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RESEARCH ARTICLE

Quantification of Bioactive Compounds in Coffea Arabica and Their Impact with Omega-3 Supplementation on Non-High-Density Lipoprotein Levels in Hyperlipidemia-Induced Rats

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ABSTRACT

This study aims to evaluate the levels of coffee lipids, diterpenes, chlorogenic acid (CGA), and caffeine in coffee samples with different roasting levels. Additionally, it aims to assess the impact of coffee extracts with/without omega-3 supplementation on lipid profile parameters. Liquid-liquid and soxhlet extraction, and quantitation were performed using HPLC-DAD. Two groups of Wistar rats were used: non-hyperlipidemic and hyperlipidemic. Blood samples were collected before and after hyperlipidemia induction after six weeks. The lipid profile: total cholesterol (TC), low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride (TG), non-high-density lipoprotein (non-HDL), and total cholesterol-to-high-density lipoprotein ratio (TC/HDL) were determined. A positive correlation between coffee lipids, diterpenes, and caffeine levels with the coffee's roasting degree was found, and a negative correlation for CGA. The study was conducted on rats fed with a healthy and hyperlipidemia-induced diet. Green coffee had a significant effect on the lipid profile of non-hyperlipidemic rats. It lowered the levels of TG, LDL, and TC/HDL, and raised HDL and non-HDL levels significantly compared with the control group. In combination, green coffee and omega-3 lowered TC, TG, LDL, non-HDL, and TC/HDL levels. Green coffee alone or in combination with (omega-3) lowered lipid profile parameters but increased non-HDL levels in non- and hyperlipidemic induced-rats.

Keywords: Caffeine, Chlorogenic acid, Coffee, Diterpenes, Lipid profile, Omega-3

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Introduction

Coffee is one of the most well-known beverages and traded products all over the world because of its physicochemical and sensory characteristics, health benefits, and physiological properties.[1,2] The worldwide production of *Coffea arabica* corresponds to 70–80%. There has been attention on investigating the possible impact of coffee's bioactive compounds on human health due to the increased intake around the world. Coffee contains a variety of substances including lipids, diterpenes, caffeine, and phenolic chemicals such as chlorogenic acid (CGA).[1] The coffee oil (coffee lipids) content ranges from 7–17%, and its' major components are triglycerides (TG) which account for 75%, followed by diterpenes (20%), and sterols (2–5%).[3] However, these values vary between non-roasted and roasted coffee beans, which denotes the roasting conditions affect the concentrations of the target compounds and the quality of coffee.[4]

The isolation and quantification of coffee oils and diterpenes, as well as the evaluation of their health impacts, have acquired a lot of attention because of their huge favorable health effects, which include anti-carcinogenic, anti-oxidant, and anti-inflammatory effects.[5] Caffeine and CGA can be isolated from the coffee beans using several techniques including hot water and organic solvent extraction. The use of water-based solvent extraction is a highly effective and popular method.[4] Numerous analytical methods, including HPLC, have been developed and used for the quantification of caffeine and CGA in coffee.[4]

It has been shown that the degree of roasting has a significant impact on the quantities of bioactive chemicals in coffee. Published studies displayed a variation in their results, with a positive, negative, and/or continuous association between roasting degree and levels of coffee bioactive components.[6,7] The variety and the complex number of molecules found in coffee, as well as their properties and health impacts, prompted the research team to investigate these rich and distinct components. A thorough investigation of the potential health effects of the selected coffee bioactive components has revealed contradictory results, notably in terms of their effect on hyperlipidemia and lipid profile markers.[8,9]

Drinking a lot of coffee, thus, consuming a lot of coffee lipids might be linked to undesired health consequences, such as dyslipidemia. It was shown that coffee consumption between 2.4–8 cups/day raised the TC, LDL, and TG concentrations after 2–11 weeks.[10] On the other hand, it has been reported that coffee decreased the blood TG levels in subjects with high cholesterol levels after 8 weeks.[8] Therefore, based on the previous studies, the impact of coffee consumption on certain lipid profile parameters, particularly non-high-density lipoprotein cholesterol (non-HDL), is yet not completely clarified. Thus, the non-HDL can be considered a valid predictor to clarify the inconsistency of the results concerning the effect of coffee consumption on the indicators of blood lipids. This inconsistency cannot be only guaranteed with this predictor, as it might be triggered by other factors that are involved in the hyperlipidemia progression.[11]

In this context, the omega-3 fatty acids (n-3FA's) are reported to have a crucial function in lipid management; they have a notable impact on plasma TG levels and raise HDL levels, but do not affect LDL or TC levels.[12–14] Coffee drinking and n-3FA supplementation have both been shown to cause significant changes in cardiovascular hemodynamics. Despite this, there is no evidence of a possible synergistic or antagonistic link between coffee consumption and n-3FA supplementation. It is crucial to understand how coffee roasting, consumption rate, and n-3FA supplementation dose affect the relationship. Moreover, it is believed that there is a relationship between the effects of coffee roasting degree in combination with n-3FA supplementation on non-HDL levels in hyperlipidemic models. The current study aims to quantify selected bioactive compounds in roasted and non-roasted *C. arabica* and evaluate their impact on the lipid profile of hyperlipidemia-induced rats when combined with n-3FA supplementation. Reportedly, no studies have investigated the extraction and quantification of coffee lipids, as well as the influence of roasting degree in combination with omega-3 (n-3FA) supplementation on lipid profile markers such as non-HDL. Hence, the current study was designed to determine the content of selected bioactive compounds in coffee, investigate the effect of roasting temperatures on their content, and investigate the potential link between coffee bioactive compounds and hyperlipidemia (total cholesterol TC, TG, LDL, high-density lipoprotein HDL, and non-HDL) in the absence or presence of n-3FA supplementation.

Materials and methods

The coffee samples were obtained from Al-Ameed Coffee Company. The caffeine standard with 99.8% purity was bought from AZ Chem Company (Ontario, Canada). The CGA standard with 98.0% purity was purchased from TCI Company (Tokyo, Japan). The atorvastatin, with a solubility of 0.127 mg/mL, was provided by JPM Company (Jordan). HPLC-grade water and methanol were purchased from Honey

Well/Riedel-de Haën™. Formic acid 96% was purchased from TEDIA Company INC., (USA). Acetonitrile was purchased from MACRON Fine Chemicals (USA). Methyl tert-butyl ether, was purchased from Poch S.A. (Poland). KOH was purchased from Gainland Chemical Company (USA). Diethyl ether was purchased from Oxford Lab Fine Chem LLP (India). Sodium sulfate was acquired from Sigma-Aldrich (Germany). The TG, cholesterol, and HDL liquicolor reagents were purchased from Human Gesellschaft company (Germany).

