OPEN ACCESS Pharmacologia

ISSN 2044-4648
DOI: 10.5567/pharmacologia.2016.264.271

Research Article
Investigation of Involvement of Neuronal NOS and Inducible NOS for Antianxiety-like Activity of Ellagic Acid in Unstressed and Stressed Mice

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Abstract

Background and Objectives: Ellagic acid, a naturally occurring polyphenolic compound has been reported to possess antianxiety activity in mice through involvement of GABAergic system and scavenging of free radicals. But involvement of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) for antianxiety activity of ellagic acid in unstressed and stressed mice has not been explored. The study was aimed to evaluate the role of nNOS and iNOS in antianxiety-like activity of ellagic acid in unstressed and stressed Swiss young male Albino mice. **Methodology:** Ellagic acid (17.5, 35 and 70 mg kg^{-1}, p.o.) and alprazolam (0.25 mg kg^{-1}, i.p.) *per se* were administered for 10 successive days to unstressed and stressed mice and their effect on anxiety was evaluated using elevated plus maze and light-dark test. Acute stress was produced by immobilization of mice for 150 min on 10th day. **Results:** Ellagic acid (70 mg kg^{-1}) and alprazolam significantly ($p<0.001$ and $p<0.05$, respectively) showed antianxiety-like activity in both unstressed and stressed mice. The drugs did not show any significant effect on locomotor activity of mice. Ellagic acid significantly ($p<0.001$) decreased plasma nitrite levels in stressed mice only. The 7-nitroindazole (nNOS inhibitor) did not significantly potentiate antianxiety effect of ellagic acid in unstressed mice. But aminoguanidine (iNOS inhibitor) significantly ($p<0.05$) potentiated antianxiety and plasma nitrite decreasing effects of ellagic acid in stressed mice. Plasma corticosterone levels were significantly ($p<0.05$) decreased by ellagic acid in stressed mice. **Conclusion:** Ellagic acid produced significant antianxiety-like activity in unstressed mice possibly not though inhibition of neuronal NOS, but produced significant anxiolytic effect in stressed mice possibly through inhibition of inducible NOS and reduction of plasma corticosterone levels.

Key words: Anxiety, ellagic acid, immobilization stress, inducible NOS, neuronal NOS

Received: May 27, 2016 Accepted: June 05, 2016 Published: June 15, 2016

Citation: Dinesh Dhingra, Ritu Chhillar and Jyotsna Bhargava, 2016. Investigation of involvement of neuronal NOS and inducible NOS for antianxiety-like activity of ellagic acid in unstressed and stressed mice. Pharmacologia, 7: 264-271.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anxiety disorders are characterized by persistent and disproportionate fear and subsequent avoidance in response to a specified object or situation and in the absence of true danger[1]. Hyperactivity of the stress response has been reported in association with anxiety disorders. Stress leads to activation of the hypothalamic-pituitary-adrenal axis (HPA) resulting in the release of hypothalamic corticotropin releasing hormone, which in turn releases adrenocorticotropin releasing hormone from anterior pituitary gland, resulting in the secretion of adrenal glucocorticoids (cortisol in humans and corticosterone in rodents) into the blood[2]. Psychiatric patients suffering from anxiety disorders exhibit increased levels of plasma cortisol[3]. The substantial rise in the level of plasma corticosterone after acute restraint stress (150 min) has been reported from our laboratory[4].

Nitric oxide is an intercellular messenger in the brain generated from L-arginine by various enzymes called nitric oxide synthases. There are three isoforms of nitric oxide synthase (NOS) neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS[5]. The NOS has been localized in brain regions involved with anxiety such as hypothalamus, amygdala and hippocampus[6]. Immobilization stress induces a generalized increase in the production of nitric oxide and cause anxious behavior in rodents[7]. Immobilization-induced stress has been observed to significantly increase plasma nitrite levels and expression of NOS in rodents. A differential role is played by neuronal and inducible isoforms of NOS in anxiety in mice under unstressed and stressed conditions. Selective inhibition of nNOS by 7-nitroindazole in unstressed mice and selective inhibition of inducible nitric oxide synthase by aminoguanidine in stressed mice produced antianxiety-like effect[8,9].

