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Article

Daily consumption of a chocolate rich in flavonoids decreases cellular genotoxicity and improves biochemical parameters of lipid and glucose metabolism.

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Abstract: In recent years, Atherosclerotic Cardiovascular Disease (ACVD), Obesity and Diabetes, have increase exponentially worldwide. In the present work, we evaluate the genoprotective effect of consuming a flavonoids-rich chocolate and the improvement in the biochemical parameters related to the prevention and treatment of cardiovascular risk and metabolic syndrome in young Mexican adults. A randomized, placebo-controlled, double-blind study was undertaken in the Autonomous University of Baja California. The treatments were a daily intake of 2 grams of dark chocolate containing 70% cooca or milk chocolate. Total phenolic compounds and flavonoids were evaluated in both chocolates. Anthropometrical and Biochemical parameters were measured in the 84 participants before and after the study. Buccal epithelial genotoxicity was also evaluated from the beginning to the end of the experiment in the participants. Result suggested that flavonoids of cocoa intake have protective effects against DNA damage, and Biochemical parameters (total cholesterol, triglycerides, and LDL-cholesterol level in blood) and anthropometrical parameters (waist circumference) were also improved after six months of daily intake of 2 grams of dark chocolate with a 70% of cocoa.

Keywords: flavonoids; dark chocolate; genotoxicity, lipid metabolism, glucose metabolism.

1. Introduction

Atherosclerotic Cardiovascular Disease (ACVD) is the leading cause of death around the world[1]. Obesity, high levels of LDL, triglycerides and low levels of HDL are associated with the development of ACVD[2]. Other affections such as Diabetes, insulin resistance and hypertension are also risk factors to develop cardiovascular diseases[3]–[5]. The incidence of all these pathologies are increasing worldwide and they and they are becoming a public health problem.
Diet is one of the major lifestyle factors that can significantly influence the incidence and progression of chronic diseases such as cardiovascular disease, diabetes and cancer. Cocoa has been consumed by humans since at least as early as 460 A.D[6]. Increasing amount of reports have shown that the consumption of cocoa and dark chocolate exerts several beneficial effects on cardiovascular health and endothelial dysfunction [7]; Observations are consistent with reports that dark chocolate lower blood pressure[7] and improve endothelium-dependent vasodilatador responses[8]. Cocoa, especially dark chocolate, contains high level of flavanols such as epicatechin and catechin, and

procyanidins. Flavanols in cocoa are present as either the monomers (-) epicatechin and (+) catechin or oligomers of epicatechin and/or catechin, called proanthocyanidins or condensed tannins. Flavonoids have been widely reported by their many benefical effects in health. For example, some reports showed that epicatechins improved vascular function, reduce BP, improve insulin sensitivity, and reduce platelet activity [9].

In recent years, increasing attention has been given to compounds that induce genetic damage by various mechanisms. There is evidence that the mutagenic events and genotoxic agents may play an important role in the cause and/or progression of human diseases other than cancer[10]. Recently, cells from the oral epithelium have been used for the evaluation of exposure to various genotoxic agents, associated with its recognized sensitivity for the assessment of DNA damage. The toxicological relevance of the micronucleus test is well defined: it is a multi-target genotoxic endpoint, assessing not only clastogenic and aneugenic events but also some epigenetic effects, which is applicable in different cell types. Micronuclei in exfoliated epithelial cells are widely used as a noninvasive biomonitoring process suitable for the detection genotoxicity.

In the present work, we evaluate the genoprotective effect of consuming a flavonoids-rich chocolate and the improvement in the biochemical parameters related to cardiovascular risk and metabolic syndrome in young Mexican adults.

2. Results

2.1 Study population

Table 1 also describes important participant characteristics such as sex, education level, physical activity and Cardiometabolic co-morbidities (diabetes and hypertension). The physical activity was classified as inactive, moderate active and active (Table 1), and it was assessed via scoring criteria of the International Physical Activity Questionnaire of World Health Organization[11]. The majority of the studied population was university students, with normal blood pressure but high triglyceride and glucose levels (Table 2). Also they presented an average body mass index (BMI) of 32.1, with an incidence of 68% (Table 1). According to the World Health Organization, for adults over 20 years old, a BMI between 30.00 and 34.9 falls into one of the category of Obesity Class I[12].

