

Title Page

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Running title: Antiatherogenic effect of licorice extract

Abstract

Aim: Ethanolic extract of licorice root has been shown to reduce low-density lipoprotein (LDL) oxidation in atherosclerotic mice and in hypercholesterolemic, as well as in normal lipidemic humans. The aim of this study was to examine the effect of licorice-root extract on carotid intima-media thickness (CIMT) in individuals with hypercholesterolemia.

Methods: Individuals with hypercholesterolemia (total cholesterol ≥ 6.18 mmol/L [240 mg/dL]) and a baseline ultrasound measurement of carotid intima-media thickness (CIMT) above 0.7 mm without significant stenosis were randomly allocated to two groups: an experimental group that consumed 0.1 gr/day of ethanolic extract of licorice root for 12 months, and a control group that received a placebo drug of similar appearance.

Results: Participants were 94 individuals (46 men and 48 women, aged 41 - 80 years, mean 54.5 ± 16.3). For 69% (35/51) in the experimental group CIMT decreased by 0.1mm or more compared to 12% (5/43) in the control group. Mean plasma total cholesterol levels were 284 ± 32 and 272 ± 28 for the study group, $p < 0.05$, and 291 ± 35 and 289 ± 31 for the control group, $p =$ not significant (NS) at baseline and one-year, respectively. Mean plasma LDL cholesterol levels were 181 ± 27 and 167 ± 23 for the study group, $p = 0.05$, and 187 ± 24 and 189 ± 22 for the control, $p =$ NS, respectively. Mean high-density lipoprotein (HDL) did not change significantly in either group.

Conclusion: Following one year of licorice consumption, mean CIMT and LDL levels were decreased. This suggests that licorice may attenuate the development of atherosclerosis and of related cerebral vascular diseases.

Key words: Licorice extract, Carotid intima media thickness, Atherosclerosis, LDL cholesterol

Introduction

The dried roots of the licorice plant *Glycyrrhiza glabra* have been consumed for the past 6000 years. They are used as flavoring and sweetening agents, and as demulcents and expectorants in the Western world; and as anti-allergic and anti-inflammatory agents in Japan and China [1].

Ethanollic extract of licorice root has been shown to reduce low-density lipoprotein (LDL) oxidation in atherosclerotic mice [2] and in hypercholesterolemic, as well as in normal lipidemic humans [2, 3]. . This shows that dietary consumption of licorice-root extract by hypercholesterolemic patients may act as an antioxidant agent against cardiovascular disease [4].

Carotid intima-media thickness (CIMT) is commonly used as a predictor of cardiovascular outcome in patients with metabolic conditions such as obesity, diabetes and cardiovascular diseases. The easy applicability and noninvasiveness of sonography render it suitable as a surrogate endpoint for measuring atherosclerotic burden in people with hypercholesterolemia risk factors [5].

The aim of the study was to analyze the anti-atherogenic effect of licorice-root extract consumption in patients diagnosed with hypercholesterolemia, according to changes in LDL and CIMT.

Methods

Study Design

This is a double-blind. The participants, physician and researcher and all those involved with the conduct of the study were blinded from the group allocation except the sonographer.

The main endpoint after one year was change in carotid intima media thickness (CIMT), and the secondary endpoint was change in laboratory for lipid parameters.

Patients

The study population consisted of 110 ambulatory patients who arbitrarily recruited and diagnosed with hypercholesterolemia (of at least the following level: total cholesterol ≥ 6.18 mmol/L [240 mg/dL]) by a family physician (Table 1). For the purpose of this study each patient underwent screening including a detailed medical history and physical examination, electrocardiography (ECG), complete blood count (CBC), and chemistry screen (e.g., glucose, sodium, creatinine), including lipid profile and liver function tests (enzyme tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT); bilirubin, and albumin). Patients were excluded if the above levels were abnormal, if diagnosed with dyslipidemia, and if they had hypertension, diabetes mellitus, or ischemic heart disease. Patients on any anti lipid oxidant (e.g., vitamin E, omega 3, olive oil/fish formula, juice supplement) were asked to stop taking them at least 2 weeks before consuming the licorice.