Plant materials

Coffee samples (green coffee, light-roast, medium-roast, and dark-roast coffee) were obtained from a local coffee company - Al-Ameed® Coffee Company (Amman, Jordan) in July 2021.

Ethical approval

All practices were conducted according to the international regulations for the care and usage of laboratory rats. The ethical approval on the study was obtained by the Institutional Review Board at Applied Science Private University, Amman, Jordan (IRB protocol: 2022-PHA-12).

Instrumentations and chromatographic conditions

All analyses were performed by using HPLC instrumentation (Hitachi Technologies - Tokyo, Japan). The Hitachi LaChrom HPLC system equipped with a pump, column oven, autosampler, PDA Detector (Tokyo-Japan), Supelcosil C-18 column (30 cm × 4 mm × 5 μm) SUPELCO, the flow rate was 1.0 mL/min. The analysis was performed at a wavelength of 273.5 nm for caffeine, and 330.0 nm for CGA. The system was organized using an organizer (L-2000), controlled by the software EZChrom Elite (Ver. 3.3.2, Agilent, CA, USA). The samples were injected and were carried out by HPLC system syringe filters (0.45 μm).

Humastar 200 biochemistry analyzer (Human company, Germany) was used for the analysis of the serum samples to measure HDL, TG, and TC. Humastar 200 is a fully automatic instrument, with dimensions 69×76×52 cm (W × D × H), frequency 50/60 Hz, voltage 220–240 or 110–120 Vac, temperature 16–30 ˚C, up to 200 t/h throughput, reaction volume 210–350 il, and the humidity <80% non-condensing. Humastar 200 has unique features including an open random-access analyzer, less than 1 L/hr water consumption, an 8-step wash station, reagent cooling, 30 reagents, 60 sample sites, an internal sample barcode reader, capacitive liquid level detector, needle shock detectors and large liquid containers with level sensors.

Preparation of mobile phase

The mobile phase was developed for the HPLC analysis of caffeine and CGA. For caffeine, the mobile phase consisted of water and methanol in the following ratio of 60:40% (v/v). The mobile phase was degassed by ultrasonic for one hour. While for CGA, the mobile phase consisted of 0.1% formic acid and acetonitrile in the following ratio 85:15% (v/v), then it was degassed by ultrasonic for one hour. [15]

Preparation of stock standard solutions

The caffeine stock solution was prepared by weighing 100.2 mg of caffeine standard, which corresponds to 100.0 mg of caffeine standard, and then dissolved in 100.0 mL of mobile phase to prepare a 1000.0 μg/mL concentration. The CGA stock solution was prepared by weighing 102 mg of CGA standard, which corresponds to 100.0 mg of CGA standard, and then dissolved in 100 mL of methanol to prepare a 1000.0 μg/mL concentration. [15]

Preparation of calibration curves solutions

The calibration curve solutions of caffeine and CGA were prepared in six concentrations: 10.0, 50.0, 100.0, 200.0, 500.0, and 1000.0 μg/mL by diluting standard solutions of caffeine with mobile phase and the CGA standard solutions with methanol.

Sample preparation, extraction, and quantitation

Extraction of coffee oils: Soxhlet extraction

The coffee oil levels were determined according to the Soxhlet extraction techniques reported by Echeverri-Giraldo et al. and Novaes et al. [16,17] The extraction procedure was developed, and a few modifications were made. Ten grams of roasted ground coffee were weighed and placed in a 500.0 mL soxhlet glass thimble; the extraction was performed using 150.0 mL of methyl tert-butyl ether as a solvent for 8 hours. After extraction, filtration was performed twice to remove any coffee traces. Afterward, the solvent was evaporated by a rotary evaporator at 55 ˚C. After solvent evaporation, coffee oil was obtained, and the weight of the coffee oil extract was gravimetrically determined.

Extraction of coffee diterpenes

The levels of total diterpenes were determined according to the extraction procedure reported by Novaes et al.[3] The procedure was developed in several steps and a few modifications were done. The coffee oil was saponified by using potassium hydroxide, which was prepared by dissolving 3.9 g of KOH in 35.0 mL of ethanol then it was added to the coffee oil and placed in the water bath for 4 hrs. Afterward, 50.0 mL of water and 50.0 mL of diethyl ether were added to the mixture resulting from the saponification step and transferred into a separatory funnel, then the solution was washed with 45.0 mL of water at three steps (15.0 mL was added at each step) with gentle shaking. The organic phase was dried with sodium sulfate. Subsequently, the organic phase was separated and then evaporated to dryness. Finally, the total diterpenes were weighed gravimetrically.

Extraction of caffeine and CGA

The coffee samples were extracted according to the Caracostea et al. method with slight modifications.[8] Four samples of C. arabica with different degrees of roasting were used. One gram of each type of coffee was weighed and then added to 100.0 mL of hot deionized water; the coffee solutions were placed in a water bath at 80 ˚C for 20.0 minutes and then ultrasonicated at 75.0 ˚C for 15 minutes to homogenize the coffee solutions. Afterward, the solutions were centrifuged for 15.0 minutes at a speed of 7900 × g. The filtration was performed for all solutions and coffee extracts were preserved at a temperature of –20 ˚C until the analysis. All extracts were filtered using a 0.45 μm porosity Nylon syringe filter before injection.

Study design

Seventy-eight male Wistar rats 180–200 g were individually maintained under standard housing conditions (room temperature 25.0˚C, and humidity 60–65% with (12:12) light-to-dark cycle at the animal house at the Applied Science Private University. Based on the diet model, animals were divided into 13 groups (6 rats per group) which were classified under two main groups: non-high fat diet (NHD), and high-fat diet (HD). The NHD rats were adopted to an adjustment phase of three weeks to a standard diet (100.0 g/kg per day) only. The HD rats were fed (lamb fat 30.0 g/kg + standard diet of 70.0 g/kg per day) to develop hyperlipidemia.[18] The HD model was confirmed by the measurement of a TC concentration greater than (55.0 mg/dL). Rats in both dietary models (NHD and HD) groups had unrestricted access to water. Rats that failed to develop the HD model were excluded from the study. Each group was divided into subgroups as follows:

A. NHD groups:

1. NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement.
2. NHDG: received green (non-roasted) coffee extract only.
3. NHDR: received medium-roasted (R) coffee extract only.
4. NHDΩ: received n-3FA (1 g/rat per day) supplement only.
5. NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω) supplement.
6. NHDRΩ: received medium-roasted coffee extract and n-3FA supplement.

