

# The Effects of Dark Chocolate Consumption on Lipid Profile, Fasting Blood Sugar, Liver Enzymes, Inflammation, and Antioxidant Status in Patients with Non-Alcoholic Fatty Liver Disease: A Randomized, Placebo-Controlled, Pilot study

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## ABSTRACT

**BACKGROUND:** Nonalcoholic fatty liver disease (NAFLD) is an important health problem worldwide and despite the rising prevalence, there is currently no satisfying therapeutic strategy. Dark chocolate (DC) is a food rich in phenolic antioxidants, which may exert favorable and modifying effects on lipid profile, insulin resistance, oxidative stress, and metabolic effects. This study aims to investigate the possible effects of DC consumption on the lipid profile, fasting blood sugar (FBS), liver transaminases (ALT, and AST), inflammatory, and antioxidant status among NAFLD patients.

**METHODS:** In this double-blind, placebo-controlled trial, 42 patients with NAFLD were randomly allocated to 2 groups: the treatment group ( $n=21$ ) whom received 30 gr dark chocolate (83%) daily and the control group ( $n=21$ ), for a 12 weeks period.

**RESULTS:** During the intervention period, taking 30 gr DC (83%) daily resulted in a significant decrease in AST ( $P=0.012$ ), body weight ( $P=0.027$ ), and BMI ( $P=0.042$ ) in the treatment group. In addition, patients who received DC had considerable changes in serum HDL ( $P=0.044$ ). However, no significant changes occurred in serum levels of ALT, hs-CRP, anthropometric measures (WC, HC, and WHR), and grades of NAFLD in both groups ( $P>0.05$ ).

**CONCLUSION:** DC consumption can decrease the level of AST in patients with NAFLD and could be a potential therapeutic approach. We recommend more investigation about potential therapeutic effects of dark chocolate to be further clarified.

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**Key Words:** Non-alcoholic Fatty Liver Disease; Dark Chocolate; liver Transaminases

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## INTRODUCTION

The liver is a main organ for human life, and the health of this organ often mirrors the overall individual health as the center of crucial metabolic functions. The liver tissue can be the target of several diseases, all able to change the hepatic functions. One of the most common causes of primary and chronic hepatic disorders worldwide is the non-alcoholic fatty liver disease (NAFLD)<sup>[1]</sup> which is an important health problem in various age groups<sup>[2]</sup>. NAFLD defines as the excessive accumulation of fat deposits, primarily in the form of triglycerides in hepatocytes<sup>[3]</sup>, characterized by histologic features similar to alcoholic fatty liver disease<sup>[4]</sup> but in the absence of history of excessive alcohol consumption<sup>[5]</sup>, and encompasses a wide spectrum of liver conditions, from simple steatosis to nonalcoholic steatohepatitis (NASH), end-stage liver failure, and, ultimately, hepatocellular carcinoma<sup>[6]</sup>, which leads to liver-related death<sup>[7]</sup>.

At the end of the current decade, population screening has estimated the NAFLD prevalence to be rising in all regions of the world, including Asia, and also NAFLD is now the liver disease related to the highest mortality and consequently increased risk of cardiovascular diseases<sup>[8]</sup>. Moreover, it accounts for up to 20% of liver function test abnormalities in most countries with modern industrialized economies<sup>[2]</sup>. Nonetheless, the actual underlying molecular and cellular mechanisms of liver injury in NAFLD are still unknown<sup>[1]</sup>. However, the NAFLD patho-physiology was firstly described by the ‘two-hit hypothesis’, and subsequently<sup>[8]</sup>, there is currently an accepted concept for NAFLD pathogenesis that is called “multiple hit” hypothesis. According to this hypothesis, multiple mechanisms, including insulin resistance, oxidative stress, inflammation, and genetic factors interact to initiate the NAFLD development<sup>[9]</sup> and also these are etiological factors associated with metabolic derangements<sup>[1]</sup>. Moreover, it has become evident that development of NAFLD is due to multiple interrelated factors including visceral obesity, dyslipidemia, diabetes, and hypertension, all of them are key risk factors related to NAFLD. Consequently, the potential therapies for NAFLD could focus on each one or more of these underlying factors<sup>[9]</sup>.