Patients were lottery randomly divided into two groups: experimental group A (n=59) and control group B (n= 51) (Table 1). Group A (experimental) consumed a daily dose (free of charge) of 0.1 grams of DGL ethanol-extraction licorice for 12 consecutive months, whereas Group B (control) consumed a placebo drug. The color,

odor, shape, taste and packaging of the placebo were the same as that of DGL ethanol-extraction licorice. The DGL ethanol-extraction licorice and placebo were supplied by the manufacturer free of charge, but the manufacturer was not involved in the design and analysis of this study.

Ethical committee

The institutional ethics committee of health maintenance organization (HMO), Kupat Holim Kelalit, which is under the administrative control of the Israeli Ministry of Health, approved the study, and its guidelines for the study have been implemented. Therefore, the study complied rigorously with data protection legislation, and was conducted according to the principles of the Helsinki Declaration, following standards of good clinical practice.

Outcome measures

CIMT was measured for all study participants before and one year after starting consumption of licorice or placebo. CIMT is defined as the distance between the luminal - intima and medial - adventitia interfaces at the distal 10 mm of the common carotid artery.

Ultrasound examination of the carotid arteries was performed with patients in the supine position, the head slightly hyper extended and turned to the opposite side. Axial and sagittal scans were obtained using a high resolution linear transducer of 5-12 or 3-9 MHz (Philips IU22, Bothell, Washington). Wall thickness and atherosclerotic plaques in the carotid arteries were searched for. CIMT was measured on the far wall of the distal common carotid artery on each side, 1-2 centimeters proximal to the bifurcation (Figure 1). An electronic software was applied, which

follows the two echogenic lines on the far wall of the carotid artery: the lumen-intima interface and the media-adventitia interface (Figure 2). Normal CIMT in adults is in the range of 0.6-0.8 mm; slightly increased, moderately increased and severely increased CIMT are in the range of 0.8-1.0 mm, 1.0-1.5 mm and above 1.5 mm, respectively (Figure 3). CIMT measurements were performed according to an international consensus statement [5], by skilled personnel working in the US Unit, Department of Medical Imaging.

Categorization of the outcome measures

The measured changes in CIMT after one year were categorized as: 0= no change; +1= 0.1 mm reduction; -1= 0.1mm increment; +2= 0.2mm reduction; -2= 0.2 mm increment; +3= 0.3 mm reduction; -3= 0.3 mm increment; +4= 0.4 mm reduction; -4= 0.4 mm increment. For both groups the baseline posterior wall CIMT was ranged from 0.7– to 2.0 mm, without any significant stenosis. Progression and regression in carotid atherosclerosis were defined as an increase and decrease, respectively in the CIMT from baseline to 12 months, following daily licorice or placebo consumption. The change was measured in the same pre-selected carotid artery segments.

Statistical analysis

All analyses were carried out with the ASPSS Version12 statistical package. Quantitative results were reported as mean \pm SD. The data were tested by analysis of variance (ANOVA) and the means were compared across groups by the Tukey test, significance was considered as $P \leq 0.05$.

Results

Only 94 participants concluded the study, (age range 41 to 80 years, mean age 54.5 ± 16.3), eight participants from each group dropped out from the study (personal reason $n=11$; medical reason $n= 5$). All 94 participants stated that they consumed the licorice supplement or the placebo for at least 90% of the time throughout 365 days.

For 69% (35/51) in the experimental group CIMT decreased by 0.1mm or more compared to 12% (5/43) in the control group. For 12 % (7/51) of the experimental group CIMT increased by 0.1mm or more compared to 67% (29/43) in the control group. For 9 participants in each study group CIMT did not change during the course of the year (Figures 4-5).

After one year mean plasma total cholesterol levels decreased from 284 ± 32 to 272 ± 28 mg/dl ($p < 0.05$), which was due to a decrease in plasma LDL cholesterol from 181 ± 27 to 167 ± 23 levels mg/dl (8% reduction), $p = 0.05$. No significant changes in mean plasma total cholesterol and LDL were detected in the control group: from 291 ± 35 to 289 ± 31 mg/dl and from 187 ± 24 to 189 ± 22 mg/dl. No significant changes in mean HDL were observed (Table 1).

