**Pharmacological Reports**
2011, 63, 660–669
ISSN 1734-1140



# Thymoquinone produced antianxiety-like effects in mice through modulation of GABA and NO levels

Neeraj Gilhotra, Dinesh Dhingra

Pharmacology Division, Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar – 125001, Haryana, India

**Correspondence:** Dinesh Dhingra, e-mail: din_dhingra@rediffmail.com

**Abstract:**
The aim of the present study was to investigate the role of GABAergic and nitriergic modulation in the antianxiety effect of thymoquinone, a major constituent of *Nigella sativa*, in mice under unstressed and stressed conditions. Thymoquinone (10 and 20 mg/kg), methylene blue (1 mg/kg) and diazepam (2 mg/kg) were administered followed by behavioral testing using an elevated plus maze, the light/dark test and the social interaction test in both unstressed and stressed mice (mice subjected to 6 h immobilization). The effects of the above-mentioned drugs on plasma nitrite, a stable metabolite of nitric oxide (NO) and brain GABA content were also studied. Diazepam (2 mg/kg) produced significant anxiolytic-like effects only in unstressed mice. However, diazepam significantly increased the GABA content in both unstressed and stressed mice as compared with their respective control groups. Thymoquinone (10 and 20 mg/kg) produced significant antianxiety effects in unstressed mice without altering nitrite levels, but only the higher dose (20 mg/kg) of thymoquinone increased the GABA content in unstressed mice. In stressed mice, thymoquinone (20 mg/kg) showed anxiolytic effects, with a significant decrease in plasma nitrite and reversal of the decreased brain GABA content. Pre-treatment with methylene blue enhanced the antianxiety effect of thymoquinone in both unstressed and stressed mice. Therefore, the present study suggests an involvement of NO-cGMP and GABAergic pathways in the anxiolytic-like activity of thymoquinone.

**Key words:**
anxiety, cGMP, GABA, methylene blue, nitric oxide, thymoquinone

**Abbreviations:**
cGMP – cyclic guanosine monophosphate, eNOS – endothelial NOS, GABA – γ-aminobutyric acid, iNOS – inducible NOS, nNOS – neuronal NOS, NO – nitric oxide, NOS – nitric oxide synthase

## Introduction

Nitric oxide (NO), an intercellular messenger in the brain generated from L-arginine by different isoforms of nitric oxide synthase (nNOS, iNOS and eNOS), plays an important role in various physiological and pathological processes [20, 54]. Nitric oxide synthase (NOS) is localized in brain regions involved with anxiety, such as hypothalamus, amygdala and hippocampus [37, 38, 56]. Inhibition of NOS by *N*-nitro-L--arginine-methyl ester [13], 7-nitroindazole and 1-(2-trifluoromethylphenyl)imidazole [58, 59] and S-ethyl-isothiourea and aminoguanidine [18, 42) have been reported to produce antianxiety activity in different animal models of anxiety. L-arginine, the donor of NO, is reported to prevent the antianxiety effect of NOS inhibitors [15]. Acute stress induces a general-

ized increase in the production of NO and causes anxious behavior in rodents [48]. Stress induced by immobilization of the rodents for 6 h has been observed to significantly increase the expression of NOS in rodents [17–19, 34, 35]. An abundance of NOS-containing cells in the medial amygdala suggests equally significant nitriergic influence on the processing of stress-related responses in these structures [53]. NOS inhibition in the medial amygdala also produces anxiolytic-like effects in the elevated plus maze [9]. Immobilization stress in rats has been reported to significantly increase plasma nitrite levels [32]. Similar short-term (6 h) immobilization stress has also been found to increase NO levels in mice in our laboratory [17–19].

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system [4]. GABA pathways have a regulatory role in the modulation of behavioral sequelae resulting from stress [49]. The basolateral complex of the amygdala, an important area for manifestation of anxiety, contains relatively large numbers of benzodiazepine/$GABA_A$ receptors [43], and infusion of benzodiazepines or $GABA_A$ agonists in the basolateral amygdala reduced fear conditioning and anxiety [28]. Short-term and prior stress downregulate GABA pathways [24, 36]. Immobilization stress induces rapid and persistent changes in the GABA-benzodiazepine-barbiturate receptor complex in animal brains [60]. Furthermore, GABA is reported to attenuate stress-induced NO release, and stress conditions known to release iNOS-mediated NO are responsible for dysregulation of GABA pathways [24]. cGMP downregulates $GABA_A$ receptor function in hippocampus, an area involved in anxiety [47].

Thymoquinone, the major bioactive constituent of seed oil of *Nigella sativa,* possesses various pharmacological activities, such as analgesic and anti-inflammatory activity [1], protection against chemical-induced carcinogenesis [25], inhibition of eicosanoid generation and membrane lipid peroxidation [27], neuroprotection [2], anticonvulsant activity [26] and suppression of oxidative stress-induced neuropathy [23]. At the molecular level, thymoquinone has been shown to downregulate tumor necrosis factor [11] and suppress nuclear factor kappa B (NF-κB) activation in brain and spinal cord [39]. Thymoquinone significantly suppressed the expression of iNOS [10]. Four weeks of daily administration of *Nigella sativa* seed oil produced antianxiety effects in rats due to an increase in brain serotonin levels [45]. There was partial involvement of benzodiazepine receptors in the antianxiety activity of thymoquinone. Non-benzodiazepine receptor blocking mechanisms were also proposed to mediate the antianxiety activity of thymoquinone [46]. Involvement of NO or nitriergic modulation has still not been explored in regard to the antianxiety effect of thymoquinone.

