*Full Length Research Paper*

# Withaferin-A displays enhanced anxiolytic efficacy without tolerance in rats following sub chronic administration

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**Withaferin-A dose-dependently (10 tob40 mg/kg) displayed anxiolytic activity, as measured by an increase in open arm exploration time in the elevated plus-maze (EPM), following intraperitoneal (i.p.) administration in rats. Acute administration of withaferin-A at 40.0 mg/kg significantly ($P<0.05$) increased open arm exploration time by 176% as compared to vehicle control, which is similar to the benzodiazepine diazepam at 1.0 and 3.0 mg/kg (191 and 200%, respectively). However, 24 h following sub chronic 5-day administration of diazepam twice daily (bid) at 3.0 mg/kg, diazepam was devoid of anxiolytic activity at 1.0 mg/kg, as measured by no difference in open arm exploration time when compared with vehicle control, while the 3.0 mg/kg dose still produced a significant ($P<0.05$) 175% increase in open arm exploration time. In contrast, following sub chronic administration of withaferin-A (40.0 mg/kg), a significant ($P<0.01$) enhancement in open arm exploration time was observed at 40.0 mg/kg (665% as compared to control). Therefore, withaferin-A resulted in anxiolysis which is similar to diazepam following acute administration in the EPM. However, following sub chronic administration unlike diazepam which showed an attenuation of anxiolytic activity, withaferin-A displayed enhanced anxiolytic efficacy and was devoid of tolerance.**

**Key words:** Withaferin-A, anxiety, benzodiazepines, nitric oxide, elevated plus-maze.

## INTRODUCTION

*Withania 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; 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, with asomniferin A, withasomnidienone, withasomnierose A–C, withanone, etc. Other constituents include 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; Ganzera et al., 2003). It has been suggested that withaferin-A in

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doses of 40 and 50 mg/kg, causes a mild anxiolytic-like behavior (Khan and Ghosh, 2010). Benzodiazepines (BZDs), such as diazepam, have been shown to produce anxiolysis both preclinically (Cook and Davidson, 1973) and clinically (Haefely, 1991). Tolerance, a phenomenon observed when the effects following an initial administration of a drug at a given dose are no longer observed or are significantly reduced after repeated administration, is a prominent side effect associated with BZD administration. BZDs have also shown tolerance to their anxiolytic effects in the conflict model (Smith et al., 1996). Therapeutically, tolerance is an undesirable side effect and may also be linked to dependence liability (Kolesnikov et al., 1993).The purpose of this study was to investigate whether repeated systemic sub chronic administration of a withaferin-A would produce tolerance to the anxiolytic effects observed following acute administration in the EPM. Withaferin-A, which has *in vivo* selectivity for nNOS and is devoid of hypertensive activity (Moore et al., 1993a) was compared with the benzodiazepine diazepam following acute and sub chronic administration in the EPM paradigm.

## MATERIALS AND METHODS

Male Wistar rats (150 to 200 g) were used in this 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 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).
The following drugs were used: L-Arginine and D-Arginine (Sigma Aldrich). All drugs were freshly prepared and given intraperitonealy (i.p.) in a volume of 0.1 ml/100 g body weight of rats at different time intervals before the testing.

### Extraction of withaferin-A and drugs

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 40 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 40 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.

### Elevated plus maze

The elevated plus maze (Pellow et al., 1985; Pellow and File, 1986) was elevated to a height of 50 cm and consisted of two open arms 50 x 10 cm, and two enclosed arms 50 x 10 x 40 cm, arranged such that the two arms of each type were opposite each other. The apparatus and procedure have been previously described (Volke et al., 1997). During a 5-min observation period, the following parameters were measured: number of open arm entries, time spent on open arms and number of closed arm entries. Subsequently, the percentage of the number of entries into the open arms of the total number of entries into all arms and percentage of time spent on open arms were calculated.

