Journal of Medicinal Plants Research Vol. 4(6), pp. 490-495, 18 March, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR09.079
ISSN 1996-0875© 2010 Academic Journals

Full Length Research Paper

Possible nitric oxide modulation in protective effects of Withaferin A against stress induced neurobehavioural changes

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Accepted 10 March, 2010

The involvement of nitric oxide (NO) in stress-induced neurobehavioral changes in rats was evaluated using the elevated plus maze and open field tests. Restraint stress (1 h) reduced both the number of entries and time spent in open arms, with both expressed vehicle- controls and these changes were reversed with diazepam (1 mg/kg) and withaferin A (10 - 50 mg/Kg, i.p), N-nitro-L -arginine methyl ester (L -NAME) (50 mg/kg)pretreatment. In the open field test, the restraint stress-induced (a) increased entry latency and (b) decreased ambulation and rearing were reversed by diazepam and withaferin A(40 mg/Kg), N-nitro-L -arginine methyl ester (L -NAME) (50 mg/kg). Biochemical data showed that withaferin A had a direct effect on the total brain nitrate/nitrite content after acute restraint stress. The root extract of *Withania somnifera* (100 - 500 mg/kg, oral) also showed anxiolytic effects in dose dependent manner. We suggest that Withaferin A in doses of 40 and 50 mg/kg causes a mild anxiolytic-like behavior and discuss the possibility that this reported effect could be caused by inhibition of nitric oxide synthase.

Key words: Anxiety, withaferin A, nNOS, L-NAME.

INTRODUCTION

Stress is conceived as any physical, psychological and/or environmental stimulus capable of altering physiological homeostasis, and the ability to cope with such stressful stimuli is a crucial determinant of health and disease (Selye, 1936; Bohus, 1990). Pathological anxiety is one of the most common emotional disorders and treatment of phobias or panic attacks is still not conclusive. Pharmacological treatment plays an important role in the therapeutic concept. Benzodiazepines have been the most widely used anxiolytics in general practice for many years (Holm, 1988 and Rang et al., 1995) and are relatively safe drugs for a short-term treatment of anxiety despite their drug dependence potential and side effects (Ballinger, 1990 and Lader, 1999). Benzodiazepine diazepam is a standard anxiolytic that is also employed in behavioural pharmacology as the reference compound for an anxiolytic-like effect even if the screened drug is not acting via benzodiazepine receptors. We also have used diazepam as a standard anxiolytics in our experiments. Nevertheless, there is considerable interest in the development of new anxiolytics. Nitric Oxide has been widely studied for its role in stress related phenomenon, which forms the basis of our experiments.

Nitric oxide (NO), a stable gaseous free radical, is now recognized as an important biomodulator. Several physiological functions have been attributed to NO and it has also been implicated in various pathological states. NO is also recognized as an intracellular messenger in central nervous system and its role as a neurotransmitter/neuromodulator has been proposed (Moncada, 1991; Gairthwaite, 1988 and Zhang, 1995). NO/cGMP pathway in ventral hippocampal region has been studied for anxiety related behaviours in rats and is synthesized by the enzyme, neuronal nitric oxide synthase (nNOS) forms NO from L-arginine in the presence

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Abbreviations: WS, *Witahnia somnifera* root extract; **WA,** witahferin A,Dz- diazepam; **nNOS,** neuronal nitric oxide synthase.

of molecular oxygen with a stoichiometric generation of L-citruline (Calixto et al., 2010). On the other hand, it has been reported that the plasma NOx levels in depressed patients were significantly higher than those in healthy controls, and were reduced after recovery from the state of depression (Suzuki et al., 2001). New synthesized compounds as well as drugs derived from traditional herbs may have a possible therapeutic relevance in the treatment of anxiety (Beaubrun and Gray, 2000). L-NAME, a inhibitor of nNOS has been widely proved to be anxiolytics, but it also have side effects (Masood et al., 2003). Especially, the use of “mild,” “natural” and tolerable phytopharmaceuticals are in public favor for this purpose (Lake, 2000). Further screening of traditional herbs which might act via nitric oxide pathway and may exert anxiolytics effects leads us to *Withannia somnifera*.