The reported induction of iNOS by immobilization stress, the observed inhibition of NF-κB, a transcriptional activator of iNOS, by thymoquinone and the reversal of the antianxiety effect of thymoquinone by flumazenil, a benzodiazepine receptor antagonist, prompted us to explore the involvement of nitriergic and GABAergic influences in the antianxiety effect of thymoquinone under both unstressed and stressed conditions. Therefore, the present study was designed to explore possible nitriergic modulation in the antianxiety effect of thymoquinone. Furthermore, taking into consideration the variations in the neurochemical levels of NO [18] and GABA [24] under conditions of stress, the investigation was extended to explore the interplay between GABA and NO in the observed effect of thymoquinone under both unstressed and stressed conditions.

## Materials and Methods

### Animals

Male Swiss albino mice (20–25 g) were employed in the present study. Animals were procured from the Disease Free Small Animal House, CCS Haryana Agricultural University, Hisar, Haryana, India. Animals were housed under laboratory conditions with an alternating light and dark cycle of 12 h each in cages at a controlled room temperature of 20–22°C. The animals were acclimatized to laboratory conditions before behavioral experiments. The experimental protocol was approved by the Institutional Animal Ethics Committee and care of the animals was carried out in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Registration No. 0436).

### Drugs

Thymoquinone, methylene blue (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and diazepam (Calmpose®; Ranbaxy Laboratories Ltd., India) were used in the present study. Corn oil and normal saline were used as vehicles for thymoquinone and methylene blue, respectively. Diazepam injection was diluted in normal saline [52].

### Behavioral paradigms

#### Elevated plus maze

The plus maze apparatus consisted of two open arms (without walls), 16 × 5 cm, and two enclosed arms, 16 × 5 × 12 cm, arranged opposite to each other. The maze was elevated to a height of 25 cm. Each mouse was placed individually at the center of the elevated plus maze with its head facing towards an open arm and observed for a period of 5 min [30, 44]. In the elevated plus maze test, the percentage of time spent in the open arms was determined as follows:

$$\% = \frac{\text{Number of seconds spent in open arms} \times 100}{\text{300 total s (5 min observation time)}}$$

#### Light and dark test

The apparatus consisted of a rectangular box (45 × 27 × 27 cm), partitioned into two compartments connected by a 7.5 × 7.5 cm opening in the wall between compartments. One compartment was painted black and covered with a roof. The other compartment had no roof and was brightly illuminated by a 60 W bulb located above the box. An animal was placed in the center of the light compartment and was observed for 5 min. The time spent in the open (white/light) compartment was recorded [7]. The percentage of time spent in the light compartment was determined as follows:

$$\% = \frac{\text{Number of seconds spent in the light compartment} \times 100}{\text{300 total s (5 min observation time)}}$$

#### Social interaction test

The social interaction arena was an open topped box (22 × 15 × 12 cm). After introduction into the test arena, mice were scored for the cumulative time spent in genital investigation, sniffing a partner, climbing over and under another conspecific, neck licking and boxing [14].

$$\% = \frac{\text{Number of seconds spent in interaction} \times 100}{\text{300 total s (5 min observation time)}}$$

### Plasma nitrite estimation

For nitrite estimation, blood was withdrawn from the tail vein of immobilized mice in all study groups immediately before setting the animal free and subjecting it to behavioral tests [17–19]. Plasma was separated by centrifugation (2500 rpm at 4°C) for 10 min. It was stored in a refrigerator and processed for estimation of nitrite content within 24 h. Plasma nitrite was measured by a spectrophotometric assay based on the Griess reaction [22].

### Brain GABA estimation

Brain GABA content was estimated using the established method of Lowe et al. [33]. Brains were rapidly removed from mice after completing behavioral testing, and isolated brains were weighed and transferred to 5 ml of ice-cold trichloroacetic acid (10% w/v), homogenized and centrifuged at 10,000 × g for 10 min at 0°C. Then, 0.1 ml of tissue extract was added to 0.2 ml of 0.15 M ninhydrin solution in a 0.5 M carbonate-bicarbonate buffer (pH 9.95), which was incubated in a water bath at 60°C for 30 min and then cooled and treated with 5 ml of copper tartrate reagent (0.16% disodium carbonate, 0.03% copper sulfate and 0.03% tartaric acid). After 10 min, a fluorescence reading was taken at excitation/emission wavelengths of 377/451 nm in a spectrofluorimeter (Shimadzu RF-1501).

### Locomotor activity

The effects of various treatments on spontaneous locomotor activity of animals were measured using an actophotometer (INCO, Ambala, India). The data are presented as the number of counts recorded by the apparatus as the light beam was interrupted between the light source and photo sensors in response to animal movements. The locomotor activity scores for each animal were recorded for a period of 10 min before and after drug treatment.

### Experimental protocol

Male Swiss albino mice (n = 6 mice per group) were employed in the present study. Stress was produced by immobilizing the mice for 6 h (8 a.m. – 2 p.m.) by taping all four of their limbs and their trunk to a wooden board [17–19]. Mice subjected to immobilization were considered as stressed mice. Mice not subjected to immobilization were considered as unstressed mice. All treatments (vehicle, 10 ml/kg; thymoquinone, 10 and 20 mg/kg; methylene blue, 1 mg/kg) were administered intraperitoneally (*ip*) in a fixed volume of 1 ml/100 g body weight in separate groups of mice. Doses and routes of administration of drugs were selected according to previous studies conducted in our laboratory [17, 18] and as reported in the literature [47]. Unstressed mice received vehicle and drugs 30 min before testing them in various behavioral paradigms. The remaining mice received vehicle and drugs 30 min before subjecting them to immobilization for 6 h [31]. When pre-treatment of methylene blue was employed, methylene blue was administered 15 min before injection of thymoquinone. For nitrite estimation, blood was withdrawn from the tail vein of immobilized mice in all study groups immediately before setting the animals free and subjecting them to behavioral tests. The sampling procedure was completed during immobilization of the mice to avoid the extra stress that would be inflicted if the mice were immobilized for a second time for the purpose of drawing blood from the tail vein [17–19]. For GABA estimations, animals were sacrificed by decapitation after behavioral testing and their brains were removed.