Table 1. Characteristics of study population at recruitment.

Characteristics	n	(%)	Observations
Participants	84	(100)	
Sex			
Men	47	(55.90)	
Women	37	(44.10)	
Education Level (completed or in progress)			According to the information provided in the surveys conducted.
Primary School	2	(2.38)	
High School	3	(3.57)	
University	79	(94.05)	
Current smokers	32	(38.09)	
Physical activity			According to the information provided in the

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			surveys conducted.
Inactive	17	(20.24)	
Moderate active	55	(65.48)	
Active	12	(14.28)	
Obesity (BMI range)			The classification was made according to of the world health organization (WHO)
Underweight (Below 18.5)	0	(0.00)	
Normal weight (18.5–24.9)	9	(10.71)	
Pre-obesity (25.0–29.9)	10	(11.90)	
Obesity class I (30.0–34.9)	57	(67.85)	
Obesity class II (35.0–39.9)	8	(9.52)	
Obesity class III (Above 40)	0	(0.00)	
Risk Factors			
Hypertension	18	(21.41)	Defined as systolic blood pressure≥ 140mmHg and/or diastolic blood pressure≥90mmHg and/or taking antihypertensive medications
Diabetes	12	(14.28)	Defined as taking antidiabetic medications and/or having fasting plasma glucose ≥120 mg/dl (≥7 mmol/l)
Dyslipidemia	52	(61.90)	Defined as having at least one of the following anomalies: total cholesterol ≥190 mg/dl (≥4.9 mmol/l), TAG ≥150 mg/dl (≥1.7 mmol/l), LDL-cholesterol ≥115 mg/dl (≥3.0 mmol/l), HDL-cholesterol <40 mg/dl for men and <46 mg/dl for women and/or taking hypolipid medications

2.2 Determination of total phenolic and flavonoids content

Total phenols content in the commercial dark chocolate and milk chocolate used for the present study are shown in Table 2. Total phenolic content was 63.7 ± 0.2 and 56.3 ± 1.5 µmol of gallic acid equivalents per 1 gram of dark chocolate and milk chocolate respectively. Flavonoids content in the same commercial dark chocolate was 34.8 ± 0.5 µmol of (-)-catechin equivalents per 1 gram, and milk chocolate presented 10.4 ± 0.8 µmol of (-)-catechin equivalents per 1 gram. The percentage of flavonoids/phenols (%) was significantly higher in dark chocolate than in milk chocolate.

Table 2. Phenolic and Flavonoid content in used dark and milk chocolates for the study.

Used Treatment	Phenolic content (µmol Gallic acid equivalents/g)	Flavonoid content (µmol catechin equivalents/ g)	flavonoids/phenols (%)
FRC	63.7 ± 0.2	34.8 ± 0.5	54.63
MC	56.3 ± 1.5	10.4 ± 0.8	18.47

FRC= commercial dark chocolate with 70% cocoa, MC = commercial milk chocolate

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2.4 Changes in measured parameters by daily chocolate consumption

Dietary variables did not significant change by the consumption of flavonoid-rich chocolate. Certain anthropometric and biochemical variables varied after the flavonoid-rich-chocolate consumption. Dietary, anthropometric and biochemical variables measured in the present studied are reported in Table 2. Every variable was measured in each participant before and after the study of milk or dark chocolate daily consumption for 6 months.

Table 3. Dietary, anthropometric and biochemical variables before and after the study of flavonoid-rich chocolate consumption for 6 months.

