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GABAergic and nitriergic influence in antianxiety-like Activity of Garlic in Mice

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ABSTRACT

Objectives: To investigate the GABAergic and nitriergic mechanism involved in the anxiolytic-like profile of ethanolic extract of garlic (GE).
Materials and Methods: Male Swiss albino mice were employed in the present study. Stress was produced in mice by immobilizing them for 6h. Elevated plus maze, light/dark box and social interaction test were used for the assessment of anxiety in mice. Concentrations of GABA in brain and nitrite level in plasma were estimated to determine the possible involvement of GABAergic and nitriergic mechanisms in the anxiolytic profile of GE.
Results: The present study showed that the GE produced significant antianxiety- like activity in unstressed and stressed mice. In unstressed mice, GE significantly increased GABA levels, but could not produce any change in nitrite levels. Meanwhile, in stressed mice, GE significantly increased GABA levels along with a significant decrease in nitrite levels. Pre-treatment with aminoguanidine, an inducible nitric oxide synthase inhibitor, significantly enhanced the anxiolytic-like activity of GE, as compared to GE and aminoguanidine alone in stressed mice, but not in unstressed mice. On the other hand, pretreatment with 7-nitroindazole, a neuronal nitric oxide synthase inhibitor, did not produce any significant change in antianxiety- like activity of GE in unstressed as well as stressed mice.
Conclusion: It has been concluded that the garlic may possess anxiolytic- like activity and possess NOS inhibiting property in stressed mice, which may add to its status to be used in stress-induced anxiety conditions.

INTRODUCTION

Stress can influence the neurobehavioral profile and precipitate an anxiety-like syndrome (Esch *et al*., 2002; Masood *et al*., 2003). Immobilization stress (60 and 120 min) has been reported to enhance anxious behavior in rodents (Heinrichs *et al*., 1994). Acute (6h) stress activates nitric oxide synthase (NOS) and enhance anxiety in rodents (Madrigal *et al*., 2002; Sevgi *et al*., 2006; Sharma *et al*., 2011). Stressor used in the present study (6h immobilization) has been found to increase TNF-α levels. This increase in TNF-α level is observed to underlie NF-κB activation and iNOS expression in brain cortex after 6h immobilization stress, as used in the present study (Madrigal *et al*., 2002). Activation of iNOS produces nitric oxide in the large quantity (Nagao *et al*., 2003). A transgenic mouse model, over-expressing gene for TNF-α has been shown to express excessive (pathological) anxiety in light and dark test (Fiore *et al*., 1998). Further, aminoguanidine, an inhibitor of iNOS, has been reported to attenuate 6h immobilization-induced anxiety in mice (Gilhotra and Dhingra, 2009). Similarly, PDTC, an inhibitor of NF-κB, is observed to produce the antianxiety-like activity and enhance the antianxiety-like activity of aminoguanidine in the same 6h immobilization model in mice (Gilhotra *et al*., 2010). Recently, inhibition of p38MAPK, a reported activator of NF-κB (Baeza-Raja and Munoz-Canoves, 2004; Vercammen *et al*., 2008) has been found to restore the similar 6h immobilization stress-attenuated anti-anxiety effect of diazepam (Sharma *et al*., 2011). In addition to reported antianxiety-like activity of aminoguanidine (a selective iNOS inhibitor), non-selective inhibition of nitric oxide synthase by L-NAME (Sevgi *et al*., 2006; Kumar and Singh, 2008) and selective inhibition of other isoforms of NOS, relevant to pathological anxiety, i.e. neuronal form of NOS, by 7-nitroindazole

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(Pokk *et al*., 2001) have also found to produce antianxiety-like activities.

In addition to the above mentioned nitriergic mechanism of 6h immobilization stress-induced anxiety, other mechanism may involve GABAergic modulation, as indicated by the observation that short term stress downregulate GABA pathways and reduce brain GABA content in mice (Manzanares *et al*., 2005). Involvement of GABAergic mechanism in 6h immobilization stress- induced anxiety is strengthened by observations that anti-anxiety effect of diazepam, a potent GABAergic agonist, is suppressed in mice, exposed to 6h immobilization stress. Both GABA and NO are actively involved in stress- induced behavioral processes including anxiety and their levels are observed to differ in unstressed and stressed mice (Gilhotra and Dhingra, 2009; Sharma *et al*., 2011). Recently, strong nitriergic influence over GABA has been observed, where SB-203580 (p38MAPK inhibitor) and PDTC (NF-κB inhibitor) were observed to restore the compromised anti-anxiety effect of diazepam in 6h immobilization- stressed mice (Sharma *et al*., 2011).