W. somnifera Dunal (WS), known as Ashwagandha or Indian ginseng has been commonly used in Indian traditional medicines for over 3,000 years. WS has been categorized as *Rasayana* in Ayurveda, which is known to augment defense against diseases, arrest aging, revitalize the body in debilitated condition, increase the capability of the individual to resist adverse environmental factors and create a sense of mental wellbeing (Bone, 1996; Bhatnagar et al., 2005). Earlier studies have reported multiple properties of WS, such as antioxidant, adaptogenic, aphrodisiac, liver tonic, astringent and, also as an anti-inflammatory and anti-ulcer agent (Gupta and Rana, 2007*)*. It has also been used to treat stress, insomnia, arthritis and age related disorders including neurodegeneration (Gupta et al., 2003 and Mishra et al., 2000). The biologically active constituents in WS are alkaloids (ashwagandhin, cuscohygrine, anahygrine, topine etc.), steroidal compounds, including ergostane type teroidallactones, Withaferin A, withanolides A–Y, withasomniferin A, withasomnidienone, withasomnierose A–C, withanone etc. Other constituents includes saponins containing an additional acyl group (Sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X) (Elsakka et al., 1990 and Ganzera et al., 2003). It has been suggested that Withaferin A can inhibit the NO production by modulation iNOS (Oh et al., 2008).So there are probable chances that Withaferin A could also modulate the nNOS thereby changing concentration of NO in brain. And considering the neuroprotective properties of WS root extract the present study was carried out to understand the possible mechanism of neuroprotection and to evaluate the effects of Withaferin A from WS root extract on rats exposed to acute restraint stress.

MATERIALS AND METHODS

Animals

Male wistar rats (150 - 200 g) were used in the study. Rats were housed at a constant temperature of 20 + 2°C under a 12 h light: 12 h dark cycle. The animals (n = 6 - 10 per group) had free access to food and water throughout the experiments. Animal care was as per Indian National Science Academy (INSA) Guidelines for the Care and Use of Animals in Scientific Research, and the study had the approval of Institutional Animal Ethical Committee (IAEC).

Stress procedure

The animals were subjected to restraint stress for 1 h at room temperature by immobilizing them in adjustable Plexiglas restrainers (INCO, Ambala). Immediately after the restraint stress procedure, the rats were exposed to the behavioral tests.

Plant material and preparation of extracts

The roots of *W. somnifera* grown in natural habitat and purchased from an authorized dealer were air-dried in shade and finely powdered. The chief botanist at Indian Agricultural Research Institute (IARI), New Delhi, India, identified the roots and a voucher specimen (accession number NISCAIR/RHM/F-3/2008/Consult /473) has been deposited at the herbarium of IARI. The root powder was exhaustively extracted with methanol: water (4:1, v/v) under reflux (WS). This extract was partitioned with chloroform and water to give WS-chloroform and WS-water, respectively. WS-chloroform was subjected to 12 successive elutions of water and acetonitrile (ACN) and these elutions were labeled from A1 to A12.

HPLC (Waters, Milford, U.S.A.) of *W. somnifera* extracts was performed using Kromasil C8 column (4.6 mm × 25 cm, 5 µm), and the mobile phase consisted of ACN and water (1:1, v/v) at a flow rate of 1 ml/min for a run time of 30 min. The HPLC of fractions was conducted using Novapak C18 column (3.9 mm × 15 cm, 4 µm) and the mobile phase consisted of potassium-dihydrogen orthophosphate (0.05 M) and ACN (3:7, v/v) at the flow rate of 1.5 ml/min for a run time of 30 min. The photodiode array (PDA) detector was set to detect at 229 nm and scan spectral data from 190 to 400 nm. Using the standard Withaferin A (Natural Remedies Private Ltd., Bangalore, India), the bioactive constituents of the extracts were quantified by external calibration method.

Drugs

The following drugs were used: *N*-nitro L-arginine methyl ester (L-NAME) and Diazepam (all from Sigma Aldrich, USA). L-NAME was dissolved in distilled water. Diazepam was suspended in distilled water with a drop of Tween 80. All drugs were freshly prepared and administered intraperitoneally (i.p.) in a volume of 1 ml/kg. The pretreatment time for diazepam and L-NAME was 30 min. The root extract was given orally and Withaferin A (isolated through above mentioned process) was administered itraperitoneally (i.p) with pretreatment time of 60 and 120 min.