Despite the rising incidence of NAFLD<sup>[5]</sup>, there is currently no satisfying therapeutic strategy (medical or surgical) for curing NAFLD<sup>[9]</sup>, regards to the NAFLD propensity to induce end-stage liver disease suggests that early interventions could potentially prevent the more serious latter stage manifestations of this disease. Hence, a multi-disciplinary and beneficial approach necessitates to finding a potential and useful therapeutic agent for controlling disease<sup>[9]</sup>. Recently, this has attracted much attention in the research on dark chocolate (DC) consumption, and especially cocoa as a nutritional factor due to its potential healing effects<sup>[10]</sup>. Historically, consumption of DC by human subjects has a long and intriguing history for its purported healing properties<sup>[11]</sup>. DC is one of the foods

**Table 1** Energy, and Nutrient Component Intake with Dark and white Chocolate (100 g)<sup>a</sup>.

Content per Dose	Dark Chocolate	White chocolate
Energy, kcal	570	289
Total fat, g	42	28.1
Carbohydrates, g	39	0
Protein, g	9	5.7

<sup>a</sup>: Energy and nutrient composition are calculated from data provided by the manufacturers.

**Table 2** Demographic characteristics of study participants before study (Wt: Weight, BMI: Body Mass Index as kg/m<sup>2</sup>).

Variable	Intervention group	Placebo group	P value
Age	38.18 ± 11.04	37.95 ± 10.34	0.944 <sup>a</sup>
Weight	88.59 ± 13.17	84.92 ± 20.64	0.49 <sup>a</sup>
BMI	30.27 ± 3.58	29.69 ± 5.76	0.691 <sup>a</sup>
Sex			0.281 <sup>b</sup>
female	3 (13.6%)	6 (27.3%)	
male	19 (86.4%)	16 (72.7%)	
Smoking			1.000 <sup>b</sup>
yes	1 (4.5%)	1 (4.5%)	
no	21 (95.5%)	21 (95.5%)	

a, b: P values for differences between the intervention and placebo groups at baseline based on Independent T Test (numerical variables) or Pearson  $\chi^2$  (categorical variables).

rich in bioflavonoids (flavanols, polyphenols, and theobromine)<sup>[12]</sup>, which has the highest antioxidant level in comparison with other food sources based on Oxygen Radical Absorbance Capacity measurement<sup>[13]</sup>. Besides nutrients such as saturated fat (60%), monounsaturated fat (35%), and linoleic acid (3%), chocolate contains important minerals such as potassium and magnesium<sup>[14]</sup> as well as cocoa, which is the main and the quintessential ingredient in chocolate<sup>[10]</sup>. Cocoa and some of its derivatives are highly complex food<sup>[14]</sup> and a rich source of the flavonoid antioxidants, catechin and epicatechin<sup>[15]</sup>. The potential health benefits of consuming chocolate have only recently been discovered<sup>[11]</sup>. Several recent studies have suggested that DC may exert favorable and modifying effects on lipid profile, decreasing total and LDL cholesterol levels and increasing HDL cholesterol levels<sup>[17]</sup>. In addition, numbers of nutritional studies have been indicated that cacao in chocolate may increase its nutritional properties, from minerals, to antioxidants, and to vasoactive compounds<sup>[15]</sup>, specifically polyphenolic compounds (flavonoid antioxidants) exhibit antihypertensive, anti-inflammatory, antithrombotic, and metabolic effects, all of them may contribute to their protective effect<sup>[17]</sup>. Moreover, these can improve insulin resistance through reducing oxidative stress, improving endothelial function, and/or changing glucose metabolism<sup>[15]</sup>. A recent study on the rat model of alcoholic steatohepatitis had shown that cocoa supplementation have a useful effect on disease by reducing the hepatic accumulation of fat, inflammation and necrosis<sup>[16]</sup>. Latter studies have mostly supported a protective relationships between cocoa or chocolate consumption and a variety of health indicators<sup>[14]</sup>. The results of these studies have shown that cocoa and DC consumption can reduce stress, stimulate brain function, fight cancerous cells, improve circulation, mood, memory, immune system, and even protect the heart<sup>[12]</sup>, and liver tissue<sup>[10]</sup>. Hence, we assumed that dark chocolate consumption may also have a therapeutic potential in preventing and controlling NAFLD complications. The current study was designed to assess the effects of regular consumption of 30 gr serving dark chocolate (DC) which contained 83% cocoa for 12 weeks on lipid profile, fasting blood sugar, liver enzymes, and inflammatory and antioxidant status among patients suffering NAFLD.