Ellagic acid, a naturally occurring polyphenolic compound is reported to be present in a number of plants such as *Punica granatum*[10], *Phyllanthus emblica*[11] and so on. It has been reported to possess a wide spectrum of pharmacological activities such as antidepressant[12], antitardive dyskinetic[13], antioxidant[14], anticancer[15], antiallergic[16], antimalarial[17], antiwrinkle[18], antiglycative and anti-inflammatory[19]. Further, ellagic acid inhibited amyloid β-42 induced neurotoxicity *in vitro* and is also a βeta-secretase inhibitor[20,21].

In addition to the above pharmacological activities, ellagic acid has been reported to possess antianxiety activity in mice through involvement of gamma-amino butyric acid (GABA)[22] and scavenging of free radicals[23]. But involvement of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) for antianxiety activity of ellagic acid in unstressed and stressed mice has not been explored. The study was aimed to evaluate the role of nNOS and iNOS in antianxiety-like activity of ellagic acid in unstressed and stressed Swiss young male Albino mice. Plasma nitrite levels were also estimated to further elucidate the involvement of nNOS and iNOS in antianxiety-like activity of ellagic acid in unstressed and stressed mice. Plasma corticosterone levels were measured to evaluate the effect of ellagic acid on increased activity of HPA axis in stressed mice.

MATERIALS AND METHODS

Experimental animals: Swiss male Albino mice (3 months old, weighing around 20-25 g) were purchased from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana, India). Estrogens have been reported to have antianxiety effect[24], so excluded female mice and used only male mice for the study. Animals were housed separately in groups of 10 per cage (Polycarbonate cage size: 29×22×14 cm) under laboratory conditions with alternating light and dark cycle of 12 h each. The animals had free access to food and water. The animals were kept fasted 2 h before and 2 h after drug administration. The animals were acclimatized for atleast 5 days before behavioral experiments which were carried out between 09:00 and 17:00 h. The experimental protocol was approved by Institutional Animals Ethics Committee of Guru Jambheshwar University of Science and Technology, Hisar.

Drugs and chemicals: Ellagic acid, sulfanilamide, N-(1-naphthyl) ethylene diamine dihydrochloride, meta phosphoric acid and tween 80 were purchased from Hi-Media laboratories Pvt. Ltd., Mumbai (India). The 7-nitroindazole and aminoguanidine were purchased from Sigma-Aldrich, St. Louis, USA. Sucrose was purchased from Qualigens Fine Chemicals, Mumbai (India). Trichloroacetic acid, Tris and EDTA disodium salt AR were purchased from SD Fine Chem Ltd., Mumbai (India).

Vehicle: Ellagic acid was suspended in 0.1% w/v gum acacia. Alprazolam and aminoguanidine were dissolved separately in normal saline (0.9% w/v sodium chloride). The 7-nitroindazole was dissolved in normal saline with few drops of tween 80. Volume of i.p. injection was 1 mL/100 g of mouse.

Selection of doses: Doses of various drugs were selected on the basis of literature that is 0.25 mg kg^{-1} for alprazolam[4], 50 mg kg^{-1} for aminoguanidine[9] and 20 mg kg^{-1} for 7-nitroindazole[8].

Immobilization stress in mice: Stress was induced in mice by immobilizing them for 150 min (10:00 am to 12:30 pm) by placing them on their back and taping all its 4 limbs and trunk on a wooden board. Mice subjected to immobilization were called as stressed mice. Unstressed mice were exposed to behavioral tests and not subjected to immobilization. Drugs were administered 45 min before immobilization session in case of stressed mice. Behavioral testing was started 10 min after setting the animals free from immobilization[4,8,9].

Behavioral models

Elevated plus maze: Effect of the drugs on anxiety of mice was evaluated using elevated plus maze. This maze apparatus consisted of two open arms 16×5 cm and two closed arms 16×5×12 cm, connected to a central platform (5×5 cm). The maze was elevated to a height of 25 cm above the floor. 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 and time spent in open arms[25]. The plus maze was carefully wiped with hydrogen peroxide and dried with sponge after each trial.

