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GC-MS Analysis of Indigofera Tinctoria and its Protective Effect on Noise Induced Behavioral and Biochemical Alterations in Rats

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Keywords

GC-MS analysis; Immunomodulators; Noise stress; Antistressor; Behavior

ABSTRACT

The aim of the present study is to investigate GC-MS analysis and protective effect of *Indigofera tinctoria (I.tinctoria)* on noise induced behavioral and biochemical changes in wistar albino rats. Noise stress was done by broadband white noise generator, 100 dBA, 4 h daily for 15 days and Indigofera tinctoria (300 mg/Kg .b.w.) administered orally. Seven compounds were isolated via GC-MS analysis, out of which l-(+)-Ascorbic acid 2,6-dihexadecanoate, N-(3-(3,4,5-trimethoxyphenyl) propionyl) piperidin-2-one and 3,6-diacetyl-9-ethylcarbazole have reported to exhibit antioxidant capacity. Upon exposure to the noise-stress, a significant ($P<0.001$) increase in immobilization with decrease in rearing, grooming, and ambulation behavior are seen in open field. This outcome suggest that sub-acute stress affect locomotor activity in rat. Sub-acute noise stress significantly increased the level of glutathione peroxidase, lipid peroxidation, nitric oxide, glutathione S transferase and decreased the level of superoxide dismutase, catalase, reduced glutathione, glutathione reductase, Vitamin C, protein thiols, suggesting that oxidative imbalance in immune organs when expose to sub-acute noise. However, Oral administration of *I.tinctoria* significantly prevented noise induced behavioral and biochemical changes. These results concluded that *I.tinctoria* supplementation could scavenge the noise induced free radical generation, suggesting its antioxidant property and can be regarded as an antistressor.

INTRODUCTION

The experiences of stress are common to all living things. Noise is a pervasive aspect of many modern community and work environments [1]. According to the International Programme on Chemical Safety [2] an adverse effect of noise is defined as a change in the morphology and physiology of an organism that results in impairment of functional capacity, or an impairment of capacity to compensate for additional stress, or increases the susceptibility of an organism to the harmful effects of other environmental influences.

Noise can cause health effects in hearing impairment, interference with spoken communication, sleep disturbance, cardiovascular disturbances, impaired task performance, disturbances in mental health, and negative social behaviour and annoyance reactions [3]. When noise exposure of any kind exceeds 90 dB, noise becomes a stressor [1]. The effects of noise on the immune status have also been reported [4]. Environmental stress plays an important role in the elevation of blood glucocorticoid level, which suppresses both innate as well as acquired immune functions and results in susceptibility to infection [5]. Corticotrophin-releasing hormone released during stress, stimulates the release of adrenocorticotropic hormone which in turn releases corticosterone from the adrenal cortex [6]. Various stresses have been associated with enhanced free radical generation causing oxidative damage. Oxidative stress arises from the imbalance between pro-oxidants and antioxidants in favor of the

former, leading to the generation of oxidative damage [7]. Generation of free radicals is an integral feature of normal cellular functions, in contrast, excessive generation and/or inadequate removal of free radical results in destructive and irreversible damage to the cell [8].

The plant *Indigofera tinctoria (I.tinctoria)* belongs to the family Fabaceae and found throughout India. *I. tinctoria* is native to India one of the oldest known centres of indigo dye production. The extract screened for phytochemical analysis was found to contain bioactive compounds like flavonoids, saponins, tannins, steroidal terpens, phenols and anthroquinone [9]. The roots, stems and leaves of *I. tinctoria* are bitter, thermogenic, laxative and are useful for hepatoprotective, anticancer, epilepsy, neuropathy, chronic bronchitis, asthma, ulcers, and skin diseases [10]. Dry leaf powder is used in the treatment of asthma [11], constipation, liver disease, heart palpitation and gout [12]. Decoction of the leaves used in bites and stings or venomous insects and reptiles to relieve the pain [13]. Plants contain several phytochemicals which possess strong antioxidant activities [14]. But the effectiveness of aqueous extract of *I.tinctoria* in averting noise-stress induced production of free radicals in animal models has not yet been reported. Hence, this present study was undertaken to evaluate the antioxidant properties of *I.tinctoria* against noise stress-induced behavioral and biochemical changes in wistar albino rats.

EXPERIMENTAL

Collection and identification

The plant *I. tinctoria* was collected (May to November 2013) from the KSG Enterprises (Tindivanam, Tamil Nadu, India) and authenticated by Dr. D. Aravind (Department of Medical Botany, and National Institute of Siddha, Chennai, India). Voucher specimens were deposited at the Herbarium of National institute of Siddha, Reg no: NIS/MB/83/2013. The collected plants were separated from unwanted materials and dried in shade. The leaves were grounded to coarse powder with the help of a suitable grinder. The powder was then stored in an airtight container, kept in a cool, dark and dry place until the analysis.

Extraction procedure

I. tinctoria dried powdered leaves of 30 g were extracted with 250 mL of sterile distilled water using the Soxhlet apparatus at 100 ˚C. The aqueous extracts were filtered with Whatman No 1 filter paper and then freeze dried and stored at 4˚C for further investigation. The extraction efficiency was quantified by determining the weight of the extracts and the percentage yield was calculated as 16%.