### Procedure

Rats were subjected to restrained stress for 1 h and then administered diazepam (0.3, 1.0 or 3.0 mg/kg) or vehicle controlled (distilled water plus one drop of Tween 80) 30 min prior to testing in EPM. On days 2 to 6 (sub chronic drug administration), animals were administered diazepam twice daily at 3.0 mg/kg, i.p (08:30 and 17:30 h). On day 7, animals were administered diazepam after RS (0.3, 1.0 or 3.0 mg/kg). Same 7 day protocol was followed with administration of withaferin-A (10, 30 or 40 mg/kg) or vehicle. For sub chronic drug administration, withaferin-A was used at 40 mg/kg i.p.

### Statistical analysis

The mean ± S.E.M. open arm exploration time (s) and total arm entries for each dose group was compared with the vehicle treated group. Dose response effects for acute vs. sub chronic dosing were compared by a one-way analysis of variance (ANOVA) followed by a Dunnett's test for post hoc comparison. Significance was measured at $P<0.05$ for all post hoc comparisons.

## RESULTS

Both diazepam and withaferin-A dose-dependently increased open arm exploration time following acute administration. Figure 1 shows that diazepam at 1.0 and 3.0 mg/kg significantly ($P<0.05$) increased open arm exploration time by 191 and 200%, respectively as compared to the vehicle (0.0 mg/kg) control ($F_{3.20} = 5.31$; $P<0.01$). Furthermore, diazepam at 3.0 mg/kg significantly ($P<0.05$) increased overall motor activity (total arm entries) by 84% as compared to vehicle control ($F_{3.20} = 4.75$; $P<0.05$). Similarly, acute administration of withaferin-A at 40 mg/kg produced significant ($P<0.05$) anxiolytic activity (Figure 2) by increasing open arm exploration time by 176% as compared to the vehicle control ($F_{3.20} = 4.29$; $P<0.05$); although, there was no significant change in overall motor activity following acute administration of withaferin-A ($F_{3.20} = 0.83$; $P>0.05$).