Discussion

Cardiovascular (CVD) diseases are leading causes of deaths and disability worldwide. The role of micronutrients has been studied extensively as a CVD risk minimizing intervention. These include antioxidants that are available over the counter as dietary supplements. Despite high commercialization of such products, scientific evidence and clinical trials supporting their use are not yet conclusive [6]. The lipid peroxidative hypothesis of atherosclerosis indicates that low-density lipoprotein (LDL) oxidation plays a crucial role in early stage of atherogenesis [7]. This hypothesis is

supported by evidence that oxidized LDL (Ox-LDL) is present in atherosclerotic lesions [8] and in human plasma from patients with cardiovascular diseases, and that it correlates with the presence of angiographically documented complicated plaques in mice [9]. Antioxidants can protect LDL from oxidation not only by their binding to the lipoprotein, but also following their accumulation in cells of the arterial wall [10]. Licorice was shown to be a very potent antioxidant against LDL oxidation [11] and, in parallel, to inhibit atherosclerosis development in Vit. E-deficient mice [12,13]. One-month consumption of ethanolic extract of licorice root by moderately hypercholesterolemic patients was shown to reduce plasma cholesterol levels by 5% and plasma LDL levels by 9% [2]. These results are similar to those of our study (reductions by 4% and 8%, respectively). However, our results were recorded after one year consumption.

The relationship between licorice consumption and cardiovascular diseases may be due to attenuation of LDL oxidation, macrophage foam cell formation and progression of atherosclerosis. The effect of licorice on arterial cell-mediated oxidation of LDL is determined by its accumulation in the lipoprotein and in arterial cells, such as macrophages. Licorice can reduce LDL lipid peroxidation by scavenging reactive oxygen/nitrogen species, chelation of transition metal ions and sparing of LDL-associated antioxidants. Licorice can also reduce oxidative stress of macrophages by inhibition of cellular oxygenases (12, 14). Oxidized LDL is highly atherogenic as it stimulates macrophage cholesterol accumulation and foam cell formation; it is cytotoxic to cells of the arterial wall and stimulates inflammatory and thrombotic processes. LDL oxidation can lead to subsequent aggregation, which further increases cellular cholesterol accumulation [15].

Recent improvements in imaging technology have made possible noninvasive assessment and identification of early vascular changes using ultrasonography. B-mode ultrasound is a noninvasive, accurate modality that accurately visualizes the arterial wall thickness. CIMT is constituted by the combined thickness of the intima and media layers of the artery wall, measured at the far wall of the distal common carotid arteries. CIMT is measured by high resolution B-mode ultrasound of extra-cranial carotid arteries, and is the most widely accepted and it is the only non-invasive surrogate marker for early atherosclerotic disease [16, 21, 22]. Increased CIMT is an intermediate stage in the continuum of atherosclerosis, which significantly correlates with coronary and cerebrovascular disease [17]. Epidemiological studies have consistently reported a predictive value of increased CIMT for myocardial infarction and stroke, independent of traditional CV factors [18,19]. This has been confirmed in a meta-analysis of 12 relevant general population-based studies [20]. The current study showed a noteworthy decrease in CIMT in the experimental group compared to the control group. Pomegranate juice consumption was also shown to lead to significant CIMT reduction, by up to 30%, after 1 year. The effect was attributed to the potent antioxidant characteristics of pomegranate juice polyphenols [21]. Two large observational studies provided solid evidence that CIMT may be used as an indicator of generalized atherosclerosis. Increased CIMT and carotid plaques are associated with high levels of atherogenic lipids and lipoproteins, plasma total homocysteine, and haemostatic factors[22] Elevated CIMT in patients with cardiovascular risk factors, such as diabetes mellitus, hypertension, smoking, male sex and advanced age is associated with an increased risk for myocardial infarction and stroke. A decrease in CIMT subsequent to drug treatment is associated with reduced incidence of vascular events [17,23].

Limitations of the study

We did not identify any placebo effects in any of the measures investigated. However, no statistical difference between experimental and control/placebo values was found, yet we suggest that a cross over study with two weeks of washout period could provide more adequate results.

Future directions

Several explanations may be proposed for our findings, including a number of mechanisms of antioxidant activity against LDL oxidation, and the pathogenesis of atherosclerosis prior to clinical presentation. Therefore, we suggest that future study should focus rather than on indirect antioxidant effect of licorice, clearance of oxidative damage may be a more effective strategy for the treatment of anti-atherosclerotic phenomenon in patients with hypercholesterolemia.

In conclusion, this study demonstrated possible anti-atherogenic capabilities of licorice in two related components of atherosclerosis: plasma lipoproteins and arterial CIMT. We suggest that a potent anti-oxidative capacity of licorice against lipid peroxidation may be the central link for the anti-atherogenic effects of licorice.

