

Inhibitory effect of walnut (*Juglans regia* L.) husk hydroalcoholic extract on LDL oxidation *in Vitro*

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Abstract:

Introduction:

Lipid oxidation, including LDL oxidation, plays a pivotal role in development of atherosclerosis. Supplementing diet with antioxidants, such as vitamin E and walnut husk hydroalcoholic extract, may prevent LDL oxidation. In the present study, we investigated the *in vitro* effects of walnut husk hydroalcoholic extract on serum LDL oxidation caused by copper sulfate.

Method:

Blood samples were obtained from healthy individuals after an overnight fasting. Serum LDL was isolated. A control group was created using the serum LDL alone, and experiment groups were created using serum LDL oxidized by CuSO₄ and walnut husk hydroalcoholic extract at concentrations of 0.2, 2 and 20 µg/mL. LDL oxidation was assessed through measuring conjugate dienes, lag times and malonedialdehyde (MDA) to provide a basis for comparing the effects of different concentrations of walnut husk hydroalcoholic extract on serum LDL oxidation in the presence of copper.

Results:

Our findings indicate that walnut husk hydroalcoholic extract reduces serum LDL oxidation, with lag times equal to 87%, 178% and 202% for concentrations of 0.2, 2 and 20 µg/mL, respectively. In other words, the inhibitory properties of walnut husk hydroalcoholic extract on LDL oxidation are proportionate to the extract concentration.

Conclusion:

The findings of the present study indicate that walnut husk hydroalcoholic extract prevents LDL oxidation. This compound may have similar effects *in vivo*.

Keywords: walnut, LDL, In Vitro

Introduction:

LDL oxidation plays an essential role in development of atherosclerosis. Dietary intake of oxidants creates oxidized LDL which favors the development and progression of atherosclerosis (1). Oxidizing

LDL diminishes its affinity for its receptor. Accumulation of oxidized LDL in macrophages creates foamy cells and atherosclerosis. Ingestion of antioxidants, such as vitamin E and herbal substances like walnut husk which is rich in antioxidants,

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may prevent LDL oxidation and consequently, atherosclerosis (2). The pathogenesis of atherosclerosis is a complicated process, but, as current evidence suggests, lipid and LDL oxidation is a critical parameter for formation of atherosclerotic plaques (3, 4). Moreover, oxidative and pro-inflammatory factors create a vicious cycle for progression of atherosclerosis as an inflammatory condition (5-7).

The plant used in the present study is "walnut" with the scientific name *Juglans regia*. It is a native flora of Iran. Walnut husk is rich in antioxidants such as phenolic compounds (8, 9), most notably naphthoquinones and flavonoids. Juglone (5-hydroxy 1,4-naphthoquinone) is a known compound present in fresh walnut leaves and husk, but it is scarce in dry walnut leaves and husk due to polymerization (10). Other substances, such as chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, myricetin, hydroxyaminic acids, quercetin, catechin and other phenolic compounds, are abundant in walnut green husk (11, 12). Walnut leaf is used for treating rheumatismal pains, fever, diabetes and cutaneous diseases; its root is used in treating diabetes, and walnut blossoms are used in treating malaria (13). In addition, walnut leaf is extensively used in traditional medicine for treating headaches, frost bites and skin conditions. It lowers the risk of cardiovascular diseases and is helpful for hemorrhoids diarrhea, fungal diseases, hypertension and elevated blood sugar (10, 14). Few studies have dealt with walnut husk and no study has been conducted so far to investigate its effects on reducing serum oxidation *in vitro*.

Theoretical evidence suggests that oxidative stress is the most important factor in development of atherosclerosis. Thus, any substance with inhibitory properties on oxidative stress may be used to prevent

atherosclerosis. It appears that treatment with walnut husk extract or other antioxidants may be helpful in suppressing oxidative stress and other injurious mechanisms. The aim of the present study is to investigate the antioxidant effects of walnut husk hydroalcoholic extract on LDL oxidation caused by copper sulfate.

Material and Methods:

Materials: Disodium ethylene diamine tetra acetate (Na_2EDTA), potassium bromide, sodium chloride, and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma Corporation.