The effects of both diazepam and withaferin-A following sub chronic bid administration differed from those of acute administration. Following sub chronic drug administration, diazepam at 3.0 mg/kg, (Figure 1)
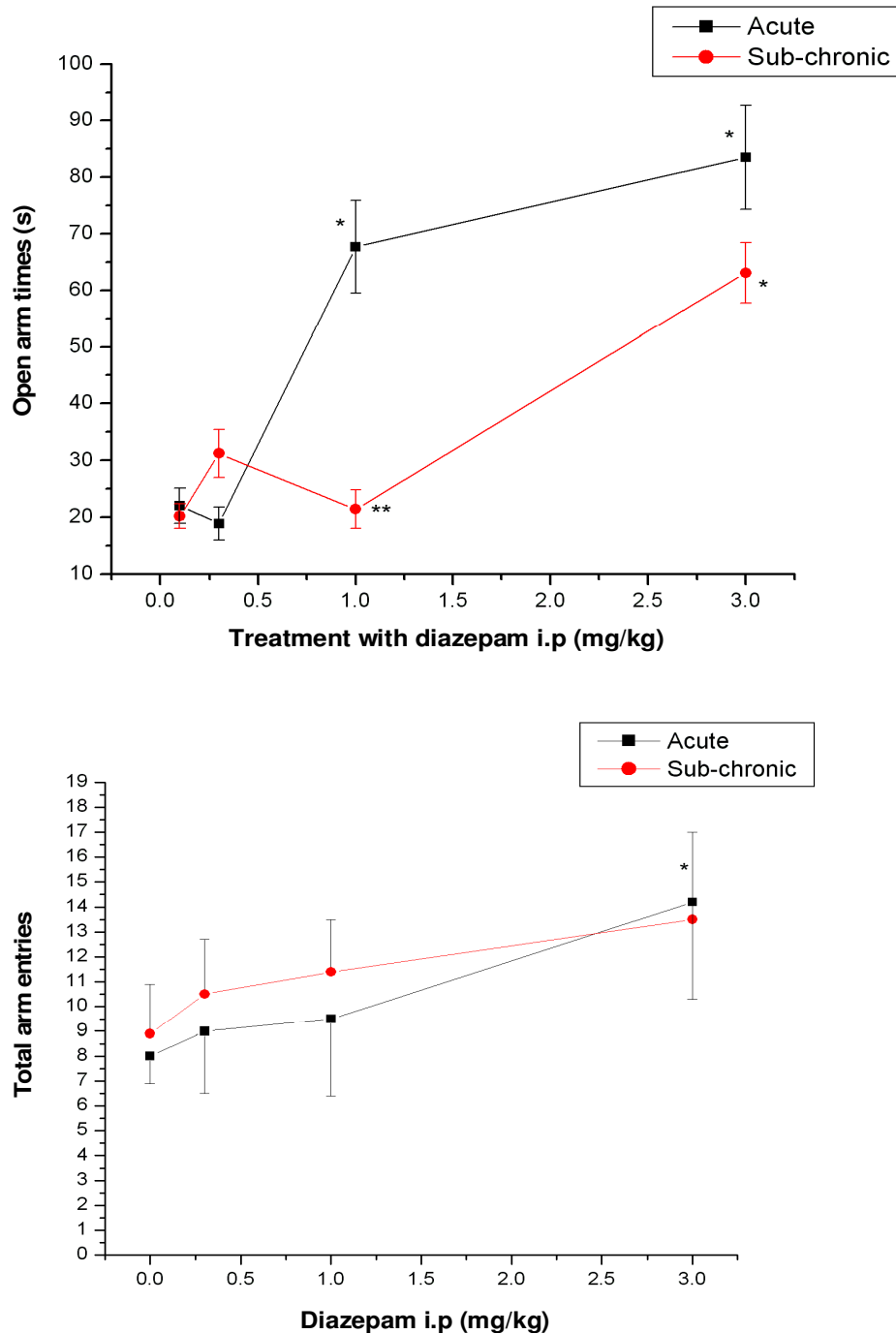
**Figure 1.** The effects of diazepam (0.0, 0.3, 1.0 and 3.0 mg/kg, i.p.) on open arm exploration time in seconds ($X$±S.E.M.; top) and total number of arm entries ($X$±S.E.M.; bottom) following acute and 5 day sub chronic (3.0 mg/kg, bid) diazepam treatment are depicted. Per dose group, n = 6. * $P$<0.05 as compared to control; **$P$<0.05 for acute as compared to subchonic treatment.

significantly ($P$<0.05) increased open arm exploration time by 175% in comparison with the vehicle (0.0 mg/kg) control ($F_{3.20}$ = 3.41; $P$<0.05). The effects on open arm exploration time following acute administration of 3.0 mg/kg diazepam and following 5 days of 3.0 mg/kg bid

dosing were not significantly different ($F_{3.20}$ = 2.28; $P$>0.05). However, following sub chronic drug administration, the animals administered 1.0 mg/kg did not show significant anxiolytic effects in open arm exploration when compared with vehicle control ($F_{3.20}$ =

