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Inhibitory Effect of *Allium sativum* on Low-Density Lipoprotein (LDL) Oxidation Induced by $CuSO_4$ *In-Vitro.*

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ABSTRACT

Cardiovascular disease is the leading cause of mortality. An increased concentration of plasma low-density lipoprotein (LDL) cholesterol constitutes a major risk factor for atherosclerosis and oxidation of low-density lipoprotein has been strongly suggested as a key factor in the pathogenesis of atherosclerosis. Thus the inclusion of some anti-oxidant compounds in daily diet may inhibit the production of oxidized LDL and may decrease both the development and the progression of atherosclerosis**.** The present work investigated the inhibitory effects of aqueous and methanolic extract of *Allium sativum* on LDL oxidation induced by $CuSO_4$ quantitatively *in vitro*. The extent of oxidation was assessed by formation of conjugated dienes, lipid peroxides and thiobarbituric acid reactive substances (TBARS). Antioxidant properties of crude (aqueous and methanolic) extracts of *Allium sativum* were also studied by six *in vitro* models viz. radical scavenging activity by DPPH reduction assay, scavenging of SO, H_2O_2 and NO, reducing power and FRAP assay. Both extracts were found to contain large amounts of phenolic compounds and flavonoids. The results indicate that the inhibitory effect of the garlic on LDL oxidation were dose-dependent at concentrations ranging from 10 to 30µg/ml. The garlic prevented the oxidation of LDL *in vitro* and it may suggest that they have a similar effect *in vivo*.

Keywords: Low-density lipoprotein oxidation, garlic, $CuSO_4$, Antioxidant capacity.

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INTRODUCTION

There is strong evidence suggesting oxidative modification of low density lipoprotein (LDL) plays an important role in initiating vascular inflammation and atherosclerotic lesion formation [1-3]. It is likely that oxidative modifications of LDL involve lipid peroxidation and the modification of apolipoprotein B-100, followed by macrophage uptake and cell accumulation of cholesterol to generate foam cells, causing early atherosclerotic lesions [4].

Once an atheroma has developed, oxidised lipids can produce surface fissures, resulting in larger and more occlusive atherosclerotic lesions [5-7]. According to the oxidation hypothesis, LDL is protected against oxidative stress by using antioxidants, thereby delaying the formation of modified LDL [8-10]. Therefore, the use of antioxidants as dietary supplements to protect LDL particles against oxidation may reduce both the development and the progression of atherosclerosis [11].

Garlic (*Allium sativum*) has distinct nutritional profile with special reference to its bioactive components and is used in different diet-based therapies to cure various lifestyle-related disorders. Commercial and non-commercial preparations of this plant are increasingly used as 'health supplements' and include garlic powder, garlic oil, water and ethanol extracts of raw garlic and an aged garlic extract. It has already been shown that Alliums such as *Allium cepa* and *Allium sativum* could decrease the formation of atherosclerotic lesions in animal models. Epidemiological data suggest that an inverse relationship exists between the intake of antioxidant and the risk of coronary artery disease.

This work was undertaken to investigate the effect of *A.sativum* on the modification of LDL induced by $CuSO_4$ *in vitro* by monitoring the formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS). Also in this study various methods were used to assess the antioxidant activity of the extracts including free radical scavenging capacity and total antioxidant activity.

MATERIALS AND METHODS

Plant material and preparation of extracts

Garlic bulbs were collected and authenticated. The dry skins of the bulb were removed before use; then the cloves were peeled and crushed finely and made into a paste by using mortar and pestle. Thirty grams of the garlic paste was then weighed and extracts were prepared using both methanol (methanolic extract) and distilled water (aqueous extract) as solvents and diluted appropriately before use.

Estimation of phytochemical constituents:

Estimation of total phenol content (TPC)

The total phenol content was determined by Folin- Ciocalteu reagent method [12] and expressed in terms of gallic acid equivalent (mg/g) [13].

Estimation of total flavonoids (TF)

The total flavonoid content was determined by aluminum chloride method [14] and expressed in terms of quercetin equivalent (mg/g).

Estimation of sugars

Estimation of sugars in the extract was done by DNSA method [15] Maltose is a reducing sugar which will reduce 3,5 – dinitro salicylic acid (DNSA) to 3 – amino – 5 – nitrosalicylic acid in alkaline medium that is orange coloured and absorbance was measured at 525 nm. Sugar content was expressed in terms of maltose equivalent (mg/g).

Estimation of tannins

The tannin content was determined by Folin-Ciocalteu reagent method [13] and expressed in terms of tannic acid equivalent (mg/g).