Light and dark test: The apparatus consisted a rectangular box (45×27×27 cm) partitioned into 2 compartments connected by a 7.5×7.5 cm opening in the wall between compartments. An animal was placed in the center of the light compartment and was observed for 5 min for time spent in this compartment[26].

Photoactometer: To rule out the effects of various drug treatments on locomotor activity, horizontal locomotor activities of control and test animals were recorded for a period of 5 min using photoactometer (INCO, Ambala, India). The difference in the locomotor activity scores were noted before and after the drug treatment[27].

Experimental design: The animals were divided into following groups having 10 mice in each group.

Groups for elevated plus maze

Groups 1 to 5: Normal saline i.p. alprazolam (0.25 mg kg^{-1}, i.p.) and ellagic acid (17.5, 35 and 70 mg kg^{-1}, p.o.), respectively were administered for 10 successive days and 45 min after the administration on 10th day, number of entries and time spent in open arms was recorded.

Groups 6 and 7: Normal saline i.p. and ellagic acid (70 mg kg^{-1}, p.o.), respectively were administered for 10 successive days and 45 min after the administration on 10th day, 7-nitroindazole (20 mg kg^{-1}, i.p.) was injected and 45 min after the injection, number of entries and time spent in open arms was recorded.

Groups 8 to 12: Normal saline i.p. alprazolam (0.25 mg kg^{-1}, i.p.) and ellagic acid (17.5, 35 and 70 mg kg^{-1}, p.o.), respectively were administered for 10 successive days and 45 min after the administration on 10th day, immobilization stress was produced according to procedure mentioned above. The animals were tested on elevated plus maze 10 min after setting them free from immobilization and number of entries and time spent in open arms was recorded.

Groups 13 and 14: Normal saline i.p. and ellagic acid (70 mg kg^{-1}, p.o.) were administered for 10 successive days and 45 min after the administration on 10th day, aminoguanidine (50 mg kg^{-1}, i.p.) was injected and 45 min after the injection, immobilization stress was produced. The animals were tested on elevated plus maze 10 min after setting them free from immobilization and number of entries and time spent in open arms was recorded.

Groups for light and dark test: These were same as groups 1-14, except the time spent in light box was recorded.

Groups for measurement of locomotor activity: These were same as groups 1-14, except the locomotor activity was measured using photoactometer.

Biochemical estimations: 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 handling of their tail. Plasma was separated using refrigerated centrifuge (Remi, Mumbai, India) at 2500 rpm for 10 min. It was stored in a refrigerator and processed for nitrite estimation within 24 h[4,8,9].

Estimation of plasma nitrite levels: Plasma nitrite was measured by using the method of Green *et al.*[28]. A mixture of 1% w/v sulphanilamide in 5% aqueous solution of m-phosphoric acid (1 part) and 0.1% w/v N-(1-naphthyl) ethylene diamine dihydrochloride (1 part) was prepared and kept at 0°C for 60 min. About 0.5 mL plasma was mixed with 0.5 mL of the above mixture and kept in dark for 10 min at room temperature. The absorbance was read at 546 nm.

Estimation of plasma corticosterone levels: The quantitative estimation of corticosterone level in the blood plasma was performed by the method of Bartos and Pesez[29]. About 1.0 mL of sample in ethanol, 0.50 mL of 0.10% solution of p-nitroso-N, N-dimethylaniline in ethanol was added and the tubes were immersed in ice water for 5 min and 0.50 mL of 0.10 N sodium hydroxide was added. The tubes were plugged with cotton-wool and were let to stand at 0°C for 5 h protected against light. To the above solution, 2.0 mL of buffer for pH 9.8, 5.0 mL of 0.10% solution of phenol in ethanol and 0.50 mL of 1.0% aqueous solution of potassium ferricyanide were added. The tubes were kept in a water bath at 20±2°C for 10 min. The solution was read at 650 nm.