**Table 3** Anthropometric characteristics of study participants (Wt: weight, WC: wrist circumference, HC: Hip circumference, BMI: Body Mass Index, WHR: Weight/Hip Ratio).

Variables	Intervention		P-value <sup>a</sup>	Placebo		P-value <sup>b</sup>
	Before	After		Before	After	
Wt	88.59 ± 13.17	87.18 ± 12.35	0.027	84.92 ± 20.64	83.74 ± 18.46	0.235
WC	103.27 ± 8.18	102.13 ± 7.43	0.267	104.61 ± 16.25	102.45 ± 11.51	0.232
HC	108.36 ± 7.14	103.36 ± 21.68	0.261	108.54 ± 9.83	108.71 ± 9.34	0.796
BMI	30.27 ± 3.58	29.84 ± 3.46	0.042	29.69 ± 5.762	29.476 ± 5.08	0.527
WHR	0.95 ± 0.03	0.94 ± 0.04	0.477	0.95 ± 0.06	0.94 ± 0.04	0.13

a, b: P values indicate differences between the intervention and placebo groups Before and After study period (Paired T Test).

**Table 4** Effects of DC consumption on serum levels of FBS, and lipid profile in patients with NAFLD.

Variables	Intervention		P-value <sup>a</sup>	Placebo		P-value <sup>b</sup>
	Before	After		Before	After	
FBS	89.15 ± 6.68	91 ± 8.60	0.215	90.71 ± 18.24	94.28 ± 15.31	0.238
Chole	198.45 ± 46.59	203.50 ± 41.40	0.693	196.95 ± 33.13	184.80 ± 28.70	0.182
TG	195.22 ± 81.11	198.95 ± 83.64	0.835	206.47 ± 104.19	172.76 ± 79.13	0.117
HDL	43.09 ± 10.64	46.72 ± 7.27	0.044	41.90 ± 7.62	44.80 ± 8.55	0.18
LDL	114.13 ± 35.40	120.59 ± 36.41	0.531	112.19 ± 23.63	101.76 ± 18.17	0.098
LDL:HDL	2.46 ± 0.73	2.98 ± 1.23	0.047	2.72 ± 0.55	2.30 ± 0.46	0.009
VLDL	38.69 ± 15.94	39.81 ± 16.66	0.755	42.85 ± 14.90	38.23 ± 13.74	0.254

a, b: P values indicate differences between the intervention and placebo groups at Before and After (Paired T Test).

**Table 5** Effects of DC consumption on serum levels of MDA, and hs-CRP in patients with NAFLD.

Variables	Intervention		P-value <sup>a</sup>	Placebo		P-value <sup>b</sup>
	Before	After		Before	After	
hs.CRP	1382.60 ± 465.60	1210.72 ± 424.60	0.065	1985.76 ± 914.81	1923.85 ± 1006.03	0.58
MDA	1.38 ± 2.93	1.23 ± 2.86	0.007	2.57 ± 1.32	2.31 ± 1.47	0.539

a, b: P values indicate differences between the intervention and placebo groups at Before and After (Paired T Test).

**Table 6** Effects of Dark chocolate supplementation on serum levels of liver enzymes and NAFLD in patients with NAFLD.

Variables	Intervention		P-value <sup>a</sup>	Placebo		P-value <sup>b</sup>
	Before	After		Before	After	
NAFLD grades	1.27 ± 0.45	1.5 ± 0.91	0.285	1.38 ± 0.92	1.95 ± 1.65	0.15
AST	39.04 ± 18.70	30.86 ± 12.74	0.012	30.95 ± 11.22	28.85 ± 9.99	0.436
ALT	54.18 ± 41.29	54.27 ± 36.23	0.986	39.61 ± 19.29	41.85 ± 20.92	0.609

a, b: P values indicate differences between the intervention and placebo groups at Before and After (Paired T Test).