### Statistical analysis

All results are expressed as the mean ± SE. All statistical analyses were performed using analysis of variance (ANOVA) followed by Tukey's test with the aid of the GraphPad InStat package, version 3.05; $p < 0.05$ was considered as significant.

## Results

### Effect of thymoquinone and methylene blue on mouse behavior in various behavioral paradigms (elevated plus maze, light/dark test, social interaction test)

In the elevated plus maze, the light-dark test and social interaction test, respectively, a significant in-

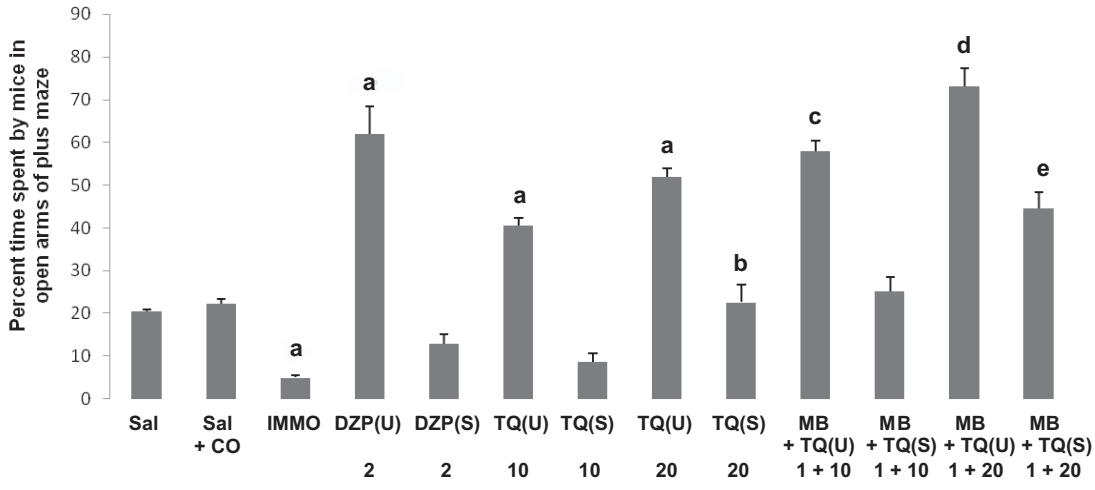


**Fig. 1.** Effect of different treatments on time spent by mice in open arms of elevated plus maze. n = 6 in each group. Values expressed as the mean ± SEM. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test, $F(12, 65) = 49.68$; $p < 0.0001$, a = $p < 0.05$ significant difference from vehicle-treated control group (unstressed mice), b = $p < 0.05$ significant difference from vehicle-treated control group (stressed mice), c = $p < 0.05$ significant difference from TQ (10 mg/kg)-treated unstressed mice, d = $p < 0.05$ significant difference from TQ (20 mg/kg)-treated unstressed mice, e = $p < 0.05$ significant difference from TQ (20 mg/kg)-treated stressed mice. Sal: Normal saline; CO: Corn oil; IMMO: immobilization; DZP(U): diazepam (unstressed); DZP(S): diazepam (stressed) TQ(U): thymoquinone (unstressed); TQ(S): thymoquinone (stressed); MB: methylene blue. Doses mentioned are in mg/kg

crease in the time spent in the open arms and in the number of open-arm entries, a significant increase in the time spent in the light compartment, and a significant increase in the time spent in social interactions indicate an anxiolytic effect. Alternatively, a significant decrease in the various parameters of these three behavioral tests above indicates an anxiogenic effect.

Six hours of acute immobilization induced a significant anxiogenic effect in unstressed mice as compared with vehicle-treated unstressed mice. Corn oil did not significantly affect the time spent in the open arms, the light compartment or social interaction in saline-treated unstressed mice. Diazepam produced significant antianxiety effects in unstressed mice as compared with the saline-treated group but did not exert a significant anxiolytic effect in stressed mice as compared to immobilization-induced stressed mice. Thymoquinone (10 mg/kg and 20 mg/kg) produced significant anxiolytic activity in unstressed mice. In stressed mice, only the higher dose (20 mg/kg) of thymoquinone produced a significant antianxiety effect. Pre-treatment with methylene blue significantly enhanced the antianxiety effect of thymoquinone (10 and 20 mg/kg) in unstressed mice as compared with those treated with thymoquinone (10 and 20 mg/kg) alone. Methylene blue treatment also enhanced the antianxiety effect of thymoquinone (10 and 20 mg/kg) in stressed mice as compared with those treated with thymoquinone alone (Fig. 1; Tabs. 1 and 2). Methylene blue alone could not produce any significant effect on anxiety of unstressed and stressed mice; hence, these data are not shown.

### Effect of thymoquinone and methylene blue on plasma nitrite levels

Immobilization stress significantly increased plasma nitrite levels in mice as compared with saline treated unstressed mice. Corn oil did not significantly affect the plasma nitrite levels in saline treated unstressed

**Tab. 1.** Effect of different treatments on time spent by the mice in the light compartment of the light/dark box

| Treatment | Dose (mg/kg) | Percentage of time spent in light compartment |
|---|---|---|
| Saline | 10 ml | 22.0 ± 1.5 |
| Saline + Corn oil | 10 + 10 ml | 24.5 ± 2.3 |
| IMMO | 6 h | 4.0 ± 0.32[a] |
| DZP (U) | 2 | 65.0 ± 3.01[a] |
| DZP (S) | 2 | 12.0 ± 2.2 |
| TQ (U) | 10 | 41.0 ± 2.6[a] |
| TQ (S) | 10 | 14.0 ± 0.56 |
| TQ (U) | 20 | 57.0 ± 2.2[a] |
| TQ (S) | 20 | 32.5 ± 0.65[b] |
| MB + TQ(U) | 1 + 10 | 62.0 ± 0.73[c] |
| MB + TQ(S) | 1 + 10 | 36.1 ± 0.65 |
| MB + TQ(U) | 1 + 20 | 74.0 ± 2.1[d] |
| MB + TQ(S) | 1 + 20 | 58.0 ± 2.3[e] |