Preparing walnut husk hydroalcoholic extract: Walnut husk hydroalcoholic extract was obtained from the Herbal Medicine Research Center at Lorestan University of Medical Sciences. In order to prepare the extract, walnut green husk was dried in shade and then ground. 100 g of the ground substance was incubated in 50% alcohol in Soxhlet extractor for 9 hours at 50°C. The resulting extract was filtrated, dried and the resulting waxy substance was preserved at 4°C (2.287%).

Blood sampling: Blood samples were obtained from healthy individuals and centrifuged at 3000 rpm for 10 minutes to have their serum isolated. In order to prevent oxidation, sodium nitrite was added at a final concentration of 0.06% wt/vol.

LDL isolation: Serum LDL was isolated using discontinuous concentration gradient ultracentrifugation at Cardiovascular Research Center at Isphahan University of Medical Sciences. Serum density was raised to 1.21 g/mL with addition of potassium bromide (0.365 g/mL). 3.5 mL sodium chloride (0.154 mol/L) and 1.5 mL concentrated serum were added to centrifuge tubes and then centrifuged at 40000 rpm for 2.5 hours at 10°C in Beckman L7-55 ultracentrifuge apparatus. The resulting yellow layer containing LDL was harvested,

deoxidized in normal saline phosphate buffer (0.01 mol/L Na₂HPO₄, 0.16 mol/L NaCl, pH 7.4) for 48 hours at 4°C, and dialyzed in 0.01% sodium nitrite and 0.01% ethylene diamine tetra acetic (EDTA). The buffer was changed three times during dialysis (15).

LDL oxidation: After LDL isolation, its protein concentration was measured using Bradford's method (16). In order to assess LDL oxidation, it was raised to 150 µg/mL with 10 mmol/L PBS pH=7.4. Subsequently, a control sample containing LDL alone, a sample containing 10 µmol/L copper sulfate without extract, and samples containing 10 µmol/L copper sulfate and walnut husk hydroalcoholic extract at concentrations of 0.2, 2 and 20 µg/mL were prepared. Solvent was added to the control and copper samples to render their volume equal to extract samples. Over a period of 5 hours, the absorption rate of ultraviolet light at 234 nm was measured for the solutions every 10 minutes to follow the oxidative change in LDL (17). In order to assess the kinetics of LDL oxidation, we plotted absorption rates of samples versus time. Using this curve, we determined the lag times and final concentration of conjugate dienes after 5 hours using a molar extinction coefficient of 29500 L/mol/cm.

Measurement of produced malondialdehyde (MDA): The final product of lipid peroxidation, MDA, was quantified using Burge and Aust's method. For this purpose, LDL samples were added copper sulfate and extract and then incubated at 37°C for 5 hours. The oxidation process was stopped with addition of EDTA with final concentration of 2 mmol/L. In

order to measure lipid peroxidation, 1.5 mL of 0.6% thiobarbituric acid and 1 mL of 10% trichloroacetic acid were added to 100 µL of the sample and boiled for 30 minutes. After cooling off, the solution was centrifuged at 1000 rpm for 15 minutes and absorption rate of the supernatant at 532 nm was measured. The obtained values with a molar extinction coefficient of 1.56×10^5 L/mol/cm were considered as the MDA produced and reported as nm/mg-LDL-protein (18, 19).

Statistical analysis: The findings of the present study are reported as mean \pm standard deviation. The significance of differences between groups was verified using Mann-Whitney test and SPSS software.

Results:

After addition of copper and the respective concentrations of walnut husk hydroalcoholic extract to the samples and reading the absorption rates every 10 minutes, the kinetic curve of LDL oxidation was drawn and the conjugate dienes and MDA were quantified. Our findings indicate that walnut husk hydroalcoholic extract significantly reduces LDL oxidation *in vitro* ($p < 0.05$). The impact of walnut husk hydroalcoholic extract on inhibition of LDL oxidation is linearly proportionate to the extract concentration.

In order to study the kinetics of LDL oxidation, absorption rates at 234 nm were plotted versus time (Diagram 1). Three distinct sections are observed in this diagram, namely the lag phase, the propagation phase (where LDL oxidation is intensified) and decomposition phase (where LDL oxidation is terminated).