## METHODS AND MATERIALS

### Design

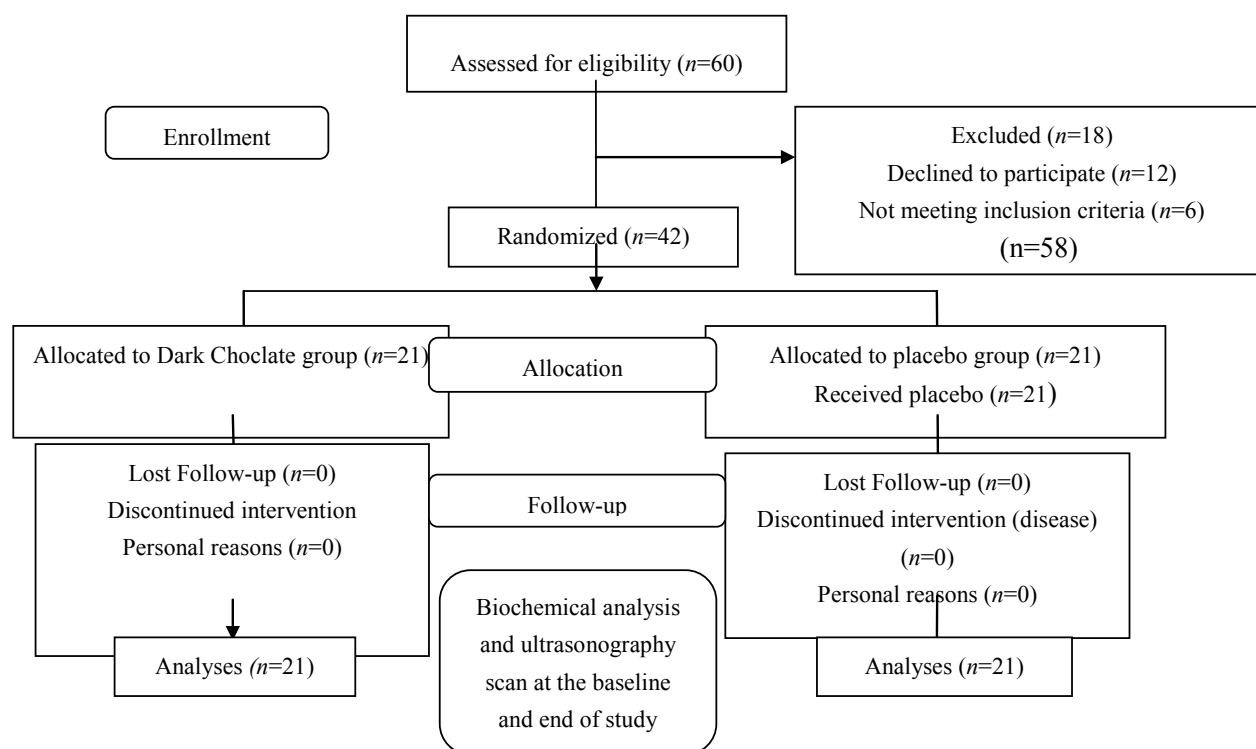
The current study is a double blinded, randomized, placebo-controlled clinical trial with two parallel groups. Participants in this study were divided randomly into the treatment group (group A) who received ninety packs of dark chocolate for a 3months period (30 gr/day), which contained 83% cocoa and covered with aluminium foils, and the control group (group B) whom received 30gr zero calorie white chocolate as placebo (produced by Shirin Asal Co.). Intervention subjects received a total of 171 kcal from a daily dose of 30 gr DC (Table 1).