Allium sativum Linn. (Family: Lilliaceae) is commonly known as garlic. Dried bulbs of garlic are incorporated in day to day practice as condiment/spice in food items. Garlic is endowed with several medicinal properties (Wealth of India, 1985). It has been reported to possess anti-stress, anti-ageing properties and reported to prevent progression of Alzheimer's disease (Ushijima *et al*., 1997; Chauhan and Sandoval 2007). Garlic and its constituents has demonstrated a wide spectrum of pharmacological properties; anti-oxidative (Ide *et al*., 1997; Imai *et al*., 1994), immunomodulating (Kyo *et al*., 2001), and hepato- protective (Wang *et al*., 1999; Sumioka *et al*., 2001) properties. Among its effects on brain, garlic has been reported to exert neuroprotection (Perez-Severiano *et al*., 2004; Saleem *et al*., 2006), prevent stress-induced organ degeneration (Saglam *et al*., 2006) and possess anti-stress property (Kyo *et al*., 1999). Recently, Dhingra and Kumar have reported anti-depressant property of garlic in mice (Dhingra and Kumar, 2008). Garlic significantly suppresses both mRNA and protein levels of iNOS (Schwartz *et al*., 2002). Further, garlic also inhibits nuclear factor kappa beta, a transcription activator of iNOS (Youn *et al*., 2005).

Therefore, in view of the existing relevant literature, we explored the potential of garlic extract for its anti-anxiety effect in behavioral paradigms, used to assess anxiety in mice, under unstressed and stressed conditions. Further, in view of relative roles of NO and GABA in stress-induced psychiatric changes including anxiety, biochemical estimations of plasma nitrite levels and brain GABA contents were also carried out to study the effect of different treatments on these parameters and resultant mice behavior in selected paradigms to assess anxiety.

MATERIALS AND METHODS

Animals

Swiss albino mice (male; 20-25 g) were employed in the present study. Animals were procured from Disease Free Small Animal House, CCS Haryana Agricultural University, Hisar, Haryana, India. Animals were provided normal diet and tap water *ad libitum* and were exposed to 12h light and 12h dark cycle. The animals were acclimatized to the laboratory condition before experiments. Experimental protocol was approved by Institutional Animal Ethics Committee. Care of the animals was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Collection of plant material and Preparation of extract from plant material

Dried bulbs of *Allium sativum* Linn.was purchased from the local markets of Hisar, Haryana, India. About 200g of chopped garlic extract were imbibed in 20% v/v ethanol for 10 days at ambient temperature and filtered. The crude extract was dried on water bath and kept in refrigerator till further use.

Drugs

Aminoguanidine (AG; Sigma Chemical Co., St. Louis, MO, USA), 7-nitroindazole (7-NI; Cayman Chemicals, USA), Diazepam (DZP; Calmpose injection, Ranbaxy Laboratories, Gurgaon, India) were used in the present study.

Behavioral testing

Elevated plus maze

The maze was a plus-shaped apparatus with an open roof, consisting of two 16×5 cm open arms, and two 16×5×12 cm enclosed arms, and elevated at a height of 25 cm. All testing was conducted between 0800 and 1700 hours in a quiet and dimly illuminated room. Each mouse was placed individually at the centre of elevated plus maze with its head facing towards an open arm and observed for 5 min to record the number of entries into open arm, closed arm and time spent in each arm (Kulkarni, 1999). A mouse was considered to have entered or spent time in an arm only when all four paws were in the respective arm. The time spent in the open arms and the number of open-arm entries were expressed as a percentage of total arm activity (open arm time / open arm time + closed arm time) × 100, and total arm entries (open arm entries / open arm entries + closed arm entries) × 100, respectively. A higher percentage of open arm time or open arm entries are taken as measures of anxiety reduction (anxiolysis).

In elevated plus maze test, percent time spent on the open arms was determined as follows:

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

In elevated plus maze test, percent entries in open arms was determined as follows:

$$\% = 100 \times \frac{\text{Number of open arm entries}}{\text{Total entries in open and closed arms}}$$

Light and dark box test

The apparatus consisted a rectangular box (45×27×27 cm), partitioned into two compartments (one light and one dark) connected by a 7.5×7.5 cm opening in the wall between compartments. An animal was placed into the center of the light compartment and was observed for 5 min for time spent in open (white/light) compartment (Crawley and Goodwin, 1980). Percent time spent in the light compartment was determined as follows:

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

Social interaction test

The social interaction arena was an open topped box (22×15×12 cm). Mice were isolated for 1 h before the test. After introduction to the test arena, mice were observed for cumulative time spent in genital investigation, sniffing a partner, climbing over and under, neck licking and boxing (File, 1980).

Biochemical estimation of plasma nitrite

For nitrite estimation, blood was withdrawn from tail vein of immobilized mice, immediately before setting the animal free and subjecting it to behavioral tests in all the groups. The sampling procedure was completed during immobilization to avoid the extra stress incurred upon mice during a new procedure of mouse immobilization for handling the tail of mice. Plasma was separated using refrigerated centrifuge at 2500 rpm for 10 min. It was stored in a refrigerator and processed for nitrite estimation within 24h (Gilhotra and Dhingra, 2009; 2011; Sharma *et al*., 2011). Plasma nitrite was measured by spectrophotometric assay based on Griess reaction (Green *et al*., 1982).