Values expressed as the mean ± SEM, n = 6 in each group. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test; $F(12, 65) = 151.9$; $p < 0.0001$, [a] $p < 0.05$; significant difference from vehicle treated control group (unstressed mice), [b] $p < 0.05$; significant difference from vehicle-treated control group (stressed mice), [c] $p < 0.05$; significant difference from TQ (10 mg/kg)-treated unstressed mice, [d] $p < 0.05$; significant difference from TQ (20 mg/kg)-treated unstressed mice, [e] $p < 0.05$; significant difference from TQ (20 mg/kg)-treated stressed mice. Sal: Normal saline; CO: Corn oil; IMMO: immobilization; DZP(U): diazepam (unstressed); DZP(S): diazepam (stressed) TQ(U): thymoquinone (unstressed); TQ(S): thymoquinone (stressed); MB: methylene blue

**Tab. 2.** Effect of different treatments on time spent by the mice during the social interaction test

| Treatment | Dose (mg/kg) | Percentage of time spent in social interaction |
|---|---|---|
| Saline | 10 ml | 26.0 ± 0.75 |
| Saline + Corn oil | 10 + 10 ml | 25.0 ± 0.77 |
| IMMO | 6 h | 7.00 ± 0.36[a] |
| DZP (U) | 2 | 58.0 ± 1.13[a] |
| DZP (S) | 2 | 11.0 ± 1.2 |
| TQ (U) | 10 | 44.0 ± 1.03[a] |
| TQ (S) | 10 | 9.20 ± 0.47 |
| TQ (U) | 20 | 62.0 ± 1.16[a] |
| TQ (S) | 20 | 28.0 ± 1.10[b] |
| MB + TQ(U) | 1 + 10 | 61.0 ± 1.18[c] |
| MB + TQ(S) | 1 + 10 | 33.0 ± 0.68 |
| MB + TQ(U) | 1 + 20 | 76.0 ± 0.96[d] |
| MB + TQ(S) | 1 + 20 | 44.0 ± 1.03[e] |

Values expressed as the mean ± SEM, n = 6 in each group. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test; $F(12, 65) = 553.98$; $p < 0.0001$, [a] $p < 0.05$; significant difference from vehicle-treated control group (unstressed mice), [b] $p < 0.05$; significant difference from vehicle-treated control group (stressed mice), [c] $p < 0.05$; significant difference from TQ (10 mg/kg)-treated unstressed mice, [d] $p < 0.05$; significant difference from TQ (20 mg/kg)-treated unstressed mice, [e] $p < 0.05$; significant difference from TQ (20 mg/kg)-treated stressed mice. Sal: Normal saline; CO: Corn oil; IMMO: immobilization; DZP(U): diazepam (unstressed); DZP(S): diazepam (stressed) TQ(U): thymoquinone (unstressed); TQ(S): thymoquinone (stressed); MB: methylene blue

**Tab. 3.** Effect of different treatments on plasma nitrite levels

| Treatment | Dose (mg/kg) | Plasma nitrite levels |
|---|---|---|
| Saline | 10 ml | 8.0 ± 0.51 |
| Saline + Corn oil | 10 + 10 ml | 10.0 ± 0.8 |
| IMMO | 6 h | 26.0 ± 1.0[a] |
| DZP (U) | 2 | 12.0 ± 0.46 |
| DZP (S) | 2 | 24.6 ± 1.4 |
| TQ (U) | 10 | 11.0 ± 0.5 |
| TQ (S) | 10 | 23.0 ± 0.7 |
| TQ (U) | 20 | 12.0 ± 0.46 |
| TQ (S) | 20 | 14.0 ± 1.01[b] |
| MB + TQ(U) | 1 + 10 | 13.0 ± 0.65 |
| MB + TQ(S) | 1 + 10 | 20.0 ± 1.7 |
| MB + TQ(U) | 1 + 20 | 9.0 ± 0.8 |
| MB + TQ(S) | 1 + 20 | 18.0 ± 0.6 |

Values are expressed as the mean ± SEM, n = 6 in each group. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test; $F(12, 65) = 47.88$; $p < 0.0001$, [a] $p < 0.05$; significant difference from vehicle-treated control group (unstressed mice), [b] $p < 0.05$; significant difference from stressed mice. Sal: Normal saline; CO: Corn oil; DZP(U): diazepam (unstressed); DZP(S): diazepam (stressed); IMMO: immobilization; TQ(U): thymoquinone (unstressed); TQ(S): thymoquinone (stressed); MB: methylene blue

**Tab. 4.** Effect of different treatments on brain GABA levels

| Treatment | Dose (mg/kg) | Brain GABA levels (µg/g of wet brain tissue) |
|---|---|---|
| Saline | 10 ml | 356 ± 3.38 |
| Saline + Corn oil | 10 + 10 ml | 364 ± 3.85 |
| IMMO | 6 h | 302.0 ± 4.0[a] |
| DZP (U) | 2 | 426 ± 3.8[a] |
| DZP (S) | 2 | 440 ± 4.8[b] |
| TQ (U) | 10 | 372.0 ± 5.49 |
| TQ (S) | 10 | 312.0 ± 3.71 |
| TQ (U) | 20 | 410.0 ± 5.63[a] |
| TQ (S) | 20 | 368.0 ± 5.26[b] |
| MB + TQ(U) | 1 + 10 | 342.0 ± 4.73 |
| MB + TQ(S) | 1 + 10 | 314.0 ± 3.34 |
| MB + TQ(U) | 1 + 20 | 385 ± 4.73 |
| MB + TQ(S) | 1 + 20 | 378.0 ± 4.7 |