The intervention period was 12 weeks. In order to blind the trial, the numbers of patient were assigned and the packages of chocolate were coded by another person unaware of the trial and random sequences. So that, the researchers and participants were unaware of randomization and allocation until the statistical analysis was completed. We called participants once every week to remind them about the DC consumption and were requested to report if there were any adverse effects of the DC consumption. In the study period, subjects were advised not to take any change in diet, lifestyle habits, and physical activity level and also advised not to use any other kind of chocolate during the study.

### Participants

This study was conducted in the Southwest of Iran from 2013 to 2014 by the research institute for infectious diseases of the digestive system

of Jundishapur University of Medical Sciences, Ahvaz, Iran. In total, 42 persons with NAFLD aged 18 years and older were enrolled in this study based on inclusion and exclusion criteria. The demographic and anthropometric characters of two groups had not any significant difference statistically (Tables 2 and 3). We included all participants with NAFLD based on one or more of the following criteria: the chronic elevation of liver enzymes (aspartate aminotransferase and alanine aminotransferase), absent or negligible history of alcohol consumption (<20 g/day), and ultrasonographic evaluation of the liver compatible with NAFLD as diagnosed by an expert radiologist. The exclusion criteria were: consumption of caffeine-containing beverages, antioxidant or vitamin supplements, history of alcohol usage (equal to or more than 20 g/day), smoking, consumption of hepatotoxic drugs such as corticosteroids, amiodarone, tamoxifen, and/or methotrexate in the last 6 months prior to study, history of any other chronic liver disease such as hereditary hemochromatosis, Wilson's disease, and  $\alpha$ 1-antitrypsin deficiency, history of jejunoileal bypass surgery or gastroplasty and using total parenteral nutrition in the past 6 months. This study was performed according to the ethical standards for human experimentation. The research protocol was approved by the institutional review board and the ethical committee of the Digestive Disease Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Registration No. REC-165) and registered at the Iranian Registry of Clinical Trials (IRCT number: IRCT201303264545N2). All subjects requested to sign an informed consent before participation in the study.



**Figure 1** Flowchart of double blind, placebo controlled, randomized clinical trial of DC supplementation in NAFLD

### Blood sampling

After explaining the aim of the study, blood samples from all patients were taken at the study entry and at the end of research to measure their liver enzyme levels, plasma concentrations of hs-CRP, malondialdehyde (MDA), lipid profile and fasting blood sugar. For this reason, 10 ml fasting venous blood samples from each participant were obtained each time in the morning of the sampling day and collected into evacuated tubes and serum were prepared after centrifugation (3,000 RPM, 4 °C, 15 minutes) by a trained examiner and were then stored frozen (-70 °C) until analysis.

Fasting blood sugar concentration was assayed using the glucoseoxidase method, by Pars Azmoon test kits. Serum Alanine transaminase (ALT) and aspartate transaminase (AST) enzyme activities, as a marker of hepatic cell damage, were determined by the kinetic method (Pars Azmoon Co, Tehran, Iran). The normal range value of ALT, based on this method was lower than 17 U/L for female and lower than 22 U/L for male; for AST, lower than 15 U/L for female and lower than 18 U/L for male considered normal; Serum levels of high-sensitivity C-reactive protein (hs-CRP) as an inflammatory factor was determined by enzyme-linked immunosorbent assay (ELISA), using LDN of Germany test kits. Triglycerides (TG), total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were assayed using an enzymatic photometric test, by Pars Azmoon test kits. With respect to Botsoglou, plasma MDA concentrations, as a marker of lipidperoxidation were also determined by using the thiobarbituric acid-reactive substance (TBARS) method (18). At baseline and the end of the intervention duration anthropometric measurements, blood samples, biochemical variables and ultrasonographic measurement were performed under similar conditions.

### Anthropometrics

The obtained demographic information of each patient included lifestyles (smoking, drinking, diet, and exercise habits), age, literacy

level, socio-economical status and medical and drug history. This information was collected by the self administered questionnaire. All anthropometric measurements were recorded by the same person to decrease error at baseline and at the end of the study.

