

***In-vivo* Antioxidant Effects of Ethyl Acetate Fraction of *Mentha spicata* L. on 4-Nitroquinoline-1-Oxide Injected Mice**

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Abstract

Antioxidant effects of ethyl acetate fraction of *Mentha spicata* (L.) were evaluated against 4-nitroquinoline-1-oxide injected mice. For this study, experiment setup consisted of 36 albino mice of either sex divided into 6 groups: Control (25% DMSO in water), ethyl acetate fraction (EAF) alone group (80, 160 mg/Kg body weight-bwt), 4-NQO (7.5 mg/Kg bwt-IP) alone and 4-NQO + EAF. EAF and vehicles were administered orally for five consecutive days. 4-NQO (7.5 mg/Kg bwt) was injected intraperitoneally on the 6th day. After 24 h, the animals were killed; liver sample was extracted and used for bio-assay. 4-NQO alone treated group decreased (27-60%) the antioxidant activities and promoted lipid peroxidation (LPO-60%) over their respective control values. Pretreatment with EAF, at the maximum dose (160 mg/Kg bwt) brought down the LPO up to 87% enhanced by 4-NQO. Among the enzymatic antioxidants, glutathione S-transferase (GST) was the most affected enzyme with 4-NQO and the least was catalase (CAT). Pretreatment with EAF (160 mg/Kg bwt), the restoration of antioxidants like glutathione peroxidase, superoxide dismutase, and CAT were found equal or less than 1.2 fold higher than that of the respective control values whereas, GST was observed to be the most restored antioxidant. Be reduced glutathione (GSH) and the least vitamin C over their control values. EAF restored the GSH and Vitamin E levels were found to be 1.2 fold higher than the respective control values.

Keywords: Antioxidant activity; Ethyl acetate fraction; Bio-assay; *Mentha spicata*; Nitroquinoline-1-Oxide; Mice.

Introduction

Reactive oxygen species (ROS) are produced in the biological system due to the exposure of various physical and chemical toxins, either directly or indirectly. Once they enter into the body, these chemicals undergo the metabolism and detoxification. During the process, intermediate metabolites, ROS and hydrogen

peroxide are generated. The overload of ROS leads to oxidative stress to macromolecules like DNA, lipids and proteins (1). As a consequence, lipid peroxidation, DNA damage, chromosome breaks, alteration in signal transduction and gene expression occur. They are implicated in numerous diseases and disorders like cataract, birth defects, reproductive anomalies, heart diseases, neurodegenerative diseases like Parkinson and Alzheimer, cardiovascular disorder, immune dysfunction, inflammation, ageing and cancer (2). The balance between

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formation and removal of reactive oxygen molecules is required to maintain the normal physiological functions (1). The biological system is endowed with an array of antioxidants like superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, reduced glutathione, Vitamin C and Vitamin E that would neutralize the activity of ROSs, and other reactive intermediate metabolites (3). The *in-vivo* system of defense may not be adequate to neutralize all the ROSs produced. There is a need for an external source of antioxidants to neutralize the free radical load in the body (4).

Fruits, vegetables, spices and oilseeds have been increasingly recognized as external sources of antioxidants. They contain vitamins, minerals, soluble/insoluble fibers, carotenoids, phenolic compounds including phenolic acids, flavonoids, isoflavonoids, lignans and tannins (5-7). These antioxidant *compounds* may act as free radical scavengers and/or activators of anti-oxidative defense enzyme systems to suppress the radical damages in biological systems (8). Therefore, there is a growing interest toward natural antioxidants of herbal resources (9). Epidemiological and *in-vitro* studies strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against ROS-induced almighty in biological systems (10).