Variables	**Intervention group**	**Beginning of the study Mean ± SD**	**End of the Study Mean ± SD**
Age	FRC	23.8 ± 3.4	24.6 ± 3.1
	MC	23.6 ± 3.5	23.8 ± 2.6
Participants	FRC	42	42
	MC	42	42
Dietary variables			
Fruit and vegetable intake (g/d)	FRC	523.7 ± 371.4	548.2 ± 387.9
	MC		
Total energy intake (kJ/d)	FRC	2214 ± 323	2298 ± 236
	MC	2208 ± 350	2310 ± 120
Total carbohydrate (%E)	FRC	45.3 ± 8.2	43.8 ± 8.9
	MC	46.7 ± 6.3	45.8 ± 7.2
Added sugar	FRC	5.3 ± 3.2	6.1 ± 2.7
	MC	5.8 ± 4.1	5.8 ± 3.8
Total Fat (%E)	FRC	36.9 ± 8.1	37.1 ± 7.6
	MC	34.8 ± 6.2	36.5 ± 5.1
Saturated Fat (%E)	FRC	20.8 ± 6.1	16.9 ± 6.9
	MC	21.2 ± 3.1	22.3 ± 5.9
Unsaturated Fat (%E)	FRC	14.6 ± 4.1	14.8 ± 4.6
	MC	14.5 ± 3.9	15.9 ± 3.1
Anthropometric variables			
BMI (Kg/m2)	FRC	32.1 ± 3.8	30.1 ± 2.2
	MC	31.4 ± 3.2	32.4 ± 2.5
Waist Circumference (cm)	FRC	98.7 ± 3.5	**90.4 ± 4.5***
	MC	96.9 ± 4.1	94.9 ± 3.9
Biochemical variables			
Total Cholesterol (mg/dl)	FRC	221.3 ± 16.7	**201.2 ± 19.5***
	MC	224.3 ± 18.9	227.4 ± 12.4
LDL-Cholesterol (mg/dl)	FRC	149.82 ± 18.4	**116.2 ± 21.1***
	MC	147.23 ± 21.1	138.9 ± 19.1
HDL-Cholesterol (mg/dl)	FRC	46.3 ± 12.5	43.2 ± 10.9

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	MC	45.4 ± 12.1	44.2 ± 13.5
Triglycerides (mg/dl)	FRC	228.25 ± 17.9	**153.26 ± 18.95***
	MC	223.5 ± 21.1	224.1 ± 23.1
HOMA-IR	FRC	2.3 ± 1.8	**1.93 ± 1.1***
	MC	2.5 ± 1.6	2.4 ± 1.5
Fasting plasma glucose (mg/dl)	FRC	114.23 ± 13.56	**91.23 ± 9.25***
	MC	112.31 ± 16.71	111.67 ± 10.9
HbA1c (%)	FRC	5.8 ± 1.0	4.6 ± 1.1
	MC	4.7 ± 1.0	4.5 ± 0.9
Systolic blood pressure (mmHg)	FRC	139.2 ± 10.5	**127.8 ± 11.2***
	MC	136.3 ± 21.5	133.9 ± 12.7
Diastolic blood pressure (mmHg)	FRC	87.24 ± 11.8	**84 ± 9.12***
	MC	87.28 ± 9.18	87.31 ± 9.44

FRC= commercial dark chocolate with 70% cocoa, MC = commercial milk chocolate. *P values for testing the differences among variables across two groups (before and after chocolate consumption) by using $\chi 2$ test. $P > 0{\cdot}05$

2.5 Frequency in nuclear abnormalities in buccal epithelial cells

The abnormalities of the nuclei in the buccal epithelial cells were 14.4% at the beginning, participants of both experimental groups showed abnormalities of broken egg nucleus, micronucleus and binucleus (Figure 1).