Experimental design

Wister strain male albino rats weighing 180–220 g were randomly selected. The animals were maintained under standard laboratory condition and fed ad libitum with food (M/S Hindustan Lever Limited, Bombay, India) and water. All the rats were housed under condition of controlled temperature (26 ± 2°C) with 12 h light and 12 h dark exposure. The animals were divided into four groups with six animals in each group. Group I served as the control, the animals of Group II animals were subjected to noise-stress for 4 h daily for 15 days (Sub-acute exposure), Group III (*Indigofera tinctoria alone*) were treated with *I.tinctoria* for 48 days and experiments were carried out on 49th day and Group IV consisted of noise stress with *I.tinctoria* -treated animals. These animals were pre-treated with *I.tinctoria* for 33 days and then exposed to noise stress for 15 days. During the noise stress period, they were also given *I.tinctoria* extract by the oral route and all the experiments were done on the 49th day. Ethical clearance was obtained before the commencement of experiments from the ethical committee (IAEC No: 22/02/2013) and the Committee for the purpose of control and supervision of experiments on animals (CPCSEA).

Noise stress induction

Broadband white noise at 100-dB intensity was produced by a white noise generator, amplified by an amplifier connected to a loud speaker fixed 30 cm above the animal cage. A sound level meter was used to measure the intensity of the noise [1].

Sample Collection

Blood samples and isolation of spleen, thymus, lymph node and bone marrow was done between 8 and 10 a.m. to avoid circadian rhythm induced changes. Stress-free blood samples were collected as per the technique described by Feldman and Conforti [15]. At the end of experimental period all the animals were exposed to mild anesthesia and blood was collected from internal jugular vein, plasma and serum was separated respectively by centrifugation at 3000 r.p.m at 4°C for 15 min. Later all the animals were sacrificed under deep anesthesia using Pentothal sodium (40 mg/kg b.w). The spleen, thymus and lymph node was excised, washed in ice cold saline and blotted to dryness. Quickly weighed and the spleen, thymus lymph node and bone marrow sample were homogenized by using Teflon glass homogenizers. 10% homogenate of this tissue was prepared in phosphate buffer (0.1 M, pH 7.0) and centrifuged at 3000g at 4°C for 15 min to remove cell debris and the clear supernatant was used for biochemical assays.

Biochemical determinations

Estimation of plasma corticosterone was determined by the procedure of Singh and Verma [16]. Protein was estimated as per the method [17]. Lipid peroxidation was determined in the immune organs [18]. Nitric oxide (NO) levels were measured as total

nitrite + nitrate levels with the use of the Griess [19]. Protein thiol was determined [20]. Superoxide dismutase (SOD) according to Marklund and Marklund [21] and catalase (CAT) according to the method of Sinha [22]. The activity of glutathione peroxidase (GPx) was estimated by the methods [23]. Reduced glutathione (GSH) in the immune organs was estimated by the method of Moron et al. [24]. The vitamin-C (ascorbic acid) content in the tissue was determined according to the method of Omaye et al. [25].

Open field Test

The open field test was widely used to measure general locomotor and preparatory activity [26]. Rat used in this study was placed in the centre of the open field, which was novel to the animal, and the following variables were scored for 5 min: (i) Immobilization: Rats had eyes open, holding its head against the gravity but without any head, body or limb movements. (ii) Grooming: Rhythmic paw movements over the face and/or head for face washing might include episodes of biting and cleaning of paws. (iii) Rearing: Standing still on upright on its hind limb only. (iv) Ambulation: When all the four limbs were in one particular square (central or peripheral) of the field.

Gas chromatography

An Agilent 6890 gas chromatograph equipped with a straight deactivated 2 mm direct injector liner and a 15 m Alltech EC-5 column (250 µ I.D, 0.25 µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10, 1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20°C per minute to 260°C and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode).

Mass Spectrometry: A JEOL GC mate II bench top double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000 software used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

Mass spectrometry library search: Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

Statistical analysis

The results are presented with mean ± S.D. There is a regional variation for all the free radical scavenging enzymes in their distribution. The results are presented with mean ± S.D. The data statistically evaluated using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests in SPSS-20.

RESULT

GC-MS analysis

The GC-MS analysis carried out for aqueous extract of I.tincoria. The results about on, these compounds identify through mass spectrometry attached with GC (**Figure 1**). The result displays 7 compounds are identified through mass spectrometry. Molecular weight, molecular formula and structure of the isolated compounds are ascertained. Result tabulated in **Table 1** shows the presence of different phytocompounds in the extract namely l-(+)-Ascorbic acid 2,6-dihexadecanoate (2.3%), 3-Indoleacetonitrile (14.8%), N-(3-(3,4,5-Trimethoxyphenyl) Propionyl Piperidin-2-One (14.68%), Methyl stearate (4.27%), 3,6-Diacetyl-9-Ethylcarbazole (47.06%), 5-Hydroxy-L-tryptophan (4.02%) and Tetrachloro-1,2-benzoquinone (12.73%).