Statistical analysis: All the results are expressed as Mean±SEM. Data were analyzed by one way ANOVA followed by Tukey's *post hoc* test, except for vehicle treated unstressed and stressed control groups which was analyzed using student's unpaired t-test in graph pad instat (GPIS) package, version 3.05, $p<0.05$ was considered as significant.

RESULTS

Effect of various treatments on time spent and number of entries in open arms of elevated plus maze: Immobilization of mice for 150 min significantly decreased time spent in open arms and number of open arm entries as compared to vehicle treated unstressed mice. Alprazolam (0.25 mg kg^{-1}, i.p.) administered for 10 successive days significantly ($p<0.05$) increased time spent in open arms and number of open arm entries by unstressed and stressed mice as compared to their respective controls. The higher doses (35 and 70 mg kg^{-1}) of ellagic acid administered for 10 successive days significantly ($p<0.05$ and $p<0.001$, respectively) increased time spent and number entries in open arms by unstressed mice as compared to vehicle treated control. In stressed mice, only the highest dose (70 mg kg^{-1}) of ellagic acid significantly ($p<0.05$) increased time spent and number entries in open arms of elevated plus maze as compared to vehicle treated stressed mice. The 7-nitroindazole (20 mg kg^{-1}) and aminoguanidine (50 mg kg^{-1}) significantly ($p<0.05$) increased time spent in open arms and number of open arm entries by unstressed and stressed mice as compared to vehicle treated unstressed and stressed mice, respectively. The 7-nitorindazole did not significantly potentiate antianxiety effect of ellagic acid (70 mg kg^{-1}) in unstressed mice as compared to 7-nitroindazole and ellagic acid (70 mg kg^{-1}) *per se*. But aminoguanidine significantly ($p<0.05$) potentiated antianxiety-like effect of ellagic acid (70 mg kg^{-1}) in stressed mice as compared to ellagic acid (70 mg kg^{-1}) and aminoguanidine *per se* (Table 1, 2).

Effect of various treatments on time spent in light compartment of light-dark box: Immobilization (150 min) of mice significantly ($p<0.05$) decreased time spent in light compartment as compared to vehicle treated unstressed mice. Alprazolam administered for 10 successive days significantly ($p<0.05$) increased time spent in light compartment by unstressed mice and stressed mice as compared to their respective controls. Ellagic acid (70 mg kg^{-1}) administered for 10 successive days significantly ($p<0.001$ and $p<0.05$, respectively) increased time spent in light compartment by unstressed and stressed mice as compared to the respective vehicle treated control groups. The 7-nitroindazole and aminoguanidine *per se* significantly ($p<0.05$) increased time spent in light compartment by unstressed and stressed mice as compared to their respective vehicle treated controls. The 7-nitorindazole did not significantly increase time spent in light compartment by ellagic acid (70 mg kg^{-1}) in unstressed mice as compared to 7-nitroindazole and ellagic acid (70 mg kg^{-1}) *per se*. But aminoguanidine significantly ($p<0.05$) increased time spent in light compartment by ellagic acid (70 mg kg^{-1}) in stressed mice as compared to ellagic acid (70 mg kg^{-1}) as well as aminoguanidine *per se*, indicating potentiation of antianxiety effect of ellagic acid by aminoguanidine in stressed mice (Table 3).

Table 1: Effect of various treatments on time spent in open arms of elevated plus maze by unstressed and stressed mice

Drugs treatments	Dose	Time spent in open arms (sec)-unstressed mice	Time spent in open arms (sec)-stressed mice
Vehicle (Normal saline) (10 days)	10 mL kg^{-1}	79.8±4.41	18.2±1.48*
Ellagic acid (10 days)	17.5 mg kg^{-1}	78.8±3.11	20.1±0.99
Ellagic acid (10 days)	35 mg kg^{-1}	99.5±3.2*	29.3±1.12
Ellagic acid (10 days)	70 mg kg^{-1}	121.4±4.16***	43.1±1.15†
Alprazolam (10 days)	0.25 mg kg^{-1}	134.7±3.28*	54.5±3.68†
Vehicle+7-NI (U)	10 mL kg^{-1}+20 mg kg^{-1}	102.4±3.27*	-
Ellagic acid (10 days)+7-NI (U)	70+20 mg kg^{-1}	108.4±2.95	-
Vehicle+AG (S)	10 mL kg^{-1}+50 mg kg^{-1}	-	64.7±3.97†
Ellagic acid (10 days)+AG (S)	70+50 mg kg^{-1}	-	92.8±3.12‡,§