B. HD groups:

1. HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement.
2. HDS: received atorvastatin (S) only.
3. HDG: received green (non-roasted) coffee extract only.
4. HDR: received medium-roasted coffee extract only.
5. HDΩ: received n-3FA supplement only.
6. HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement.
7. HDRΩ: received medium-roasted coffee extract and n-3FA supplement.

The NHD and HD rats were orally administrated via an intra-gastric tube with coffee extract alone or in combination with omega-3 supplements for three weeks. Different coffee extracts were administrated by oral gavage. In the current method, a bulb-tipped gavage needle made of stainless steel, or a supple cannula attached to a plastic syringe was used to provide the mixture into the stomach. Atorvastatin standard of 20.0 milligrams was dissolved in 100.0 mL of distilled water to achieve 0.5 mg/mL, then administered to HDS rats for three weeks. One gram of n-3FA (contains 120 mg of Docosahexaenoic Acid TG (DHA) + 180 mg of Eicosapentaenoic Acid TG (EPA) (Jamieson, Canada)) was given for both NHD as well as HD-omega-3 treated groups.

Coffee dose

Approximately, 6.0 g of coffee were dissolved in 120.0 mL of water and each rat was given a dose of 2.0 mL of the prepared solution (equivalent to a dose of 100.0 mg of coffee). This investigation

was regulated and conducted in a similar protocol with few modifications which has been published previously.[19]

Lipid profile analysis

Serum samples of lipid profile were assayed on day 1, day 21, and at the end (day 45) of the experiment. Blood samples (0.2 mL per animal) were collected into heparinized tubes by puncturing the retro-orbital plexus. The TC, LDL, TG, and HDL levels were quantified using enzymatic kits (Humastar, Germany). Non-HDL levels were calculated using Eq. (1):[20]

$$Non - HDL = TC - HDL \quad (1)$$

Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 27.0 (Chicago, IL, USA). One Way ANOVA test was used to investigate if there were any significant differences in the mean values for each parameter between the different groups of the experiment. The Post hoc (Duncan's test) was used to determine the significant differences among groups by analyzing multiple comparisons.

Results and discussion

Impact of degree of roasting on coffee oil and diterpenes levels

Several studies have investigated the extraction and quantification of coffee oils and diterpenes which have a great emphasis on the right method of extraction from *C. arabica*,[16] as well as the impact of roasting degree on its level, due to the seemingly indisputable health benefits of coffee oil in humans.[6,10] The quantitation of coffee oil and diterpenes was performed gravimetrically using several extraction techniques. The coffee oil was extracted based on the procedure that was described by Echeverri-Giraldo et al. with a few modifications,[16] while the diterpenes were extracted as described by Novaes et al.[3] In the present study, soxhlet extractions were performed on samples of coffee with different roasting degrees to study how roasting influences its levels. The methyl tert-butyl ether was used as a solvent for the extraction of coffee oil and diterpenes, which was accomplished with a soxhlet extraction procedure. The contents of coffee oil and diterpenes in coffee samples were extracted and determined using 10.0 g of ground coffee each time, Fig. 1. The highest amount of coffee oil was found in the medium roasted while the lowest amount was found in green coffee. For diterpenes,

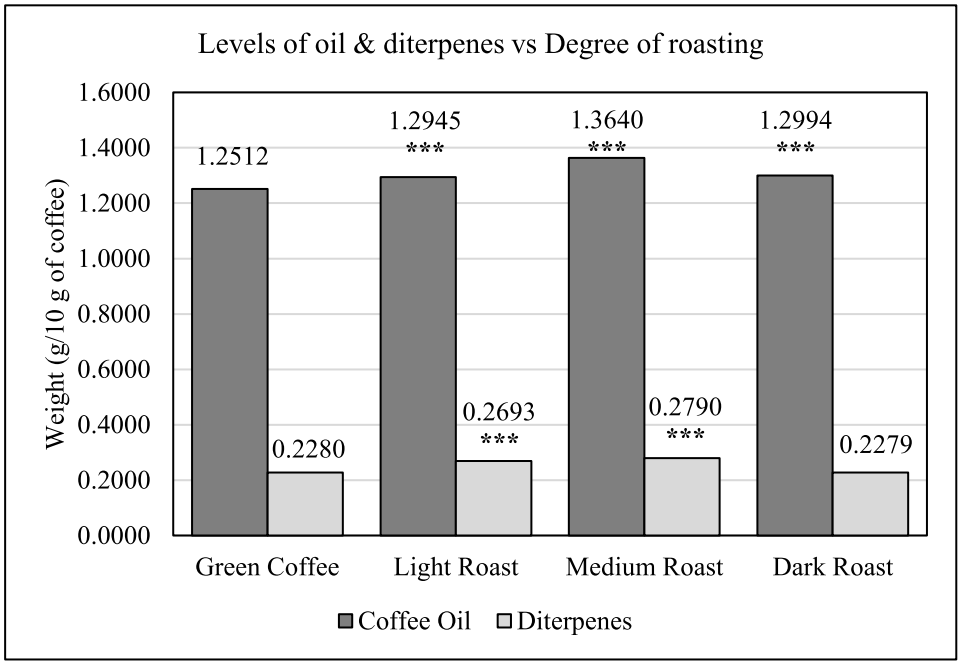

Fig. 1. The contents of coffee oil and diterpenes in green and roasted coffee (in grams).
Note: The results are represented as mean (n = 6 for each group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with the green coffee group (Duncan's Multiple Comparisons Test).