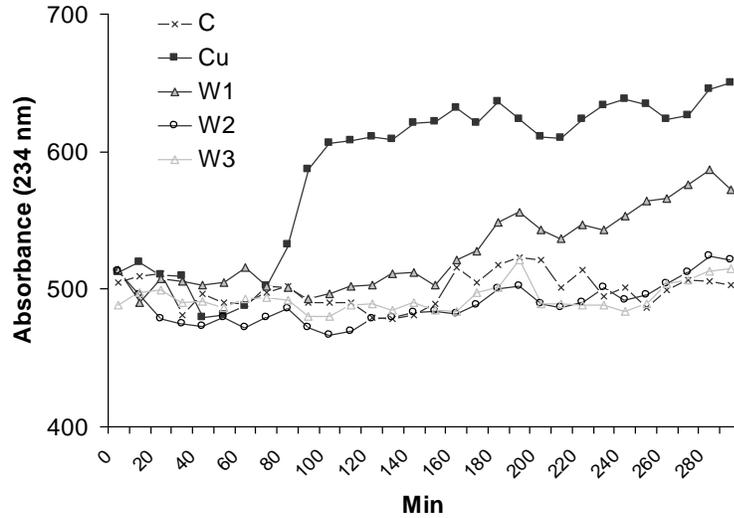


Diagram 1: Kinetics of inhibition of LDL oxidation by walnut husk hydroalcoholic extract in 10 mmol/L PBS solution, pH=7.4, at 37°C for 5 hours. Each point is the average of three trials. C: n-LDL; Cu: copper + n-LDL; W1: n-LDL + 0.2 µg/mL walnut husk hydroalcoholic extract; W2: n-LDL + 2 µg/mL walnut husk hydroalcoholic extract; W3: n-LDL + 20 µg/mL walnut husk hydroalcoholic extract.

Oxidative changes of LDL were determined with measuring UV absorption rate of the solution at 234 nm after 5 hours. Using this, the final concentration of conjugate dienes was calculated using the molar extinction coefficient of 29500 L/mol/cm (Diagram 2).

The findings indicate the impact of walnut husk hydroalcoholic extract to be significant. Furthermore, we determined the lag times using the kinetic curve (Diagram 3).

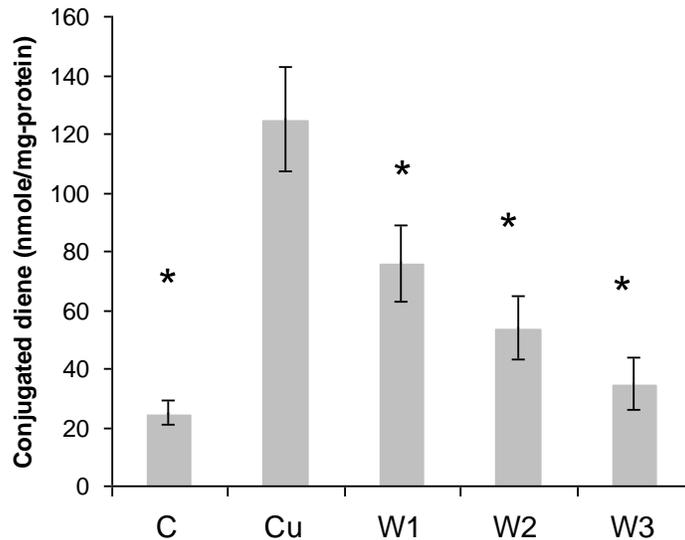


Diagram 2: Comparing the effect of walnut husk hydroalcoholic extract on inhibition of conjugate dienes resulting from LDL oxidation. Each point is the average of three trials. Asterisk (*) represents significance compared to copper ($p<0.05$). The abbreviations are similar to those used in Diagram 1.

