Nutrition 32 (2016) 649–655

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

Evaluation of therapeutic effect of omega-6 linoleic acid and thymoquinone enriched extracts from *Nigella sativa* oil in the mitigation of lipidemic oxidative stress in rats

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ARTICLE INFO

Article history:
Received 20 August 2015
Accepted 5 December 2015

Keywords:
Methanolic extract
Volatile oil
Nigella sativa
Antioxidant
Reactive oxygen species
Hyperlipidemia

ABSTRACT

Objective: *Nigella sativa* belongs to the Ranunculaceae family. The therapeutic role of methanolic extract (ME) and volatile oil (VO) fractionated from *N. sativa* seed oil was investigated for antiperoxidative and antioxidant effects in atherogenic suspension fed rats.

Methods: We examined the protective effects of ME and VO on the enzymatic and nonenzymatic antioxidants status in erythrocytes and the livers of atherogenic suspension fed rats. As a marker of lipid peroxidation, we estimated the conjugated diene, lipid hydroperoxide, and malondialdehyde concentrations in plasma in the following groups of rats: normolipidemic control, hyperlipidemic control, hyperlipidemic methanolic extract, and hyperlipidemic volatile oil. ME 500 mg or VO 100 mg/kg body weight of male rat was orally administrated for 30 d.

Results: Pretreatment of hyperlipidemic rats with these test extracts resulted in a significant decrease ($P < 0.001$) in the level of lipid peroxidation markers, conjugated diene, lipid hydroperoxide, and malondialdehyde (16–50%) compared to the hyperlipidemic control rats. In addition, ME and VO significantly ($P < 0.001$) elevated the hepatic and erythrocyte superoxide dismutase, catalase, and glutathione reductase activities (19–58%) compared to the hyperlipidemic rats. In liver homogenate of hyperlipidemic-ME and hyperlipidemic-VO, the glutathione-S-transferase activity was protected by 93% and 89%, and in erythrocytes, the glutathione peroxidase activity was protected by 90% and 77%, respectively. Interestingly, reduced glutathione level and activities of ATPases were protected to near normal levels. Pretreatment of rats with the test extracts replenished effectively ($P < 0.001$) the plasma total antioxidant power by an average of 88% against free radicals.

Conclusions: The lipidemic oxidative stress was effectively mitigated by antiperoxidative activities of ME and VO. Thus, these test extracts, especially ME, may be used as antioxidant as well as hypolipidemic agents in the form of natural food supplement to prevent or treat diseases caused by free radicals.

Introduction

Cardiovascular disease (CVD) is one of the leading causes of disability and premature death worldwide. If proper attention and care are not taken in time, it is estimated that by 2030 about 23.6 million people will die of CVD [1]. The increased level of cholesterol in plasma causes atherosclerosis, and thereby promotes the risk of coronary heart disease (CHD) [2].

Diets rich in cholesterol and fats are involved in the production of free radicals. In comparison to other free radicals, reactive oxygen species (ROS), mainly hydroxyl radical ($HO^{\bullet}$), greatly causes lipid peroxidation, and consequently, results in deterioration of tissues.

Two well-known reactions, namely Haber–Weiss reaction (eq. 1) and Fenton reaction (eq. 2) are sources for $HO^{\bullet}$, produced predominantly from H_2O_2 [3]:

The authors acknowledge University Grants Commission (grant number, F. 19-33/1/2006 (CU)), New Delhi for predoctoral fellowship. Dr. S. Ahmad carried out the experiments along with interpretation of data, and prepared the manuscript. Dr. Z. H. Beg participated in the interpretation of the data and writing of the manuscript. The authors declare no conflict of interest in the research article.