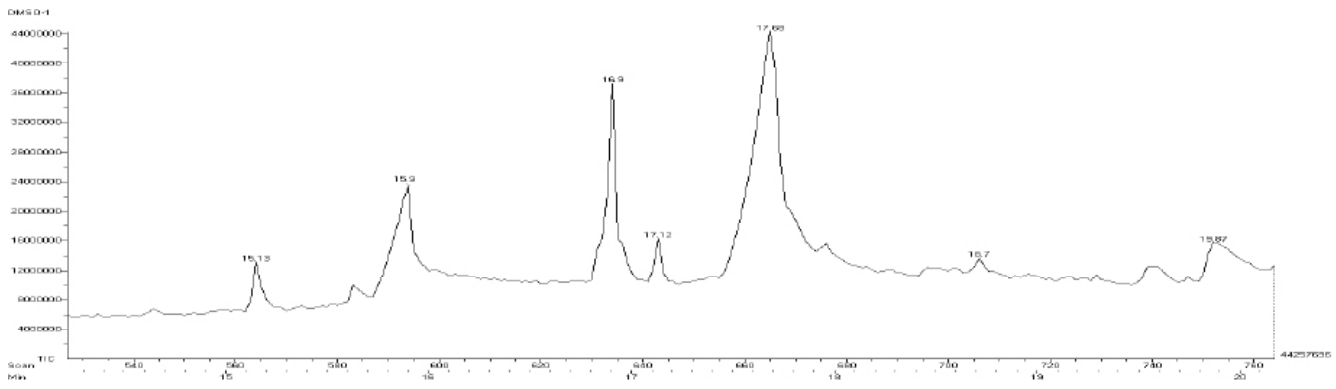
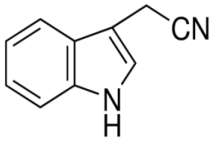
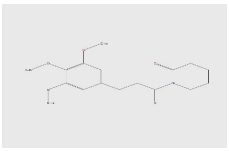
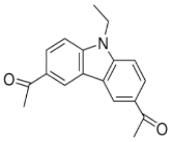
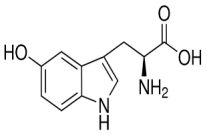
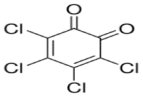


Figure 1. GC-MS Chromatogram of aqueous leaf extract of *Indigofera tinctoria.*

Table 1. Phytocomponents identified in the aqueous extract of *Indigofera tinctoria* by GC-MS analysis.

S.No	Compound Name and Formula	RT	Peak %	Structure
1.	l-(+)-Ascorbic acid 2,6-dihexadecanoate $C_{38}H_{68}O_8$ MW : 652.94	15.13	2.3	
2.	3-Indoleacetonitrile $C_{10}H_8N_2$ MW: 156.18	15.19	14.8	
3.	N-(3-(3,4,5-Trimethoxyphenyl)propionyl)piperidin-2-one $C_{17}H_{23}NO_5$ MW: 321.36	16.9	14.68	
4.	Methyl stearate $C_{19}H_{38}O_2$ MW: 298.50	17.12	4.27	
5.	3,6-Diacetyl-9-Ethylcarbazole $C_{18}H_{17}NO_2$ MW: 279.34	17.68	47.06	
6.	5-Hydroxy-L-tryptophan $C_{11}H_{12}N_2O_3$ MW: 220.22	18.7	4.02	
7.	Tetrachloro-1,2-benzoquinone $C_6Cl_4(=O)_2$ MW: 245.88 S	19.87	12.73	

Open Field Test

The results of the open field tests are given in **Table 2**. After fifteen days of exposure to noise, in OFT there was significant reduction in ambulation in central squares, peripheral squares, rearing, grooming and fecal bolus whereas a significant increase in immobilization was observed when compared to control. This noise-stress induced change in the peripheral, central, immobilization, grooming and rearing though significantly improved in *I.tinctoria* treated rats in all these parameters whereas markedly showed a decrease from controls.

Table 2. Protective effect of *I. tinctoria* (300 mg/kg animal body weight) on ambulation (squares) in open field behavior in albino rats exposed to noise-stress.

Parameters	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Peripheral	65.333 ± 7.08	42.5 ± 6.28a***	68.5 ± 7.28b***	54.833 ± 4.08a*b*
Central	4.768 ± 1.12	1.035 ± 0.64a***	4.186 ± 1.14b***	2.683 ± 0.80a**b*
Immobilization	18.851 ± 3.35	37.335 ± 7.58a***	17.36 ± 3.02b***	24.833 ± 2.85b**
Rearing	20.833 ± 4.87	10.5 ± 1.87a***	22 ± 3.84b***	15.333 ± 2.58 a*b*
Grooming	11.5 ± 1.97	5.5 ± 1.87a***	10.166 ± 1.94b**	9.5 ± 2.16b*
Fecal bolus	1.833 ± 0.75	4.016 ± 0.91a***	1.516 ± 0.56b***	2.833 ± 0.75b*

Note: Values are expressed as mean ± S.D of six animals. a–compared with control; b- compared with noise; Significance at *p<0.05; Significance at **p<0.01; and Significance at ***p<0.001.

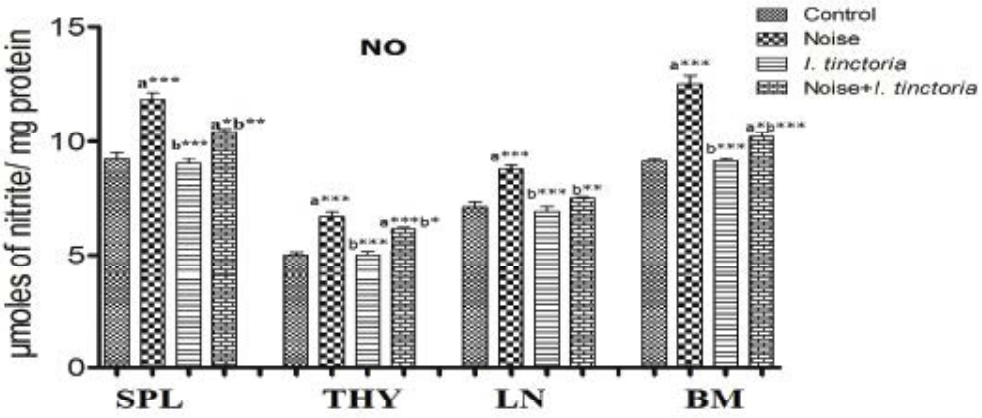
Biochemical analysis

Corticosterone: The results of the corticosterone estimation are given in **Table 3** as on mean ± S.D. Noise-stress exposed rats display a significant (p<0.001) elevation in the corticosterone level. Though treatment with the *I.tinctoria* during noise exposure could significantly (p<0.001) reduce the corticosterone level when compared with noise-stress group.

