.05 ± 5.79 mg% at baseline to 149.25 ± 5.53 mg% at 3 months, whereas, HDL levels increased from 36.8 ± 5.33 mg% to 42.3 ± 5.43 mg% at 3 months. These changes were accompanied by significant reductions in the levels of triglycerides after the study 3 months from 272.4 ± 28.5

Table 1 (Group 1). Fasting Lipid Levels in the 12 Subjects Using Statin Medications at Baseline and During Each of the 3 Months Study While Consuming One Cholevel Tablet Three Times Daily. Values Given Represent the Mean in mg% ± Standard Deviations

Volunteers (n=12)	Before Treatment	After 1 Month	After 2 Months	After 3 Months	Improvements (%)
Cholesterol (mg %)	287 ± 17.9 p value=	249.5 ±13.0 0.005	229.4 ±14.0 0.0004	217.7 ± 11.0 0.0001	24.2
LDL (mg %)	169.83 ±7.17 p value=	153.25 ±6.80 0.05	143.33 ±6.39 0.004	136.50 ±7.35 0.0005	20
HDL (mg %)	40.08 ±7.03 p value=	44.67 ±4.92 -	46.50 ±5.02 -	48.83 ±5.56 0.04	26.1
Triglycerides (mg %)	258 ±30.56 p value=	224.08 ±18.06 0.002	213.5 ±17.34 0.0004	206.42 ±16.23 0.0001	18.8

Table 2 (group 2). Fasting Lipid Levels in the 20 Subjects at Baseline and During Each of the 3 Months Study While Consuming One Cholevel Tablet Three Times Daily. Values Given Represent the Mean in mg% ± Standard Deviations

Volunteers (n=20)	Before Treatment	After 1 Month	After 2 Months	After 3 Months	Improvements (%)
Cholesterol (mg %)	282.2 ±13.8 p value=	268.3 ± 14.0 0.08	254.6 ± 12.8 0.01	241.7 ±10.6 0.005	14.3
LDL (mg %)	175.05 ± 5.79 p value=	167.10 ±6.08 0.08	157.20 ±6.19 0.01	149.25 ±5.53 0.02	15
HDL (mg %)	36.80 ±5.33 p value=	38.50 ±5.32 0.08	40.45 ±5.38 0.08	42.30 ±5.43 0.02	15.3
Triglycerides (mg %)	272.40 ±28.50 p value=	259.20 ±26.25 0.08	246.35 ±23.63 0.08	235.95 ±21.91 0.005	13

Table 3 (group 3). Fasting Lipid Levels in the 9 Control Subjects at Baseline and During Each of the 3 Months Study While Consuming One Placebo Tablet Three Times Daily. Values Given Represent the Mean in mg% ± Standard Deviations

Volunteers (n=9)	Before Treatment	After 1 Month	After 2 Months	After 3 Months	Improvements (%)
Cholesterol (mg %)	267.4 ±23.4 p value=	264.6 ±22.2 -	264.6 ±19.3 -	261.3 ±21.7 -	2.2%
LDL (mg %)	167.3 ±14.9 p value=	165.0 ±13.1 -	164.7 ±15.0 -	165.7 ±13.5 -	1%
HDL (mg %)	38.1 ±2.7 p value=	39.3 ± 3.6 -	40.8 ±2.8 -	39.3 ±2.3 -	3.3%
Triglycerides (mg %)	274.6 ± 17.5 p value=	273.1 ±12.5 -	272.4 ± 15.3 -	270.7 ±14.0 -	1.3%

mg% at baseline to 235.95 ± 21.91 mg% at 3 months (Table **2** and Table **4**).

Group 3 (Control): No significant changes were observed in the control group (Table **3** and Table **4**).

Table 4. Fasting Lipid Levels after 3 Months Study While Consuming in the Three Groups

	Improvements (%)			
Treatments	**Cholesterol**	**LDL**	**HDL**	**Triglycerides**
Cholevel + statins	24 ± 3 *	20 ± 4 *	26 ± 31 *	18.8 ± 12 *
Cholevel	14.3 ± 2 *	15 ± 2 *	15 ± 4 *	13 ± 2 *
Control	2.2 ± 3	1 ± 4	3.3 ± 3	1.3 ± 3

*Significant result.

DISCUSSION

The results disclose that Cholevel is safe and well tolerated by all 32 studied subjects and is therapeutically efficient as substantial and incremental reductions of cholesterol levels were observed during each of the study 3 months. At 3 months, baseline cholesterol levels in group 1 (Cholevel + statins) subjects were reduced by 24% which is comparable to that of 25% observed during simvastatin therapy [5]. Volunteers who consumed Cholevel without statins, (group 2) showed significant reduction in cholesterol levels (14.3%) after three months of the clinical test. Combination therapy of Cholevel with statins is efficient and can be helpful to those hyperlipidamics who don't fully respond to statin medications.

A high level of safety of Cholevel was also disclosed with very high concentrations of 17.3 g/kg to yield the LD50. Concentrations as high as 360 ug/ml did not show any sign of cellular toxicity as evidenced by LDH-release. Extracts of loquat leaves were completely atoxic for normal cells [13] as were extracts of olive leaves at doses as high as 1200 mg/kg for 60 days in animals [23].

Nevertheless, the antioxidant properties of Cholevel were evidenced at concentrations as low as 10 μg/ml and were more significant at concentrations of 50– 0.4 μg/ml. Higher concentrations did not substantially add to such properties of Cholevel. Moreover, a low concentration of 0.1 mg/ml did increase the bioviability of the co-cultured human hepatocytes and monocytes. Extracts of loquat leaves have been evidenced as the natural antioxidants superior to other tested antioxidant herbs [13,22]. Antioxidant properties of olive leaf extracts have been widely documented [4,12,24] and such antioxidant properties of both herbs contribute to the reported hypoglycaemic effects of loquat [16,17,19] and olive leaves [4,12,32].

We propose that the loquat component of Cholevel is in accord with recommendations in traditional Arabic herbalism [15,33] and has primarily a statins-like effect that reduces cholesterol production in the liver. The olive component of Cholevel seems to have primarily a Zetia-like effect that reduces cholesterol intestinal absorption. The main active ingredient in olive leaf was reported to be oleuropeoside which disclosed distinct hypolipidemic, hypotensive and hypoglycaemic properties at a dose of 16 mg/kg [4,12,32,34]. As experienced in good clinical practice, smaller doses of synergistic drugs may yield a better therapeutic efficacy with least side effects. This could explain the fact that Cholevel was well tolerated by all studied subjects and no adverse effect could be traced.

Taken together, results obtained indicate safety and hypolipidemic properties of Cholevel, consisting of extracts from *Eriobotrya japonica* and *Olea europaea*, traditionally used in the Greco-Arab medicine [9, 9,11,35,36].

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