Using a tape measure with standard protocols the participants were standing in an upright position without shoes, height was recorded with a precision of 0.1cm. Weight was measured by using digital scales with the participants wearing minimal clothing and without shoes, weight was measured to the nearest 0.1 kilograms. Body mass index (BMI) was calculated as weight (Kg) divided by height in squared meters. Using non-stretchable tape, without any pressure applied to the surface of the body, waist circumference (WC) and hip circumference (HC) were measured. These measurements were recorded with a precision of 0.1 cm for WC and HC measured at the narrowest, and at the maximum level over light clothing, respectively (Table 3).

### Hepatic examination

Ultrasonography evaluation of the liver for all the subjects was performed at entry and the end of the study period by a single expert radiologist, blinded to the treatment method of the patients (US; General Electric LOGIQ 400 CL- Using probe 3.5/5 MHz). The grade of hepatic steatosis, defined as the percentage of hepatocytes with fat droplets stages were measured for each patient and then the degree of steatosis was graded from I to III.

### Statistical analyses

Our trial carried as a pilot study due to lack of similar study. All statistical analyses were performed with Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) Program version 18 for windows. At first, normal distribution of all variables was checked with the Kolmogorov-Smirnov test. We compared the means of variables of each group with each other by using both independent sample t-test and ANCOVA in the adjusted models. The end values



of each variable were also compared with the baseline values of it by using paired sample t-test. The differences with  $P$  values  $< 0.05$  were considered as significant.

## RESULTS

From 60 selected patients with NAFLD for participating in this clinical trial, 18 cases were lost during follow up (9 in intervention group and 9 in placebo groups) due to personal reasons (Figure.1). Thus, 42 participants completed the intervention period of the study. The study groups were comparable regarding this variable, because the equal number from each group failed follow up. The participants did not report any side effect due to DC consumption. Patients' mean age in the intervention and control groups were  $37.95 \pm 10.34$  y and  $38.18 \pm 11.04$  y, respectively. The number and percentage of females in the intervention and control group were 6 (27.3%) and 3 (13.6%), respectively. At the beginning of the study, demographic characteristics of 42 participants had not any statistically significant difference between two groups ( $P > 0.05$ ) (Table 2). In addition, there were no significant differences in drug history, past medical history and/or physical activity levels among both groups at the baseline (data not shown). The anthropometric records and biochemical characteristics about FBS and lipid profile of the 42 participants before and after intervention are displayed in Tables 3, 4, and 5. At the end of the study period, intra-group differences in the DC group showed a significant reduction of weight ( $P = 0.027$ ) and BMI ( $P = 0.042$ ) (Table 3) and also revealed a significant elevation of serum HDL levels ( $P = 0.044$ ). Additionally, significant alterations in the LDL/HDL ratio occurred in the 2 groups (Table 4). The serum levels of AST were also significantly lower than baseline in the intervention group at the end of the intervention ( $P = 0.012$ ), whereas the serum levels of ALT, AST/ALT ratio (AAR) as well as the grades of NAFLD during the intervention's period did not change significantly in both groups ( $P > 0.05$ , Table 6). After 12 weeks, serum MAD levels as a marker of antioxidant status, markedly decreased in the intervention group ( $P = 0.007$ ), but no significant changes occurred in placebo group (Table 5). However, in the intra group comparison of variables, serum levels of FBS, other lipid profiles and hs-CRP as well as the grade of hepatic steatosis had not any significant difference in either of the 2 groups ( $P > 0.05$ , Table 3, 4, 5).

## DISCUSSION

The findings of this study provide information about the influence of DC consumption on the reduced weight, BMI, and serum AST levels in NAFLD patients. However, it did not alter the serum levels of hs-CRP, oxidative stress status, and grades of hepatic steatosis in adult patients with NAFLD. Although DC therapeutic effects as a hepato-protective agent is still not well understood, evidences of several trials are limited with conflicting results, to our knowledge, no clinical trial have yet to be carried out to further investigate the effects of DC on NAFLD. In accordance with the results obtained, a daily dosage of 30 gr DC (83%) for 12 weeks in patients with NAFLD produced a significant decrease in body weight and BMI. Pathogenesis of NAFLD is associated with the presence of excess weight and obesity, as well as the location of fat storage are linked with the pathogenesis of NAFLD<sup>[19]</sup>. As previously mentioned, the risk developing of NAFLD increases by accumulation of visceral fat stores in both obese and non-obese individuals so weight loss could be a main strategy to improve liver enzymes, insulin sensitivity, reducing inflammation, and liver histology<sup>[19]</sup>.