Table 3. Protective effect of *I. tinctoria* (300 mg/kg animal body weight) on and corticosterone level in albino rats exposed to noise-stress.

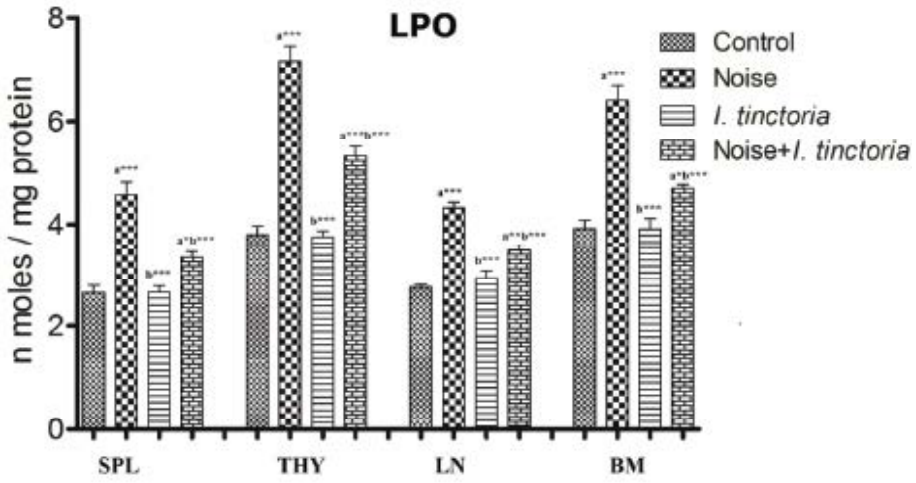
Parameters	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Corticosterone (mg/dl of plasma)	39.368 ± 4.57	76.557 ± 5.17a***	38.690 ± 4.19b***	49.101 ± 4.79a**b***

Free radical and Stress marker: The results of nitric oxide level in spleen, thymus, popliteal lymph nodes and bone marrow are summarized with mean ± S.D (**Figure 2**). The nitric oxide levels are similar in control animal compared with *I.tinctoria* alone 48 days treated animals. On the other hand noise alone 4 h daily for 15 days treated animal's (spleen, thymus, popliteal lymph nodes and bone marrow) significantly (p<0.001) increased in nitric oxide levels. However *I.tinctoria* treated during noise exposed animals are significantly decreased NO level in immune organs. The results of lipid peroxidation in the spleen, thymus, lymph nodes and bone marrow are summarized as mean ± S.D (**Figure 3**). The LPO level of *I.tinctoria* alone treated animals similar to the control animals. Whereas in sub-acute exposure of noise group significantly (p<0.001) increased LPO level in the immune organs. Though treatment with the *I.tinctoria* during noise exposure could reduce the LPO level markedly, still the LPO level was significantly elevated from controls. The results are showed protein thiol level with mean ± SD (**Figure 4**). The protein thiol level in *I. tinctoria* treated animals is similar to the control animals. The protein thiol levels in noise stress group are decreased significantly (p<0.001) in all immune organ compare with control. Although treatment with the *I.tinctoria* during noise exposure could increase the protein thiol level markedly, still the protein thiol level was significantly decreased from controls.



Values are expressed as mean ± S.D of six animals. Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001. (SPL-Spleen, THY-Thymus, LN-Lymph node and BM-Bone Marrow)

Figure 2. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on Nitric oxide (NO) level in albino rats exposed to noise-stress (µ moles of nitrite/mg protein).



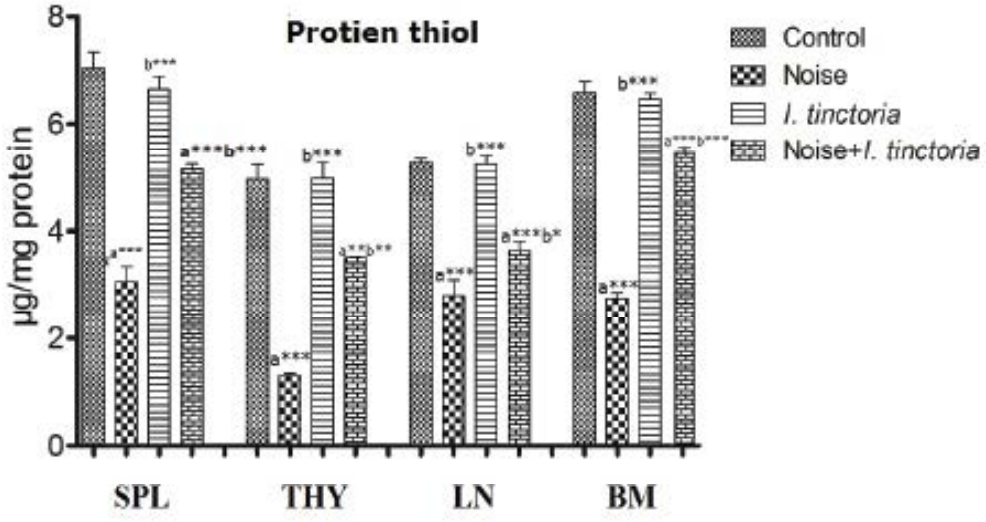
Values are expressed as mean ± S.D of six animals. Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

Figure 3. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on lipid peroxidation (LPO) level in albino rats exposed to noise-stress (nmMDA/mg protein).