Brain GABA Estimation

Brain GABA content was estimated using method of Lowe *et al*., (1958).

Experimental protocol

Swiss albino mice (male; 20-25g; n = 6 each) were employed in the present study. Stress was produced in mice by immobilizing them for 6h by taping all its four limbs and trunk on a wooden board (Gilhotra and Dhingra, 2009; 2011; Sharma *et al*., 2011; Gilhotra *et al*., 2010). Mice subjected to immobilization were called as stressed mice. Unstressed mice were exposed to elevated plus maze and light-dark test for normal duration (5 min), sufficient to assess the anxiety levels in rodents (Calatayud *et al*., 2004) and not subjected to immobilization and mentioned accordingly in the manuscript. All treatments (Vehicle, garlic extract (20, 40 and 80 mg/kg), aminoguanidine (50 mg/kg) (Gilhotra *et al*., 2010), 7-nitroindazole (20 mg/kg) (Gilhotra *et al*., 2010)) were administered intraperitoneally in a fixed volume of 1ml/100g body weight. For nitrite estimation, blood was withdrawn from tail vein of immobilized mice immediately before setting the animal free and subjecting it to behavioral tests in all the groups. The sampling procedure was completed during immobilization to avoid the extra stress incurred upon mice during an altogether new procedure of mouse immobilization for handling the tail of mice (Gilhotra and Dhingra, 2009; 2011; Sharma *et al*., 2011; Gilhotra *et al*., 2010). The apparatus was thoroughly cleaned using 5% ethanol before placing each mouse in the cage.

Locomotor activity

The effects of various treatments on spontaneous locomotor activity of animals were measured by using an actophotometer (INCO, Ambala, India). The data are presented as the number of counts, recorded by the apparatus as light ray is interrupted between 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.

Statistical analysis

All the results are expressed as Mean ± S.E.M. Data were analyzed by analysis of variance (ANOVA) in Graph Pad Instat (GPIS) package, version 3.05. $p<0.05$ was considered as significant.

RESULTS

In elevated plus maze, light-dark test and social interaction test, significant increase in percentage of time spent in open arms and number of open arm entries; significant increase in percentage of time spent in light compartment and significant increase in percentage of time spent in social interaction indicate anxiolytic-like effect respectively. On the other hand, significant decrease in various parameters of these behavioral models indicates anxiogenic effect.

In elevated plus maze test, 6h immobilization significantly decreased percentage of time spent in open arms and number of open arm entries in mice as compared to vehicle-treated unstressed mice. Diazepam (2 mg/kg) significantly increased percentage of time spent in open arms and number of open arm entries in unstressed mice, but not in stressed mice. GE (80 mg/kg) produced a significant antianxiety- like activity in unstressed as well as stressed mice. AG (50 mg/kg), an inhibitor of inducible isoform of nitric oxide synthase, per se, increased percentage of time spent in open arms and number of open arm entries in stressed mice, but not in unstressed mice. Pre-treatment with AG (50 mg/kg) significantly enhanced antianxiety effect of GE (40 mg/kg), as compared to GE (40 mg/kg) as well as AG (50 mg/kg) alone in stressed mice, but not in unstressed mice. 7-NI (20 mg/kg), an inhibitor of neuronal isoform of nitric oxide synthase, per se, significantly produced anti-anxiety activity in unstressed mice, but not in stressed mice. Pre-treatment with 7-NI (20 mg/kg) did not produced any significant change in antianxiety- like activity of GE (40 mg/kg) in unstressed and stressed mice (Fig. 1 and 2).

In light-dark test, 6-h immobilization significantly decreased percentage of time spent by mice in light compartment as compared to vehicle- treated unstressed mice. Diazepam (2 mg/kg) significantly increased percentage of time spent by

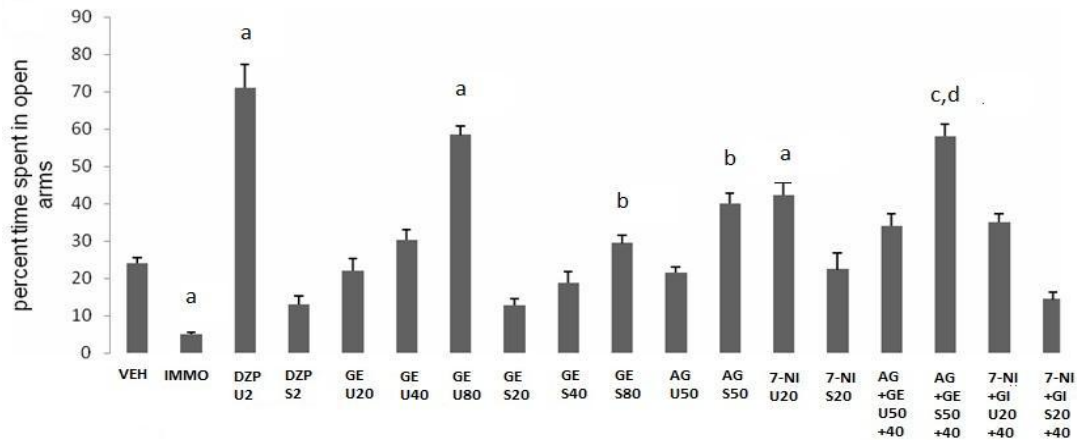

Fig. 1: Effect of different treatments on time spent by mice in open arms of plus maze.