Elevated plus maze test

The elevated plus maze consisted of two opposite arms 40 × 40 crossed with two similar closed arms with walls of 40 cm height. The arms were connected so that the maze had a plus sign look. The entire maze was elevated 50 cm above ground level and placed in a quiet, dimly lit room (Pellow et al., 1985; Bhattacharya and Satyan, 1997). Naive or pretreated rats were placed individually in the center of the maze facing the closed arms. The following parameters were measured: number of open-arm entries, and time spent on open-arm entries and closed-arm entries.

Subsequently, the percentages of open-arm entries and the time spent on open arms were calculated from open-arm entries and

time spent on open arms divided by the total number of entries in both open and closed arms and time spent on open arm exploration divided by total time spent in both open and closed arms, respectively.

Open field test

The open field apparatus consisted of a square arena 96 × 96 cm with 60 cm high walls. The walls painted were white and the floor green. The floor was divided into 16 squares by parallel and intersecting white lines (Bhattacharya and Carli, 1997). Rats were placed singly in one corner of the open field and (a) latency, (b) ambulation and (c) rearing were observed during a 5-min exposure period for both naïve and pretreated animals.

Brain nitrates and nitrites (NO*x*) assay

Brain NO*x* contents were determined as described by Tracey et al. (1995). Brain samples were homogenized in 5-ml distilled water and centrifuged at 10 000×*g* for 15 min at 4°C. Fifty microlitres of supernatant was mixed with 20 µl of 0.11 mM FAD and 20 mU of nitrate reductase. Samples were allowed to incubate for 1 h at room temperature in the dark. Then 5 µl of 1 M $ZnSO_4$ was added to the samples in order to precipitate the proteins. Samples were centrifuged at 6000×*g*, for 5 min at 4°C and the supernatants were removed. One hundred microlitres of Griess reagent (1:1 mixture of 1% sulphanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)-ethylenediamine) was added to 50 µl of supernatant and the mixture was incubated for 10 min at room temperature. Absorbance was measured at 540 nm in a microassay plate by microscan MS 5605A (Electronics Corporation of India) and converted to NO*x* content using a nitrate standard curve. Brain supernatant protein was estimated by Lowry's method (Lowry et al., 1951). The data were expressed as nmol NO*x*/mg protein.

Statistics

The data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test for post hoc comparisons. A *P* value of at least 0.05 was considered as the level of significance in all statistical tests.

RESULTS

Effects of drugs on elevated plus maze test in rats

Analysis of the elevated plus maze data revealed that the numbers of both the entries and the time spent in the open arms were significantly different across the groups. Withaferin A (10, 20, 30, 40 and 50 mg/kg) prior to restraint stress significantly increased the number of entries and also increased the time spent in the open arms ($P < 0.01$), the effect on both parameters being dose-dependent. A lower dose of Withaferin A (5 mg/kg) did not produce a similar response in open-arm activity, and though there was a 27% increase in the time spent in the open arms when compared to the control group, these differences were not statistically significant ($P > 0.05$, data not shown). On the other hand, pretreatment with the nitric oxide synthase inhibitor, L-NAME (50 mg/kg), modified the restraint stress effects on the elevated plus maze parameters. The root extract of *W. somnifera* (through oral route) increased both the no. of entries and the time spent in the open arms significantly ($P < 0.01$), when compared to the control stress group (Table 2). In stressed rats (restraint stress), diazepam enhanced elevated plus maze activity markedly ($P < 0.01$) and similar effects were seen with nitric oxide synthase inhibitors that were able to influence the elevated plus maze parameters to a significant extent. These results are summarized in Tables 1 and 2.