Free radical scavenging enzymes

Superoxide dismutase (SOD): The results summarized in **Table 4** as on mean ± S.D. The sub-acute stress induced a significant (p<0.001) decrease in the SOD levels in immune organs compare with control. Though treatment with the *I.tinctoria* during noise exposure could increase the SOD level markedly, still the SOD level was significantly decrease from controls. Moreover, *I.tinctoria* when given to normal animals they did not alter the level of SOD from controls.

Catalase (CAT): The result of Catalase in the immune organ (spleen, thymus, popliteal lymph nodes and bone marrow) are summarized in **Table 5** as on mean ± S.D. The Catalase level of *I.tinctoria* alone treated animals are similar to the control animals



Values are expressed as mean ± S.D of six animals. Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.0

Figure 4. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on Protein thiols level in albino rats exposed to noise-stress (µg/mg protein).

Table 4. Protective effect of *I.tinctoria* (300 mg/kg animal body weght) on SOD level in albino rats exposed to noise-stress (units/mg protein).

Organ	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Spleen	2.599 ± 0.28	1.592 ± 0.12 a^{***}	2.526 ± 0.18 b^{***}	2.021 ± 0.20 a^{**} b^{*}
Thymus	2.165 ± 0.09	1.592 ± 0.13 a^{***}	2.069 ± 0.05 b^{***}	1.916 ± 0.06 a^{**} b^{***}
Lymph node	2.019 ± 0.16	1.192 ± 0.10 a^{***}	1.906 ± 0.12 b^{***}	1.610 ± 0.10 a^{***} b^{**}
Bone marrow	2.119 ± 0.16	1.168 ± 0.10 a^{***}	1.973 ± 0.13 b^{***}	1.559 ± 0.16 a^{***}

Note: Values are expressed as mean ± S.D of six animals. a- compared with control; b- compared with noise; Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

Table 5. Protective effect of I.tinctoria (300 mg/kg animal body weight) on catalase level in albino rats exposed to noise-stress (µ moles of H2O2 consumed/mg protein).

Organ	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Spleen	33.405 ± 4.37	18.977 ± 1.71 a^{***}	34.914 ± 2.50 b^{***}	27.748 ± 3.37 a^{*} b^{**}
Thymus	40.386 ± 4.97	19.929 ± 1.39 a^{***}	38.028 ± 3.62 b^{***}	35.862 ± 4.44 b^{***}
Lymph node	39.080 ± 2.85	17.511 ± 2.37 a^{***}	40.352 ± 2.48 b^{***}	31.637 ± 2.82 a^{***} b^{***}
Bone marrow	32.534 ± 2.35	16.467 ± 2.17 a^{***}	30.928 ± 2.40 b^{***}	21.474 ± 3.23 a^{***} b^{*}

Note: Values are expressed as mean ± S.D of six animals. a- compared with control; b- compared with noise; Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.0

and there is no significance. The noise stress significantly decrease the CAT level in immune organ. However treatment with the *I.tinctoria* during noise exposure could increase the CAT level markedly, still the CAT level was significantly decrease from controls.

Glutathione peroxidase (GPx): The Glutathione peroxidase results summarized in **Table 6**. The enzymatic GPx levels of I.tinctoria alone treated animals are similar to the control animals and there is no significance changes observed. The sub-acute noise exposure increase the GPx levels in all the immune organ and significance (p<0.001) changes observed when compare with control. Though treatment with the IT during noise exposure could reduce the GPx level markedly, still the GPX level was significantly elevated from controls.

Table 6. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on glutathione peroxidase (GPX) level in albino rats exposed to noise-stress (µg of GSH consumed/mg protein).

Organ	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Spleen	10.273 ± 0.585	18.931 ± 1.550 a^{***}	10.772 ± 0.724 b^{***}	13.609 ± 0.208 a^{***} b^{***}
Thymus	8.086 ± 0.811	16.338 ± 1.442 a^{***}	8.011 ± 0.686 b^{***}	12.340 ± 1.231 a^{***} b^{***}
Lymph node	10.015 ± 0.792	14.709 ± 1.678 a^{***}	10.020 ± 0.414 b^{***}	11.835 ± 0.424 a^{*} b^{***}
Bone marrow	8.556 ± 0.676	12.619 ± 0.290 a^{***}	8.106 ± 0.690 b^{***}	11.710 ± 0.111 a^{***} b^{*}

Note: Values are expressed as mean ± S.D of six animals. a- compared with control; b- compared with noise; Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

Glutathione S Transferase (GST): The results of the corticosterone estimation given in **Table 7** with mean ± S.D. The GST

levels in *I.tinctoria* treated animals are similar to the control animals. Whereas in sub-acute noise exposed animals GST levels are significantly (p<0.001) increased when compared with control. Prior *I.tinctoria* treated with noise-stress, elevated GST levels are significantly reduced when compared with noise-stress group.

Table 7. Protective effect of I.tinctoria (300 mg/kg animal body weight) on Glutathione S Transferase (GST) level in albino rats exposed to noise-stress (CDNB conjugate formed/min/mg protein.