n = 6 in each group. Values expressed as Mean ± S.E., Data was analyzed by ANOVA followed by Tukey's *Post hoc* Test, F (17, 90) = 34.96; P < 0.0001, a = significant difference from vehicle treated control group, b = significant difference from stressed mice, c,d = significant difference from GE (40 mg/kg) and AG (50 mg/kg) - treated stressed mice. **Veh:** vehicle; **IMMO**: immobilization; **DZP (U):** diazepam (unstressed); **DZP(S):** diazepam (stressed) **GE(U):** garlic extract (unstressed); **GE(S):** garlic extract (stressed); **AG(U):** aminoguanidine (unstressed); **AG(S):** aminoguanidine (stressed). **7-NI(U):** 7-nitroindazole (unstressed); **7-NI(S):** 7-nitroindazole (stressed). Doses mentioned are in mg/kg.

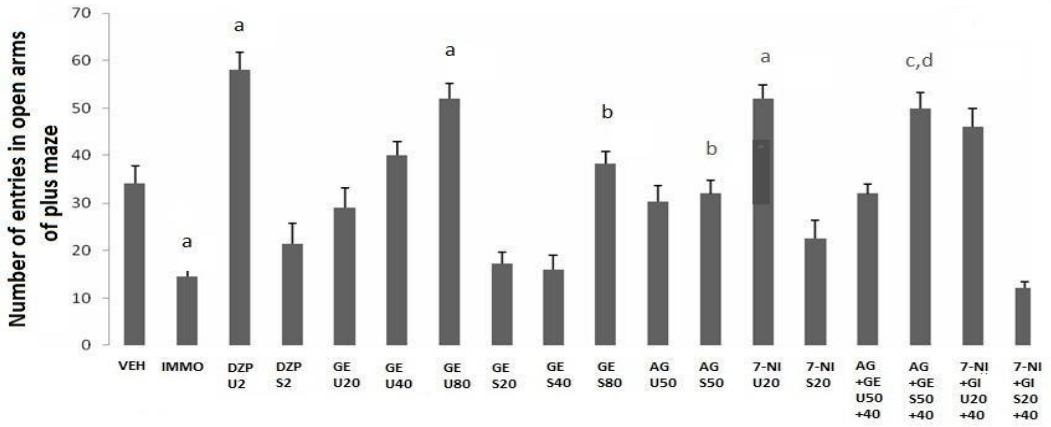

Fig. 2. Effect of different treatments on number of entries by mice in open arms of plus maze.

n = 6 in each group. Values expressed as Mean ± S.E., Data was analyzed by ANOVA followed by Tukey's *Post hoc* Test, F (17, 90) = 19.43; P < 0.0001, a = significant difference from vehicle treated control group, b = significant difference from stressed mice, c,d = significant difference from GE (50 mg/kg) and AG (50 mg/kg) - treated stressed mice.**Veh:** vehicle; **IMMO**: immobilization; **DZP (U):** diazepam (unstressed); **DZP(S):** diazepam (stressed) **GE(U):** garlic extract (unstressed); **GE(S):** garlic extract (stressed); **AG(U):** aminoguanidine (unstressed); **AG(S):** aminoguanidine (stressed). **7-NI(U):** 7-nitroindazole (unstressed); **7-NI(S):** 7-nitroindazole (stressed). Doses mentioned are in mg/kg.

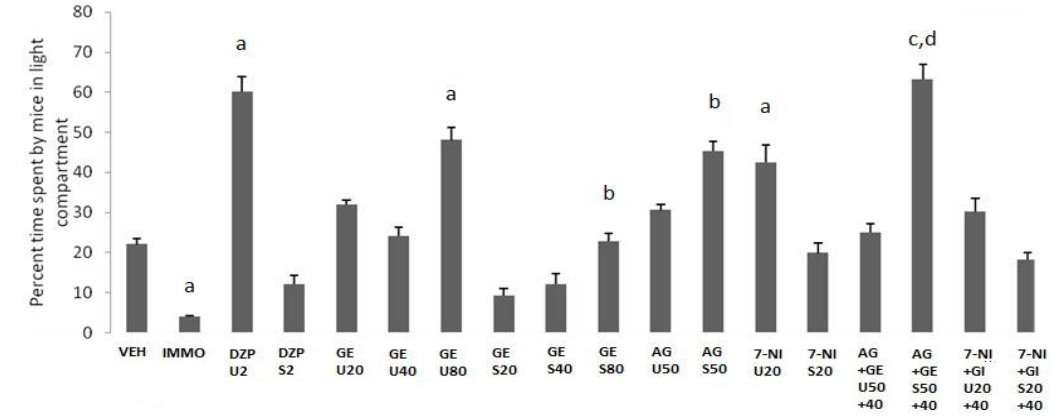

Fig. 3. Effect of different treatments on time spent by mice in light compartment of light/dark box.