Evaluation of antioxidant activity

α, α-diphenyl- β-picryl-hydrazyl (DPPH) radical scavenging assay

The free radical scavenging activity was measured using 2, 2-diphenyl-1-picryl-hydrazyl or 1, 1-diphenyl-2-picryl-hydrazyl by the method of McCune and Johns [16]. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the nonradical form DPPH-H. The absorbance was measured at 517nm [13]. DPPH scavenging activity was expressed in terms ascorbic acid equivalent (mg/g).

Nitric oxide (NO) radical scavenging assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured using the Griess reagent at 540 nm [17, 13]. NO radical scavenging activity was expressed in terms of ascorbic acid equivalent (mg/g).

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)- s-triazine (TPTZ). Decrease in the absorbance (593 nm) is proportional to the antioxidant content. [13]. The antioxidant capacity was expressed in terms of ascorbic acid equivalent (mg/g).

Estimation of reducing power (RP)

The reducing power was determined by the method of Athukorala *et al.* (2006) [18]. Reducing power may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. Absorbance was measured at 700 nm [13]. RP was expressed in terms of ascorbic acid equivalent (mg/g).

Superoxide anion (SO) radical scavenging assay

The superoxide anion scavenging activity was measured as described by Robak and Gryglewski(1998) [19]. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture [13]. SO anion scavenging activity was expressed in terms of gallic acid equivalent (mg/g).

Hydrogen peroxide (H_2O_2) radical scavenging assay

The ability of plant extracts to scavenge hydrogen peroxide is determined according to the method of Ruch *et al.* (1989) [20] .H_2O_2 is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (OH^-). Decrease in absorbance at 230 nm was determined [13]. H_2O_2 radical scavenging activity was expressed in terms of ascorbic acid equivalent (mg/g).

Isolation of LDL

Blood samples after an overnight fasting were collected in plain tubes. To obtain fresh serum, blood samples were left to coagulate and then centrifuged (3000 rpm for 10 min at 4°C). The LDL fraction was isolated from fresh serum by heparin citrate method [21] briefly 5 ml of 0.064M Na Citrate buffer, pH5.04 with 50,000IU/l heparin was mixed with 0.5 ml of serum, vortexed, and centrifuged at 1000g for 10 minute. The supernatant was removed and LDL precipitate was dissolved in 1 ml 1% triton X-100.

Oxidation of LDL and Continues monitoring of formation of conjugated dienes

After isolation of total LDL, the protein content of LDL was measured [22]. LDL concentration was adjusted to 200 µg/ml of protein with 10 mM PBS, pH 7.4 and then aliquots of *A.sativum* extracts were added to the solution. The oxidative modification of LDL was initiated by addition of freshly prepared 1 mM $CuSO_4$ solution at 37°C in a water bath for 5 h. [23] Lipoprotein oxidation in samples was quantified by conjugated diene formation measured by change in absorbance at 234 nm (delta A 234) [24,25].

Estimation of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation end products were determined as TBARS according to modified method of Ohkawa *et al.* (1979) [26]. The thiobarbituric acid reactive substances (TBARS) assess lipid hydro peroxide. Lipid hydro peroxide reacts with trichloroacetic acid and thiobarbituric acid to form pink colour adduct that was measured at 532 nm. The results were expressed as nmol MDA/mg LDL-protein.

Statistical Analysis

The results are expressed as mean ± SD. All measurements were replicated three times.

RESULT AND DISCUSSION

Some natural antioxidants present in dietary sources, although in a small amount, can contribute powerfully to increasing the oxidative resistance of LDL. The oxidative modification of LDL is the major factor that stimulates the development and progression of atherosclerosis [27]. Therefore, in the present investigation, antioxidant activity of methanolic and aqueous extracts of *A. sativum* was assessed using *in vitro* model.

Table 1 represents the phytochemical constituents in *Allium sativum* extracts. It revealed presence of high content of phenolics, flavonoids and tannins in aqueous than in the methanolic extracts. Phytochemical studies have demonstrated the presence of several chemicals, including flavonoids and sulphur-containing compounds: diallyl sulphate, alliin, ajoene, and allicin [28]. Since phenolics and flavonoids are responsible for the antioxidant activity, presence of high amount present in the extract indicates good antioxidant activity.

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [29]. Aqueous extract again displayed striking DPPH radical scavenging activity than methanolic extract (Table 2) that might be attributed to their hydrogen donating ability. The total antioxidant activity and hydrogen peroxide radical scavenging activity was found higher in aqueous extract. Allicin, the main component in aqueous extract from raw garlic scavenges hydroxyl radicals and inhibit lipid peroxidation [30]. The antioxidant properties of allicin may explain, at least in part, the ability of these extracts to inhibit Cu^{2+}-induced lipoprotein oxidation in humans (Table 2). Interestingly, it has been shown that other garlic compounds such as S-allylcysteine ,N-acetyl-S-allylcysteine, S-allylmercaptocysteine, alliin, allixin [31,32] and S-ethylcysteine, N-acetylcysteine,diallyl sulfide, and diallyl disulfide [33] are able to inhibit Cu^{2+}-induced LDL oxidation.