Organ	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Spleen	2.436 ± 0.408	5.155 ± 0.650 a^{***}	2.479 ± 0.299 b^{***}	3.291 ± 0.075 a^{**} b^{***}
Thymus	1.790 ± 0.179	4.457 ± 0.187 a^{***}	1.663 ± 0.366 b^{***}	3.120 ± 0.270 a^{***} b^{***}
Lymph node	1.366 ± 0.276	3.875 ± 0.458 a^{***}	1.251 ± 0.271 b^{***}	2.528 ± 0.368 a^{***} b^{***}
Bone marrow	1.436 ± 0.399	2.370 ± 0.242 a^{***}	1.503 ± 0.206 b^{***}	1.967 ± 0.229 a^{*} b^{*}

Note: Values are expressed as mean ± S.D of six animals. a- compared with control; b- compared with noise; Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

Glutathione Reductase (GR): The results of GR in the Immune organ (spleen, thymus, popliteal lymph nodes and bone marrow) are summarized in **Table 8** with mean ± S.D. In *I.tinctoria* alone treated animals GR levels are along with control animals. The sub-acute stress significantly (p<0.001) decreases the GR levels in immune organs. But in *I.tinctoria* with noise treated group GR levels are significantly increased.

Table 8. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on Glutathione reductase (GR) level in albino rats exposed to noise-stress (nM of NADPH oxidized/min/mg protein).

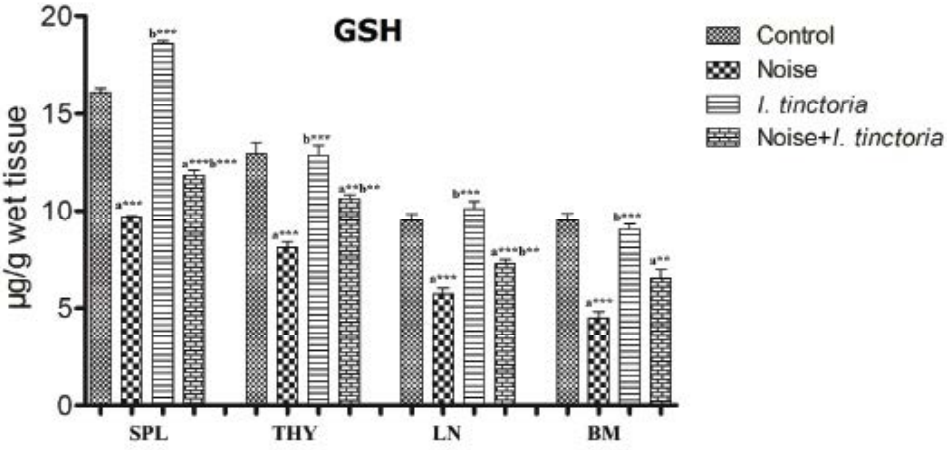
Organ	Control	Noise	Indigofera tinctoria	Noise + Indigofera tinctoria
Spleen	0.132 ± 0.015	0.074 ± 0.003 a^{***}	0.129 ± 0.004 b^{***}	0.101 ± 0.004 a^{***} b^{***}
Thymus	0.079 ± 0.013	0.042 ± 0.004 a^{***}	0.075 ± 0.006 b^{***}	0.055 ± 0.005 a^{***} b^{**}
Lymph Node	0.080 ± 0.006	0.035 ± 0.004 a^{***}	0.094 ± 0.006 b^{***}	0.067 ± 0.006 a^{***} b^{**}
Bone Marrow	0.063 ± 0.004	0.032 ± 0.004 a^{***}	0.0623 ± 0.002 b^{***}	0.050 ± 0.005 a^{***} b^{***}

Note: Values are expressed as mean ± S.D of six animals. a- compared with control; b- compared with noise; Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

Non enzymatic

Reduced Glutathione (GSH): The results are summarized with mean ± S.D (**Figure 5**). The GSH level of *I.tinctoria* alone treated animals is associated to the control animals. During noise exposure, GSH level in the immune organs are decreased significantly (p<0.001) when compared with control. Although *I.tinctoria* with noise stressed group significantly increased GSH level when compared with sub-acute noise exposed animal.

Figure 5. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on Reduced glutathione (GSH) level in albino rats Exposed to noise-stress (µg of GSH/mg of protein).



Values are expressed as mean ± S.D of six animals. Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

Vitamin C (Vit C): The results are summarized as on mean ± S.D (**Figure 6**). The non-enzymatic Vitamin C level of *I. tinctoria* alone treated animals is similar to the control animal. On the other hand noise exposed animal's (spleen, thymus, popliteal lymph nodes and bone marrow) Vitamin C level decreased and the significance is p<0.001. But in *I.tinctoria* with noise treated group VIT C level are significantly increased when compare with noise.