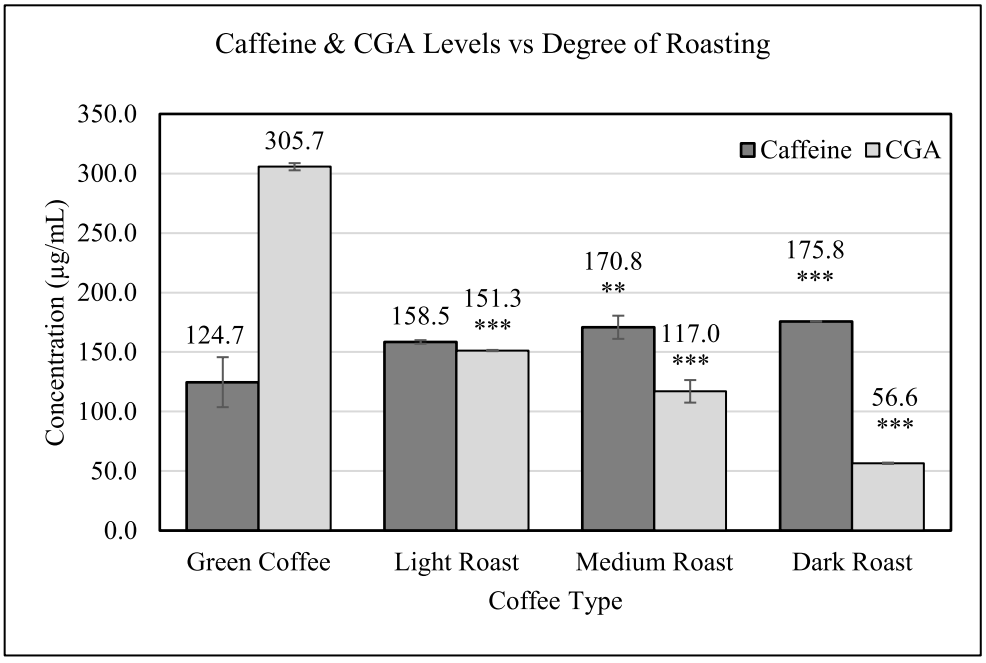

Fig. 2. The caffeine and CGA concentrations in green and roasted coffee.
Note: The results are represented as mean (n = 6 for each group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with the green coffee group (Duncan's Multiple Comparisons Test).

the highest amount was found in the medium roasted while the lowest amount was found in the green coffee.

The results indicated that as the roasting degree increases the content of coffee oil increases till it reaches the dark roasted coffee where it declines, Fig. 1. Furthermore, the content of diterpenes showed a similar relation as a function of the roasting degree, then there was an observed reduction in the content of diterpenes. As the temperature of roasting increases, the levels of diterpenes increases. The green coffee contained 0.228 g of total diterpenes, then in light-roasted coffee it went up to 0.2693 g, and reaching a maximum of 0.279 g in medium-roasted coffee before it falls down to 0.2279 g in the dark-roasted coffee due to the exposure to harsh and high temperatures where it starts to partially degrade and break down. In comparison with a previous study, the levels of diterpenes, in terms of diterpenes' mass per coffee mass or diterpenes' mass per coffee oil mass, were lower than the results reported by Novaes et al.[3] The roasted coffee groups showed significant higher mean coffee oil contents than in the green coffee group ($P < 0.001$). While for the dark-roasted coffee, significant higher mean diterpenes contents were observed in the light and medium roasted coffee groups ($P < 0.001$) as shown in Fig. 1.

Effect of degree of roasting on caffeine and CGA levels

The HPLC was used to measure the caffeine and CGA levels in various coffee samples following hot water extraction. The concentration is based on the results from an HPLC instrument as well as their average concentrations. The HPLC chromatograms for caffeine and CGA standard solutions are displayed in the supplementary files: Supplementary S1 and Supplementary S2. The caffeine and CGA concentrations (expressed as triplicates) in the coffee samples with different levels of roasting are illustrated in Fig. 2.

The highest caffeine concentration was observed in dark roasted coffee (175.79 ± 0.257 μg/mL) while the lowest caffeine concentration was observed in green coffee (124.70 ± 20.99 μg/mL). However, the results indicated that caffeine concentration increases as the roasting degree progresses to increase as seen in. Fig. 2. The highest CGA concentration was observed in green coffee (305.69 ± 3.02 μg/mL) while the lowest concentration was observed in dark roasted coffee (56.56 ± 0.56 μg/mL), this indicates an inverse relationship between the roasting degree and the CGA concentration; where the roasting degree increases, the CGA concentration decreases. The medium-roasted coffee group showed a significant higher mean caffeine content than in the green

Table 1. Baseline means and ranges of the biochemical parameters and body weight for NHD and HD (after 2 weeks of high fat-diet) study groups.

Group	Item	N	Minimum	Maximum	Mean	SD
NHD	BW (g)	36	160.00	200.00	178.33	10.30
	TC (mg/dl)	36	36.00	53.00	44.83	6.48
	TG (mg/dl)	36	86.00	145.00	112.50	23.76
	HDL (mg/dl)	36	18.80	28.30	23.47	3.40
	LDL (mg/dl)	36	24.00	57.10	37.57	13.18
	non-HDL (mg/dl)	36	17.20	24.90	21.37	3.17
	TC/HDL ratio	36	1.80	2.00	1.90	0.06
HD	BW (g)	42	140.00	250.00	198.33	14.33
	TC (mg/dl)	42	35.00	61.00	59.16	6.20
	TG (mg/dl)	42	69.00	146.00	114.83	17.60
	HDL (mg/dl)	42	22.50	36.80	27.30	4.35
	LDL (mg/dl)	42	34.3	60.7	47.30	9.90
	non-HDL(mg/dl)	42	20.8	48.6	31.80	8.90
	TC/HDL ratio	42	1.68	3.18	2.24	0.54

NHD: non-hyperlipidemic control groups, HD: Fat diet induced hyperlipidemic groups, BW: body weight, TC: total cholesterol, TG: triglycerides, HDL: high-density lipoprotein –cholesterol, LDL: low-density lipoprotein –cholesterol, Non-HDL: non-high-density lipoprotein – cholesterol, TC/HDL ratio: total cholesterol to high-density lipoprotein –cholesterol ratio, N: sample size, SD: standard deviation.

coffee group ($P < 0.01$). While the dark-roasted coffee showed a significant higher mean caffeine content than in the green coffee group ($P < 0.001$). With regard to the CGA levels, the roasted coffee groups showed significant lower mean CGA contents than in the green coffee group ($P < 0.001$).

Biological experimental part

The baseline characteristics of the standard diet-fed rats (NHD) and high-fat diet-fed (HD) rats are illustrated in Table 1.

The difference in mean body weight (BW) changes between NHD and HD study groups at the end of the experiment

No significant differences in the means BW were observed in all NHD or HD study groups at the end of the study ($P \geq 0.005$) as illustrated in Table 2.

The mean differences in serum levels of lipid profile parameters in NHD and HD study groups at baseline and the end of the study

Total cholesterol (TC)

According to ANOVA results, significant mean differences in TC levels in the NHD study groups were noted ($P = 0.009$) at the end of the experiment as presented in Table 3. The Post-hoc multiple comparisons by Duncan's test indicated that mean TC levels for the NHDR group were significantly different from

Table 2. The difference in mean body weight changes between groups within the two sections (standard and fat-induced diet) at the end of the experiment (n = 6 per group).