n = 6 in each group. Values expressed as Mean ± S.E., Data was analyzed by ANOVA followed by Tukey's *Post hoc* Test, F (17, 90) = 43.04; P < 0.0001, a = p < 0.05 significant difference from vehicle treated control group, b = p < 0.05 significant difference from stressed mice, c,d = p < 0.05 significant difference from GE (40 mg/kg) and AG (50 mg/kg) - treated stressed mice. **Veh:** Vehicle; **IMMO**: immobilization; **DZP (U):** diazepam (unstressed); **DZP(S):** diazepam (stressed) **GE(U):** garlic extract (unstressed); **GE(S):** garlic extract (stressed); **AG(U):** aminoguanidine (unstressed); **AG(S):** aminoguanidine (stressed). **7-NI(U):** 7-nitroindazole (unstressed); **7-NI(S):** 7-nitroindazole (stressed). Doses mentioned are in mg/kg.

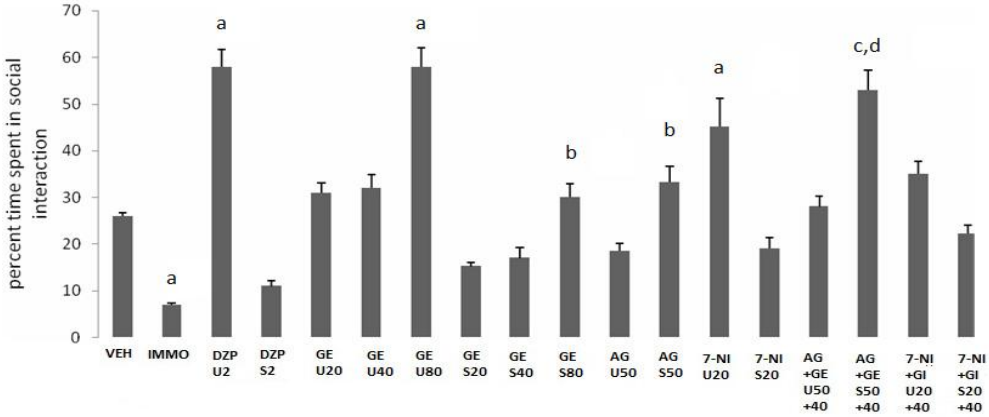

Fig. 4: Effect of different treatments on time spent by mice in social interaction.

n = 6 in each group. Values expressed as Mean ± S.E., Data was analyzed by ANOVA followed by Tukey's *Post hoc* Test, F (17, 90) = 34.39; P < 0.0001, a = p < 0.05 significant difference from vehicle treated control group, b = p < 0.05 significant difference from stressed mice, c,d = p < 0.05 significant difference from GE (40 mg/kg) and AG (50 mg/kg)- treated stressed mice. **Veh:** vehicle; **IMMO**: immobilization; **DZP (U):** diazepam (unstressed); **DZP(S):** diazepam (stressed) **GE(U):** garlic extract (unstressed); **GE(S):** garlic extract (stressed); **AG(U):** aminoguanidine (unstressed); **AG(S):** aminoguanidine (stressed). **7-NI(U):** 7-nitroindazole (unstressed); **7-NI(S):** 7-nitroindazole (stressed). Doses mentioned are in mg/kg.

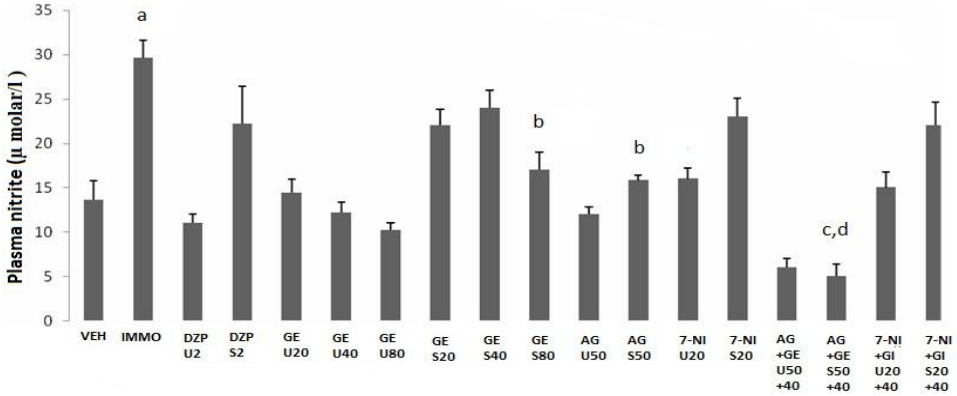

Fig. 5: Effect of different treatments on plasma nitrite levels.

