Journal of Food and Nutrition Research, 2021, Vol. 9, No. 1, 45-49
Available online at http://pubs.sciepub.com/jfnr/9/1/7
Published by Science and Education Publishing
DOI:10.12691/jfnr-9-1-7

Regulation of CD36 Gene Expression by *Hibiscus Sabdariffa* Tea Extracts to Affect the Atherosclerosis Biomarkers in Saudi Women

Asmaa Talal Al-Malki[1 *], Heba Abbas Ahmed Sindi[2], Mohammed Hussain Al-Qahtani[3]

[1]Department of Food and Nutrition Science, Faculty of Science, Taif University, Taif, Saudi Arabia
[2]Department of Food and Nutrition, Faculty of Human Sciences and Designs, King Abdul-Aziz University, Jeddah, Saudi Arabia
[3]Centre of Excellence in Genomic Medicine Research (Cegmr) King Abdulaziz University (Kau), Jeddah, Saudi Arabia
*Corresponding author: asma@tu.edu.sa

Received November 20, 2020; Revised December 24, 2020; Accepted January 07, 2021

Abstract **Scope:** Cardiovascular diseases (CVDs) share a major part of the total deaths (about 46%) in Saudi Arabia. There are indications of an association between the bioactive components in *Hibiscus sabdariffa*, in affecting the atherosclerosis biomarkers, the major cause of (CVDs). An intervention study aimed to see the effect of *Hibiscus sabdariffa* tea extracts on regulating CD36 gene expression in human to prevent atherosclerosis. **Methods and results:** The blood sample were twice collected. In addition, anthropometric measurements were determined at baseline, and a usual diet was followed while consuming the *H. sabdariffa* tea twice a day for six consecutive weeks. Following termination of exposure period blood samples were withdrawn. The results revealed that the OxLDL levels were significantly decreased (P=0.013, P< 0.05). Another result of special interest was that the CD36 gene expression significantly experienced, a down regulation in the gene level was detected (P= 0.003, P<0.05), whereas the CD36 protein levels significantly registered an increase (P=0.05, $P\leq$ 0.05). There was an obvious positive correlation between the CD36 gene expression and the OxLDL. **Conclusion:** In conclusion, a possible effect of *Hibiscus sabdariffa* tea extracts has potential in affecting the atherosclerosis biomarkers evidently rejected by regulating the CD36 gene expression. Further evaluation is suggested to examine the effect of pure anthocyanins glucoside.

Keywords: *CD36 gene expression, anthocyanin, atherosclerosis, bioactive components, antioxidants*

Cite This Article: Asmaa Talal AL Malki, "Regulation of CD36 Gene Expression by *Hibiscus Sabdariffa* Tea Extracts to Affect the Atherosclerosis Biomarkers in Saudi Women." *Journal of Food and Nutrition Research*, vol. 9, no. 1 (2021): 45-49. doi: 10.12691/jfnr-9-1-7.

1. Introduction

Globally each year nearly 30-41% of all deaths are caused by cardiovascular diseases (CVDs), between 1990 and 2013, climbing from 12.3 to 17.3 million deaths and according to an analysis of data from 188 countries, these figures are expected to grow by 2030 up to 23.6 million. [1,2] according to an analysis of data from 188 countries. [2] Moreover, the distribution of major causes of death in the world, including CVDs, and the proportions of cardiovascular deaths caused by ischemic heart disease, cerebrovascular disease, inflammatory heart disease, rheumatic heart disease, hypertensive heart disease, and other cardiovascular diseases have been quoted by the World Health Organization (2011). [1] Additionally, in Saudi Arabia the relative cardiovascular disease related deaths make up around 46% of total deaths, for all ages, both male and female. [3] However, the oxidative modification of low-density lipoprotein (OxLDL) is known to be involved in the development of cardiovascular diseases, especially Atherosclerosis, and can lead to heart attack or death. Moreover, when the oxidized LDL in blood plasma is increased, the CD36 expression is up-regulated, where CD36 is a fundamental factor in atherosclerosis due the formation of foam cells. [4] Kao *et al.* (2009) aimed to evaluate the effect of *Hibiscus* anthocyanin-rich extracts on foam cell formation and the gene expression of scavenger receptor, CD36, and its upstream transcription factor, PPARγ, on OxLDL treated mouse macrophage J774A.1 cell. The results of this study showed that CD36 was inhibited by anthocyanins. [5] Anthocyanins can be described as secondary metabolites, as well as an abundant flavonoid group in the plant kingdom that is widely consumed and has several health benefits. Therefore, increasing consumption of anthocyanins lowers the risk of cardiovascular disease (CVD). [6] So, the previous study clarified the effect of anthocyanins contained *H. sabdariffa* that may prevent cardiovascular disease (CVD) especially atherosclerosis. The present research focuses on the antioxidant effect of *Hibiscus sabdariffa* on bioactive components, especially anthocyanins effect on gene expression regulation to prevent atherosclerosis.