n = 10 in each group, values are expressed as Mean±SEM, data was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, $F(13, 126): 150.65$, $p<0.0001$, *$p<0.05$ significant difference from vehicle treated unstressed mice, ***$p<0.001$ significant difference from vehicle treated unstressed mice, $^{\dagger}p<0.05$ significant difference from immobilization-induced stressed mice, $^{\ddagger}p<0.05$ significant difference from ellagic acid (70 mg kg^{-1}) treated stressed mice, $^{\S}p<0.05$ significant difference from aminoguanidine treated stressed mice, AG (S): Aminoguanidine (stressed mice) and 7-NI(U): 7-NI (unstressed mice)

Table 2: Effect of various treatments on open arm entries of elevated plus maze by unstressed and stressed mice

Drugs treatments	Dose	Open arm entries (count/5 min)-unstressed mice	Open arm entries (count/5 min)-stressed mice
Vehicle (Normal saline) (10 days)	10 mL kg^{-1}	5.0±0.45	3.1±0.28*
Ellagic acid (10 days)	17.5 mg kg^{-1}	5.3±0.51	3.3±0.39
Ellagic acid (10 days)	35 mg kg^{-1}	7.1±0.62*	4.9±0.44
Ellagic acid (10 days)	70 mg kg^{-1}	9.1±0.53***	6.3±0.29†
Alprazolam (10 days)	0.25 mg kg^{-1}	9.3±0.50*	6.1±0.46†
Vehicle+7-NI (U)	10 mL kg^{-1}+20 mg kg^{-1}	8.4±0.31*	-
Ellagic acid (10 days)+7-NI (U)	70+20 mg kg^{-1}	8.5±0.43	-
Vehicle+AG (S)	10 mL kg^{-1}+50 mg kg^{-1}	-	6.1±0.38†
Ellagic acid (10 days)+AG (S)	70+50 mg kg^{-1}	-	8.2±0.36‡,§

n = 10 in each group, values are expressed as Mean±SEM, data was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, F (13, 126): 21.867, p<0.0001, *p<0.05 significant difference from vehicle treated unstressed mice, ***p<0.001 significant difference from vehicle treated unstressed mice, †p<0.05 significant difference from immobilization-induced stressed mice, ‡p<0.05 significant difference from ellagic acid (70 mg kg^{-1}) treated stressed mice, §p<0.05 significant difference from aminoguanidine treated stressed mice, AG(S): Aminoguanidine (stressed mice) and 7-NI(U): 7-NI (unstressed mice)

Table 3: Effect of various treatments on time spent in light compartment of light/dark box by unstressed and stressed mice

Drugs treatments	Dose	Time spent in light compartment (sec)-unstressed mice	Time spent in light compartment (sec)-stressed mice
Vehicle (Normal saline) (10 days)	10 mL kg^{-1}	119.5±4.18	86.3±2.47*
Ellagic acid (10 days)	17.5 mg kg^{-1}	122.3±5.15	89.4±3.20
Ellagic acid (10 days)	35 mg kg^{-1}	140.2±4.10*	105.3±3.90
Ellagic acid (10 days)	70 mg kg^{-1}	151.2±5.05***	136.4±3.15†
Alprazolam (10 days)	0.25 mg kg^{-1}	175.4±3.66*	145.6±5.08†
Vehicle+7-NI (U)	10 mL kg^{-1}+20 mg kg^{-1}	152.7±4.90*	-
Ellagic acid (10 days)+7-NI (U)	70+20 mg kg^{-1}	155.1±3.21	-
Vehicle+AG (S)	10 mL kg^{-1}+50 mg kg^{-1}	-	129.9±3.76†
Ellagic acid (10 days)+AG (S)	70+50 mg kg^{-1}	-	158.9±4.20‡,§