n = 6 in each group. Values expressed as Mean ± S.E., Data was analyzed by ANOVA followed by Tukey's *Post hoc* Test, F (17, 90) = 11.78; P < 0.0001, a = p < 0.05 significant difference from vehicle treated control group, b = p < 0.05 significant difference from stressed mice, c,d = p < 0.05 significant difference from AG (50 mg/kg) and GE (40 mg/kg)- treated stressed mice. **Veh:** vehicle; **IMMO**: immobilization; **DZP (U):** diazepam (unstressed); **DZP(S):** diazepam (stressed) **GE(U):** garlic extract (unstressed); **GE(S):** garlic extract (stressed); **AG(U):** aminoguanidine (unstressed); **AG(S):** aminoguanidine (stressed). **7-NI(U):** 7-nitroindazole (unstressed); **7-NI(S):** 7-nitroindazole (stressed). Doses mentioned are in mg/kg.

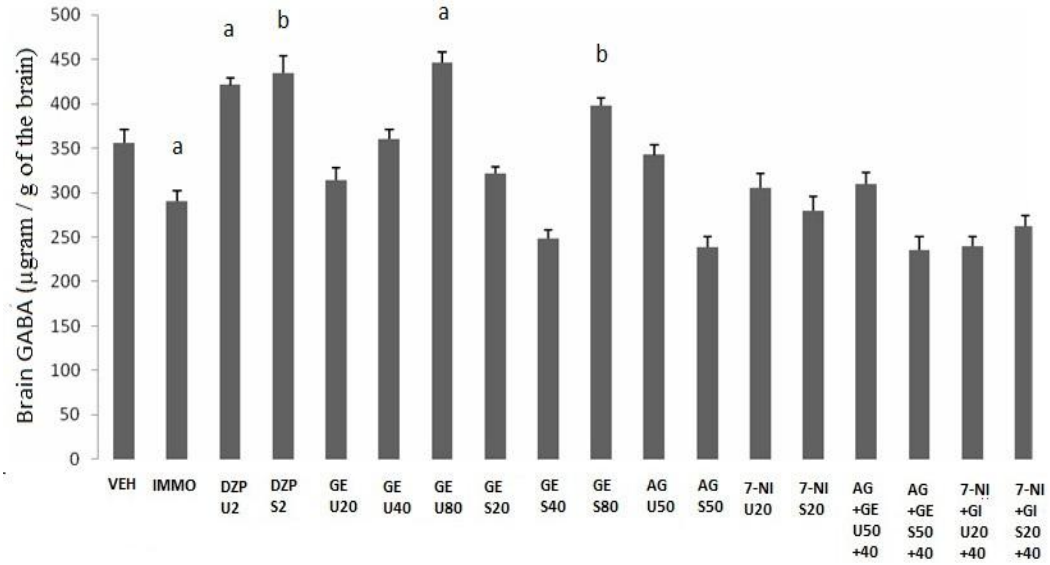

Fig. 6: Effect of different treatments on brain GABA levels.

n = 6 in each group. Values expressed as Mean ± S.E., Data was analyzed by ANOVA followed by Tukey's *Post hoc* Test, F (17, 90) = 29.12; P < 0.0001, a = p < 0.05 significant difference from vehicle treated control group, b = p < 0.05 significant difference from stressed mice. **Veh:** vehicle; **IMMO**: immobilization; **DZP (U):** diazepam (unstressed); **DZP(S):** diazepam (stressed) **GE(U):** garlic extract (unstressed); **GE(S):** garlic extract (stressed); **AG(U):** aminoguanidine (unstressed); **AG(S):** aminoguanidine (stressed). **7-NI(U):** 7-nitroindazole (unstressed); **7-NI(S):** 7-nitroindazole (stressed). Doses mentioned are in mg/kg.

unstressed mice in light compartment, but not in case of stressed mice. GE (80 mg/kg) produced a significant antianxiety-like activity in unstressed as well as stressed mice. AG (50 mg/kg) per se, significantly increased percentage of time spent in light compartment by stressed mice as compared to vehicle- treated stressed mice, but not in unstressed mice. Pre-treatment with AG (50 mg/kg) significantly enhanced antianxiety effect of GE (40 mg/kg) in stressed mice as compared to GE (40 mg/kg) as well as AG (50 mg/kg) alone in stressed mice, but not in unstressed mice. 7-NI (20 mg/kg) per se significantly produced anti-anxiety activity in unstressed mice, but not in stressed mice. Pre-treatment with 7-NI (20 mg/kg) did not produced any significant change in antianxiety- like activity of GE (40 mg/kg) in unstressed and stressed mice (Fig. 3).