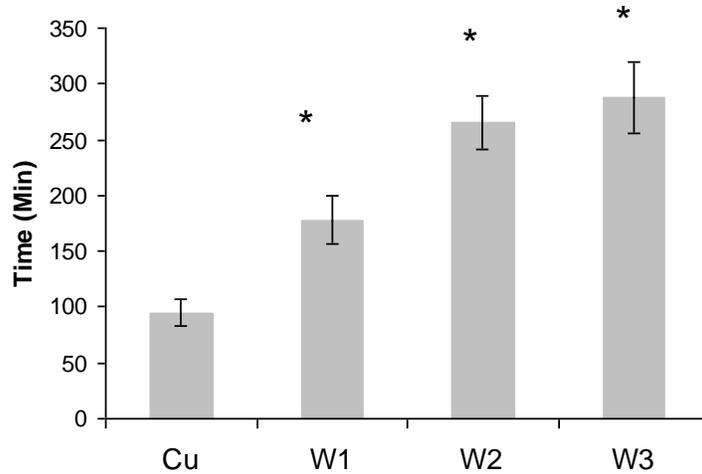


Diagram 3: The effect of walnut husk hydroalcoholic extract on lag times. Each point is the average of three trials. Asterisk (*) represents significance compared to copper ($p < 0.05$). The abbreviations are similar to those used in Diagrams 1 and 2.

In order to study the MDA produced, we followed the protocol mentioned above to read the absorption rates of samples at 532 nm using the molar coefficient and reported the produced MDA as nm/mg-LDL-protein. According to our findings, the effect of

different solutions of walnut husk hydroalcoholic extract in reducing the MDA produced is significant compared to the sample containing copper sulfate without the extract (Diagram 4).

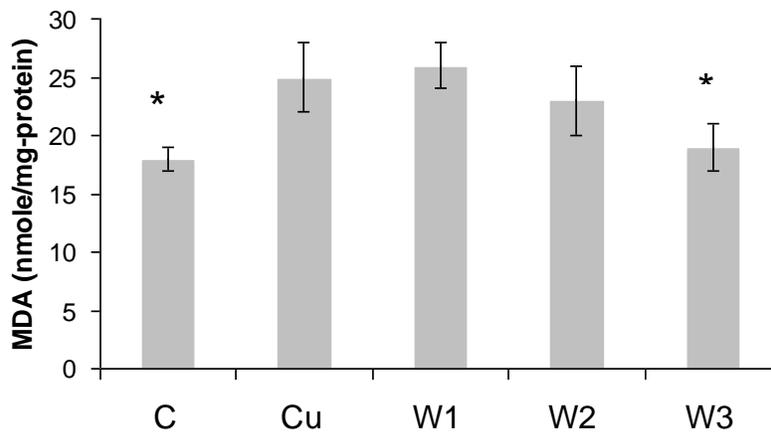


Diagram 4: The effect of walnut husk hydroalcoholic extract on inhibition of MDA production resulting from LDL oxidation. Each point is the average of three trials. Asterisk (*) represents significance compared to copper ($p < 0.05$). The abbreviations are similar to those used in Diagrams 1 and 2.

Conclusion:

As indicated by findings of the present study, walnut husk hydroalcoholic extract inhibits LDL oxidation efficiently. LDL oxidation in vessel walls is the primary

cause of development and progression of atherosclerosis (2). Previous studies suggest that individuals with atherosclerosis have a relative deficiency in antioxidants compared to healthy individuals (3). Copper produces

free radicals via the Fenton and Habrois reaction (20, 21) which is probably the mechanism through which LDL oxidation occurs, increasing lipid peroxides and conjugate dienes and reducing the antioxidants in LDL structure (e.g. vitamin E) due to their interaction with free radicals created by copper (2). Our findings indicate that walnut husk hydroalcoholic extract reduces conjugate dienes and MDA significantly, while increasing lag times, as well. Walnut husk hydroalcoholic extract is a potent antioxidant; although few studies have been conducted to investigate its effects, there is evidence confirming its antioxidant and antibacterial properties, as

well as its use in cancer treatment (13, 22). One study measured the antioxidant parameters (including reducing power, free radical scavenging power, and total antioxidant capacity) of husks of 5 walnut species in Portugal to conclude that the antioxidant properties of walnut husk are comparable, and in some cases superior, to other antioxidants such as vitamin E, beta-carotene and butylated hydroxyanisole (BHA) at similar concentrations (22-26).

Conclusion: The findings of the present study indicate that walnut husk hydroalcoholic extract inhibits LDL oxidation efficiently.

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