Values are expressed as the mean ± SEM, n = 6 in each group. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test; $F(12, 65) = 91.08$; $p < 0.0001$, [a] $p < 0.05$; significant difference from vehicle-treated control group (unstressed mice), [b] $p < 0.05$; significant difference from stressed mice. Sal: Normal saline; CO: Corn oil; IMMO: immobilization; DZP (U): diazepam (unstressed); DZP(S): diazepam (stressed); TQ(U): thymoquinone (unstressed); TQ(S): thymoquinone (stressed); MB: methylene blue

mice. Diazepam did not have any effect on plasma nitrite levels in both unstressed and stressed mice. Thymoquinone (10 and 20 mg/kg) *per se* in unstressed mice did not produce any change in basal plasma nitrite levels. However, the higher dose of thymoquinone (20 mg/kg) significantly attenuated the immobilization-induced increase in plasma nitrite levels in stressed mice. Pre-treatment with methylene blue did not produce any significant change in the effect of thymoquinone on plasma nitrite levels in unstressed and stressed mice (Tab. 3). Methylene blue *per se* did not produce any significant change in plasma nitrite levels in unstressed and stressed mice; consequently, these data are not shown.

### Effect of thymoquinone and methylene blue on brain GABA content

Brain GABA content was significantly lower in stressed mice as compared with that in unstressed mice. Corn oil did not significantly affect the brain GABA levels in saline treated unstressed mice. Diazepam enhanced the GABA content in unstressed and stressed mice. The higher dose of thymoquinone (20 mg/kg) significantly increased the GABA content in unstressed and stressed mice as compared with their respective control groups. Methylene blue pretreatment failed to bring about any further change in brain GABA content in thymoquinone-treated stressed mice (Tab. 4).

### Effect of different treatments on locomotor activity

Immobilization significantly decreased the locomotor activity of mice as compared with saline treated unstressed mice. Thymoquinone (10 and 20 mg/kg) did not significantly affect the spontaneous locomotor activity in unstressed and stressed mice as compared to vehicle-treated unstressed and stressed mice. Methylene blue treatment did not significantly alter the locomotor activity of thymoquinone-treated unstressed and stressed mice (Tab. 5).

**Tab. 5.** Effect of different treatments on locomotor activity of mice

| Treatment | Dose (mg/kg) | Locomotor activity counts |
|---|---|---|
| Saline | 10 ml | 345.2 ± 11.7 |
| Saline + Corn oil | 10 + 10 ml | 338.4 ± 8.6 |
| IMMO | 6 h | 138.4 ± 10.6[a] |
| DZP (U) | 2 | 318 ± 7.2 |
| DZP (S) | 2 | 151.4 ± 6.8 |
| TQ (U) | 10 | 324.6 ± 14.4 |
| TQ (S) | 10 | 126.8 ± 12.1 |
| TQ (U) | 20 | 331.2 ± 14.2 |
| TQ (S) | 20 | 117.3 ± 9.3 |
| MB+ TQ (U) | 1 + 10 | 356.8 ± 13.1 |
| MB + TQ (S) | 1 + 10 | 135.1 ± 9.4 |
| MB + TQ (U) | 1 + 20 | 351.2 ± 11.6 |
| MB + TQ (S) | 1 + 20 | 131.4 ± 10.8 |

Values are expressed as the mean ± SEM, n = 6 in each group. Data were analyzed by ANOVA followed by Tukey's *post-hoc* test; $F(12, 65) = 94.26$; $p < 0.0001$, [a]$p < 0.05$; significant difference from vehicle-treated control group (unstressed mice). Sal: Normal saline; CO: Corn oil; IMMO: immobilization; DZP (U): diazepam (unstressed); DZP(S): diazepam (stressed); TQ(U): thymoquinone (unstressed); TQ(S): thymoquinone (stressed); MB: methylene blue

## Discussion

In the present study, thymoquinone at both doses administered (10 and 20 mg/kg) showed significant anxiolytic activity in unstressed mice, but only the higher dose (20 mg/kg) produced significant antianxiety activity in stressed mice. Diazepam produced a significant anxiolytic effect in unstressed mice, but the antianxiety effect of diazepam was observed to be compromised in stressed mice. This is in agreement with our recent report [17]. The anxiolytic effect of thymoquinone (20 mg/kg) was comparable to that of diazepam (2 mg/kg) in unstressed mice. Furthermore, methylene blue (1 mg/kg) potentiated the anxiolytic effect of thymoquinone in unstressed and stressed mice as compared with their respective control groups. The antianxiety-like effect of thymoquinone and diazepam seem not to be associated with any motor effects because these drugs did not significantly change locomotor function of treated mice as compared to control mice. This confirms the assumption that the antianxiety-like effect of these drugs is specific.

Forced immobilization is one of the best explored models of stress in rodents. This model combines emotional stress (escape reaction) and physiological stress (muscle work), resulting in both restricted mobility and aggression. We have used physical immobilization for 6 h as a stressor in mice and found that stress-exposed mice showed more anxious behavior as compared with unstressed mice. This finding is in agreement with earlier reports that acute (6 h) stress activates NOS and enhances anxiety in rodents [17–19, 21, 48]. Acute immobilization stress, as used in the present study, is reported to increase expression of iNOS in the brain cortex and leads to production of the stable nitric oxide metabolites (nitrite and nitrate) in both plasma and brain [35]. Furthermore, physical or psychological stress-induced changes in the brain correlate with the production of NO metabolites in both peripheral (plasma) and central (brain) compartments [34].