In social interaction test, 6h immobilization significantly decreased percentage of time spent by mice in social interaction as compared to vehicle- treated unstressed mice. Diazepam (2 mg/kg) significantly increased percentage of time spent in social interaction by unstressed mice, but not by stressed mice. GE (80 mg/kg) produced a significant antianxiety- like activity in unstressed as well as stressed mice. AG (50 mg/kg) per se, produced a significant antianxiety- like activity in stressed mice, but not in unstressed mice. Pre- treatment with AG (50 mg/kg) significantly enhanced antianxiety effect of GE (40 mg/kg) in stressed mice as compared to GE (40 mg/kg) as well as AG (50 mg/kg) alone in stressed mice, but not in unstressed mice. 7-NI (20 mg/kg) per se, significantly produced anti-anxiety activity in unstressed mice, but not in stressed mice. Pre-treatment with 7-NI (20 mg/kg) did not produced any significant change in antianxiety-like activity of GE (40 mg/kg) in unstressed and stressed mice (Fig. 4).

Immobilization stress significantly increased plasma nitrite levels in stressed mice, as compared to vehicle- treated unstressed mice. Diazepam (2 mg/kg) did not produce any significant change in plasma nitrite levels in both unstressed and stressed mice. GE (20, 40 or 80 mg/kg) did not produce any change in basal plasma nitrite levels in unstressed mice as compared to vehicle- treated unstressed mice. In stressed mice, only high dose of GE (80 mg/kg) significantly attenuated the immobilization stress- induced increase in plasma nitrite levels. AG (50 mg/kg) per se administration did not produce any significant change in plasma nitrite levels in unstressed mice as compared to that in vehicle- treated unstressed mice, but it significantly decreased immobilization- induced increase in plasma nitrite levels in stressed mice. Further, pre-treatment with AG (50 mg/kg) significantly decreased plasma nitrite levels in GE (40 mg/kg)- treated stressed mice as compared to mice, treated with GE (40 mg/kg) and AG (50 mg/kg) alone. 7-NI (20 mg/kg) per se, did not produce any significant change in plasma nitrite levels in both unstressed and stressed mice as compared to their respective vehicle- treated controls. Similarly, pre-treatment with 7-NI (20 mg/kg) in GE (40 mg/kg)- treated stressed mice did not produce any significant change in plasma nitrite levels, as compared to that in stressed mice treated with GE (40 mg/kg) and 7-NI (20 mg/kg) alone (Fig. 5).

Immobilization stress significantly lowered brain GABA content in stressed mice as compared to vehicle- treated unstressed mice. Diazepam (2 mg/kg) significantly enhanced the brain GABA content in unstressed as well as stressed mice as compared to respective vehicle- treated controls. GE (80 mg/kg) significantly increased brain GABA content in unstressed as well as stressed mice. AG and 7-NI per se failed to produce any change in brain GABA levels in unstressed as well as stressed mice. Further, Pre-treatments with AG (50 mg/kg) and 7-NI (20 mg/kg) failed to bring any change in GABA levels in brains of unstressed as well as stressed mice, treated with GE (40 mg/kg) (Fig. 6).

None of the drug treatment was able to produce any significant change in locomotor activity, so results are not shown here.

DISCUSSION

Forced immobilization is one of the best explored models of stress in rats. As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress (Bhattacharya and Bhattacharya, 1982). Therefore, we have used physical immobilization for 6h as stressor for mice and found that stress exposed mice were more anxious in their behavior as compared to unstressed mice (Gilhotra and Dhingra, 2009; 2011; Sharma *et al*., 2011). Further, immobilization stress, as used in the present study, is reported to increase expression of iNOS in brain cortex and leads to production of the stable nitric oxide metabolites (nitrite and nitrate) in both plasma and brain (Madrigal *et al*., 2002; Lee *et al*., 2007) and plasma nitrite levels are found to increase significantly in mice, exposed to 6h immobilization stress (Gilhotra and Dhingra, 2009; 2011; Sharma *et al*., 2011). On the other hand, reports exploring the effect of longer stress on animal behavior are not consistent, rather contradictory to each other; for example, 72h sleep-deprived mice showed an anxiogenic behavior in elevated plus maze (Kumar and Singh, 2008) and opposite results were found by another study (Pokk and Vali, 2002), indicating reduced anxiety in mice induced by 24 h small platform stress.

Diazepam (2mg/kg) produced significant anxiolytic- like effect in unstressed mice, but could not exert significant anxiolysis in stressed mice. This effect is independent of its effect on locomotor activity, as noticed in the present study and also supported by the literature (Kurt *et al*., 2004). This observation is supported by our earlier reports (Gilhotra and Dhingra, 2011). Recently, Sharma *et al*. have reported the observed the underlying nitriergic influence, responsible for this compromised effect of diazepam in stressed mice (Sharma *et al*., 2011). GE (80 mg/kg) produced a significant anti-anxiety activity in unstressed mice, which was accompanied by a significant increase in brain GABA content. Agents, that act as agonists of GABA receptors (known as GABA analogues or GABAergic drugs) or increase the available amount of GABA typically have relaxing, anti-anxiety and anti-convulsive effects (Chapouthier and Venault, 2001; Foster and