Effects of drugs on the open-field behavior in rats

Analysis of open-field test data showed that (a) latency of entry, (b) ambulation and (c) rearings were significantly different across all (groups ($F(16,102) = 13.18$, $P < 0.01$ for latency, $F(16,102) = 7.03$, $P < 0.001$ for ambulation, and $F(16,102) = 14.89$, $P < 0.01$ for rearing, respectively; one-way ANOVA). Withaferin A induced a marked decrease in the latency of entry into the open field, whereas ambulation and rearing were appreciably increased, the data for the latter two parameters being statistically significant ($P < 0.01$, Dunnett's test) (Table 3). Pretreatment with diazepam (1 mg/kg) attenuated the restraint stress effects on the open field behavior, that is the latency of entry was decreased, and both ambulation and rearing activity were increased. Withaferin A (40 and 50 mg/kg), given prior to restraint stress, also showed diazepam-like effects, that is the latency of entry was reduced and there was a general increase in both ambulatory and rearing activity ($P < 0.01$) in each case. L-NAME (50 mg/kg) induced effects on restraint stress-induced open field behavior, that is the latency of entry was decreased and both ambulation and rearing were increased. Whereas the root extract (500 mg/kg, orally) in dose dependent manner reduced latency of entry and increased ambulation and rearing ($P < 0.01$) when compared to that of the control group. In non-stressed rats, none of the drugs used was able to influence the open field test parameters to any significant extent.

Effect of restraint stress and L-name on brain NO activity

Biochemical assay for total nitrates and nitrites (NO*x*) in the brain supernatant were significantly different across the groups. Whereas in the case of Withaferin A group, the value for NO*x* was 0.473 ± 0.034 nmol/mg protein (33% inhibition as compared to control and the values for the control groups 0.613 ± 0.050 nmol/mg protein. While the value for L-NAME (50) was 0.273 ± 0.024 nmol/mg protein (a 63% inhibition compared to control). The brain NO*x* data were significantly different across all groups $F(3,15) = 20.28$, $P < 0.001$ (one-way ANOVA) and

Table 1. Effect of Withaferin A on elevated plus maze test parameters in rats.

Treatment (mg/kg i.p)	Elevated plus maze parameters mean		mean ± S.E
	n	Open-arm entries (%)	Time spent (%)
Control (Saline/0.5%)	10	23.2 ± 2.3	10.3 ± 1.2
WA-10	9	28.2 ± 3.0	15.9 ± 1.3
WA-20	10	38.0 ± 2.5	26.2 ± 5.1
WA-30	10	41.3 ± 3.9	21.4 ± 3.3
WA-40	8	58.1 ± 3.1	20.6 ± 2.4
WA-50	8	48.3 ± 4.7	13.0 ± 1.5
DZ(1)	6	54.0 ± 2.5	29.2 ± 5.1
L-NAME(50)	8	38.0 ± 2.6	22.2 ± 3.2

P < 0.01- compared to control group.

Table 2. Effect of root extract of WS on elevated plus maze test parameters in rats.

Treatment (mg/kg oral)	Elevated plus maze parameters mean		mean ±S.E
	n	Open-arm entries (%)	Time spent (%)
Control	10	0.86 ± 0.14	22.57 ± 3.88
WS(50)	9	1.14 ± 0.14	28.86 ± 5.47
WS(100)	6	2.00 ± 0.22	40.43 ± 3.49
WS(200)	6	2.29 ± 0.29	46.71 ± 2.53
WS(500)	6	3.14 ± 0.26	51.71 ± 3.08
DZ(1)	6	2.43 ± 0.37	42.14 ± 1.65
L-NAME	9	2.24 ± 0.27	38.11 ± 1.46

P < 0.01- compared to control groups.

Table 3. Effect of withaferin A on open-field test parameters in rats.

Treatment (mg/kg i.p)	Open field parameters (mean ± S.E)			
	n	Latency(s)	Ambulation	Rearing
Control (Saline/0.5%)	10	4.5 ± 0.8	40.6 ± 5.1	21.2 ± 2.3
WA-10	9	2.3 ± 0.9	52.5 ± 10.8	18.8 ± 1.5
WA-20	6	1.5 ± 0.2	45.6 ± 1.4	20.6 ± 1.7
WA-30	6	1.1 ± 0.1	52.6 ± 3.1	20.0 ± 2.1
WA-40	6	0.6 ± 0.10	64.5 ± 5.3	28.3 ± 1.6
WA-50	6	0.8 ± 0.10	82.0 ± 14.0	48.6 ± 5.0
DZ(1)	9	1.0 ± 0.2	56.4 ± 11.6	26.1 ± 3.0
L-NAME(50)	8	2.0 ± 0.5	67.4 ± 10.7	24.1 ± 2.1

P < 0.01- compared to control groups.

post-hoc comparisons by Dunnett's test showed that the data for both the Withaferin A groups were significantly different from the data for control group (P < 0.05).