Numerous studies that investigated the impact of DC on body weight revealed conflicting results. In agreement with our results, Massolt and colleagues found the effects of DC (85% cocoa) in suppressing appetite and possibly reduced weight gain after eating 30 gr chocolate in 12 females<sup>[20]</sup>. It seems that the DC could<sup>[21]</sup> increase glucose uptake, increase fatty acids and glucose oxidation, inhibit fat synthesis, and enhance lipolysis in adipose tissue<sup>[22]</sup> probably by increasing Nitric oxide (NO) bioavailability. Moreover, in another study, Naoko et al. has been found the cacao effective for reduction of visceral adipose tissue in rats, possibly by altering the enzyme expression genes and transport molecules involved in fatty acid synthesis and thermogenesis in liver and white adipose tissue<sup>[23]</sup>. However, several studies have shown that the daily dark chocolate was not associated with any weight change<sup>[24]</sup>.

The results of this study confirmed that a 12 weeks course of DC at a dosage of 30 gr/day can increase the serum levels of HDL-C in NAFLD patients. However, the serum levels of TC, LDL, and VLDL were not affected after DC supplementation. It is evident that postprandial hepatic lipid metabolism may alter in patients with NAFLD<sup>[25]</sup>. Furthermore, inhibition of LDL peroxidation may play a key role in its anti-atherogenic properties<sup>[26]</sup>. The lipid-lowering effects of cocoa have been studied in numerous studies, while evidences of cocoa's effects on the lipid profile appear to be limited and conflicting<sup>[21]</sup>. Our findings are in agreement with those reported by Hamed MS et al., who have shown that seven days of DC consumption rose serum levels of HDL by 9% in 28 healthy volunteers<sup>[27]</sup>. Mellor et al. have observed that the daily consumption of 45 gr flavonoid-rich chocolate over 16 weeks significantly increases serum HDL in type-2 diabetic (T2DM) patients<sup>[28]</sup>. Also in a cross over study, Mathur et al. observed the effects of cocoa supplementation (36.9 g of dark chocolate bar and 30.95 g of cocoa powder drink) on lipid profile in healthy subjects for 6 weeks. Findings from this study show that cocoa may significantly increase HDL-C, and have no affect on LDL-C levels in healthy subjects for 6 weeks<sup>[29]</sup>. However, some studies have shown the positive effects of dark chocolate on blood lipids (decreasing LDL-C and increase of HDL-c) and other studies has reported the in effectiveness of DC<sup>[11]</sup>. Reasons for this differences may partly be due to the differences in diseases, subjects studied, kind and doses of dark chocolate.

The study data showed that the LDL/HDL ratio in both groups had markedly changed at the end of the intervention. These results were not expected and there is no clear explanation for these results. Since the average change was not so large, it seems that there was a statistical difference without any clinical importance. A large body of evidence suggests that pro-inflammatory biomarkers have been involved in the NAFLD progression<sup>[30]</sup>. Based on recent studies, reports illustrated that hs-CRP as one of the important pro-inflammatory markers is associated with histo-pathologic features of NAFLD independent of other risk factors<sup>[31]</sup>. In addition, the available evidence established that NAFLD subjects have a higher serum level of hs-CRP than healthy individuals<sup>[32]</sup>. Thus, in this clinical trial, we assessed the effectiveness of regular DC consumption on serum hs-CRP levels in NAFLD patients. The results of our study observed no significant changes in serum hs-CRP levels in the intervention group. Interestingly, cocoa constituents, such as flavanols include epicatechin, catechin, and procyanidins<sup>[21]</sup>. These flavanols exhibit anti-inflammatory properties, which can modulate TNF- $\kappa$ B expression gene and reducing the inflammatory biomarkers and ROS production<sup>[21]</sup>. Several studies in this line suggest that DC consumption lead to reduced inflammatory markers, while others report that DC is ineffective on inflammatory markers<sup>[29, 33]</sup>.