Mentha spicata L. (Lamiaceae) is an important aromatic spice which is cultivated throughout the world. Its essential oil is widely used in food, cosmetic and pharmaceutical industries (11). *M. spicata*, selected for the present study as it possess large amounts of total antioxidant activity which is better than some well-known antioxidants like Quercetin, Beta carotene and Vitamin C (12). It is also known to possess antimicrobial, antimutagenic and anti-inflammatory properties due to the presence of caffeic acid, rosmarinic acid and α -tocopherol and anti-histaminic activity due to 5-O-desmethylnobiletin, cirsilinoleol, thymosin, thymonin and sideritoflavone (13-16). It was found to be antiallergic, antiplatelet (17), cytotoxicity (18), chemopreventive (19) and H₂O₂ scavenging activities (7). Therefore, the present study focused on evaluating the *in-vivo* antioxidant effects of ethyl acetate fraction

from ethanol extract of *Mentha spicata* L. on 4-nitroquinoline-1-oxide-induced oxidative stress in mice.

Experimental

Swiss albino of either sex (10-12 weeks old) weighing 25-30 grams were used in this study. Animals obtained from the King Institute, Chennai, India and maintained (Committee for the Purpose of Control and Supervision of Experiments on Animals -CPCSEA) at the Institute's animal house under standard environmental conditions (temperature: 22 ± 2°C and 12/12 h (light/dark) period). The Institute's ethical committee approved all experiments.

Chemicals

Malondialdehyde (MDA), 4-Nitroquinoline-1-oxide (4-NQO), Giemsa stain and May-Grunwald stain were purchased from Sigma Aldrich (Sigma, USA). Folin's phenol reagent, 5,5-dithiobis (2-nitro benzoic acid) (DTNB), 1-chloro 2,4-dinitrobenzene (CDNB) and reduced glutathione were purchased from SISCO Research Laboratories Pvt. Ltd. (Mumbai, India). All other solvents used in the experiments were of analytical grade.

Extraction from leaves of mentha spicata

Plant material was commercially obtained from a local market (Chennai) and identified at the Center for Advanced Studies in Botany, University of Madras (voucher number-855). Eight hundred grams of shadow dried leaf powder was immersed in 4 L of 95% ethanol and left for 24 h under constant stirring and filtered. Concentrated ethanol extract was further fractionated sequentially by hexane, chloroform, ethyl acetate and water. Extract method briefly given in the early publication (14).

Experimental group for antioxidant properties of Mentha spicata

Thirty-six mice were divided into six groups of either sex. The concentrated ethyl acetate fraction (EAF) was dissolved in 25% DMSO and administered by oral gavage to the mice for 5 consecutive days. Group 1, the control, was administered 25% DMSO, groups 2 and 3

received doses of 80 and 160 mg/Kg bwt EAF, respectively, group 4 was administered 4-NQO alone (7.5 mg/Kg bwt, intraperitoneally - IP), groups 5 and 6 received the doses of 80 and 160 mg/Kg bwt of EAF for 5 days, and after 2 h, 4-NQO (7.5 mg/Kg bwt) was injected. After 24 h of 4-NQO treatment, all the animals were sacrificed by cervical dislocation. Mouse liver was immediately excised and stored in the physiological saline until use.

Bio-assays

Liver homogenate (10%) was made in 0.1 M of Tris-HCl buffer (pH 7.4), using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was used for the following bioassays: total protein (20), lipid peroxidation (LPO) (21), glutathione peroxidase (GPx) (22), glutathione-s-transferase (GST) (23), superoxide dismutase (SOD) (24), catalase (CAT) (25), reduced glutathione (GSH) (26), Vitamin E (27) and Vitamin C (28) were estimated by using conventional procedures.

Statistical analysis

In the present study, results were shown as mean \pm standard error for each group with six mice. Results were performed through one-way ANOVA using SPSS Software Version 12.0 with Student-Neuman-Keuls test (SNK TEST) to assess the differences among the groups. The significant values considered at $p \leq 0.05$.

