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Nanocurcumin Restores Arsenic-Induced Disturbances in Neuropharmacological Activities in Wistar Rats

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Abstract

The present study was carried out to examine the ameliorative potential of nanocurcumin against arsenic induced (sub-chronic) alterations in central nervous system in male Wistar rats. Nanocurcumin was synthesised and the hydrodynamic diameter, *zeta* potential and particle size were~76.60 nm, (-) 30 mV and 95nm, respectively. Experimental rats sub-chronically exposed to sodium (meta) arsenite (As; 10 $mg.kg^{-1}$; 70 days; *p.o*) induced significant ($p<0.05$) reduction in superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione and favoured free radical generation and induced lipid peroxidation in brain tissue. The exposure resulted in significant ($p<0.05$) decrease in voluntary- and involuntary motor activities and enhanced anxiety levels. However, experimental rats receiving nanocurcumin (15 $mg.kg^{-1}$; *p.o*) showed significant ($p<0.05$) recovery in enzymatic - and non-enzymatic antioxidant defence system and restoration of redox balance and overcome arsenic induced depression in motor activities and elevated anxiety levels. Further, Arsenic induced elevation in pro-inflammatory cytokines, cyclooxygenase-2 activity and prostaglandin-E_2 in brain and angiotensin-II levels (plasma) was significantly ($p<0.05$) ameliorated by nanocurcumin. Additionally, quantitative real -time polymerase chain reaction revealed a fivefold decrease in Nox_2 expression in brain following nanocurcumin administration. Thus, the study concludes that nanocurcumin can serve as a potential therapeutic candidate to counter arsenic induced redox imbalance and neuropharmacological disturbances and there exists a vast scope to exploit its utility after appropriate clinical modelling.

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1. Introduction

Arsenic (As) is omnipresent, exists in both organic and inorganic (*i*As) forms[1]. Drinking groundwater contamination from geological sources serves as major source of 'As' exposure[2]. The routes of exposure include ingestion, inhalation, direct dermal contact, or placental transmission from the mother to foetus[3]. Most of the ingested and inhaled 'As' gets absorbed through the gastrointestinal tract and lungs into the bloodstream[4]. Absorption may involve two mechanisms *viz.*, trivalent 'As' *via* diffusion[5] and pentavalent 'As' *via* energy-dependent transport system[6].The trivalent arsenicals bind to the sulfhydryl (-SH) groups of dihydrolipoamide to inhibit pyruvate dehydrogenase[7] either, directly attacking -SH groups or indirectly through the generation of reactive oxygen species (ROS)[8]. Arsenic interferes with enzymes namely, catalase (CAT)[9], super oxide dismutase (SOD), and antioxidant glutathione (GSH)[10,11] which indirectly increases the level of ROS.

'As' is well-known neurotoxicant and has neuro-developmental and neurocognitive effects[12]. Exposure to 'As' and its metabolites methyl arsonic acid (MMA) [III] suppresses the N-methyl D-asparate receptors in the hippocampus, which play a pivotal role in synaptic plasticity, learning and memory, leading to neurobehavioral disorders and cognitive dysfunction[13]. Arsenic exposure can alter metabolism of various neurotransmitters such as monoamines, acetylcholine, gamma aminobutyric acid and glutamate[14]. Exposure to 'As'caused an increase in the level of proinflammatory cytokines like interferon-γ (IFNγ), interferon-1β (IL-1β) and tumor necrosis factor-α (TNFα)[15], cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE_2) in brain[16].