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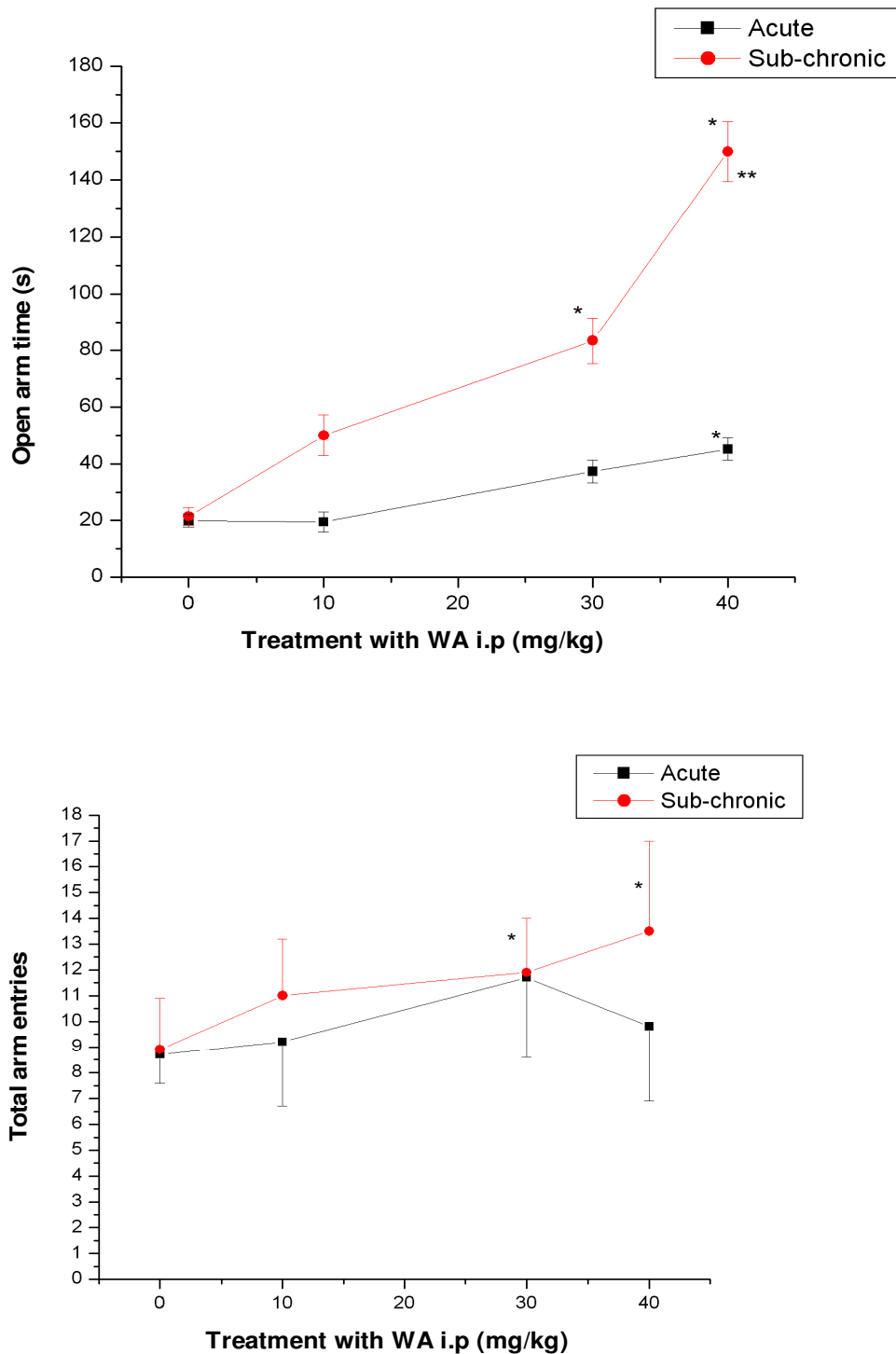
**Figure 2.** The effects of WA (10.0, 30.0 and 40.0 mg/kg, i.p.) on open arm exploration time in s ($X$±S.E.M.; top) and total number of arm entries ($X$±S.E.M.; bottom) following acute and 5-day sub chronic (40.0 mg/kg, bid) WA are depicted. Per dose group, n = 6. *$P<0.05$ as compared to control; **$P<0.05$ for acute as compared to sub chronic treatment.

2.28; $P>0.05$). There was a significant ($P<0.05$) 65% decrease in open arm exploration time, when acute effects of 1.0 mg/kg were compared with the effects following sub chronic administration ($F_{7.40}$ = 4.10; $P<0.01$). For overall motor activity, the 3.0 mg/kg dose was no longer significantly different from vehicle control ($F_{3.20}$ = 1.24; $P>0.05$).