Results and Discussion

The enhanced LPO level by 4-NQO was observed to be 1.6 fold higher than the control value, 1.84 nmoles of MDA formed/mg protein (Figure 1). Ethyl acetate fraction alone tested group showed no effect on the LPO level (group 1 = group 2 = group 3, $p > 0.05$; Figure 1). Pretreatment with EAF significantly decreased the LPO increased by 4-NQO (group 4 > group 5 > group 6, $p < 0.05$ (Figure 1)). This decreased level was comparable to the control value (1.84 nmoles of MDA formed/mg protein) only at the highest dose treated, *i.e.*, at 160 mg/Kg bwt.

The enzymatic antioxidants were found to be decreased with 4-NQO in the range of 1.4-1.7 fold lesser than that of respective control

values (Table 1). Pretreatment with EAF, even at the lowest dose (80 mg/Kg bwt), effectively increased all of the four enzymatic antioxidants *viz.*, GPx, GST, SOD and CAT to the levels comparable to their control values. In fact, at the highest dose of 160 mg/Kg bwt, the levels of GST and SOD were significantly greater than their control values (GST-2.55 nmoles of CDNB conjugated/min/mg protein and SOD - 5.36 units/min/mg protein). Besides, ethyl acetate alone group had no changes in the levels of antioxidants (Table 1).

Similarly, 4-NQO-decreased non-enzymatic antioxidant was found to be in the range of 1.6-2.5 folds lesser than that of respective control values (Figure 1). Pretreatment with EAF, at a dose of 80 mg/Kg bwt, the level of GSH was significantly increased but it was still lower than the control value (group 5 > 4 and < 1, $p < 0.05$). At dose, 160 mg/Kg bwt the enhanced level of GSH was significantly greater than the control value (group 6 > 1, $p < 0.05$). Vitamin E level was effectively enhanced even at the lowest dose, *i.e.*, 80 mg/Kg bwt (group 5 = 1, $p < 0.05$). At the highest dose (60 mg/Kg bwt), its level was found to be higher than the control value (group 6 > 1, $p < 0.05$). Vitamin C increased with the increasing dose of EAF and at dose, 160 mg/Kg bwt, its level was comparable with the control value (group 6 = 1, $p > 0.05$).

4-NQO is an oral synthetic carcinogen and widely used as mutagen (29). It is also used as UV-mimetic and S-dependent clastogenic agent (30). Earlier reports indicate that 4-NQO induced oxidative stress in the biological system by enhancing LPO and suppressing body defense mechanisms during the exposure (31). In the present study, the altered activities of enzymatic (decreased 27-41%), non-enzymatic (decreased 38-60%), antioxidants and promoted (60%) LPO were observed in the 4-NQO alone treated group when compared with respective control values (Table 1 and Figure 1). It showed an impaired anti-oxidative capacity and sustained oxidative stress emerged following the exposure of 4-NQO. Pretreatment with EAF, at the lowest dose (80 mg/Kg bwt) reduced LPO by about 56%. At dose of 160 mg/Kg bwt, the reduction of lipid peroxidation reached up to 87%. The decreased lipid peroxidation by the EAF

Table 1. Protective effect of ethyl acetate fraction of *Mentha spicata* against 4-NQO altered enzymatic antioxidants in liver of mice.

Groups	Treatment (Dose: mg/Kg bwt)	GPX	GST	SOD	CAT
1	Control	43.41 ±	2.55 ±	5.36 ±	5.36 ±
2	EAF (80)	41.25 ±	2.46 ±	5.61 ±	364.23 ±
3	EAF (160)	46.99 ±	2.66 ±	6.42 ±	374.22 ±
4	4-NQO (7.5)	27.02 ±	1.51 ±	3.36 ±	249.22 ±
5	EAF (80) + NQO (7.5)	37.09 ±	2.66 ±	5.19 ±	372.57 ±
6	EAF (160) + NQO (7.5)	49.98 ±	3.42 ±	6.27 ±	390.83 ±