Curcumin, the principal *curcuminoid* of turmeric is derived from the rhizome of *Curcuma longa* (Linn.). The United States Food and Drug Administration considered this molecule as '*Generally Recognized as Safe*'[17]. Moreover, the curcumin molecule exhibits a multi-targeting ability in various pathological conditions, which allows translation into a therapeutic or nutraceutical agent[18]. Curcumin is an excellent scavenger of ROS[19], which has the potential to inhibit the production of superoxide anion ($O_2^{\bullet}$) radical[20]. Further, curcumin being an antioxidant can act as a good neuroprotectant[21]. Curcumin counteracts the cell damage *via* reduction of ROS production and lipid peroxidation (LPO) and through the enhancement of the phase- II detoxification enzyme such as CAT, SOD and GPx[22]. In spite of the several medical/therapeutic properties, curcumin could not be used clinically due to the following limitations. These include low hydrophilicity and intrinsic dissolution rate(s), low physico-chemical instability, poor bioavailability[23,24] rapid metabolization, low penetration, and targeting efficacy[25-27]. However, nanocurcumin has the advantages of high drug loading capacity and high bioavailability in aqueous media. Similarly, encapsulation of curcumin in chitosan nanoparticles retains its antioxidant properties and metal chelating properties *in vivo* during experimental toxicosis[28]. In the present study, we hypothesized that nano sizing of curcumin would ameliorate the 'As' induced (sub-chronic) alterations in redox homeostasis in the central nervous system and its consequences on certain neuropharmacological activities in experimental rats.

2. Materials and Methods

2.1 Chemicals, Reagents and Assay Kits

Sodium (meta) arsenite and curcumin, bovine serum albumin and glutathione reductase were procured from Sigma-Aldrich (St. Louis, MO, USA). 2-thiobarbituric acid and protease inhibitor cocktail from Merck (Darmstadt, Germany), while pyrogallol from S.D. Fine Chemicals (Mumbai, India).

Enzyme linked immunosorbent assay (ELISA) kits for IL-1β, IL-6 and TNF-α manufactured by Ray Biotech, (Georgia, USA) were used. Assay of PGE_2 in the brain tissue homogenate and angiotensin-II (Ang-II) in plasma were carried out using ELISA kits manufactured by Elabscience® Biotechnology (Wuhan, China). Sandwich ELISA kits (Bioassay Technology Laboratory, Shanghai, China) were employed to measure COX-2. Other chemicals or reagents were of analytical grade and procured from either Hi-Media® or S.D Fine Chemicals, Mumbai, India. Millipore (reverse osmosis) water was used for preparing buffers and solutions required for the various laboratory procedures.

2.2 Synthesis of Nanocurcumin

Nanocurcumin (NC) was synthesized by solvent (-) anti-solvent precipitation technique using ethanol and millipore water. Curcumin (100 $g.L^{-1}$) was dissolved in ethanol and then millipore water was added to at a ratio of 1:10 and mixed well. Then, the mixture was subjected

to ultrasonication (Labman®, PRO 650, Chennai India) at an amplitude of 45µm for 5s with a pause of 3s for 30 min for three cycles to get a homogeneous suspension. Later the suspension was lyophilized to obtain NC in amorphous form.

2.2.1 Characterization of Nanocurcumin

Dynamic light scattering (DLS) was used to determine the hydrodynamic radius or diameter of the nanoparticles (nanoPartica SZ-100, Horiba, Kyoto, Japan). *Zeta* potential was determined in *zeta* mode with an electrode voltage of 3.4 V at 25°C (Nanopartica®, SZ-100, Horiba). Scanning electron microscopy (SEM) was carried out (Hitachi-TM3030Plus, Tokyo, Japan) by placing a pinch of NC on a carbon coated copper grid and samples were coated with gold using sputter coater (K-550 X, Emitech, Kent, UK).