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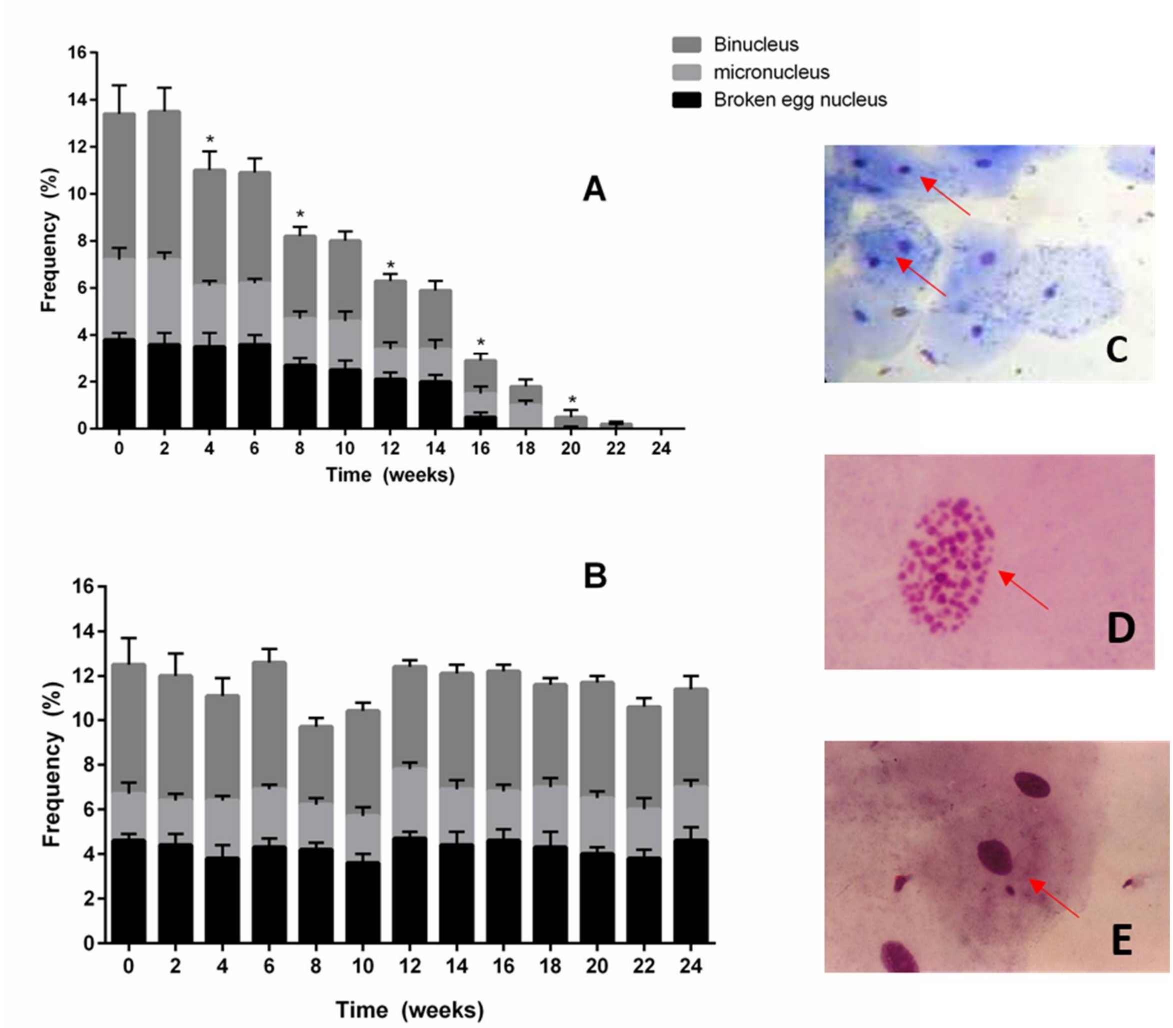

Figure 1. Frequency in nuclear abnormalities in buccal epithelial cells during consumption of dark chocolate with 70% (A) cocoa and milk chocolate (B). ** P>0.05 frequency was compared with the frequency obtained in the previous week*

3. Discussion

3.1 Determination of total phenolic and flavonoids content

Results showed that 54.6% of phenolic content in the used dark chocolate were from the flavonoids group; this percentage of flavonoids was significant higher compared with the 18% of flavonoids contained in milk chocolate. The relation flavonoids/polyphenols showed that the used commercial dark chocolate with 70% cocoa was significant better source of flavonoids compared with the used milk chocolate. Moreover, taking into account that 2 grams of dark chocolate were provided to each participant in the study; approximately 69.6µmol of (-)-catechin equivalents of flavonoids were provided to each participant daily in the treatment with commercial dark chocolate with 70% cocoa (FRC), and 20.8 µmol of (-)-catechin equivalents of flavonoids were provided to participant in milk chocolate treatment (MC) .

3.2 Characteristics of study population

84 participants were recruited for the present study most of them were university students (94%), of which 38 % were current smokers, and 65 % of them reported having a habit of moderate physical activity. 67% presented a BMI in a range of 30-34.91 due to they were classified with Obesity class I.

half of them were randomly included to the treatment where commercial dark chocolate with 70% cocoa was provided and the other half were treated with milk chocolate. The principal reported risk factor by participants was dyslipidemia, as 61.90 % of participants presented at least one of the following anomalies: total cholesterol ≥190 mg/dl (≥4.9 mmol/l), TAG ≥150 mg/dl (≥1.7 mmol/l), LDL-cholesterol ≥115 mg/dl (≥3.0 mmol/l), HDL-cholesterol <40 mg/dl for men and <46 mg/dl for women and/or taking hypolipid medications.