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http://dx.doi.org/10.1016/j.nut.2015.12.003
0899-9007/

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\cdot} \quad (1)$$

$$Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^- + HO^{\cdot} \quad (2)$$

Claster et al. [4] has reported that red cells are more prone to peroxidative damage in the presence of polyunsaturated fatty acid (PUFA) within the membrane, an oxygen rich environment and iron rich hemoglobin. In fact, in erythrocytes, autoxidation of oxygenated hemoglobin and hemicrom iron donate one electron to O_2 and form superoxide anion ($O_2^{\bullet -}$) and thus dismutation of O_2 generates excessive amount of H_2O_2 that results in the production of $HO^{\bullet}$ [3,5,6].

Lipid peroxidation reaction due to ROS results in loss of membrane integrity and cell death [7]. An end product of membrane lipid peroxidation, malondialdehyde (MDA) has shown to cross-link erythrocyte and thus causes impairment of membrane related functions [8]. Many membrane components including ATPases may be the possible target for oxidants. ATPases have sulfhydryl groups that are susceptible to oxidation and it makes them defective in their activities. The oxidative attack on ATPases has been suggested due to lipid peroxidation [9]. Decrease in the activities of ATPases due to free radical attack [10] shows some pathologically important correlations with CHD [11]. On the other hand, low-density lipoprotein (LDL), especially small-dense LDL, is highly susceptible to oxidation, leading to the formation of more stable compounds, including MDA [12]. The MDA in turns binds to ε-amino group of apo B-100 present in LDL, and thus provides it with net negative charges. The change in this domain may cause the failure of binding with the apoB receptor. If this binding fails, the residence time of LDL particles in the circulation will increase, which can cause blockage of arteries. Therefore, the lipidemic oxidative stress may greatly contribute to the development of CVD.

The body possesses various types of defense mechanisms, so it can remain unharmed against the deadly attack of ROS. Protection to the cells against free radicals, breakdown products of lipids, and proteins is provided by enzymatic and nonenzymatic antioxidants. On the other hand, exogenous natural antioxidants such as polyphenolic compounds also have good potential to decrease oxidative stress, and thus protect against deadly diseases such as CVDs [13,14].

Thymoquinone (TQ) (2-isopropyl-5-methylbenzo-1,4-quinone), principal constituent of *Nigella (N.) sativa,* has been shown to carry hypolipidemic [15], antioxidant [16,17], and antiinflammatory [18] properties, while omega-6 linoleic acid [19] and omega-6 linoleic acid in the presence of palmitic acid have been shown to possess the hypolipidemic action [20]. In our recent study, hypolipidemic activity of methanolic extract (ME) rich in omega-6 linoleic acid, and volatile oil (VO) rich in TQ fractionated from *N. sativa* seed oil was investigated in lipidemic oxidative stressed rats [21]. In the present study, putative protective effects of ME and VO were investigated in atherogenic suspension fed rats, regarding changes in erythrocyte and liver superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), glutathione reductase (Gred), and glutathione-S-transferase (GST) activities, along with reduced glutathione (GSH) and oxidized glutathione (GSSH) levels. Concentrations of conjugated diene (CD), lipid hydroperoxide (LOOH), and MDA as plasma lipid peroxidation markers were also estimated. Besides protective effects of ME and VO on erythrocyte membrane-bound enzymes, ATPases activities were also investigated in rats.

Material and methods

Chemicals

Export quality and edible seed oil of *N. sativa,* also called black cumin, was purchased from a local store. Ouabain, 1,1,3,3-tetramethoxypropane, and adenosine triphosphate were provided from Sigma-Aldrich Inc. (St. Louis, MO, USA), and phenazine methosulfate, NADP, NADPH, 2,4,6-tripyridyl-S-triazine, triphenyl phosphine, xylenol orange, nitroblue tetrazolium salt, glutathione reduced, and N-ethylmaleimide were provided from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Glutathione oxidized and 5,5'-dithiobis (2-nitrobezoic acid) (DTNB) were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). 1-Chloro 2,4-dinitrobenzene (CDNB) was purchased from Central Drug House, Pvt. Ltd. (Mumbai, India), and hemoglobin assay kit was bought from Ranbaxy Diagnostics (New Delhi, India). Rat food was purchased from Ashirwad Industries (Chandigarh, India). All other chemicals and reagents required for the study were of analytical grade.