2.3 Experimental Animals

Male Wistar rats (n= 36) of 7–8 weeks of age and weighing ~120- 150 g were procured from Sri Raghavendra Enterprises, Kenchanapura, Bengaluru, Indiaand were acclimatized to animal facility (CPCSEA Registration No. 493/GO/ReBiBt-S/Re-L/01/CPCSEA), Veterinary College, Hebbal, Bengaluru. Animals were maintained in groups of four in polypropylene cage (18"×10"×8") under a constant temperature (24±2°C) and humidity (55±5%) with 12 hrs of light:dark cycle. Experimental rats were given free access to standard rodent chow (Amrut®, Ms. Pranav Agro Industries Ltd, India) and to water *ad libitum* throughout the experimental study. The study was approved by the Institutional Animal Ethics Committee (IAEC) *vide* Letter No. VCH/IAEC/2019/114 dated 03.12.2019 and the experimental study was carried out as per the guidelines of the Committee for Prevention of Cruelty and Supervision of Experiment on Animals (CPCSEA), New Delhi, India.

The rats were randomly divided into four equal groups (n=6/group), *viz.*, Group-I served as control; Group-II was administered sodium (meta) arsenite @ 10 mg.kg^{-1} body weight through oral gavage daily during morning hours for a period of 70 consecutive days. Group-III received sodium (meta) arsenite and NC @15 mg.kg^{-1} b.wt (*p.o*) for similar duration. Experimental rats in Group-IV received a similar dose of NC alone. All the experimental animals were sacrificed on day-71 (morning hours) after overnight fasting using standard anaesthetic protocol (xylazine hcl@ 10 mg.kg^{-1} and ketamine hydrochloride @ 40 mg.kg^{-1}; *i.p*). Blood samples were withdrawn by cardiac puncture. Brain tissue was quickly dissected out and washed with phosphate buffer saline, weighed and stored at -80°C until further analysis. Additionally, 12 rats were maintained under standard laboratory conditions similar to untreated control and these rats were served as reference positive control (Diazepam hydrochloride; DZP), each time utilizing six rats after appropriate wash out period while carrying out neuropharmacological tests.

2.4 General Observations

All the experimental rats were observed twice daily for clinical signs of toxicity and mortality if any during the course of study period. Clinical signs observed include changes in gait, posture, skin, fur, eyes, mucus membranes, nature of secretions and excretions and response to handling.

2.5 Assessment of Neuropharmacological Activity

The neuropharmacological activity in experimental rats with respect to voluntary motor activity (spontaneous motor activity), involuntary motor activity (forced locomotor) and anxiety levels were evaluated on day 35 as well as on day 70. The rats which served as reference positive control (diazepam hcl; 1.0 mg.kg^{-1}; *i.p*) were subjected to different neuropharmacological assessment after 30 min. of administration.

The spontaneous motor activity, a measure of exploratory behaviour of experimental animals was measured using actophotometer (Photoactometer, INCO®, Haryana, India) for a period of 5 min. according to Kulkarni [29].

The forced locomotor activity, a measure of strength and coordinated movement of experimental rats were studied by using rota rod apparatus as described by Dunham and Miya[30].

2.6 Evaluation of Anxiety-/Depression- like Behaviour

Experimental animals were subjected to their anxiety-/depression- like behaviour on day 35 or day 70 by elevated plus maze according to Belovicova *et al.*[31].

2.7 Body Weight and Scarification

The body weights of the experimental rats were recorded before the initiation of the study and at term before they were sacrificed [*i.e.,* morning hours on day 71) under anaesthesia (xylazine hydrochloride @ 10 $mg.kg^{-1}$; *i.p*). The change in body weight (g) in each experimental group was expressed as *per cent* change.

2.8 Preparation of Brain Tissue Homogenate

Brain from each of the experimental rats was minced with scissors, transferred into a centrifuge tube and homogenized in a homogenizer (Heidolph® Silent Crusher M; Schwabach Germany) by using about 1 ml (10-fold) extraction buffer (ice-cold 50 mM potassium phosphate buffer, pH 7.4) at 3-5°C. The homogenate was then centrifuged at 10,000 x*g* at for 10 min and the supernatant was stored at -80°C until further assays.

2.9 Evaluation of Biochemical Parameters

2.9.1 Protein Estimation

The protein content in the supernatant of brain homogenates was determined by following the method described by Lowry *et al.*[32] using bovine serum albumin as standard.