2. Material and Methods

2.1. Kits and Chemicals

ImProm- II ™Reverse Transcription system (Promega, USA), RNeasy Mini Kits (Qiagen, Germany), Human soluble cluster of Differentiation 36 (sCd36) Elisa kit 96 test (No. MBS753477), ELISA kit for Oxidized Low-Density Lipoprotein (OxLDL) 96 well strip plate (No. SEA527Hu),) USCN, China).

2.2. Preparation of *H. sabdariffa* Extract

Dried *H. sabdariffa* herbal tea bags were obtained from the local market (Al Wefak Al Saudi for Food Ind, Egypt). Each tea bag contains 2g of grounded leaves. *H.sabdariffa* tea was prepared by adding 240 ml of boiling water (100°C) to herbal tea bag. Each bag was infused for 10 min, to this one cube of sugar (5 g) may be added. [7]

2.3. Research Design and Plan of the Study

The research was seeking to assess the effectiveness of a commercially available *H. sabdariffa* tea on reducing the risk of atherosclerosis in human intervention study. The volunteers belonged to King Abdul-Aziz University and the study secured the ethical approval, Ref 20-15 and preceded applying strictly the recommendation of the research from the ethics committee of King Abdul-Aziz University. Sixteen female volunteers were asked to sign on a consent form before beginning of the study.

The study setup, include where an anthocyanins rich tea was consumed by healthy volunteers selected, enrolled and recruited as students, staff and the general population. A three-day washout period norm was followed before starting the intervention. Participants were asked to avoid certain foods base on a low-flavonoid diet before the studies period.

This was explained to them and were assisted by a list for their guidance. Each participant was asked to fast for 8-12 hours before visiting the researcher at the end of the three-day wash-out time. In addition, subjects were also made to prepare and present a dietary record for three days, before the intervention. This was to determine if results were not altered or changed due to a dietary pattern. Before the intervention, blood sample were collected in resting state and in no test drink condition. Record of anthropometric measurements at baseline were separately maintained. Participants were followed to follow their usual diet throughout the intervention. The exposure included consuming of *H. sabdariffa* tea, one cup, about approximately of 240 ml 1 tea bag, twice a day for six weeks' duration. Thus, the total time for the study was six weeks. At the end of the intervention period, all the subjects were made to fast overnight and the blood sample were drawn.

2.4. Determination of OxLDL Levels

The determination of OxLDL concentration in serum was performed with ELISA kit for Oxidized Low-Density Lipoprotein (OxLDL) 96 - well strip plate (No. SEA527Hu).

2.5. Determination of sCD36

The determination of CD36 concentration in the serum was performed with the human soluble cluster of Differentiation 36 (sCd36) Elisa kit 96 tests.

2.6. (RT-PCR) Analysis

Total RNA was extracted using the RNeasy Mini Kits (Qiagen, Germany). The ImProm-II™ Reverse Transcription System kit was used in this study. It includes reverse transcriptase and an optimized set of reagents designed for efficient synthesis of first-strand cDNA in preparation for PCR amplification in the RT-PCR.

2.7. Statistical Analysis

Data was analyzed as mean±standard deviation (± STD) from individual magnitude. Statistical differences were analyzed with a T-test after examining the normality distribution of the samples. The relationship between OxLDL and sCD36 were tested by Pearson correlation. SPSS program version (20) was used, and $P < 0.05$ was considered significant in all types of statistical analysis.