In the present study, although diazepam (2 mg/kg) served to increase brain GABA levels in both unstressed and stressed mice, it produced significant anxiolytic effects in unstressed mice but was unable to exert significant antianxiety effects under stressful conditions. The observed lack of antianxiety effect of diazepam in stressed mice may be adequately explained by two sets of observations: (a) the immobilization stress-induced disturbances in GABAergic receptors and benzodiazepine coupling to these receptors; and (b) the immobilization stress-induced strong anxiogenic nitriergic influence and resultant NO-cGMP enhanced endogenous anxiety accompanied by decreased GABAergic influence. It is well known that behavioral effects of drugs acting at the GABA-benzodiazepine-barbiturate complex may vary between stressed and unstressed animals [6]. Furthermore, immobilization stress is accompanied by an increase in the level of endogenous anxiety and induces persistent changes in the GABA-benzodiazepine-barbiturate complex in the brain of stressed animals [60]. Immobilization stress for 6 h, as used in the present study, has been shown to produce subsensitivity of central GABA receptors [51]. Similar results have been reported with chronic mild restraint stress that produces a decrease in benzodiazepine receptor binding sites [40]. Moreover, immobilization stress for 6 h has previously been reported to act as a nitriergic stimulus and to enhance endogenous anxiety [17, 18, 35]. Furthermore, iNOS-derived NO activates an endogenous NO-sensitive guanylyl cyclase, resulting in increased levels of cGMP [3, 41]. There is evidence

suggesting that the role of the NO/cGMP signaling pathway is the effect of NO on anxiety [12]. Inhibition of the nitric oxide–cGMP pathway by inhibition of NOS has been reported to produce antianxiety effects [50]. The role of cGMP is also indicated by the important observation in the hippocampus, an area involved in anxiety, that cGMP may downregulate $GABA_A$ receptor function and that NO-induced cGMP synthesis induces hyperexcitability [16, 47]. Stress-restress-mediated glucocorticoid release activates iNOS, followed by a reactive downregulation of hippocampal NMDA receptors and dysregulation of inhibitory GABA pathways [24]. NO analogues have been found to reduce GABA-gated currents *via* cGMP-dependent pathways, which lead to anxiety [61]. L-arginine (100 mg/kg, *ip*), an NO donor, has been reported to abolish the anxiolytic-like effect of diazepam (2 mg/kg, *ip*) [57]. Moreover, it has been shown that benzodiazepine anxiolytics do not protect against various emotional changes produced by stress stimuli in mice [55]. Furthermore, diazepam has no effect on NO under stressed conditions; as a result, the effect of diazepam is suppressed under stressed conditions. Therefore, the inability of diazepam to modify the stress-induced increase in nitriergic influence may be responsible for the compromised effect of diazepam in stressed mice.

Prior to the present study, there was only one report on the antianxiety activity of thymoquinone, suggesting a partial involvement of benzodiazepine receptor modulation [46]. This report pertains to the effect of thymoquinone under unstressed conditions using animal model tests such as the head dip and Y-maze. In the present study, we evaluated the involvement of nitriergic and GABAergic systems in the antianxiety effect of thymoquinone under both unstressed and stressed conditions. The anxiolytic effect of thymoquinone observed in unstressed mice is in line with an earlier report on the antianxiety effect of thymoquinone in unstressed mice [46]. Potentiation of the anxiolytic activity of thymoquinone by methylene blue suggests the involvement of guanylate cyclase in the antianxiety effect of thymoquinone. Methylene blue is an inhibitor of a component of the NO signaling pathway, i.e., guanylate cyclase [29]. Methylene blue (administered at a dose of 7.5 mg/kg, intravenously in rats) inactivated NO extracellularly through generation of superoxide anions and was found to produce anxiolysis through the NOS-NO-cGMP pathway [12]. In the present study, methylene blue (1 mg/kg) *per se* did not produce any significant effect on mouse behavior. The target of methylene blue, at the dose used in the present study, is cGMP, a downstream effector of NO [8]. Therefore, methylene blue did not have any significant effect on NO. Hence, lower doses of methylene blue (less than 7.5 mg/kg) did not exert any significant antianxiety effect in unstressed and stressed mice. Therefore, potentiation of the antianxiety effect of thymoquinone by methylene blue suggests the involvement of cGMP in the manifestation of the antianxiety effect of thymoquinone in mice. Thymoquinone (20 mg/kg) significantly attenuated the immobilization-induced increase in plasma nitrite levels and immobilization-induced decrease in GABA content in stressed mice, suggesting that a decrease in NO and increase in GABA may be responsible for the antianxiety effect of thymoquinone in stressed mice.

In unstressed mice, thymoquinone (20 mg/kg)- and diazepam (2 mg/kg)-induced increases in GABA are accompanied by a significant anxiolytic effect, which may further be attributed to the absence of a strong nitriergic influence in unstressed mice, as evident by the insignificant change in plasma nitrite levels produced by these drugs. The absence of nitriergic influence in unstressed mice has also been demonstrated in other reports [5, 19]. The observed pattern of behavioral and biochemical effects of thymoquinone and diazepam under unstressed and stressed conditions further suggests that the nitriergic stimulus in stressed mice is sufficient to disturb benzodiazepine-GABA receptor function. These observations are strengthened by earlier reports of disturbance in benzodiazepine-GABA receptor function by stressful stimuli, including immobilization [6, 60].

Thus, the inability of diazepam to show anxiolytic effects under stressed conditions presented here show a stress-induced disturbance in the GABA-benzodiazepine-barbiturate complex as well as strong nitriergic influence, although the exact mechanism behind this inability has yet to be explored fully. Furthermore, thymoquinone showed significant antianxiety-like activity in mice through possible modulation of NO and GABA.