2.9.2 Estimation of $O_2^{\bullet-}$ Radical Formation

Free radical ($O_2^{\bullet-}$) generation was estimated indirectly in terms of formazan (blue colour) formed due to the reduction of nitroblue tetrazolium (NBT) as an index of $O_2^{\bullet-}$ generation and measured (formazan) by using a spectrophotometer at a wavelength of 540 nm[33].

2.10 Estimation of Enzymatic Antioxidants

2.10.1 SOD Activity

The assay of SOD activity was determined according to the method described by Madesh and Balasubramanian[34] by using a spectrophotometer. The absorbance was read at 570 nm, and the activity was expressed as unit/mg protein.

2.10.2 CAT Activity

The CAT activity in the tissue homogenate was determined according to the method described by Aebi[35] by using a spectrophotometer 240 nm. The activity was expressed as mmol H_2O_2 utilized/min/mg protein.

2.10.3 Glutathione Peroxidase (GPx)

GPx activity was determined by the method of Paglia and Valentine[36]. The change in absorbance was read at 340 nm and expressed as µmol of NADPH oxidized to NADP/min/mg protein.

2.10.4 Glutathione Reductase (GR)

The GR activity was measured following the method of Goldberg and Spooner[37]. It was recorded at 340 nm and expressed as µmol NADPH oxidized to NADP/min/mg protein.

2.11 Assessment of Non-enzymatic Antioxidant

2.11.1 Reduced Glutathione (GSH)

The GSH content was measured in brain homogenate by quantifying free -SH groups by using 5-5' dithiobis 2-nitrobenzoic acid reagent according to Sedlak and Lindsay[38].

2.12 Assessment of Oxidative Damage

2.12.1 Lipid Peroxidation (LPO)

The peroxidative damage to brain tissue in terms of LPO was measured as a thiobarbituric acid reactive substance called malondialdehyde formed per gram of tissue according to Paula *et al.*[39].

2.13 Assay of Inflammatory Cytokines- and Mediators

The level of IL-1β, IL-6, TNF-α, COX-2 and PGE_2 in brain tissue homogenate and Ang-II in plasma were measured by using ELISA kits as per the standard protocol described by manufacturers.

2.14 Quantitative Real-Time PCR Analysis of Nox_2

Brain tissues of all the experimental animals were preserved in RNA later® solution. These samples were kept at room temperature for 18 hr to allow RNA later enters into tissue and then stored at -80°C until analysis. Tissues were homogenized with 1ml ice-cold Trizol™ reagent using mortar and pestle. The purity of the isolated RNA was quantified by NanoDrop® spectrophotometer and only RNA samples with an A260/A280 ratio of 1.8-2.0 were used for reverse transcription. The synthesis of *c*DNA was carried out using commercial kit (Ms.TaKaRa Bio Inc., Japan). Moloney murine leukemia virus (MMLV) reverse transcriptase was used for *c*DNA synthesis from 1 µg total RNA. Previously standardized primers (Ms. Shrimpex Biotech Services Pvt. Ltd., Chennai, India) were used for gene expression studies (Table 1). The *c*DNA was subjected to amplification with the respective primers at various annealing temperatures to optimize the PCR conditions. Quantitative real-time polymerase chain reaction (*q*RT-PCR) was analysed by QuantStudio™ 3 (Ms. Applied Biosystems®, Thermo Fisher Scientific, USA) after validating the primer for its specificity.