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Table 1: Clinical and demographic characteristics of the study patients

| | Experimental Group A n=51 | Control group B n=43 | p-value |
|--|--|--|----------------|
| Gender | 24 men | 22 men | |
| Age (years) | 56.5 ± 17.9 | 52.5 ± 15.4 | NS |
| BMI | 30.8 ± 0.99 | 30.1 ± 1.17 | NS |
| <i>Mean levels of cholesterolemia (mg/dl):</i> | | | |
| Total cholesterol : beginning of study | 292 ± 32 | 290 ± 30 | NS |
| Total cholesterol : after 12 months | 280 ± 51 | 291 ± 35 | 0.02 |
| LDL Cholesterol : beginning of study | 189 ± 22 | 188 ± 22 | NS |
| LDL Cholesterol : after 12 months | 172 ± 27 | 187 ± 34 | 0.01 |
| HDL Cholesterol : beginning of study | 40 ± 12 | 38 ± 15 | NS |
| HDL Cholesterol : after 12 months | 39 ± 19 | 38 ± 10 | NS |

Figures

Figure 1: Intima-media thickness (IMT) is the blood-intima (upper arrow) to media-adventitia (lower arrow) interfaces width.

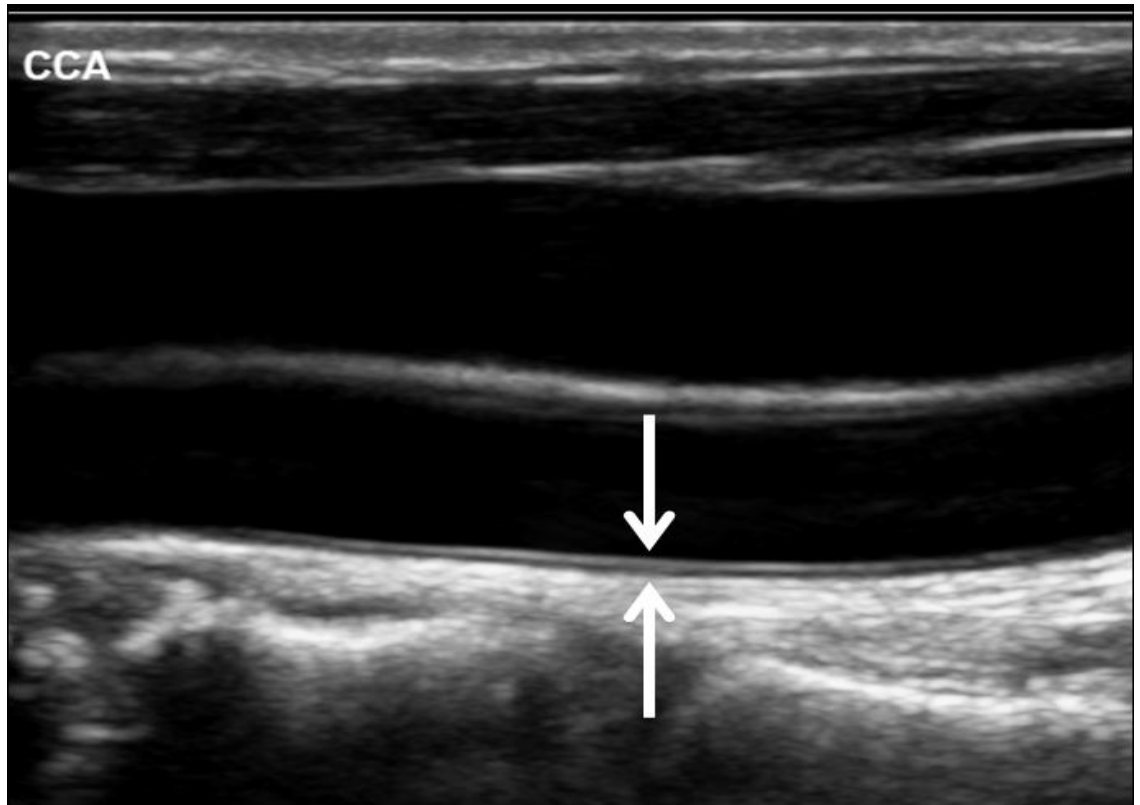


Figure 2: Electronic measurement of the IMT is performed by placing the cursors on the interfaces, along a centimeter at the distal common carotid artery. A normal IMT is demonstrated (0.6 mm width), with 100% success (see Success=100% at the side of the image).