n = 10 in each group, values are expressed as Mean±SEM, data was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, F (13, 126): 41.82, p<0.0001, *p<0.05 significant difference from vehicle treated unstressed mice, ***p<0.001 significant difference from vehicle treated unstressed mice, †p<0.05 significant difference from immobilization-induced stressed mice, ‡p<0.05 significant difference from ellagic acid (70 mg kg^{-1}) treated stressed mice, §p<0.05 significant difference from aminoguanidine treated stressed mice, AG (S): Aminoguanidine (stressed mice) and 7-NI(U): 7-NI (unstressed mice)

Table 4: Effect of various treatments on plasma nitrite levels of unstressed and stressed mice

Drugs treatments	Dose	Plasma nitrite levels (μg mL^{-1})-unstressed mice	Plasma nitrite levels (μg mL^{-1})-stressed mice
Vehicle (normal saline) (10 days)	10 mL kg^{-1}	18.79±0.63	27.28±1.06*
Ellagic acid (10 days)	17.5 mg kg^{-1}	18.82±0.35	28.82±0.69
Ellagic acid (10 days)	35 mg kg^{-1}	17.76±0.41	19.92±0.74†
Ellagic acid (10 days)	70 mg kg^{-1}	17.43±0.32	14.44±0.63†††
Alprazolam (10 days)	0.25 mg kg^{-1}	17.94±0.59	24.49±0.57
Vehicle+7-NI (U)	10 mL kg^{-1}+20 mg kg^{-1}	13.71±0.48*	-
Ellagic acid (10 days)+7-NI (U)	70+20 mg kg^{-1}	12.54±0.41	-
Vehicle+AG (S)	10 mL kg^{-1}+50 mg kg^{-1}	-	13.80±0.52†
Ellagic acid (10 days)+AG (S)	70+50 mg kg^{-1}	-	9.76±0.39‡,§

n = 10 in each group, values are expressed as Mean±SEM, data was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, F (13, 126): 88.38, p<0.0001, *p<0.05 significant difference from vehicle treated unstressed mice, †p<0.05 significant difference from immobilization-induced stressed mice, †††p<0.001 significant difference from immobilization-induced stressed mice, ‡p<0.05 significant difference from ellagic acid (70 mg kg^{-1}) treated stressed mice, §p<0.05 significant difference from aminoguanidine treated stressed mice, AG (S): Aminoguanidine (stressed mice) and 7-NI(U): 7-NI (unstressed mice)

Effect of various treatments on plasma nitrite levels of mice: Immobilization stress significantly (p<0.05) increased plasma nitrite levels in stressed mice as compared to vehicle treated unstressed mice. Alprazolam administered for 10 successive days did not produce any significant change in plasma nitrite levels in both unstressed and stressed mice. Ellagic acid (35 and 70 mg kg^{-1}) administered for 10 successive days significantly (p<0.05 and p<0.001, respectively) attenuated the immobilization stress-induced increase in plasma nitrite levels. Ellagic acid did not significantly decrease plasma nitrite levels in unstressed mice, but 7-nitroindazole significantly (p<0.05) decreased plasma nitrite levels in unstressed mice. The combination of 7-nitroindazole with ellagic acid did not produce any significant effect on plasma nitrite levels as compared to 7-nitroindazole and ellagic acid (70 mg kg^{-1}) *per se*. Aminoguanidine significantly (p<0.05) decreased plasma nitrite levels and potentiated (p<0.05) plasma nitrite decreasing effect of ellagic acid (70 mg kg^{-1}) in stressed mice as compared to ellagic acid (70 mg kg^{-1}) and aminoguanidine *per se* (Table 4).