Real-time PCR (RT-PCR) was carried out by using TB Green premix Ex Taq-II [(2X) (TaKaRa®, Japan)]. The optimum annealing temperature as determined by PCR for Nox_2 using the specific primers was 63°C. Each sample was run in triplicate by using a 20µl reaction mixture. The 20µl reaction mixture consists of 10µl TB Green premix, 1µl from 10pmol working solutions of each of gene-specific forward and reverse primers, 0.5µl of *c*DNA, and the volume was made up to 20µl with nuclease-free water. RT-PCR reaction started with initial incubation at 95°C for 30s followed by 40 cycles of amplification with denaturation at 95°C for 5s, annealing at 63°C for Nox_2 for 5s and extension at 72°C for 5s for each cycle. To assess the specificity of the amplified product, a dissociation curve was generated at temperature 60°C through 95°C. The result obtained was expressed as threshold cycle (CT). This value corresponds to the cycle number when the fluorescence of the reporter dye appreciably higher than the background fluorescence. The CT value adjusted automatically by the instrument was used for the generation of CT values. Relative Quantification (RQ) value was determined ($RQ = 2^{-\Delta\Delta CT}$) and compared between different experimental groups of rats.

2.15 Statistical Analysis

All data are presented as the mean ± standard error of mean. The results were analyzed for statistical significance by One-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* multiple comparison test of significance using Statistical Package for Social Sciences (SPSS) software (IBM®SPSS® software, version 21.0.0, 2012, Armonk, NY, USA). Significance was set at 95%. Graph Pad Prism (GraphPad® software Inc., version 8.4.3; San Digo, CA, USA) was employed for graphical presentation of data.

Table 1. Nox_2 Primer sequence

Type of Gene	Primer sequences	Size (bp)	Reference
Nox_2	F 5'- CCAGTGAAGATGTGTTCAGCT-3'	195	[67]
	R 5'- GCACAGCCAGTAGAAGTAGAT-3'		
GAPDH	F 5'-TGATTCTACCCACGGCAAGTT-3'	107	NM_017008.4

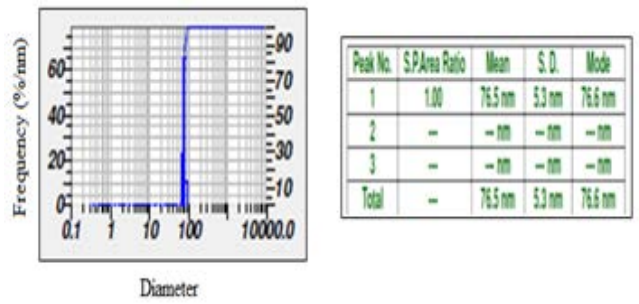
3. Results

3.1 Characterization of Nanocurcumin

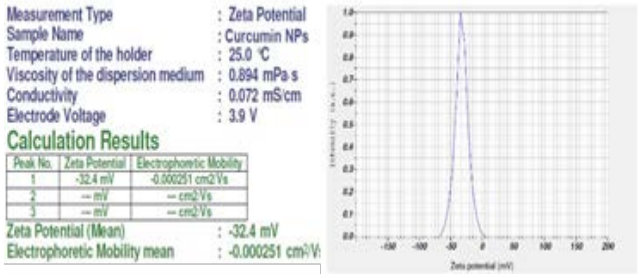
The hydrodynamic diameter of the synthesized nanoparticles was in the range of 76.60 nm (Figure 1a) with *zeta* potential of -32.4 mV (Figure 1b), indicating the stability of NC. The SEM revealed an average individual particle size of 95 nm (Figure 1c).