Body weight (g)					
	NHD			HD	
Group	Mean	± SD	Group	Mean	± SD
NHD-C	223.33	16.33	HD-C	232.30	14.90
NHDG	228.33	8.16	HDS	229.17	42.71
NHDR	210.00	17.60	HDG	236.67	22.51
NHDΩ	224.17	8.01	HDR	229.17	20.10
NHDGΩ	215.83	13.57	HDΩ	221.67	22.51
NHDRΩ	229.17	11.58	HDGΩ	238.33	24.22
			HDRΩ	240.00	22.36
P	0.113		*P*	0.821	

NHD: Non-high fat diet, HD: high-fat diet, NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement, NHDG: received green (non-roasted) coffee extract only, NHDR: received medium-roasted coffee extract only, NHDΩ: received n-3FA (1 g/rat per day) supplement only, NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω)supplement, NHDRΩ: received medium-roasted coffee extract and n-3FA supplement, HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement, HDS: received atorvastatin (S) only, HDG: received green (non-roasted) coffee extract only, HDR: received medium-roasted coffee extract only, HDΩ: received n-3FA supplement only, HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement, HDRΩ: received medium-roasted coffee extract and n-3FA supplement, P: value for ANOVA test at the end of the experiment, SD: standard deviation.

other study groups, Table 3. In the HD group, significant differences in the mean TC levels were observed ($P = 0.007$). The post-hoc multiple comparisons by Duncan's test indicated that the mean TC levels for the (HDRΩ) group were significantly different from other study groups.

Triglycerides (TG)

Table 4 indicates that a significant difference in the mean TG levels between NHD groups was noted ($F = 8.918$, $P < 0.001$), as well as between HD groups ($F = 9.98$, $P < 0.001$) at the end of the experiment. Among the NHD groups, post-hoc multiple comparisons by Duncan's test indicated that the mean TG levels for control and NHDR groups were higher than other groups. The lowest mean level was found in NHDΩ and NHDG-treated groups (49.00 ± 19.03; 60.33 ± 7.30, respectively). In HD groups, the post-hoc multiple comparisons by Duncan's test indicated that the mean TG levels for HDΩ were significantly lower than other groups while the mean levels were the highest in HDR.

High density lipoprotein (HDL)

There was no significant difference in the mean HDL levels between HD groups ($P = 0.583$), while

Table 3. Effect of roasted, green coffee extracts, and their combination with omega-3 supplements on total cholesterol levels in experimental rats.

Total Cholesterol (mg/dl)					
	NHD			HD	
Group	Mean	± SD	Group	Mean	± SD
NHD-C	40.33*2	4.27	HD-C	58.00*3	6.90
NHDG	53.67*3	12.09	HDS	47.50*2	8.60
NHDR	47.83*3	10.40	HDG	55.00*2,*3	9.80
NHDΩ	39.00*2	5.66	HDR	48.83*2	10.44
NHDGΩ	37.00*1	5.80	HDΩ	48.17*2	5.19
NHDRΩ	44.00*2	5.73	HDGΩ	44.50*1	5.47
			HDRΩ	41.83*1	6.79
P	0.009		*P*	0.007	

NHD: Non-high fat diet, HD: high-fat diet, NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement, NHDG: received green (non-roasted) coffee extract only, NHDR: received medium-roasted coffee extract only, NHDΩ: received n-3FA (1 g/rat per day) supplement only, NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω)supplement, NHDRΩ: received medium-roasted coffee extract and n-3FA supplement, HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement, HDS: received atorvastatin (S) only, HDG: received green (non-roasted) coffee extract only, HDR: received medium-roasted coffee extract only, HDΩ: received n-3FA supplement only, HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement, HDRΩ: received medium-roasted coffee extract and n-3FA supplement, *P*: value for ANOVA test at the end of the experiment, *1,*2,*3 Subset for alpha =0.05 detected by Duncan's test of post-hoc multiple comparisons for determining the differences of TC levels among different NHD groups at the end of the experiment, SD: standard deviation.

Table 4. The difference in mean TG change between NHD and HD study groups at the end of the experiment (n = 6 per group).

Triglycerides (mg/dl)					
	NHD			HD	
Group	Mean	± SD	Group	Mean	± SD
NHD-C	129.17*3	36.76	HD-C	114.80*3	19.20
NHDG	60.33*1	7.30	HDS*1	78.10*1	8.15
NHDR	113.20*3	34.70	HDG*2	118.10*3	13.16
NHDΩ	49.00*1	19.03	HDR	129.33*3	37.76
NHDGΩ	87.00*2	12.57	HDΩ *1	72.60*1	7.40
NHDRΩ	91.00*2	15.05	HDGΩ *1	78.00*1	15.82
			HDRΩ*1	83.60*2	15.81
F	8.918		F	9.98	
P	<0.001		*P*	<0.001	

NHD: Non-high fat diet, HD: high-fat diet, NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement, NHDG: received green (non-roasted) coffee extract only, NHDR: received medium-roasted coffee extract only, NHDΩ: received n-3FA (1 g/rat per day) supplement only, NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω)supplement, NHDRΩ: received medium-roasted coffee extract and n-3FA supplement, HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement, HDS: received atorvastatin (S) only, HDG: received green (non-roasted) coffee extract only, HDR: received medium-roasted coffee extract only, HDΩ: received n-3FA supplement only, HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement, HDRΩ: received medium-roasted coffee extract and n-3FA supplement, F: variation between sample means/variation within the samples, *P*: value for ANOVA test at the end of the experiment, *1,*2,*3 Subset for alpha =0.05 detected by Duncan's test of post-hoc multiple comparisons for determining the differences of TG levels among different NHD groups at the end of the experiment, SD: standard deviation.

there was a significant difference among NHD groups (F = 2.71, $P = 0.0387$). The post-hoc multiple comparisons by Duncan's test indicated that the mean HDL was significantly lower in the NHDΩ group (22.13 ± 3.86), while the highest mean was for the NHDG group (30.05 ± 6.21) as indicated in Table 5.

Low density lipoprotein (LDL)

Based on the results of ANOVA, the mean LDL levels were significantly different among both NHD and HD study groups (Table 6). Among NHD groups, there were significant differences in the mean LDL levels (F = 7.435, $P < 0.001$) at the end of the experiment. The post-hoc multiple comparisons by Duncan's test indicated that the mean LDL levels for the HDR group were significantly different from other groups.

Notably, the mean LDL levels were lower in HDΩ, HDGΩ, and HDRΩ groups than in other groups. Concerning the HD group, there was also a significant difference in the mean LDL levels (F = 13.08, $P < 0.001$) at the end of the experiment. Duncan's test indicated that the mean LDL levels for group control and HDR were significantly different Table 6.

Non–high density lipoprotein (non-HDL)

There was a significant difference in the mean non-HDL levels (F = 11.94, $P < 0.001$) at the end of the experiment among the NHD groups, Table 7. The post-hoc multiple comparisons by Duncan's test indicated that the mean levels of non-HDL for NHDGΩ and NHDRΩ treated groups were significantly different from other groups particularly the NHDG and the NHDR groups as shown in Fig. 3A. Similar results were observed at the end of the experiment between HD with a significant difference in mean non-HDL levels (F = 13.81, $P < 0.001$). Duncan's test indicated that the mean levels of non-HDL for group HDGΩ and HDRΩ treated groups were significantly different from other groups, particularly the HDG group Fig. 3B.