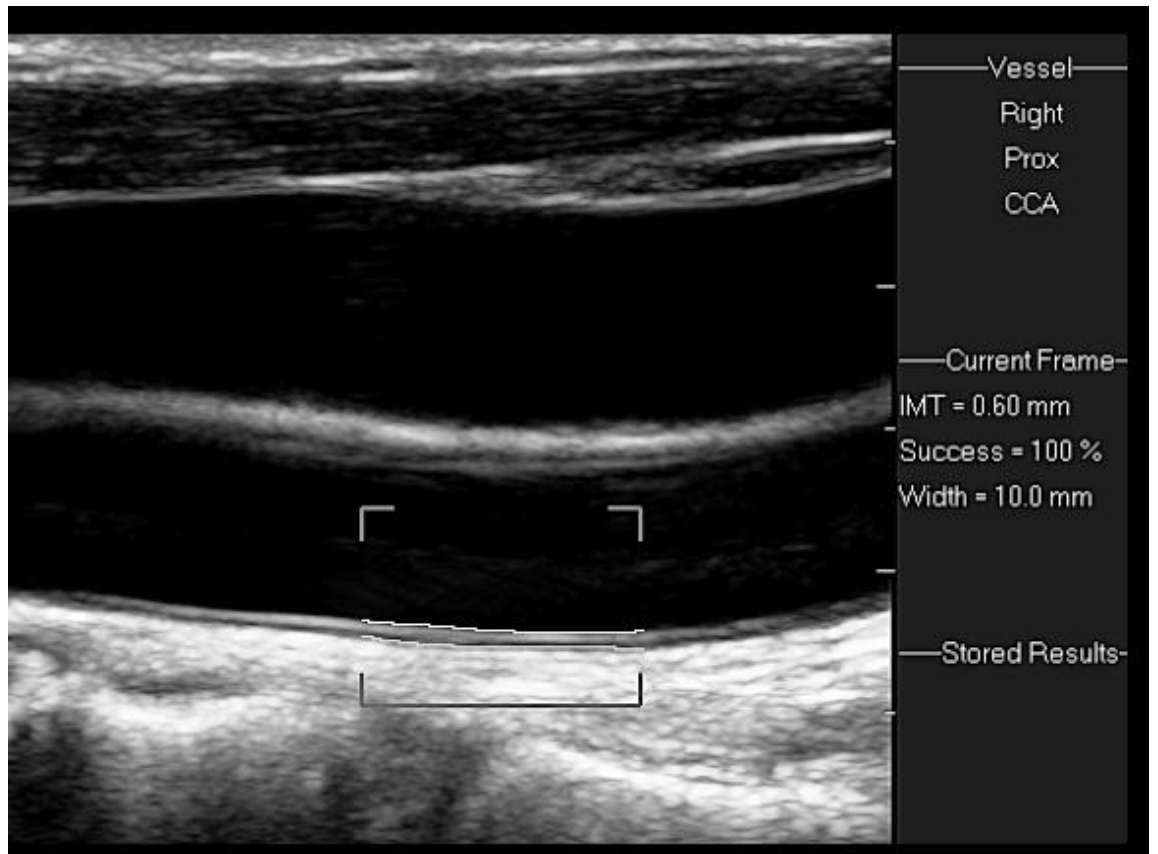


Figure 3: Moderately enlarged IMT. A 1.35 mm width on the distal CCA is measured, with 99% success.