Table 1: Phytochemical constituents in *Allium sativum* extracts

Tests	Standard equivalent in methanolic extract (µg/g)	Standard equivalent in aqueous extract (µg/g)
Total phenol content	41.86 ±5.64	272.3 ±11.63
Total flavonoids	14.4 ±5.76	25.91 ±2.88
Sugar content	0.84±0.216	4.06 ±0.40
Tannin content	69.32±21.33	634.5±29.88

(The results obtained were expressed as Mean ± S.D. of triplicates)

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Table 2: Antioxidant activity of *Allium sativum* extracts

Tests	Standard equivalent in methanolic extract (µg/g)	Standard equivalent in aqueous extract(µg/g)
Total antioxidant activity	1835.34±106.73	2294.3±112.29
DPPH Scavenging assay	93.01±13.32	366.24±15.103
NO Radical scavenging assay	8.58±1.16	6.08±0.7
FRAP assay	1.5±0.09	1.3±0.017
Reducing power assay	10.36±0.64	13.82±0.98
SO radical scavenging assay	14.41±0.305	7.433±0.355
H_2O_2 radical scavenging assay	1.57±0.023	1.83±0.005
H radical scavenging assay	315.92±0	66.35±13.33

(The results obtained were expressed as Mean ± S.D. of triplicates)

The toxicity and damage caused by NO and O_2 is multiplied as they react to produce reactive per-oxynitrile ($ONOO^-$) which leads to serious toxic reactions with biomolecules. In our study the crude aqueous extract of *Allium sativum* showed a remarkable nitric oxide radical scavenging activity (Table 2). It is well documented that NO plays a crucial role in the pathogenesis of inflammation where it is secreted as a mediator and this may explain the use of *Allium sativum* extract for the treatment of inflammatory diseases [34,35].

Superoxide anion plays an important role in plant tissues and is involved in the formation of other cell-damaging free radicals [36]. Both garlic extracts exhibited excellent superoxide anion scavenging activity, which can be correlated with its high flavonoid content and its stronger odor that correlates with its sulfide content.

Reducing power of plant extract was reported to be directly correlated with its antioxidant activity and is based on the presence of reductants like quercetin-3, 5-diglucoside and cyanidin-3-sophoroside-5-glucoside which exerts antioxidant activity by breaking the free radical chain and donating a hydrogen atom[37,38]. Reducing power and sugar content is highest in aqueous garlic extract.

Figure 1: Inhibitory effects of garlic extracts on generation of conjugate dienes with respect to time.