At the end of the experiment and among the normal groups, the NHDGΩ treated group showed a significant lower mean non-HDL level than in the NHDG group ($P < 0.05$). While among the hyper groups, the HDGΩ group ($P < 0.01$) and HDRΩ group ($P < 0.001$) showed significant lower mean non-HDL levels than in the HDG group.

Table 5. The difference in mean HDL-C change between NHD and HD study groups at the end of the experiment (n = 6 per group).

HDL (mg/dl)					
	NHD			HD	
Group	Mean	± SD	Group	Mean	± SD
NHD-C	23.50*2	2.49	HD-C	26.38	1.80
NHDG	30.05*3	6.21	HDS	27.30	4.77
NHDR	24.20*2	5.97	HDG	30.75	8.66
NHDΩ	22.13*1	3.86	HDR	30.86	5.10
NHDGΩ	24.83*2	3.86	HDΩ	29.87	5.68
NHDRΩ	28.53*2	3.93	HDGΩ	31.60	5.10
			HDRΩ	30.61	4.85
F	2.71		F	0.790	
P	0.0387		*P*	0.583	

NHD: Non-high fat diet, HD: high-fat diet, NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement, NHDG: received green (non-roasted) coffee extract only, NHDR: received medium-roasted coffee extract only, NHDΩ: received n-3FA (1 g/rat per day) supplement only, NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω)supplement, NHDRΩ: received medium-roasted coffee extract and n-3FA supplement, HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement, HDS: received atorvastatin (S) only, HDG: received green (non-roasted) coffee extract only, HDR: received medium-roasted coffee extract only, HDΩ: received n-3FA supplement only, HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement, HDRΩ: received medium-roasted coffee extract and n-3FA supplement, F: variation between sample means/variation within the samples, *P*: value for ANOVA test at the end of the experiment, *1,*2,*3 Subset for alpha =0.05 detected by Duncan's test of post-hoc multiple comparisons for determining the differences of HDL levels among different NHD groups at the end of the experiment„ SD: standard deviation.

Table 6. The difference in mean LDL change between NHD and HD study groups at the end of the experiment (n = 6 per group).

LDL (mg/dl)					
	NHD			HD	
Group	Mean	± SD	Group	Mean	± SD
NHD-C	42.50*3	18.07	HD-C	48.17*3	10.90
NHDG	11.62*1	9.43	HDS	18.25*1	4.87
NHDR	39.75*3	21.50	HDG	42.45*1,*2	12.09
NHDΩ	10.48*1	2.49	HDR	43.04*1,*2	21.38
NHDGΩ	16.32*1	6.72	HDΩ	13.57*1	7.21
NHDRΩ	20.93*2	8.29	HDGΩ	11.44*1	5.85
			HDRΩ	13.35*1	6.30
F	7.435		F	13.08	
P	<0.001		*P*	<0.001	

NHD: Non-high fat diet, HD: high-fat diet, NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement, NHDG: received green (non-roasted) coffee extract only, NHDR: received medium-roasted coffee extract only, NHDΩ: received n-3FA (1 g/rat per day) supplement only, NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω)supplement, NHDRΩ: received medium-roasted coffee extract and n-3FA supplement, HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement, HDS: received atorvastatin (S) only, HDG: received green (non-roasted) coffee extract only, HDR: received medium-roasted coffee extract only, HDΩ: received n-3FA supplement only, HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement, HDRΩ: received medium-roasted coffee extract and n-3FA supplement, F: variation between sample means/variation within the samples, *P*: value for ANOVA test at the end of the experiment. *1,*2,*3 Subset for alpha =0.05 detected by Duncan's test of post-hoc multiple comparisons for determining the differences of LDL levels among different NHD and HD groups at the end of the experiment, SD: standard deviation.

Several studies were undertaken to evaluate the content of coffee oil as roasting degrees advanced, with increased yields of coffee oil obtained at high roasting temperatures due to heat, which can assist in oil removal in the material.[7,21] The yield of coffee oils increases with higher roasting temperatures.[7] This is believed to be attributed to the high temperature which causes a pyrolysis reaction on the texture of coffee so solvent is easier to penetrate at the time of extraction take place. Based on the results of this investigation, there is a strong correlation between the degree of roasting temperature and the levels of coffee oil. Specifically, we observed that the highest levels of coffee oil were present in the medium-roasted coffee, while the dark-roasted coffee showed a slight decline in levels. These results align with this investigation's initial expectations and signify a positive outcome.

This investigation showed a similar trend as seen in the coffee oil levels. A slight increase in the diterpenes levels as the roasting temperatures increased to a certain point then a reduction in the diterpenes levels was observed in the dark roasted coffee. This observation corresponds with the findings of a previous study that indicated several new diterpene chemicals,[6] including dehydrocafestol and dehydrokahweol, which are the breakdown products of cafestol and kahweol found in roasted coffee due to the dehydration of diterpenes during the roasting process. Other studies have shown that diterpenes, kahweol, and cafestol levels depend on the intensity of the roasting process, as they can either maintain their original level or sometimes be slightly increased during the roasting process due to the stability of the lipid product. However, after 8 minutes of roasting at 230 ˚C, a reduction of diterpenes will appear due to the breakdown of the lipids, consequently, the reduction of diterpenes will not have a positive relationship with roasting since the degradation products will only be formed after 8 minutes of roasting.[16] Moreover, around 80% of diterpenes found in green coffee were lost after the roasting process was performed at higher temperatures.[22]

Table 7. The difference in mean non-HDL change between NHD and HD study groups at the end of the experiment (n = 6 per group).

non-HDL (mg/dl)					
	NHL			HL	
Group	Mean	± SD	Group	Mean	± SD
NHD-C	16.83*1,*2	3.46	HD-C	30.48*3	3.10
NHDG	20.41*2	3.69	HDS	20.20*1,*2	4.29
NHDR	24.86*2	2.63	HDG	21.26*2	4.84
NHDΩ	18.36*1,*2	3.34	HDR	17.96*1,*2	6.32
NHDGΩ	12.17*1	3.02	HDΩ	18.30*1,*2	5.61
NHDRΩ	15.47*1	2.11	HDGΩ	12.56*1	1.98
			HDRΩ	10.50*1	1.10
F	11.94		F	13.81	
P	< 0.001		*P*	< 0.001	