Following i.p injections, both 10.0 and 40.0 mg/kg

withaferin-A (Figure 2) significantly ($P<0.05$) increased open arm exploration time by 409 and 665%, respectively as compared to the vehicle control ($F_{3.20} = 4.80$; $P<0.01$). In addition, open arm exploration time following sub chronic injections was significantly ($P<0.05$) greater by 172% than following acute administration of withaferin-A at 40.0 mg/kg ($F_{7.40} = 12.13$; $P<0.01$). Following sub chronic drug administration, withaferin-A demonstrated a significant ($P<0.05$) increase in overall motor activity at 10.0 and 40.0 mg/kg by 37 and 63%, respectively as compared to the vehicle control ($F_{3.20} = 2.93$; $P<0.05$).

## DISCUSSION

Both diazepam and withaferin-A produced a dose dependent anxiolytic effect as measured by open arm exploration time in the EPM following acute administration. However, sub chronic administration of diazepam, which acutely produced anxiolytic effects at 1.0 mg/kg, was devoid of anxiolytic activity at 1 mg/kg, suggesting tolerance to its anxiolytic effects. In contrast, withaferin-A showed enhanced anxiolytic efficacy following sub chronic administration when compared to its effects following acute administration.

In this study, the benzodiazepine diazepam demonstrated anxiolytic effects by increasing open arm exploration time in the EPM. These findings are in agreement with previous studies that have shown diazepam to produce consistent anxiolytic effects both preclinically (Cook and Davidson, 1973) and clinically (Haefely, 1991). This study further demonstrated that the withaferin-A produced anxiolytic effects by increasing open arm exploration time. These findings are in agreement with recent reports that showed that withaferin-A have demonstrated anti-anxiety effects similar to benzodiazepines in preclinical models (Khan and Ghosh, 2010). In addition, the anxiolytic activity of withaferin-A was independent of benzodiazepine receptor activation (Bhatnagar et al., 2005). Furthermore, in rodents, withaferin-As were devoid of benzodiazepine-like side effects such as motor in-coordination and amnesia (Bhatnagar et al., 2005).

In addition, the benzodiazepine diazepam has been shown to produce tolerance to its anxiolytic effects following sub chronic dosing in the conflict assay (Smith et al., 1996) and in the EPM in this study. Tolerance is an undesirable side effect for therapeutic purposes and may also be linked to dependence liability (Kolesnikov et al., 1993). This study shows that withaferin-A was devoid of tolerance to its anxiolytic effects. The similar open arm times in the vehicle control groups in the acute and sub chronic EPM paradigms in this study as well as previous EPM studies in rats show that a single prior experience in the EPM has no effect on open arm time or total arm entries following retesting (Pellow et al., 1985).

Therefore, this findings are most likely due to the inherent pharmacological properties of diazepam and withaferin-A.

Tolerance to benzodiazepines has been associated with down regulation of the $GABA_A$-benzodiazepine receptor complex (GBR) (Sher et al., 1983). The reason for lack of tolerance following sub chronic administration of a withaferin-A is presently unknown, but may be related to the mechanism of action of withaferin-A, namely, enzymatic inhibition rather than a receptor mediated interaction. By enzymatically mediating the release of NO, and not directly affecting receptor site(s), down regulation and resulting tolerance at a receptor site may be avoided.

Another important consideration concerning our results of enhanced anxiolytic efficacy following sub chronic administration of withaferin-A involves reversible vs. irreversible inhibition of NOS by various withaferin-A. Based on these findings, it might be expected that repeated administration of an irreversible withaferin-A would produce an increase in NOS inhibition, an increased duration of effect and possible enhanced anxiolytic activity following sub chronic administration. However, these enhanced effects would not be expected with a reversible inhibitor such as withaferin-A. It is unclear as to why repeated administration of withaferin-A in this study produced enhanced anxiolytic efficacy. Enzymatic studies are currently underway to investigate the effects of sub chronic administration of withaferin-A on NOS activity.

In conclusion, acute inhibition of nitric oxide synthase by withaferin-A resulted in anxiolysis which is similar to diazepam. However, following sub chronic administration unlike diazepam, withaferin-A displayed enhanced anxiolytic efficacy without tolerance. These findings suggest that withaferin-As may represent a novel class of anti-anxiety agents which may produce continued anxiolytic efficacy following repeated administration, without tolerance liability.

## ACKNOWLEDGEMENTS

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