EAF: Ethyl Acetate Fraction; Mean ± Standard Error; LPO: nmoles of MDA formed/mg protein; GPx: moles of reduced glutathione oxidized/min/mg protein; GST: nmoles of CDNB conjugated/min/mg protein; SOD: units/min/mg protein; CAT: μ moles of H_2O_2 consumed/min/mg protein.

suggests the prevention of free radical formation in the process of 4-NQO toxicity. High phenolic and flavonoid content were observed in EAF which may be responsible for the reduction of lipid peroxidation (12). The major phenolics and flavonoids of *M. spicata* were of caffeic acid, rosmarinic acid, α -tocopherol, apigenin, acacetin, luteolin, thymonin, thymosin, sideritoflavone, cirsilineol, sorbitolin, 5-O-desmethylnobiletin and diosmetin (32, 33, 13). The restoration of 4-NQO enhanced LPO by EAF was observed to be better than aqueous fraction obtained from ethanol extract of *M. spicata* (34).

On other hand, the most altered enzymatic antioxidant with 4-NQO was found to be GST and the least was CAT. GST is an important enzymatic antioxidant, which is involved in detoxification process of genotoxins by catalyzing the formation of thiol group of glutathione to electrophilic genotoxins. These thiol compounds are also utilized to scavenge the potentially toxic compounds including those produced as a result of oxidative stress. Catalase is also one of the most efficient enzymes that protect cells from the overload of hydrogen peroxide generated under the oxidative stress (35). Pretreatment with EAF (160 mg/Kg bwt), the restoration of GST was found to be better among the enzymatic antioxidants when compared to the control value (Table 1). The restoration of other antioxidants like GPx, SOD and CAT with EAF, was equal or less than 1.2 fold higher than that of the respective control values (Table 1). Similarly, the most affected non-enzymatic antioxidant by 4-NQO was found to be GSH and the least was vitamin C over their control values, respectively

(Figure 1). GSH is the major low molecular weight antioxidant in cells and its levels are often decreased due to oxidative stress (36). It plays a major role in the protection of biological system against proliferation of lipid peroxidation under the threat of genotoxins. Pretreatment with EAF at highest dose enhanced the GSH and Vitamin E levels by over 100 percent of the mutagen-induced values which were also found to be 1.2 fold higher than the respective control values (Figure 1). In contrast, the enhanced level of vitamin C with EAF was found near to control level. EAF effectively restored all antioxidant levels when compared to aqueous fraction of ethanol extract of *M. spicata* (34). These results indicate that ethyl acetate fraction plays a major role in protecting the cells from genotoxic insults. In fact, the non-enzymatic antioxidants such as Vitamin E, Vitamin C and GSH are the major antioxidant involved in the metabolism of genotoxins (37). Apart from its well-documented anti-oxidant property, *M. spicata* possessed many other pharmacological activities including cytotoxicity, anti-inflammatory, anti-bacterial and radical scavenging effects (14, 15, 38). Antigenotoxicity of EAF was found to be higher than that of the other fractions such as hexane, chloroform and aqueous obtained from ethanol extract of *M. spicata* (16, 34).

In conclusion, pretreatment with EAF ameliorate 4-NQO altered the antioxidants in which exception of Vitamin C, EAF increased all the antioxidants in the range 15-34 percent. Particularity, increasing GST and Vitamin E (34% and 23%, respectively) stood in contrast with the other antioxidants (14-17%). Furthermore, ethyl