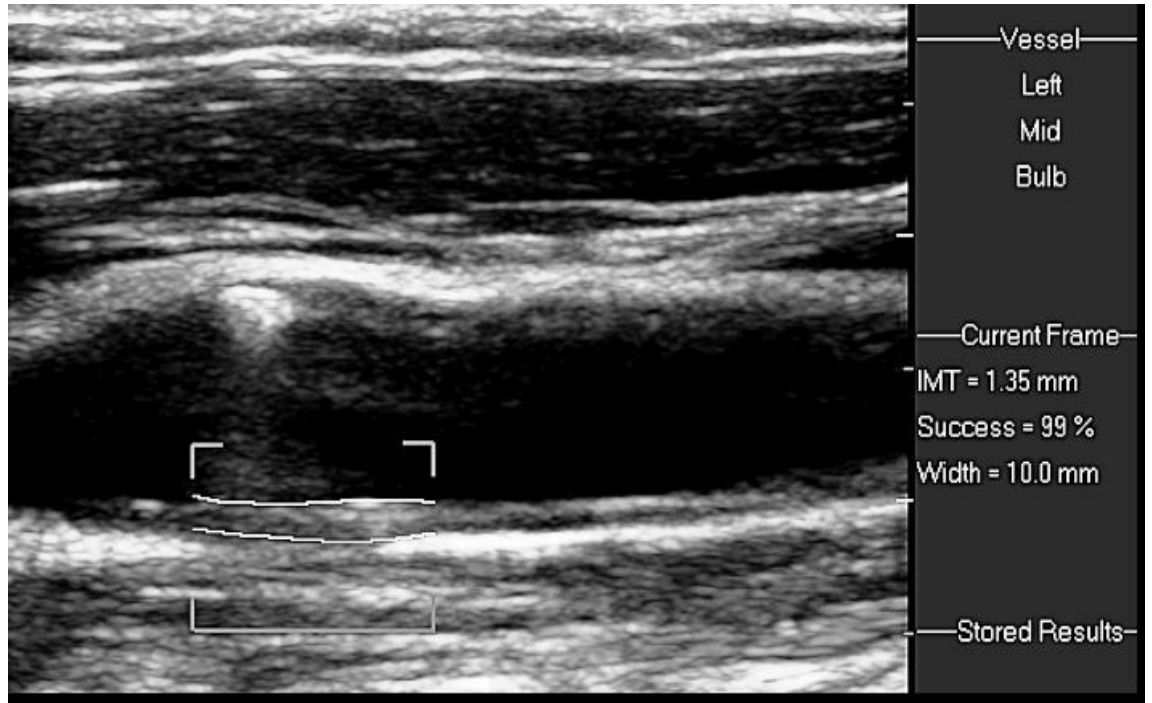


Figure 4: The change in CIMT between the experimental group (black) and control group (grey) after one year of licorice consumption and placebo consumption, respectively.

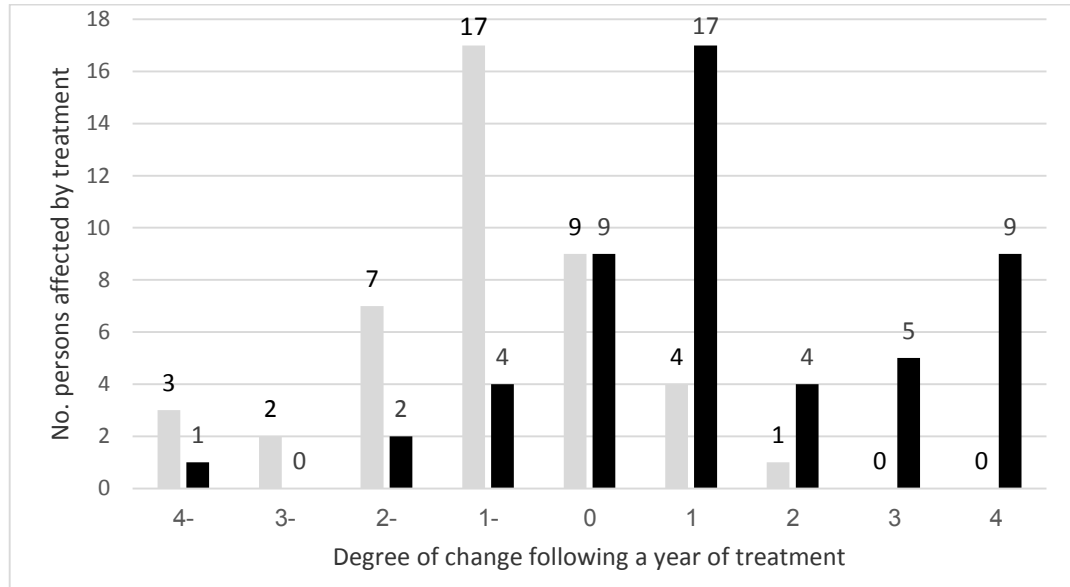


Figure 5: The change in percentage in CIMT between the experimental group (black) and control group (grey) after one year of licorice and placebo consumption,

respectively