The findings of our study are in agreement with those reported by Monagas and colleagues, who have indicated that serum hs-CRP levels were unaffected by regular cocoa consumption (40 gr/d) in patients at high risk of cardiovascular disease for 4 weeks<sup>[34]</sup>. In contrast, di Giuseppe *et al.* in a cohort study reported that the serum hs-CRP levels are markedly lower in healthy individuals consuming a daily 20-g serving of dark chocolate<sup>[35]</sup>. It is also evident that serum levels of MDA as one of the markers of lipid per-oxidation, are elevated in NAFLD patients<sup>[36]</sup>. In this study, we found that DC consumption for 12 weeks may improve oxidative stress status by effectively decreasing serum MDA levels in patients with NAFLD ( $P = 0.007$ ). The accumulating results of previous studies showed that cocoa consumption declines the lipid oxidation product formation such as MDA. In agreement with our results, in T2DM patients receiving 10 grams cocoa powder and 10 grams milk powder twice daily for 6 weeks, a significant inhibition of lipid peroxidation has been reported; but cocoa administration did not influence serum MDA levels in T2DM patients<sup>[37]</sup>. A large body of evidence suggests that such effects may be due to flavonoids contents of Cocoa<sup>[37]</sup>. Polyphenol content in cocoa exerts an antioxidant effect that scavenge reactive oxygen species, chelate  $Fe^{2+}$  and  $Cu^{+}$ , inhibit enzymes, and up-regulate antioxidant defenses<sup>[38]</sup>. Highly elevated serum AST and ALT levels are well-known markers of NAFLD, and liver diseases<sup>[16]</sup>. In the current study, 12 weeks DC consumption significantly decreased the serum AST levels in NAFLD patients ( $P = 0.012$ ), but there were no significant effect on serum levels of ALT and AST/ALT Ratio. One possible reason might be the oxidative stress suppressing effects of DC. Because liver enzyme AST is related to oxidative stress and elevated all-cause mortality<sup>[39]</sup>. In this regard, Abrokwhah *et al.* showed that oral gavage of aqueous cocoa suspension (1 ml/day) for 48 days had no effects on AST, ALT and Alkp in rat<sup>[40]</sup>. However, our literature review did not find any clinical trials that have directly assessed the effect of DC intake on hepatic enzymes and liver function in NAFLD patients. We failed to find any histological changes in degree of steatosis in our NAFLD patients after DC intake. One possible reason might be ultrasound diagnostic method, which could not as accurate as liver biopsy identify the histologic changes, and it may be a possible reason for absence of significant differences between two groups in our trial. In contrast to our study, McKim *et al.* found that Cocoaextract (400 mg/kg per day) continuously for 4 weeks improves liver function by blunting severe fat accumulation, mild inflammation, and necrosis in earlyalcohol-inducedliverinjury in the mice<sup>[41]</sup>. The point that should be mentioned is that the pathogenesis of alcoholic fatty liver is not similar to NAFLD and this study is the first clinical trial that evaluated the effects of DC on NAFLD.

Our study had several limitations. Firstly, due to ethical issues, we did not performed liver biopsy for diagnosing and determination of NAFLD grade, which is a more precise diagnostic method<sup>[42]</sup>. Secondly, the sample size of the current study was relatively small and follow-up duration was not long enough to consider the effects of DC on the hepatic system. Thirdly, High-performance liquid chromatography is needed to provide better information on flavonoid antioxidant content in DC.

In conclusion, the results of this study suggest that 30 gr of 83% dark chocolate for 12 weeks has been able to reduce weight, BMI, serum levels of AST, and MDA in NAFLD patients, but did not affect the serum levels of hs-CRP, ALT, AST/ALT Ratio and grade of hepatic steatosis. Therefore, dark chocolate supplementation might be considered as a good adjuvant therapeutic option to ameliorate oxidative stress alongside other treatments for this disease. Although

this study verifies our hypothesis, longer duration studies with larger sample size are required to elucidate the potential mechanisms of chocolate supplementation in NAFLD patients and to confirm the current data.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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