3. Results and Discussion

3.1. Effect of *Hibiscus sabdariffa* on OxLDL

The early step in atherosclerosis and the development of complications includes an increase in the level of low-density lipoprotein oxidation that promotes endothelial dysfunction, [8] which begins when LDL infiltrates the intima and is readily oxidized by resident macrophages or endothelial cells. Moreover, C-reactive protein (CRP) and OxLDL can synergistically act to increase monocytes inflammatory properties through Monocyte Chemoattractant Protein-1 (MCP-1), Prostaglandin E2(PGE2), and Matrix metalloproteinase-1(MMP-1) production, as well as attract further circulating monocytes through the release of (MCP-1) to adhere the activated dysfunctional endothelial cells and extravasate to the intima to scavenge OxLDL. [9]

However, Monocyte/macrophage CD36 plays a critical role in the development of atherosclerotic lesions by its capacity to bind and endocytose oxidized low-density lipoprotein (OxLDL) macrophages as a scavenger receptor, and ultimately becomes implicated in the differentiation of resident macrophages into foam cells that constitute the atherosclerotic lesion core. [9] The OxLDL can serve as ligands for the nuclear hormone receptor PPARγ, once engaged and activated. It drives the expression of CD36 gene, and its expression on macrophages is increased and the uptake of OxLDL "loads" the cells with cholesterol, creating foam cells. [10]

The current study investigates the effect of consuming *H. sabdariffa* tea on decreasing the OxLDL levels of the participants, and the results showed that there was a statistically significant difference (P=0.013, P< 0.05) in OxLDL levels of the participants before and after a period of consuming *H. sabdariffa* tea, which means that the tea had an appositive effect in lowering OxLDL levels (Figure 1).

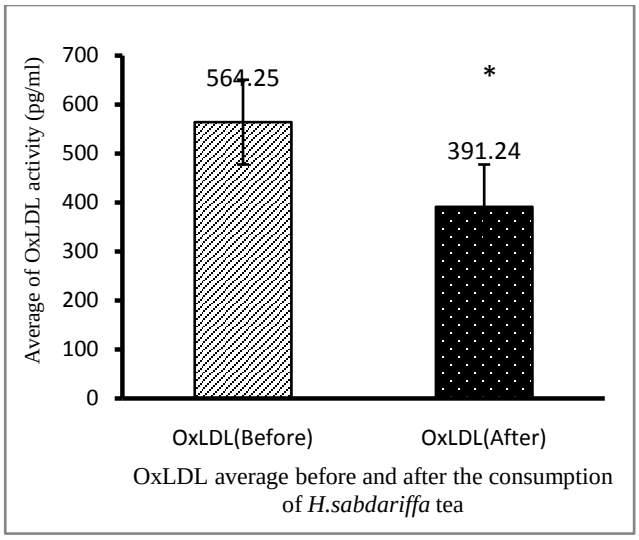

Figure 1. The total average of the OxLDL levels before and after consumption of *H. sabdariffa* tea, (*) there is a statistically significant difference

These results are in agreement with a study conducted by Yi *et al*. (2010). Which compared the inhibitory effect of diverse anthocyanins against oxidized low-density lipoprotein-induced endothelial injury, since some anthocyanins, including delphinidin, cyanidin, cyanidin-3-glc and peonidin-3-glc are reported to influence the function of endothelial cells and have an inhibitory effect on oxidative injury in endothelial cells. However, the inhibitory effects of 21 anthocyanins on OxLDL-induced endothelial injury in human vascular endothelial cell lines was measured through cell viability, which found that 20 anthocyanins could significantly inhibit OxLDL significantly ($P < 0.05$) over 100 μM, and delphinidin and delphinidin-3-glucoside were found to be the most potent anthocyanidin and anthocyanin. Furthermore, the different positions of various substituents of OH moieties and hydroxylation at C3′, C4′ and C3 significantly increased the inhibitory effects of anthocyanins. [11]