3.3 Determination of total phenolic and flavonoids content

According to results, the content of phenolics was slightly less in milk chocolate compared with dark chocolate, however it has been reported that sugar content could affect the measurement of phenolic content by Folin-Ciocalteu method. Interestingly, the flavonoid content was 3 fold higher in the dark chocolate compared with the milk chocolate used for the present study. Since the percentage of flavonoids/phenols (%) was significantly higher in the dark chocolate than in the milk chocolate, the used dark chocolate was a better source of flavonoids compared with the commercial milk chocolate used in the present study.

According to the flavonoid content (Table 2) in the daily chocolate intake and to that there were no significant difference in the intake of fruits and vegetables reported by the participants (Table 3), it was assumed that the flavonoid intake of participants treated with the commercial dark chocolate with 70% cocoa was significantly higher than the flavonoid intake of participants treated with the commercial milk chocolate.

2.4 Changes in measured parameters by daily dark chocolate consumption

Dietary variables did not significant change by the consumption of flavonoid-rich chocolate. However, certain anthropometric and biochemical variables varied after the flavonoid-rich-chocolate consumption (Table 2). The waist circumference of participants was significant lower ($P>0.05$) after de study, change could not be attributed to changes in dietary since Total energy intake, and the proportion of carbohydrates and lipids were similar before and after the study. Interestingly, total blood cholesterol, triglycerides and LDL-cholesterol significantly decreased after six months of daily intake of dark chocolate with high content of flavonoids ($P<0.05$). There has been previously reported that dark chocolate intake could improve LDL levels in blood [13]. Moreover, it was also reported that the consumption of flavonoids could significantly increase lipid oxidation[14], [15], thus flavonoids intake might improve lipid biochemical parameters by the modulation of reverse cholesterol transport in gut and liver[16]. Regarding to HOMA-IR and fasting plasma glucose both parameters were significantly decreased after 6 months of daily intake of the flavonoid-rich chocolate. HOMA-IR is a homeostatic model assessment (HOMA) to determine insulin resistance (IR) in β-cells. It is calculated with the proportion of plasma glucose and insulin levels. According to the results of the present study, and in accordance with previous reports[17], daily flavonoid-rich chocolate intake was significantly associated with a lower HOMA-IR ($P<0.05$). Some flavonoids, such as genistein and epicatechin has been previously associated with a lowering blood glucose concentration[14], [18]. Finally, and not less important blood pressure was significantly improved by the consumption of flavonoid-rich chocolate compared with milk

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chocolate (Table 3). Cocoa flavonoids have been previously reported for their effect in the improvement of cardiovascular parameters, such as blood pressure and platelet aggregation[19].

2.5 Frequency in nuclear abnormalities in buccal epithelial cells

The abnormalities of the nuclei in the buccal epithelial cells were 14.4% at the beginning of the study. The participants of both experimental groups showed abnormalities of broken egg nucleus, micronucleus and binucleus (Figure 1), it was expected to find nuclear abnormalities in buccal epithelial cells of participants because most of them presented risk factors that have been related with genotoxicity such as the habit of smoke, obese state and high glucose levels in plasma[20]. However the frequency of nuclear abnormalities significantly decreased with the daily consumption of flavonoid-rich chocolate compared with milk chocolate intake. This effect was a novelty finding, because flavonoids consumption in dark chocolate has not been related with antigenotoxic effect before. The decreasing effect of genotoxicity might be related with the decreasing effect of oxidative stress in cells due to the antioxidant capacity and activity of flavonoids, and the modulation of expression of CYP450[21].

4. Materials and Methods

4.1 Study Design and participants

A randomized, placebo-controlled, double-blind study was undertaken in the Autonomous University of Baja California. The study was approved by the Ethics Committee of faculty of Medicine and Psychology of Autonomous University of Baja California, and written consent was obtained from all subjects prior to enrollment in the study.