## References:

1. Abdel-Fattah AM, Matsumoto K, Watanabe H: Antinociceptive effects of *Nigella sativa* oil and its major com-

ponent, thymoquinone, in mice. Eur J Pharmacol, 2000, 400, 89–97.
2. Al-Majed AA, Al-Omar FA, Nagi MN: Neuroprotective effects of thymoquinone against transient forebrain ischemia in the rat hippocampus. Eur J Pharmacol, 2006, 543, 40–47.
3. André M, Latado H, Felley-Bosco E: Inducible nitric oxide synthase-dependent stimulation of PKGI and phosphorylation of VASP in human embryonic kidney cells. Biochem Pharmacol, 2005, 69, 595–602.
4. Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C et al.: International Union of Pharmacology. XV. Subtypes of γ-aminobutyric acid A receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev, 1998, 50, 291–313.
5. Beijamini V, Guimaraes FS: Activation of neurons containing the enzyme nitric oxide synthase following exposure to an elevated plus maze. Brain Res Bull, 2006, 69, 347–355.
6. Boix F, Fernandez TA, Tobea A: The anxiolytic action of benzodiazepines is not present in handling- habituated rats. Pharmacol Biochem Behav, 1988, 31, 541–546.
7. Crawley J, Goodwin FK: Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav, 1980, 13, 167–170.
8. Dawson VL, Dawson TM: Physiological and toxicological actions of nitric oxide in the central nervous system. In: Nitric oxide: biochemistry, molecular biology, and therapeutic implications. Eds. Ignarro L, Murad F, Academic Press, London, 1995, 323–330.
9. De Oliveira RW, Forestiero D, Manfrim CM, Guimaraes FS: Anxiolytic effect induced by nitric oxide synthase inhibitors microinjected at medial amygdala of rats. Psychopharmacology, 2006, 184, 166–172.
10. El-Mahmoudy A, Matsuyama H, Borgan MA, Shimizu Y, El-Sayed MG, Minamoto N, Takewaki T: Thymoquinone suppresses expression of inducible nitric oxide synthase in rat macrophages. Int Immunopharmacol, 2002, 2, 1603–1611.
11. El-Mahmoudy A, Shimizu Y, Shiina T, Matsuyama H, Nikami H, Takewaki T: Macrophage-derived cytokine and nitric oxide profiles in type I, type II diabetes mellitus: effect of thymoquinone. Acta Diabetol, 2005, 42, 23–30.
12. Eroglu L, Cagalyan B: Anxiolytic and antidepressant properties of methylene blue in animal models. Pharmacol Res, 1997, 36, 381–385.
13. Faria MS, Muscara MN, Moreno JH, Teixeira SA, Dias HB, De Oliveira B, Graeff FG, De Nucci G: Acute inhibition of nitric oxide synthesis induces anxiolysis in the plus maze test. Eur J Pharmacol, 1997, 323, 37–43.
14. File SE: The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs. J Neurosci Methods, 1980, 2, 219–238.
15. Forestiero D, Manfrim CM, Guimarães FS, De Oliveira RM: Anxiolytic -like effects induced by nitric oxide synthase inhibitors microinjected into the medial amygdala of rats. Psychopharmacology (Berl), 2006, 184, 166–172.
16. Garthwaite J: Glutamate, nitric oxide and cell-cell signaling in the nervous system. Trends Neurosci, 1991, 14, 60–67.
17. Gilhotra N, Dhingra D: GABAergic and nitriergic modulation by curcumin for its antianxiety-like activity in mice. Brain Res, 2010, 1352, 167–175.
18. Gilhotra N, Dhingra D: Involvement of NO-cGMP pathway in antianxiety effect of aminoguanidine in stressed mice. Prog Neuropsychopharmacol Biol Psychiatry, 2009, 33, 1502–1507.
19. Gilhotra N, Jain H, Dhingra D: Differential effects of nitric oxide synthase inhibitors on anxiety in unstressed and stressed mice. Indian J Exp Biol, 2010,48, 365–372.
20. Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H: Biological significance of nitric oxide-mediated protein modifications. Am J Physiol Lung Cell Mol Physiol, 2004, 287, L262–L268.
21. Goyal R, Kumar A: Protective effect of alprazolam in acute immobilization stress-induced certain behavioral and biochemical alterations in mice. Pharmacol Rep, 2007, 59, 284–290.
22. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnock JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [N–$^{15}$N]-labelled nitrate in biological fluids. Anal Biochem, 1982, 126, 131–138.
23. Hamdy NM, Taha RA: Effects of *Nigella sativa* oil and thymoquinone on oxidative stress and neuropathy in streptozotocin-induced diabetic rats. Pharmacology, 2009, 84, 127–134.
24. Harvey BH, Oosthuizen F, Brand L, Wegener G, Stein DJ: Stress-restress evokes sustained iNOS activity and altered GABA levels and NMDA receptors in rat hippocampus. Psychopharmacology, 2004, 175, 494–502.
25. Hassan M, El-Dakhakhny M: Effect of some *Nigella sativa* constituents on chemical carcinogenesis in hamster cheek pouch. J Egypt Soc Pharmacol Exp Ther, 1992, 11, 675–677.
26. Hosseinzadeh H, Parvardeh S: Anticonvulsant effects of thymoquinone, the major constituent of *Nigella sativa* seeds, in mice. Phytomedicine, 2004, 11, 56–64.
27. Houghton PJ, Zarka R, Heras BDL, Hoult JRS: Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leukocytes and membrane lipid peroxidation. Planta Med, 1995, 61, 33–36.
28. Jasnow AM, Huhman KL: Activation of $GABA_A$ receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. Brain Res, 2001, 920, 142–150.
29. Kontos HA, Wei EP: Hydroxyl radical-dependent inactivation of guanylate cyclase in cerebral arterioles by methylene blue and by LY83583. Stroke, 1993, 24, 427–434.
30. Kulkarni SK: Handbook of Experimental Pharmacology, 3rd ed., Vallabh Prakashan, Delhi, 1999, 135–137.
31. Kumari B, Kumar A, Dhir A: Protective effect of non-selective and selective COX-2-inhibitors in acute immobilization stress-induced behavioral and biochemical alterations. Pharmacol Rep, 2007, 59, 699–707.
32. Lee CY, Cheng HM, Sim SM: Mulberry leaves protect rat tissues from immobilization stress-induced inflammation. Biofactors, 2007, 31, 25–33.