Table 5: Effect of ellagic acid and alprazolam on plasma corticosterone levels in unstressed and stressed mice

Treatment for 10 days	Dose	Corticosterone levels (μg dL^{-1})-unstressed mice	Corticosterone levels (μg dL^{-1})-stressed mice
Vehicle (Normal saline)	10 mL kg^{-1}	5.76±0.81	14.38±0.76*
Ellagic acid	17.5 mg kg^{-1}	5.98±0.54	13.65±0.64
Ellagic acid	35 mg kg^{-1}	5.32±0.66	10.03±0.41†
Ellagic acid	70 mg kg^{-1}	5.09±0.50	6.64±0.52†
Alprazolam	0.25 mg kg^{-1}	5.46±0.86	14.15±0.66

n = 10 in each group, Values are expressed as Mean±SEM, data was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, F (9, 90): 37.64, p<0.0001, *p<0.05 significant difference from vehicle treated unstressed mice and †p<0.05 significant difference from immobilization-induced stressed mice

Table 6: Effect of various treatments on locomotor activity of unstressed and stressed mice

Drugs treatments	Dose	Locomotor activity counts-unstressed mice	Locomotor activity counts-stressed mice
Vehicle (Normal saline) (10 days)	10 mL kg^{-1}	337.1±15.15	309.5±5.35*
Ellagic acid (10 days)	17.5 mg kg^{-1}	344.4±15.98	314.5±4.40
Ellagic acid (10 days)	35 mg kg^{-1}	336.4±8.65	317.2±3.41
Ellagic acid (10 days)	70 mg kg^{-1}	342.3±15.05	304.6±4.67
Alprazolam (10 days)	0.25 mg kg^{-1}	265.8±12.35*	225.2±5.70†
Vehicle+7-NI (U)	10 mL kg^{-1}+20 mg kg^{-1}	342.6±6.33	-
Ellagic acid (10 days)+7-NI (U)	70+20 mg kg^{-1}	345.1±8.40	-
Vehicle+AG (S)	10 mL kg^{-1}+50 mg kg^{-1}	-	311.4±3.20
Ellagic acid (10 days)+AG (S)	70+50 mg kg^{-1}	-	311.6±3.71

n = 10 in each group, values are expressed as Mean±SEM, data was analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test, F (13, 126): 13.49, p<0.0001, *p<0.05 significant difference from vehicle treated unstressed mice, †p<0.05 significant difference from immobilization-induced stressed mice, AG (S): Aminoguanidine (stressed mice) and 7-NI(U): 7-NI (unstressed mice)

Effect of various treatments on plasma corticosterone levels of mice: Immobilization stress significantly (p<0.05) increased plasma corticosterone levels in stressed mice as compared to that vehicle treated unstressed mice. Alprazolam administered for 10 successive days did not produce any significant change in corticosterone contents of stressed mice as compared to vehicle treated stressed mice. Ellagic acid (35 and 70 mg kg^{-1}) administered for 10 successive days significantly (p<0.05) decreased corticosterone levels in stressed mice as compared to vehicle treated stressed mice (Table 5).

Effect of various treatments on locomotor activity of mice: Immobilization significantly (p<0.05) decreased locomotor activity of stressed mice as compared to vehicle treated unstressed mice. Alprazolam administered for 10 successive days significantly (p<0.05) decreased locomotor activity of unstressed and stressed mice as compared to respective vehicle treated unstressed and stressed mice. Ellagic acid, aminoguanidine, 7-nitroindazole and their combinations used in the present study did not significantly affect the spontaneous locomotor activity of unstressed and stressed mice as compared to respective vehicle treated unstressed and stressed mice (Table 6).