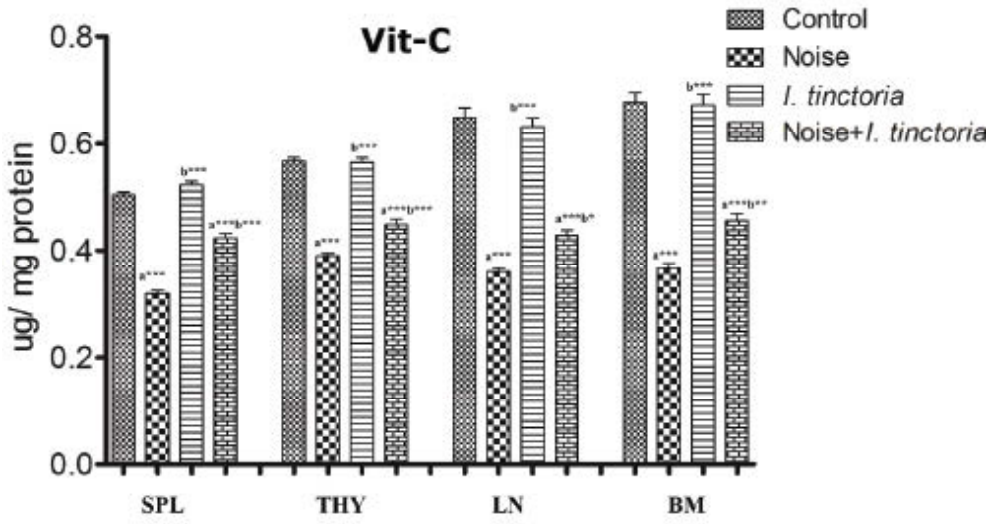
DISCUSSION

GS-MS chromatogram

Plants are considered a vast source of several pharmacologically active principles and compounds that are commonly

used in home remedies against multiple ailments [27]. The more precise information in qualitative phytochemical analysis can be obtained by gas-chromatography coupled with mass spectrometry (GC-MS) [28]. The plant GS-MS chromatogram showed 7 compounds (**Table 1**). The mass spectrums with the data base gave more than 90% match as well as confirmatory compound structure match.

Figure 6. Protective effect of *I.tinctoria* (300 mg/kg animal body weight) on Vitamin C (Vit-C) level in albino rats Exposed to noise-stress (µg/mg protein).



Values are expressed as mean ± S.D of six animals. Significance at *p<0.05; Significance at **p<0.01; and c- Significance at ***p<0.001.

I-(+)-Ascorbic acid 2,6-dihexadecanoate comprising of 2.3% in the extract, has been reported to have an antioxidant, anti-inflammatory and antinociceptive properties. It even exhibited antibacterial activity against Staphylococcus aureus, Escherichia coli, etc. It also enhances sperm quality and prevents sperm agglutination thus making them more motile with forward progression [29].

3-Indoleacetonitrile one of the foremost (14.8%) compound present in *I.tinctoria* extract is known to exhibit light-induced auxin-inhibitory substance, it also have tryptophan dioxygenase inhibitors pyridyl-ethenyl-indoles as potential anticancer and immunomodulators [30].

N-(3-(3,4,5-Trimethoxyphenyl) propionyl) piperidin-2-one comprises 14.68% of the peak percentage. This compound is derived from piperidine group. Piperidine is very important pharmacophore because it is presence in numerous alkaloids, pharmaceuticals, agrochemicals and as synthetic intermediates. Piperidines are known to have CNS depressant action at low dosage levels and stimulant activity with increased doses. In addition, the nucleus also possesses analgesic, ganglionic blocking and anesthetic properties as well [31].

Methyl stearate (4.27%) is fatty acid compounds identified possess many biological activities is reported to have antidiarrheal, cytotoxic and anti-proliferative activities [32].

3.6-Diacetyl-9-Ethylcarbazole (47.06%) compound is derived from Carbazole group and this one is a major compound present in I. tinctoria extract. Carbazole is an aromatic heterocyclic organic compound. It has been reported to have an antibacterial, anti-tumor, antidiabetic, free radical scavenging, neurotropic [33], anti-inflammatory, antifertility, insecticidal.

5-Hydroxy-L-tryptophan possess 4.02% of the peak percentage. India one of the oldest known centre of indigo dye production and it's derived from 5-Hydroxy-L-tryptophan. 5-HTP act as sleep disorders, depression [34], anxiety, migraine and tension-type headaches, fibromyalgia, binge eating associated with obesity, premenstrual syndrome (PMS), premenstrual dysphoric disorder (PMDD), attention deficit-hyperactivity disorder (ADHD), and along with prescription drugs to treat seizure disorder and Parkinson's disease [35]. 5-HT and 5-HTP modulate GPG-generated rhythmic activity, including locomotion, swimming, respiration, and chewing, in many invertebrates and vertebrates [36].

Tetrachloro-1,2-benzoquinone compound presence 12.73% in *I.tinctoria* extract. Derived from Benzoquinone (torpedoed). Tetrachloro-1,2-benzoquinone possesses special effects on white and red blood cell counts and it enhance activity of delta-aminolevulinic acid dehydratase in erythrocytes [37].

Behavioral analysis

The open field model (OFT) examines anxiety and locomotor related behavior characterized by the normal aversion of the animal to an open, brightly lit area. Animals removed from their acclimatized cage and placed in environment express anxiety and fear, by showing alteration in all or some parameters. Anxiolytic treatments reduce such fearful behavior of animals in open field [38]. In the present study, noise exposed animals shows increased immobilization in OFT. Along with these changes, decrease in rearing, grooming, fecal bolus and ambulation squares also revealed that the noise exposed group express anxiety, fear and

altered locomotory activity. The noise stress-induced behavioral changes in OFT were prevented in *I.tinctoria* treated group (**Table 2**). In GC-MS analysis found 5-Hydroxy-L-tryptophan (4.02%) presence in the plant extract. 5-HTP has been reported to have a sleep disorders, depression anxiety, migraine and tension-type headaches the presence of 5-HTP may act as an anxiolytic or recuperating normal function [34].