Methanolic extract preparation

Methanolic extract (ME) was fractionated from *N. sativa* seed oil. The procedure of ME extraction was followed as described in our previously reported article [21]. In brief, 10 g of *N. sativa* seed oil was added to 100 mL of methanol, and stirred for 100 min at ambient temperature. The upper methanolic layer containing pharmaceutically important constituents was carefully removed, and then methanol was allowed to evaporate at 45°C. The ME thus extracted was stored under nitrogen in dark colored bottle at 4°C for the future study.

Volatile oil preparation

Volatile oil (VO) was extracted from *N. sativa* seed oil. The method for VO extraction with slight modification is essentially the same as employed by Kanter et al. [17]. In short, 400 mL of distilled water was taken in a distillation flask that contained 12 g of *N. sativa* oil. The temperature of the distillation flask was adjusted to the boiling point. The distillate of about 150 mL containing VO was collected in a dark glass bottle. The distillate was properly treated with 50 mL of diethyl ether. Moisture was removed by adding anhydrous sodium sulphate, and finally diethyl ether was allowed to evaporate at 40°C. The obtained VO by this procedure was stored in a dark colored bottle at 4°C for the future use.

Animals and treatment

All animal protocols were approved by the Board of Studies of the Biochemistry Department, and Animal Ethics Committee of Jawahar Lal Nehru Medical College, AMU, Aligarh. Normal male Wistar rats weighing 180 to 210 g from inbred colony were maintained in the central animal facility of Jawahar Lal Nehru Medical College, and allowed free access to standard rat food and water. A suspension of 10% ME, and 2% percent VO was prepared in 12.5% DMSO, followed by homogenization with saline. The atherogenic suspension administrated to the rats was prepared as described previously [15] and was basically composed of 0.5% cholesterol, 3% coconut oil, and 0.25% cholic acid, and prepared by mixing in a Potter-Elvehjem homogenizer. The experimental rats were grouped as below:

Normolipidemic Control (NLP-C): this normal control group of five rats orally received 1.0 mL of saline containing 12.5% DMSO in two equal divided doses of 0.5 mL each in the morning and evening, per rat per day, for a period of 30 d.

Hyperlipidemic Control (HLP-C): this experimental hyperlipidemic control group containing four rats orally received 1.0 mL of saline containing 12.5% DMSO, followed by the administration of atherogenic suspension in two equal divided doses of 0.5 mL each per rat per day in the morning and evening for 30 d.

Hyperlipidemic Methanolic Extract (HLP-ME): before the administration of 1.0 mL of atherogenic suspension, 1.0 mL of ME suspension in two equal doses of 0.5 mL each in the morning and evening was orally given to a group of four rats for 30 d.

Hyperlipidemic Volatile Oil (HLP-VO): before the administration of 1.0 mL of atherogenic suspension, 1.0 mL of VO suspension in two equal doses of 0.5 mL each in the morning and evening, was orally given to a group of four rats for 30 d.

All groups of rats were kept overnight fasted after the treatment of drugs. Blood was withdrawn from the anaesthetized rats by cardiac puncture, and kept in heparinized tubes. The blood plasma and erythrocytes were fractionated by centrifugation at 2500 rpm for 30 min. The upper portion of the sample, namely plasma, was carefully removed, and saved at 4°C. The lower portion of the sample was again centrifuged at 1500 rpm after rinsing with two parts of physiological saline. This procedure was repeated a second time. A portion of washed erythrocytes was utilized to isolate erythrocyte membranes using the procedure described by Hanahen and Ekholm [22]. The method of Lakshmi and Rajagopal [23] was used to prepare the hemolysates from washed erythrocytes.