Kao *et al*. (2009) also investigated the effect of *Hibiscus* anthocyanin-rich extracts (0.05-0.2 mg/ml) on foam cell formation and the gene expression of scavenger receptors on OxLDL treated mouse macrophage J774A.1 cell. Oxidized low-density lipoprotein (OxLDL) was previously thought to atherogenesis promote through foam cell formation; However, it showed that the natural extract may inhibit OxLDL-mediated foam cell formation and prevents lipid accumulation in cells treated with OxLDL (50μg/ml), where it provides artificial antioxidant efficacy regarding to its anthocyanins that scavenge free radical and reduce the production of oxidative LDL whenever endogenous antioxidants are consumed when oxidative modification of LDL occurs, LDL oxidation can be effectively inhibited by *H.sabdariffa* extract. [5]

According to Hopkins *et al.* (2013), the most common explanation for the mechanisms of hypotensive and anticholesterol effects in a comprehensive review of animal and human studies is the antioxidant effect of anthocyanins where they inhibit LDL-C oxidation, which impedes atherosclerosis and is an important cardiovascular risk factor. [12] These findings are in agreement with the current study, in that the daily consumption of *H. sabdariffa* tea substantially decreased OxLDL levels.

3.2. Alteration in Gene Expression and *Hibiscus sabdariffa*

Anthocyanins and their aglycones, which are rich in *hibiscus* extracts, show an effect on gene expression related to atherosclerosis, as seen in the scavenger receptor CD36 in mouse macrophages. [5] Since CD36 scavenger receptor is up-regulated by OxLDL, which was shown to be necessary for the differentiation of macrophages into foam cells. Moreover, the endocytosed OxLDL induces important and complex transcriptional changes in monocytes-derived-macrophages that includes an up-regulation of the CD36 expression. [9]

Bilberry anthocyanin-rich extract alters expression of genes related to atherosclerosis development in the aorta of apo E-deficient mice; a greater focus will be on CD36 gene expression where it is a critical factor in the formation of atherosclerotic foam cells since the increased oxidized LDL in blood plasma has been shown to up-regulate CD36 expression, and the impaired regulation of LDL is caused by oxidative stress. As antioxidants, anthocyanins can reduce oxidation through a free radical scavenging effect, thus inhibiting expression of CD36. Dietary anthocyanins can also reduce LDL oxidation and oxidative damage in endothelial cells. [4]

The CD36 gene expression (RT-PCR) analysis for the participants from whole blood samples after the RNA was purified and nucleic acid was determined in our study revealed that the consumption of *H. sabdariffa* tea for six weeks showed significant down regulation in the gene level (P= 0.003, P<0.05), but CD36 protein levels were significantly increased (P=0.05, $P \leq 0.05$). This increase in CD36 protein levels is due to the post translation modification (PTM), which refers to the covalent and generally chemical (enzymatic) modification of proteins during or after protein biosynthesis, and has wide effects broadening its range of functionality. Some types of post-translational modification are consequences of oxidative stress. [13]

As for Mauray *et al*. (2012) study, that the bilberry anthocyanin-rich extract supplemented diet that affected the expression of 1261 genes was used, while 707 were down regulated. The genes are involved in cell adhesion/migration, angiogenesis and lipid metabolism. Moreover, bilberry anthocyanin-rich extract impacted other relevant processes affecting atherosclerosis, including the response to oxidative stress and lipid transport, where by the gene expression levels were down-regulated. However, a nutritional dose of bilberry anthocyanin-rich extract can also affect the expression of numerous aortic gene encoding proteins that are involved in oxidative stress, inflammation, trans endothelial migration and angiogenesis. [14]

In this context, Mauray *et al.* (2010) aimed to investigate the impact of bilberry anthocyanin-rich extract (BE) supplementation (0.02%) on gene expression in the livers of apo mice for two weeks with a standard diet. This study suggested that anthocyanin supplementation modulated the expression of genes associated with cardiovascular disease and affected cholesterol biosynthesis related genes. [15] Kao *et al*. (2009), also investigated the effect of *Hibiscus* anthocyanin-rich extracts (0.05-0.2 mg/ml) on foam cell formation and the gene expression of

scavenger receptors in OxLDL treated mouse macrophage J774A.1 cell. Thus, the macrophage uptake of OxLDL was inhibited and may involve CD36 downregulation, as CD36 mRNA expression in mouse macrophage J774A.1 cell was significantly down regulated by antioxidant efficacy of *Hibiscus* anthocyanin-rich extracts. [5] There is a view that atherosclerosis may be prevented by modulating genes, involved in adhesion and the endothelial barrier function. In fact, a mixture of oligomeric procyanidins on human vein endothelial cells (HUVECs) in-vitro, has indeed induced significant gene expression changes in both resting and TNF-alpha- stimulated cells. [16]