DISCUSSION

In the study, ellagic acid (70 mg kg^{-1}, p.o.) administered for 10 successive days showed significant antianxiety-like activity in unstressed and stressed mice, employing elevated plus maze and light-dark test. Forced immobilization is one of the best explored models of stress in mice. As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress[30]. The elevated plus maze is considered one of the most widely validated tests for assaying new benzodiazepine-like anxiolytic agents[31]. This test is based on the observation that rodents tend to avoid elevated areas and avoidance of the open arms in elevated plus maze is interpreted as anxiety behavior. Single dose (acute) administration of ellagic acid (35 and 70 mg kg^{-1}) in unstressed and stressed mice did not show significant antianxiety activity (data not shown), but administration for 10 successive days significantly increased number of entries and time spent in open arms of elevated plus maze by both unstressed and stressed mice, as compared to the respective control groups, indicating antianxiety effect. In order to further corroborate the anxiolytic activity observed in the elevated plus maze test. In this study light-dark test is also used in which exploration of light compartment is inhibited by anxiety[26]. Ellagic acid (70 mg kg^{-1}) administered for 10 successive days significantly increased the time spent in light compartment of light-dark box, thus indicating an antianxiety-like effect. Ellagic acid did not affect the locomotor activity of mice, hence indicating that antianxiety-like effect of ellagic acid is specific and not false positive. Alprazolam (0.25 mg kg^{-1}) administration for 10 successive days significantly showed anxiolytic activity in both unstressed and stressed mice. But it significantly decreased locomotor activity

of unstressed and stressed mice as compared to respective vehicle treated groups. This observation is in line with the earlier report where repeated administration of alprazolam (0.25 mg kg^{-1}) showed sedative effect, hence, decreased the locomotor activity[32].

Administration of 7-nitroindazole (nNOS inhibitor) to ellagic acid (70 mg kg^{-1}) pretreated mice did not significantly potentiate its antianxiety-like activity in unstressed mice as compared to ellagic acid and 7-nitroindazole *per se*, indicating that antianxiety-like activity of ellagic acid in unstressed mice might not be through inhibition of neuronal NOS. On the other hand, aminoguanidine (iNOS inhibitor) significantly potentiated antianxiety-like effect of ellagic acid (70 mg kg^{-1}) in stressed mice as compared to ellagic acid and aminoguanidine *per se*, indicating that ellagic acid might produce antianxiety-like activity in stressed mice through inhibition of inducible NOS.

Immobilization for 150 min increased anxiety and impaired motor activity of mice as compared to unstressed mice. This finding is in agreement with earlier reports that acute stress activates iNOS and enhances anxiety in rodents[4,8,9]. Acute immobilization stress as used in the 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[7]. Immobilization stress significantly increased plasma nitrite levels as compared to vehicle treated unstressed mice, as reported earlier from the laboratory[4,8,9]. Ellagic acid (35 and 70 mg kg^{-1}) significantly decreased the plasma nitrite levels in stressed mice, but showed no such effect in unstressed mice. The 7-nitroindazole and aminoguanidine *per se* significantly decreased plasma nitrite levels in both unstressed mice and stressed mice respectively as compared to the respective vehicle treated controls. Potentiation of plasma nitrite levels decreasing effect of ellagic acid by aminoguanidine in stressed mice further supports the involvement of inducible NOS inhibition in antianxiety-like activity of ellagic acid in stressed mice. On the other hand, combination of ellagic acid with 7-nitroindazole did not produce any significant effect on plasma nitrite levels, which rules out the involvement of neuronal NOS inhibition in antianxiety-like activity of ellagic acid in unstressed mice.

Stress in rats brings about transient activation of the HPA axis, as measured by increased adrenal gland weight with subsequent increase in plasma corticosterone levels[30]. In the study, immobilization stress significantly increased plasma corticosterone levels as compared to vehicle treated unstressed mice. Ellagic acid (35 and 70 mg kg^{-1}) significantly decreased plasma corticosterone levels in stressed mice as compared to vehicle treated stressed mice. Thus, in addition to the involvement of iNOS inhibition for antianxiety-like activity of ellagic acid in stressed mice, it might also act by reducing the hyperactivity of HPA axis in stressed mice as indicated by decrease in plasma corticosterone levels.

CONCLUSION

Ellagic acid showed significant antianxiety-like activity in unstressed mice possibly not through inhibition of nNOS but produced significant anxiolytic effect in stressed mice possibly through inhibition of iNOS and reduction of elevated plasma corticosterone levels. Thus, ellagic acid may be explored further for treatment of anxiety disorders.

ACKNOWLEDGMENT

Gift sample of alprazolam from Gnosis Pharmaceuticals Pvt. Ltd., Kala Amb, Sirmour (H.P., India) is gratefully acknowledged.

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