DISCUSSION

Restraint stress has influenced the neurobehavioral profile of the organism and precipitated an anxiety-like syndrome which was reversible by diazepam, L-NAME and Withaferin A in our experiments. In the elevated plus maze test, the increases in both (a) number of entries and (b) time spent in the open arms are indices of anxiolytic activity, and our results with diazepam (1 mg/kg) are consistent with an anti-stress effect of the agent. Similarly, in the open field test, diazepam (1 mg/kg) reduced entry latency and increased ambulation and rearing behavior. This clearly proves that our experimental procedure for restraint stress is working which can be easily tested for Withaferin A and L-NAME.

Table 4. Effect of root extract of WS on open-field test parameters in rats.

Treatment (mg/kg oral)	Open field parameters (mean± S.E)			
	n	Latency(s)	Ambulation	Rearing
Control	10	5.4 ± 0.4	42.6 ± 4.1	20.2 ± 1.3
WS(50)	9	5.1 ± 0.89	45.5 ± 10.9	19.8 ± 1.2
WS(100)	6	4.5 ± 0.2	48.6 ± 1.4	21.6 ± 2.7
WS(200)	6	4.1 ± 0.4	55.6 ± 3.1	21.0 ± 3.1
WS(500)	6	2.6 ± 0.20	64.0 ± 3.3	28.3 ± 1.6
DZ(1)	6	1.9 ± 0.10	72.0 ± 11.0	27.6 ± 4.0
L-NAME(50)	9	2.0 ± 0.4	59.4 ± 9.6	20.1 ± 2.0

P < 0.01- compared to control groups.

The Withaferin A consistently reversed the restraint stress-induced suppression of (a) percentage open-arm entries and percentage time spent in the open arms in the elevated plus maze and (b) the decreased latency of entry and increase in rearing/ambulation in the open field test (Tables 1 and 3). These effects were very similar to those seen with diazepam and thus are highly suggestive of an anti-stress/adaptogenic profile for Withaferin A. The observation that inhibition of NO synthesis by L-NAME (50 mg/kg, i.p.) produced the same effects on restraint stress-induced changes in the elevated plus maze and the open field activity is consistent with this hypothesis. Further, since L-NAME (50 mg/kg) did not have any significant effect on the general behavioral parameters such as sedation and catalepsy, it is likely that the drug influenced or interacted with the stress system to induce these behavioral effects.

Further root extract of *W. somnifera* did significantly influence (P > 0.01) most of the behavioral parameters in the elevated plus maze and the open field open field tests in restraint stress situations, indicating that the NO generated from pathways other than via neuronal nitric oxide synthase could also be involved in the NO-stress interactions (Tables 2 and 4). The paradoxical effect of the extract could also possibly be due to the partial inhibition of the overall NO activity in the brain, resulting in levels of NO that are adequate to produce anti-stress effects, and qualitatively similar to those seen with the anti-stress agent, diazepam. Biochemical data also showed that, in Withaferin A-treated animals, the NO*x* levels in the brain supernatants were lower than in control animals, after restraint stress (see results), suggestive of lower levels of NO activity in the CNS. Taken together, the results of the present study indicate that NO may act as an endogenous anti-stress agent/adaptogen in the CNS and that NO-ergic mechanisms could play a crucial modulatory role in stress-induced neurobehavioural effects. In Summary it is proposed that anxiolytic properties of WS are owing to neurochemical alterations of nNOS. So Withaferin A can be developed further as anxiolytic drug in place of diazepam that has lots of side effects. Withaferin A might affect the expression of nNOS via AKT pathway or directly inhibit its activity in competitive or non-competitive manner. We are in the process of performing *insilico* studies using docking software these inhibition mechanism in details.

ACKNOWLEDGEMENTS

Funding for the research was provided by the School of Bio Sciences and Technology, VIT University, Vellore. The authors thank Dr Anna Oomen, Senior Scientist, Department of Neurochemistry, Christian Medical College, Vellore for giving permission to use their animal house.

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