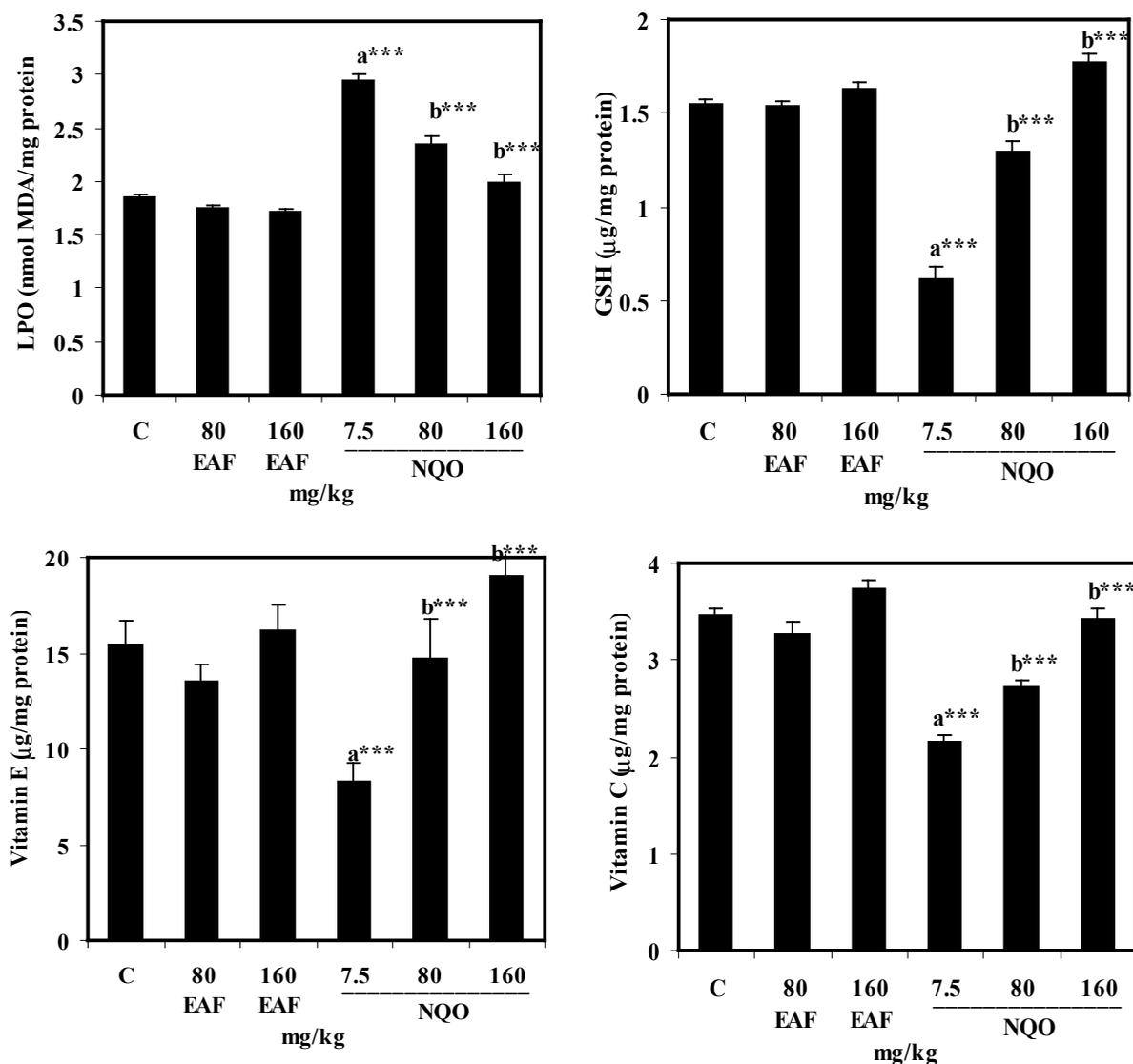


Figure 1. Protective effect of ethyl acetate fraction from ethanol extract of *Mentha spicata* against 4-NQO altered lipid peroxidation and non-enzymatic antioxidants in liver of mice.

acetate fraction showed better *in-vitro* and *in-vivo* antioxidant than that of aqueous fraction of *M. spicata* (12, 34). Antioxidant effects of EAF might be due to the synergetic effects of phenolic compounds.

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