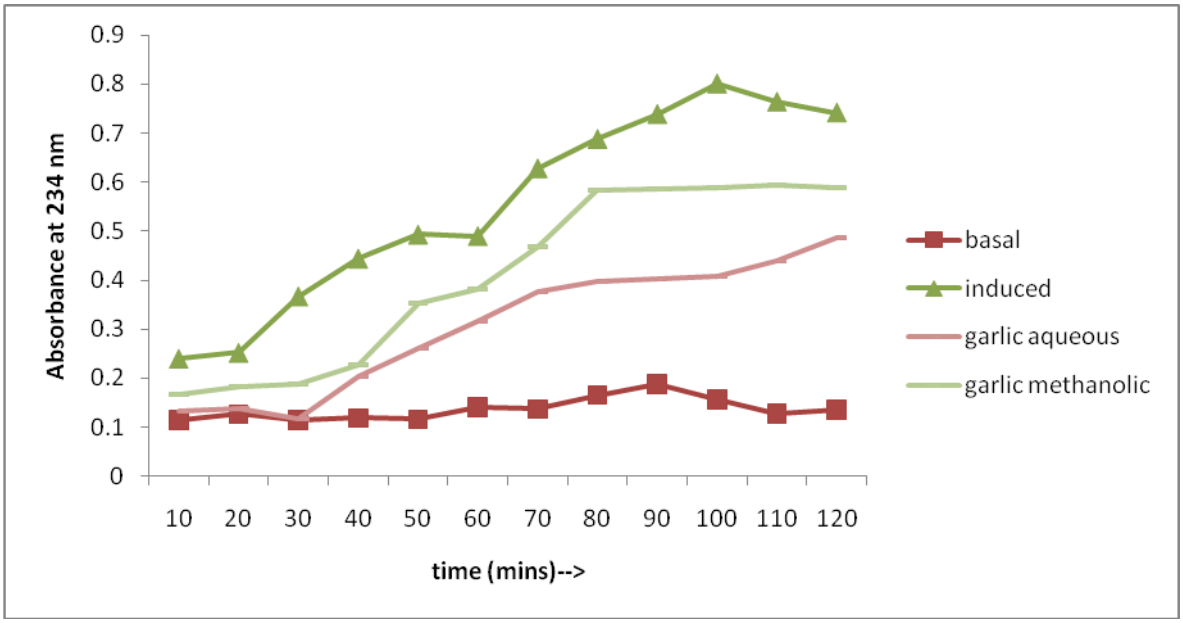

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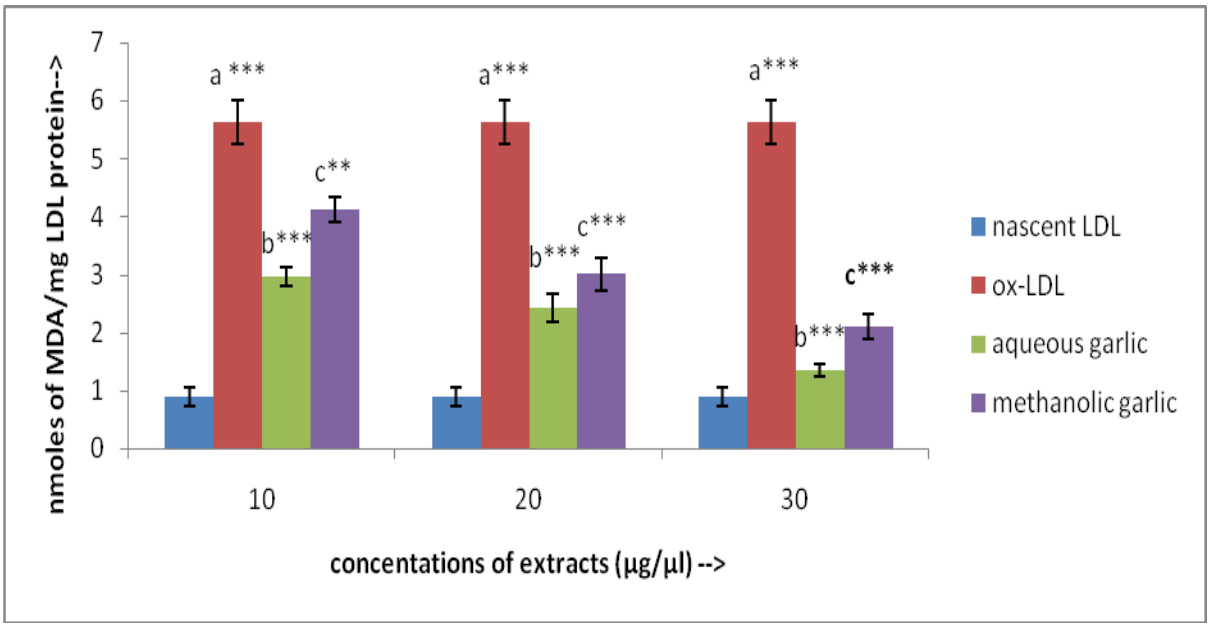
Figure 2: Inhibitory effects of garlic extracts on generation of lipid hydroperoxides. The extract concentration was 10,20 and30µg/µL. Bars represent mean ± standard deviation for triplicates. Comparisons are made between: a-nascent LDL and oxidized LDL; b-oxidized LDL and Aqueous garlic extract; c-oxidized LDL and methanolic garlic extract. Statistical significance:P<0.01,***P<0.001.**



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The oxidative modification of LDL induced by copper ions is shown to be related to free radical reaction, although the exact mechanism has not been elucidated yet. It is suggested that LDL oxidation may require the generation of super oxide anion and probably the ultimate generation of hydroxyl radicals by the Fenton reaction [1]. After oxidation of polyunsaturated fatty acids of LDL by copper ions, there was an elevation of lipid peroxides and depletion of vitamin E [39]. Fig. 1 and 2 clearly shows that aqueous garlic extract can alleviate LDL oxidation by inhibiting the formation of conjugated dienes and lipid peroxides than methanolic extract. Garlic extract is a complex mixture of phytochemicals. There are two possibilities one being complexing Cu^{2+} and thus preventing any initiating events or second preventing the depletion of lipid soluble antioxidants [23].

In conclusion our study demonstrates the ability of garlic as potent antioxidant to prevent oxidation of LDL. This protective effect may be associated with its antioxidant properties and hence it can be recommended as a good alternative to reduce the risk of coronary heart disease and other free radical associated health problems.

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