NHD: Non-high fat diet, HD: high-fat diet, NHD-C (Control): rats fed with standard diet; neither received coffee extract nor n-3FA supplement, NHDG: received green (non-roasted) coffee extract only, NHDR: received medium-roasted coffee extract only, NHDΩ: received n-3FA (1 g/rat per day) supplement only, NHDGΩ: received green (non-roasted) coffee extract and n-3FA (Ω)supplement, NHDRΩ: received medium-roasted coffee extract and n-3FA supplement, HD-C: rats fed with high-fat diet; neither receives coffee extract nor n-3FA supplement, HDS: received atorvastatin (S) only, HDG: received green (non-roasted) coffee extract only, HDR: received medium-roasted coffee extract only, HDΩ: received n-3FA supplement only, HDGΩ: received green (non-roasted) coffee extract and n-3FA supplement, HDRΩ: received medium-roasted coffee extract and n-3FA supplement, F: variation between sample means/variation within the samples, *P*: value for ANOVA test at the end of the experiment. *1,*2,*3 Subset for alpha =0.05 detected by Duncan's test of post-hoc multiple comparisons for determining the differences of non-HDL levels among different NHD and HD groups at the end of the experiment, SD: standard deviation.

Several studies reported consistent findings with the current study.[9,15,23] This trend was explained by assuming that the caffeine concentration increases due to the destruction of liquid and acidic components during the roasting process, resulting in a higher percentage of non-liquid substances such as caffeine, fat, and minerals.[9] On the other hand, some researchers indicated that there is an inverse relationship between the caffeine concentrations and the degree of roasting temperature.[23] Whereas other studies reported that the caffeine levels may not change significantly and will remain constant during the roasting process as described previously.[4] Furthermore, the caffeine content and the extraction's efficiency of caffeine from coffee depends on several factors besides to the degree of roasting, each factor can affect the amount of caffeine differently. These factors include the effect of extraction time, extraction temperature, solvent-to-coffee ratio, and mixing speed.[24]

The CGA levels demonstrated a negative correlation with the degree of roasting, which agrees with the findings of previous studies since the CGA was proven to be thermally unstable when exposed to higher degrees of temperature.[15] The conversion of CGA into melanoidin molecules explains the inverse relationship between CGA concentration and the roasting process.[8] This relationship was explained previously;[4] when the degree of roasting temperature rises, the carbon-carbon bonds break, which would result in structural changes as well as chemical changes, such as the dehydration of quinic acids and the creation of lactone rings. CGA is the other name for polyphenols; it is a natural compound that possesses diverse health benefits due to its anti-oxidative properties. The CGA prevents several diseases such as cancer, aging, and cardiovascular and neurological disorders.[25] Furthermore, it is essential in diabetes because it reduces glucose release after meals. Water extraction was chosen to extract CGA from coffee.

Despite omega-3 supplements being used extensively for the reduction of hyperlipidemia consequences,[26,27] opposite results have been reported as well.[28] On the other hand, numerous consequences including arrhythmia, hypercholesterolemia, headache, and triglyceridmeia occur with the extensive consumption of coffee.[29] However, data concerning the supplemental adverse effect of omega-3 in combination with coffee consumption is still unknown. Salamat et al. reported that green coffee has a lipid-lowering effect compared to roasted coffee which showed a good agreement with the current study.[30] A more recent explanation has emerged that relates to the anti-oxidant properties of green coffee. Interestingly, the anti-oxidant effects of green coffee are consistent with an early hypothesis attempting to explain the anti-hyperlipidemic and weight loss associated with green coffee consumption.[31] In this context, worse hyperlipidemic findings have been attributed to the roasting degree of coffee beans when compared to green coffee.[32] It was pointed out that the potential effects of coffee roasting degree might be due to the decline in antioxidant constituents such as CGA.[33] This study appeared to be of concern to prompt future studies to search further about the adverse effects of roasted coffee on human health.[34]

The TG-lowering effect of n-3FA supplements alone without coffee consumption has been previously reported in humans and animals.[35,36] Moreover, in a sample of patients that are marked with high levels of TG, n-3FA supplements reduced the levels of TGs in plasma, while non-HDL increased the levels of HDL and LDL.[37,38] While in animal model, it was indicated that rats transport cholesterol mainly in HDL particles caused by the low amount of cholesteryl ester transfer protein (CETP).[39]