Biochemical estimation

Oxidative stress is the general phenomenon of oxidant exposure and antioxidant depletion, or oxidant-antioxidant balance [39]. ROS/RNS, also known as free radicals, are normal by products of cellular aerobic metabolism, these unstable molecules can impair cellular lipids, proteins and nucleic acids in DNA if the balance of corresponding antioxidants is disrupted. Excess free radicals may cause cell death by initiating lipid peroxidation of nuclear and cell membranes, destroying the integrity of the cell and leading to necrotic cell death. Oxidative stress occurs in a cell or tissue when the concentration of ROS generated exceeds the antioxidants capability of that cell.

NO is a free radical with a diversity of cellular origins and potential functions. Nitric oxide (NO) is generated continuously in the living body, and acts as important physiological mediator. In the present study, significant increase NO level is justifies that noise to generate free radical in immune organs. Increased in the NO level may due to the oxidative stress induced through noise exposure. Same result suggested by Reha et al. [40] who reported that, elevation of NO level indicator of oxidative stress in rat. Nitric oxide is thought to react with superoxide anion to gain a radical property, which is also a potent source of oxidative injury [41]. Increased LPO was presumably associated with increased nitric oxide, conforming the fact that the noise stress-induced generation of free radicals. LPO is a free-radical-mediated process. The increased amount of LPO during sub-acute noise stress in this study is in agreement with the findings of Aravind et al. [42]. The increase level of lipid peroxidation is taken as direct evidence for oxidative stress [7]. The significant decrease in the protein thiols observed in this study may be due to the oxidation of proteins in oxidative process.

These antioxidant enzymes include catalase, SOD and GPx – enzymes that are important in the elimination of free radicals. In the present study SOD levels are significantly decreased in the noise group. Noise induced increase in lipid peroxidation could be the decrease the SOD activity, as SOD would have to remove excess ROS. This is certainly represent noise is a stress and its affect the normal physiology function through the oxidative imbalance. Because SOD is an important enzyme family in living cells for maintaining normal physiological conditions and for coping with stress. The action of SOD is to protect the biological integrity of the cells and tissues against harmful effects of superoxide free radicals [43]. In the present studies shows, decrease in catalase level in the noise stress group. This is validate that, reduction in enzyme activities with longer duration may be related to consumption of enzymes against oxidative stress. The decreased activity of the ant oxidases could additionally be attributed to their nature of synergistic functioning, which may partly explain the mechanism of their reduced activity [44]. Increase in the GPx enzyme system in order to prevent the accumulation of free radical [45]. Further concluded that, the increase in GPx enzymes suggested that there were an oxidative stress in workers at noisy environment. The findings of this study that alteration is present in these enzyme levels after sub-acute exposure to noise, which reveals that noise stress in not easily adapted to our body.

Glutathione directly quenches ROS such as lipid peroxides. The antioxidative enzyme, glutathione peroxidase, catalyzes the conversion of H_2O_2 to water by using reduced glutathione (GSH) and reduced NADPH as cofactors. In this study the GSH are significantly decreased in noise stress owing to oxidation of proteins in oxidative process and it is also justified by the decreased levels of one of the major thiol substance GSH. This correlates with the report of Sokolovski et al. [46] that alterations in the glutathione system occur, when rats were exposed to white noise of 90 db. The decreased GSH level in noise expose group may be due to increase level of lipid oxidation products which may be associated with the less availability of NADPH required for the activity of glutathione reductase. The decreased GSH level in association with decreased GR activity may support the explanation as evidence for this present study [47]. GR is a glutathione regenerating enzyme that permits the conversion of oxidized glutathione (GSSG) to reduced glutathione by the oxidation of NADPH to $NADP^+$ [48]. GSH reduction can also explain the decreased concentration of Vitamin C, which enters the cell mainly in its oxidized form where it is reduced by GSH [49]. Vitamin C is a hydrophilic reducing agent which directly reacts with super oxides, hydroxyls and various lipid hydro peroxides more effectively than any other water soluble antioxidant [50]. Therefore the decrease in the vitamin C levels could justify the altered immune function in sub-acute noise stress [4].

Pre administration of *I.tinctoria* to the noise exposed rats normalized the activities of enzymatic and non-enzymatic anti-oxidant, thereby preventing free radical mediated oxidative damage in immune organ. In GC-MS analysis *I.tinctoria* contain one of the major compound with 47.06% is 3,6-Diacetyl-9-Ethylcarbazole derived from carbazole group. Carbazole is an aromatic heterocyclic organic compound. This compound is known to possess free radical scavenging capacity [33]. In addition 2.3% peak percentage of l-(+)-Ascorbic acid 2,6-dihexadecanoate were determine. Okwu et al. [51] who reported that l-(+)-Ascorbic acid 2,6-dihexadecanoate has anti stressor activity and 3-Indoleacetonitrile was reported to have immunomodulatory activity [30]. These reports further supporting our studies, *I.tinctoria* contain antistressor and in vivo antioxidant capacity in rat immune organs.

CONCLUSION

Normally, the antioxidant mechanism fails either through overproduction of free radicals or insufficient activities of scavenging

enzymes. From these findings it can be strong to conclude that noise stress can cause free radical damage in immune organs. Noise stress altered behavioral and biochemical abnormalities were prevented by *I.tinctoria* supplementation. Therefore the present study suggests that *I.tinctoria* supplementation may exert antioxidant effect and can be regarded as a protective drug against stress.

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TRANSPARENCY DECLARATION

We declare that we have no conflicts of interest.

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