Camp, 2006). GE (80 mg/kg) also showed significant antianxiety effect in stressed mice, which was accompanied by a significant increase in brain GABA content and a significant decrease in plasma nitrite levels. This effect may be attributed to inhibitory effect on NO production (Schwartz *et al*., 2002), since, stressor (6h immobilization), used in the present study, leads to increase in expression of NO through activation of iNOS and AG, an inhibitor of iNOS, is observed to show anxiolytic- like activity (Madrigal *et al*., 2002; Gilhotra and Dhingra, 2009). Further, garlic is also reported to inhibit the iNOS and NF-κB (Youn *et al*., 2005). To date, there is not even a single report regarding protective effect of garlic in anxiety under unstressed and stressed conditions in mice. We have explored the possible impact of iNOS and NF-κB inhibiting property of GE on anxiety, using 6h immobilization-induced anxiety model; that involve TNF, NF-κB and iNOS (Madrigal *et al*., 2002) and furthermore, iNOS inhibitor (Gilhotra and Dhingra, 2009) and NF-κB inhibitor (Gilhotra and Dhingra, 2009; Sharma *et al*., 2011) have shown antianxiety- like activities in the above said model. In addition, GABA modulation property of garlic was also investigated in anxiety condition, keeping in mind that GABA modulators play an established therapeutic role in anxiety (Shwartz *et al*. 2005) and drugs, that act as agonists of GABA receptors (known as GABA analogues or GABAergic drugs) or increase the available amount of GABA typically have relaxing, anti-anxiety and anti-convulsive effects (Chapouthier and Venault, 2001; Foster and Kemp, 2006). In the present study, the noted antianxiety effect of GE was accompanied by a significant attenuation of immobilization-induced increase in plasma nitrite levels in stressed mice. Acute immobilization-induced increase in plasma nitrite levels is reported to be decreased by AG, a selective iNOS inhibitor (Gilhotra and Dhingra, 2009). Here, in the present study, the expression of antianxiety- like effect of GE in mice under stressed conditions and enhancement of this effect by pre-treatment with AG as compared to AG and GE alone, indicate the role of iNOS in the noted antianxiety effect of GE. It is noteworthy that 7-NI, a selective nNOS inhibitor did not produced any change in the antianxiety- like activity of GE in unstressed as well as stressed mice. 7-nitroindazole per se has produced antianxiety-like effect in unstressed mice and failed to do so in stressed mice. Though, expression of nNOS is also found to increase after restraint stress (De Oliviera *et al*., 2000). But, nNOS is more sensitive to autoinhibitory effects of NO on enzyme activity (Griscavage *et al*., 1995). 7-Nitroindazole has also failed to block the stress-induced hippocampal NOS activation (Harvey *et al*., 2004). It is also advocated that overproduction of NO following stress more likely involve iNOS and not nNOS. Further, increased nNOS expression following restraint stress may be more effectively targeted by 7-nitroindazole under conditions of severe stress (Harvey *et al*., 2004), and may not do so in acute stress, used in the present study. In the present study, selective inhibitor of different isoforms of NOS i.e. AG (inhibitor of iNOS) and 7-NI (inhibitor of nNOS) have produced antianxiety- like activities. However, they have produced differential effects in unstressed and stressed mice; AG, showing antianxiety- like activity in stressed mice and 7-NI, showing antianxiety- like activity in unstressed mice. These observations are supported by our earlier observations on these agents in the same model (Gilhotra *et al*., 2010). Further, when given as pretreatment 7-nitroindazole did not significantly enhance the effect of GE (40 mg/kg), suggesting the non-involvement of nNOS in antianxiety-like effect of GE in unstressed and stressed mice.

In addition to changes in nitrite levels, 6h immobilization stress also produced a significant decrease in brain GABA content. Diazepam (2 mg/kg) significantly increased the brain GABA content in unstressed as well as stressed mice. Similarly, GE (80 mg/kg) has been able to increase the brain GABA content in unstressed as well as stressed mice. However, antianxiety- like effects of GE (80 mg/kg) and diazepam (2 mg/kg) are not exerted equally in both unstressed and stressed mice; GE (80 mg/kg) producing antianxiety- like activity in both unstressed and stressed mice, while diazepam (2 mg/kg), producing antianxiety- like activity in only unstressed mice. In unstressed mice, GE (80 mg/kg)- and diazepam (2 mg/kg)- induced increases in GABA content may be responsible for their significant antianxiety- like effect, which may further be attributed to the absence of a strong nitriergic influence in unstressed mice, as compared to strong nitriergic influence, observed in stressed mice (Sharma *et al*., 2011). The absence of nitriergic influence in unstressed mice has also been demonstrated in other reports (Gilhotra *et al*., 2010). The observed pattern of behavioral and biochemical effects of GE 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 (Boix *et al*., 1988; Weizman *et al*., 1990).

Observed insignificant effects of GE, diazepam, AG, 7-NI on locomotor activity suggested the negligible interference of locomotion in observed behavior of mice in behavioral paradigms.

CONCLUSION

In conclusion, the above study suggests the garlic may exert antianxiety- like activity in mice by increasing GABA content and overcoming nitriergic influence on GABAergic environment, as evident by enhancement of the effect of GE by NOS inhibitors in unstressed and stressed mice.

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