33. Lowe IP, Robins E, Eyerman GS: The fluorimetric measurement of glutamic decarboxylase and its distribution in brain. J Neurochem, 1958, 3, 8–16.
34. Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Leza JC: Stress-induced increase in extracellular sucrose space in rats is mediated by nitric oxide. Brain Res, 2002, 938, 87–91.
35. Madrigal JLM, Olivia HO, Moro MA, Lizasoain I, Lorenzo P, Castrillo A: The increase in TNF-α levels is implicated in NF-κB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. Neuropsychopharmacology, 2002, 26, 155–163.
36. Manzanares PAR, Isoardi NA, Carrer HF, Molina VA: Previous stress facilitates fear memory, attenuates GABAergic inhibition, and increases synaptic plasticity in the rat basolateral amygdale. J Neurosci, 2005, 25, 8725–8734.
37. McHugh SB, Deacon RM, Rawlins JN, Bannerman DM: Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. Behav Neurosci, 2004, 118, 63–78.
38. McNaughton N, Corr PJ: A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. Neurosci Biobehav Rev, 2004, 28, 285–305.
39. Mohammad A, Afridi DM, Garani O, Tucci M: Thymoquinone inhibits the activation of NF-kappaB in the brain and spinal cord of experimental autoimmune encephalomyelitis. Biomed Sci Instrum, 2005, 41, 388–393.
40. Mosaddeghi M, Burke TF, Moerschbaecher JM: Chronic brief restraint decreases in vivo binding of benzodiazepine receptor ligand to mouse brain. Mol Chem Neuropathol, 1993, 18, 115-121.
41. Nagao K, Takenaka S, Yamaji R, Inui H, Nakano Y: Nitric oxide synthase induction, cGMP levation, and biopterin synthesis in vascular smooth muscle cells stimulated with interleukin-1β in hypoxia. J Biochem, 2003, 133, 501–505.
42. Nakashima MN, Ajiki K, Nakashima K, Takahashi M: Possible role of nitric oxide in anxiety following transient cerebral ischemia in mice. J Pharmacol Sci, 2003, 91, 47–52.
43. Niehoff DL, Kuhar M; Benzodiazepine receptors: localization in rat amygdala. J Neurosci, 1983, 3, 2091–2097.
44. Pellow S, Chopin P, File SE, Briley M: Validation of open-closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods, 1985, 14, 149–167.
45. Perveen T, Haider S, Kanwal S, Haleem DJ: Repeated administration of *Nigella sativa* decreases 5-HT turnover and produces anxiolytic effects in rats. Pak J Pharm Sci, 2009, 22, 139–144.
46. Raza M, El-hadiyah TM, Al-shabanah OA: *Nigella sativa* seed constituents and anxiety relief in experimental models. J Herbs Spices Med Plants, 2006, 12, 153–164.
47. Robello M, Amico C, Bucossi G, Cupello A, Rapallino MV, Thellung S: Nitric oxide and $GABA_A$ receptor function in the rat cerebral cortex and cerebellar granule cells. Neuroscience, 1996, 74, 99–105.
48. Sevgi S, Ozek M, Eroglu L: L-NAME prevents anxiety-like and depression-like behavior in rats exposed to restraint stress. Methods Find Exp Clin Pharmacol, 2006, 28, 95–99.
49. Shiah IS, Yatham LN: GABA function in mood disorder: an update and critical review. Life Sci, 1998, 63, 1289–1303.
50. Spolidório PC, Echeverry MB, Iyomasa M, Guimarães FS, Del Bel EA: Anxiolytic effects induced by inhibition of the nitric oxide-cGMP pathway in the rat dorsal hippocampus. Psychopharmacology (Berl), 2007, 195, 183–192.
51. Sun AY, Li DX, Wang YL: Stress-induced changes of central GABAergic function in rats. Zhongguo Yao Li Xue Bao, 1991, 12, 387–390.
52. Talarek S, Orzelska J, Listos J, Fidecka S: Effects of sildenafil treatment on the development of tolerance to diazepam-induced motor impairment and sedation in mice. Pharmacol Rep, 2010, 62, 627–634.
53. Tanaka M, Ikeda T, Hayashi S, Iijima N, Amaya F, Hisa Y: Nitriergic neurons in the medial amygdala project to the hypothalamic paraventricular nucleus of the rat. Brain Res, 1997, 777, 13–21.
54. Thomas M, Feron O: Nitric oxide synthases: which, where, how, and why? J Clin Invest, 1997, 100, 2146–2152.
55. Tsuji M, Takeda H, Matsumiya T: Different effects of $5\text{-}HT_{1A}$ receptor agonists and benzodiazepine anxiolytics on the emotional state of naive and stressed mice: a study using the hole-board test. Psychopharmacology (Berl), 2000, 152,157–166.
56. Vincent SR: Nitric oxide: a radical neurotransmitter in the central nervous system. Prog Neurobiol, 1994, 42, 129–160.
57. Volke V, Soosaar A, Kõks S, Vasar E, Männistö PT: L-Arginine abolishes the anxiolytic-like effect of diazepam in the elevated plus-maze test in rats. Eur J Pharmacol, 1998, 351, 287–290.
58. Volke V, Wegener G, Bourin M, Vasar E: Antidepressant- and anxiolytic-like effects of selective neuronal NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole in mice. Behav Brain Res, 2003, 140, 141–147.
59. Volke V, Soosar A, Koks S, Bourin M, Mannisto PT, Vasar E: 7-Nitroindazole, a nitric oxide synthase inhibitor, has anxiolytic-like properties in exploratory models of anxiety. Psychopharmacology (Berl), 1997, 131, 399–405.
60. Weizman AM, Bidder FF, Gavish M: Food deprivation modulates gamma-butyric acid receptors and peripheral benzodiazepine binding sites in rats. Brain Res, 1990, 535, 96–100.
61. Wexler EM, Stanton PK, Nawy S: Nitric oxide depresses $GABA_A$ receptor function via coactivation of cGMP-dependent kinase and phosphodiesterase. J Neurosci, 1998, 18, 2342–2349.

**Received:** September 9, 2010; **in the revised form:** January 18, 2011; **accepted:** January 26, 2011.