In brief, a random sample was recruited between November 2015 and January 2018. The inclusion criteria for the enrollment in the study were 1) Being Mexican of Mexican parents, 2) Being young adults from 20 to 35 years old, and 3) To have at least 3 of 5 risk factors: Glucose greater than 100 mg/dl, Triglycerides greater than 160 mg/dl, LDL greater than 130 mg/dl, HDL lower than 45 mg/dl, and Body mass index (BMI) greater than 29. Exclusion criteria for participation in the study were: to take antihypertensive, hypocholesterolemic or weight-loss medications. The elimination criteria were to start taking antihypertensive, hypocholesterolemic or weight-loss during the experiment and to decide to leave the study. Trained research staff provided the participants detailed instructions of the study, assisted them in completing questions on dietary information and then checked the completeness and accuracy of responses. The dietary intervention was carried out during 6 months with a weekly follow-up of the participants. During the weekly interviews a dietary survey was carried out, questions of the study were answered and it was checked that the participants did not fall into elimination criteria. The study began with 92 participants after data cleaning, particularly for poorly completed dietary data. During the study 8 people was eliminated from the study because of elimination criteria and the rest of the participants finished the study (n=84).

4.2 Chocolate consumption (independent variable)

The commercial dark chocolate of 70% cocoa content or milk chocolate was provided to participants by the research staff weekly packaged in daily portions, the daily dose for each patient was 2g of chocolate. Weekly a semi-quantitative survey was completed by the participants, including

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questions on habitual daily consumption of chocolate during the previous week. The participants reported their frequency of consumption ranging from 1 to 7 days of consumption. They also selected the serving size based on how many packaged portions take every day.

4.3 Determination of total phenolic content

Total phenolic content determination was made using the Folin-Ciocalteu method [22], Briefly, Extraction was performed with 80% methanol in water (v/v), then total polyphenols content was determined by using Coulter DU 520 Spectrophotometer at 750 nm. A standard curve was developed using Gallic acid (Sigma–Aldrich, Michigan, USA).

4.4 Determination of flavonoids content

Flavonoids were evaluated in the 80% methanol in water (v/v) extracts with a colorimetric method according to previous reports [22], using a Coulter DU 520 Spectrophotometer at 510 nm. A standard curve was developed with (-)–catechin (Sigma-Aldich, Michigan, USA).

4.5 Epithelial genotoxicity, biochemical and anthropometric parameters measure (dependent variables)

After 4 weeks of daily consumption of flavonoid-rich chocolate a blood sample was obtained for each participant in order to measure biochemical parameters. Standard laboratory assays were used to measure Biochemical parameters with IDEXX Catalyst Dx® equipment. Anthropometric parameters were also measure every 4 weeks with a digital weighing scale and a measuring tape. Genotoxicity was measured in each participant by micronuclei assay. Briefly, exfoliated cells were collected by a non-invasive sampling method from oral mucosa. Buccal cavity cells are obtained by scraping the cheeks with a tongue depressor. The samples are transferred dropwise to pre-cleaned slides. Then, the slides are air-dried and fixed with 80% methanol. After that slides were stained with hematoxylin for 3-5 minutes and then with eosin for 5-8 minutes. Genotoxic damage was determined by the observation of slides in the microscope; the cellular malformations were observed and counted.

4.6 Statistical analyses

Data were analyzed by using MiniTab and GraphPad Prism (GraphPad Software Inc., CA, USA) software. Results are reported as mean ± SEM. Statistical differences for mean data obtained from present study were analyzed by two way ANOVA. Correlation was made using MiniTab MiniTab General Linear Model.

5. Conclusions

In conclusion, flavonoids of cocoa have protective effects against DNA damage, it is suggested that the reducing genotoxic stress effect is related with the antioxidant activity of flavonoids and the modulation of CYP450. However, further in vivo studies are needed to determine their mechanism of action of antigentotoxic effect. Biochemical parameters (total cholesterol, triglycerides, and LDL-cholesterol level in blood) and anthropometrical parameters (waist circumference) were also improved after six months of dark chocolate with a 70% of cocoa intake. Interestingly daily flavonoid-rich chocolate intake also improve fasting plasma glucose levels and insulin resistance parameter (HOMA-IR). These effects were attributed to the proportion of flavonoids in the chocolate which was 3- fold greater than in milk chocolate. Results suggested a potential beneficial effect of daily dark chocolate consumption in lipid and glucose metabolism.

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