In conclusion, *H. sabdariffa* is an anthocyanin rich plant, certainly has a pharmacological value especially as an antioxidant. The aim of the study was to confirm this aspect in anthocyanins and further focused on its efficacy in regulating the CD36 gene expression in human. The current study found that *H. sabdariffa* tea inhibits the OxLDL and CD36 gene expression and argued that there is an obvious correlation in relation to the two tested parameters.

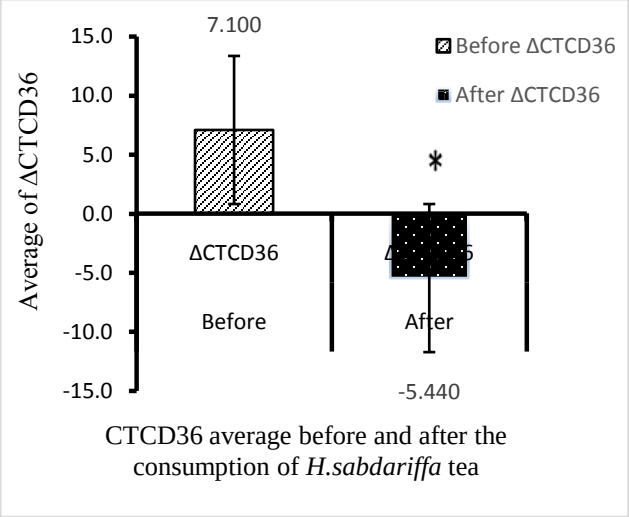

Figure 2. The total average of the ΔCTCD36 levels before and after consumption of *H. sabdariffa* tea, (*) represent statistically significant difference

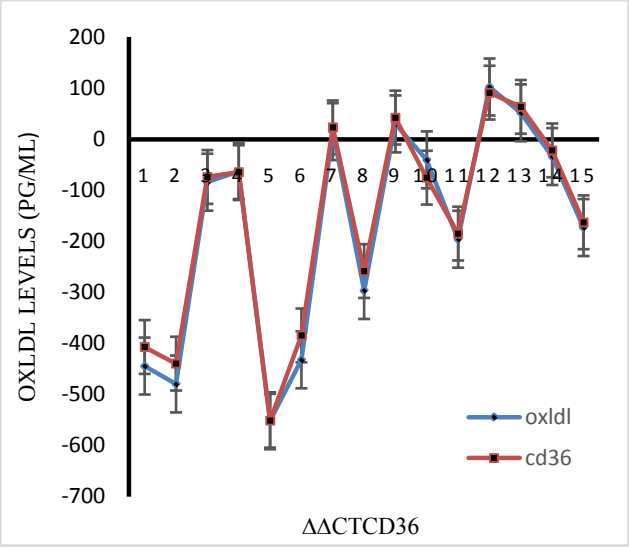

Figure 3. The correlation Between CD36 gene expression and OxLDL Levels after the consumption of *H. sabdariffa* tea

Acknowledges

The author wishes to thank Dr. Mohammed Hussain Al-Qahtani and Dr. Heba Abaas Sindi for their Supervision of this research.

My thanks to the staff of king Abdul-Aziz University, the Center of Excellence in Genomic Medicine Research and King Fahd Center for Medical Research.

Special thanks to the members of staff of Home Economic college- Food and Nutrition department.

Acknowledge the funding support of to king Abdul-Aziz City for Science and Technology for the financial support for the project which enables me in provision of requirements and necessary substances for the research.

Abbreviations

CD36	Cluster of Differentiation 36
CVDs	Cardiovascular Diseases
OxLDL	Oxidative modification of low-density lipoprotein
PPAR	Peroxisome Proliferator -activated receptor
PTM	Post Translation Modification
ROS	Reactive Oxygen Species
SPSS	Statistical Package for Social Sciences

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