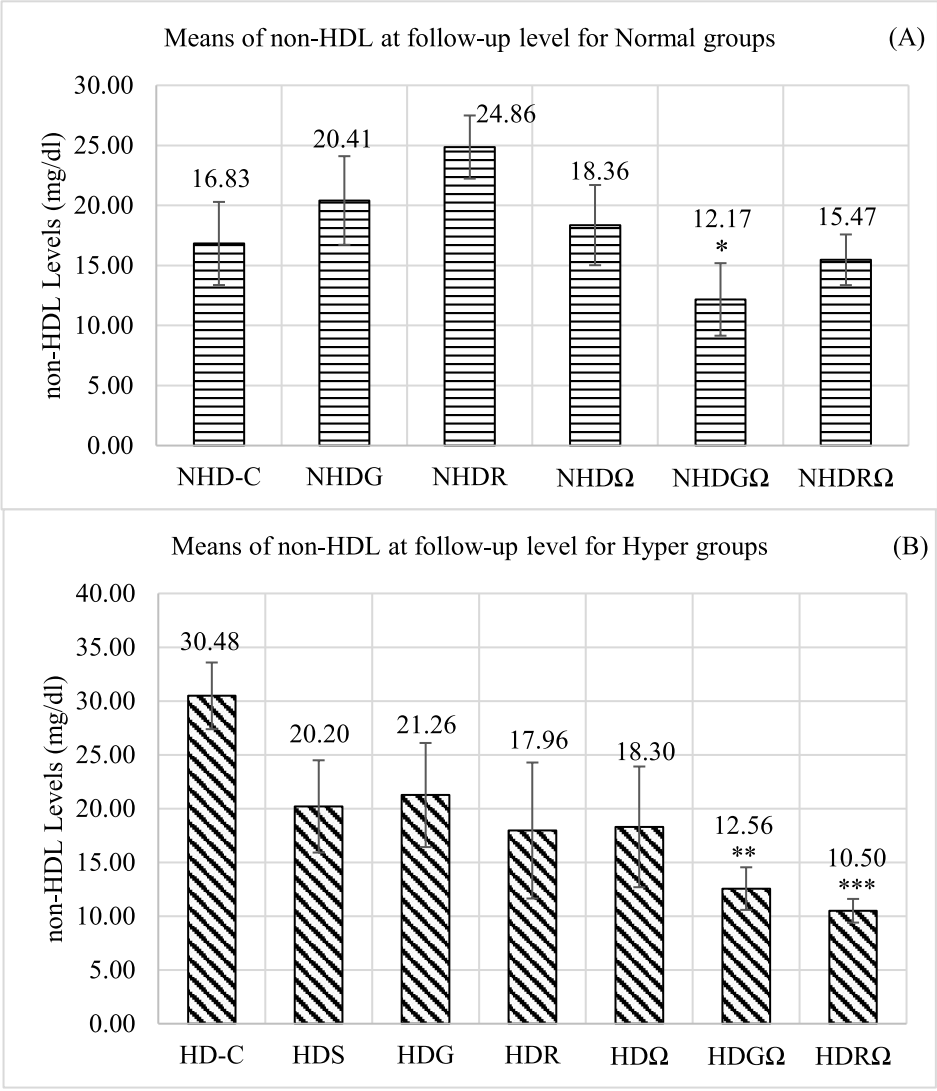

Fig. 3. The means of non-HDL at follow-up level for (A) normal experimental groups and (B) hyper experimental groups.
Note: The results are represented as mean (n = 6 for each group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with the green coffee group (Duncan's Multiple Comparisons Test).

Furthermore, the current study' results indicated that green coffee, alone or in combination with n-3FA, has a significant effect on lipid profile when compared to roasted coffee. This can be attributed to green coffee's high content of CGAs, one of the polyphenols that have anti-oxidative and hypolipidemic effects.[40] It can lower plasma TG levels and raise HDL levels; however, it does not affect LDL or TC levels.[12] Although the effects of n-3FA polyunsaturated FAs (PUFAs) from fish oil on non-HDL-C have not been well investigated, some evidence suggests that n-3FA from fish oil may lower non-HDL.[41] In addition, roasted coffee has a rich content of coffee oils and diterpenes which exhibit hypercholesteremic activity.[1] Furthermore, the impact of green coffee alone was more observable in non-hyperlipidemic rats (normal rats) than in hyperlipidemic rats which can be explained by the fact that green coffee alone was not enough to alter the lipid profile in hyperlipidemic rats. This result can give a dose-dependent effect of green coffee that the CGA concentration was relatively low to improve the lipid profile in hyperlipidemic rats which is consistent with the findings of Nguyen et al.[42]

Concerning non-HDL, the results were inconsistent, and no data have been yet available about the combined effect of either roasted or unroasted coffee beans with n-3FA supplementation on the lipid profile. Therefore, and despite that the current study was designed on a small animal sample, it provides insight into the combined effect of the two preparations on health, particularly blood lipids. Notwithstanding

these limitations, the study suggests that green coffee in combination with n-3FA supplements significantly reduces non-HDL levels. This evidence might be related to the higher levels of CGA in green than in roasted coffee.

Conclusion

Coffee oil, diterpenes, CGA, and caffeine were determined in different types of coffee. The physical properties of these compounds were affected by the roasting process. The contents of coffee oil, diterpenes, and caffeine increased with higher roasting temperatures, while CGA levels decreased. The potential biological effect of coffee extracts on lipid profile was also examined. The study found that green coffee with or without n-3FA supplementation had a significant lipid-lowering effect in non-hyperlipidemic rats compared to hyperlipidemic rats. This effect could be attributed to the high concentration of CGA in green coffee as compared to other roasted coffee extracts.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the figures and tables in the manuscript are ours. Furthermore, figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- No human studies are present in the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The ethical approval was approved by the Institutional Review Board at the Applied Science Private University, Amman, Jordan (IRB protocol: 2022-PHA-12).

Authors' contribution statement

Conception: S.H.A. & M.S.A.; Design: S.H.A. & M.S.A.; Acquisition of data: S.M.A., A.Q.D., & H.F.; Analysis: S.M.A., S.H.A., R.A.I., & T.A.; Interpretation: S.H.A., K.W.O., B.A.M., M.A., R.F.K., & M.S.A.; Drafting the MS: All authors; Revision and proofreading: S.H.A. & M.S.A.

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القياس الكمي للمركبات النشطة بيولوجيا في القهوة العربية وتأثيرها مع مكملات أوميغا 3 على مستويات البروتين الدهني غير عالي الكثافة في الفئران المصابة بفرط الدهنيات

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المستخلص

تهدف هذه الدراسة إلى تقييم مستويات دهون القهوة، والديتربينات، وحمض الكلوروجينيك (CGA)، والكافيين في أنواع مختلفة من القهوة. بالإضافة إلى ذلك، يهدف إلى تقييم تأثير مستخلصات القهوة مع أو بدون مكملات أوميجا 3 على معايير مستوى الدهون. تم إستخدام طرق استخلاص السائل والسائل والسوكسليت، وتم إجراء القياس الكمي باستخدام-HPLC DAD . تم استخدام مجموعتين من فئران ويستار: غير مفرطة الدهون وفئران الدهون. تم جمع عينات الدم قبل وبعد تحريض فرط شحميات الدم بعد ستة أسابيع. معلمات ملف الدهون بما في ذلك الكوليسترول الكلي(TC)، والبروتين الدهني منخفض الكثافة (LDL)، والبروتين الدهني عالي الكثافة (HDL)، والدهون الثلاثية (TG)، والبروتين الدهني غير عالي الكثافة(non-HDL) ، والكوليسترول الكلي تم تحديد نسبة البروتين الدهني إلى عالي الكثافة (TC/HDL). تم العثور على علاقة إيجابية بين مستويات الدهون في القهوة، والديتربينات، والكافيين مع درجة تحميص القهوة؛ ومع ذلك، تم العثور على علاقة سلبية لCGA. أظهرت الدراسة التي أجريت على الفئران التي تم تغذيتها بنظام غذائي صحي وتلك المصابة بفرط دهنيات الدم أن القهوة الخضراء كان لها تأثير كبير على مستوى الدهون في الفئران غير المصابة بفرط دهنيات الدم. لقد خفضت مستويات LDL ،TG، وTC/HDL، ورفعت مستويات HDL و non-HDL بشكل ملحوظ مقارنة بالمجموعة الضابطة. الإستخدام المزدوج للقهوة الخضراء وأوميغا 3 خفضت مستويات non-HDL، LDL، TG، TC، وTC/HDL. القهوة الخضراء وحدها أو بالاشتراك مع (أوميغا 3) خفضت مؤشرات الدهون ولكنها زادت مستويات non-HDL في الجرذان الدهنية المستحثة وغيرالدهنية.

الكلمات المفتاحية: كافيين، كلوروجينيك أسيد، قهوة، دايتربينات، مؤشرات الدهون، أوميجا-3.