3.2 General Observations and Terminal Body Weight

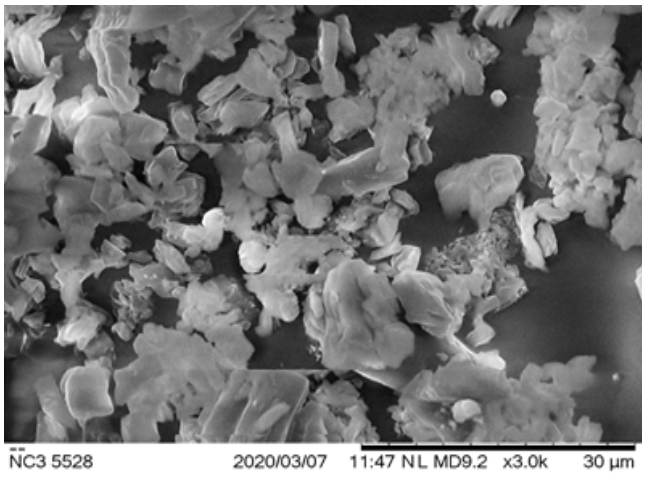
Sub-chronic exposure to 'As' did not exhibit any acute signs of illness or mortality in none of the experimental groups. On the other hand, upon close clinical examination there was minor degree of weakness in rats which received 'As' (Group II) alone. The mean body weight (b.wt.) of rats receiving 'As' showed a marked decrease in growth over other experimental groups. Arsenic exposed rats showed



a



b



c

Figure 1. Characterization of synthesized nanocurcumin. **a.** Determination of the size of nanocurcumin particles by DLS; **b.** *Zeta* potential of the nanocurcumin; **c.** SEM of nanocurcumin.

a significant (p<0.05) decline in b.wt (60.12±11.83g) when compared to control (Group I) (Figure 2). Administration of NC (Group III) resulted in significant (p>0.05) increase in b.wt compared to 'As' exposed group (Group II). However, b.wt. of NC alone administered rats (Group IV) showed a significant (p<0.05) increase over experimental rats belonging to untreated control group I.

3.3 Neuropharmacological Activities

Sub-chronic exposure to 'As' significantly (p<0.05) decreased voluntary locomotor activity when compared to control (Group-I) or NC group (Group-IV) on day 35 or 70 of the experimental study. The mean voluntary locomotor activity on day 35 and day 70 were 43.8±8.8 and 25.75±5.07, respectively in 'As' treated rats while the corresponding values in NC co-administered group were 98.25±10.87 and 89.25±11.75 (Figure 3a).

The mean forced locomotor activity (fall-off time) in 'As' exposed rats on day 35 and day 70 were 33.62±7.34 s and 51.91±1.55 s respectively, where as in NC administered group IV on corresponding days were 65.86±10.61 s and 100.28±5.91 s, respectively. Further, reduced involuntary locomotor activity in 'As' exposed rats recorded on day

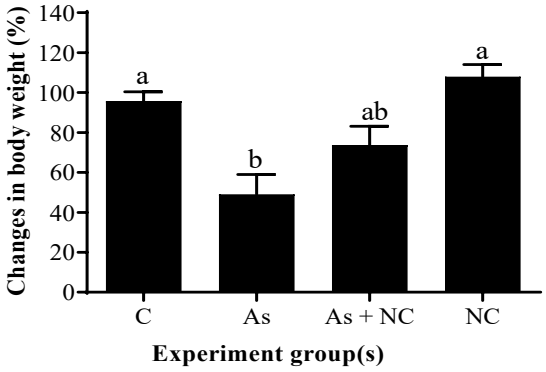


Figure 2. Percent change in body weight (n=6 each).

Note: Bars with dissimilar letters vary significantly between experimental groups; p<0.05; [C = Control, As = Arsenic, As+NC = Arsenic + Nanocurcumin, NC = Nanocurcumin].

70 was statistically distinct from DZP (positive control) treated rats. NC administration significantly (p<0.05) restored the 'As' induced reduction in forced locomotor activities (Figure 3b).

The anxiety level assessed by elevated plus maze revealed that the time spent in open arm in control (Group-I) and 'As' treated (Group-II) were 24.5±1.32 s and 46±5.77 s, respectively on day 35, while the equivalent values on day 70 were 21.8±3.74 s and 2.25±0.47 s, respectively. Thus, sub-chronic exposure to 'As' augmented the anxiety level. However, experimental data obtained on day -70 was subjected to analysis in relation to DZP control indicated that NC administration had significant (p<0.05) influence in reducing anxiety level in rats (Figure3c, 3d).

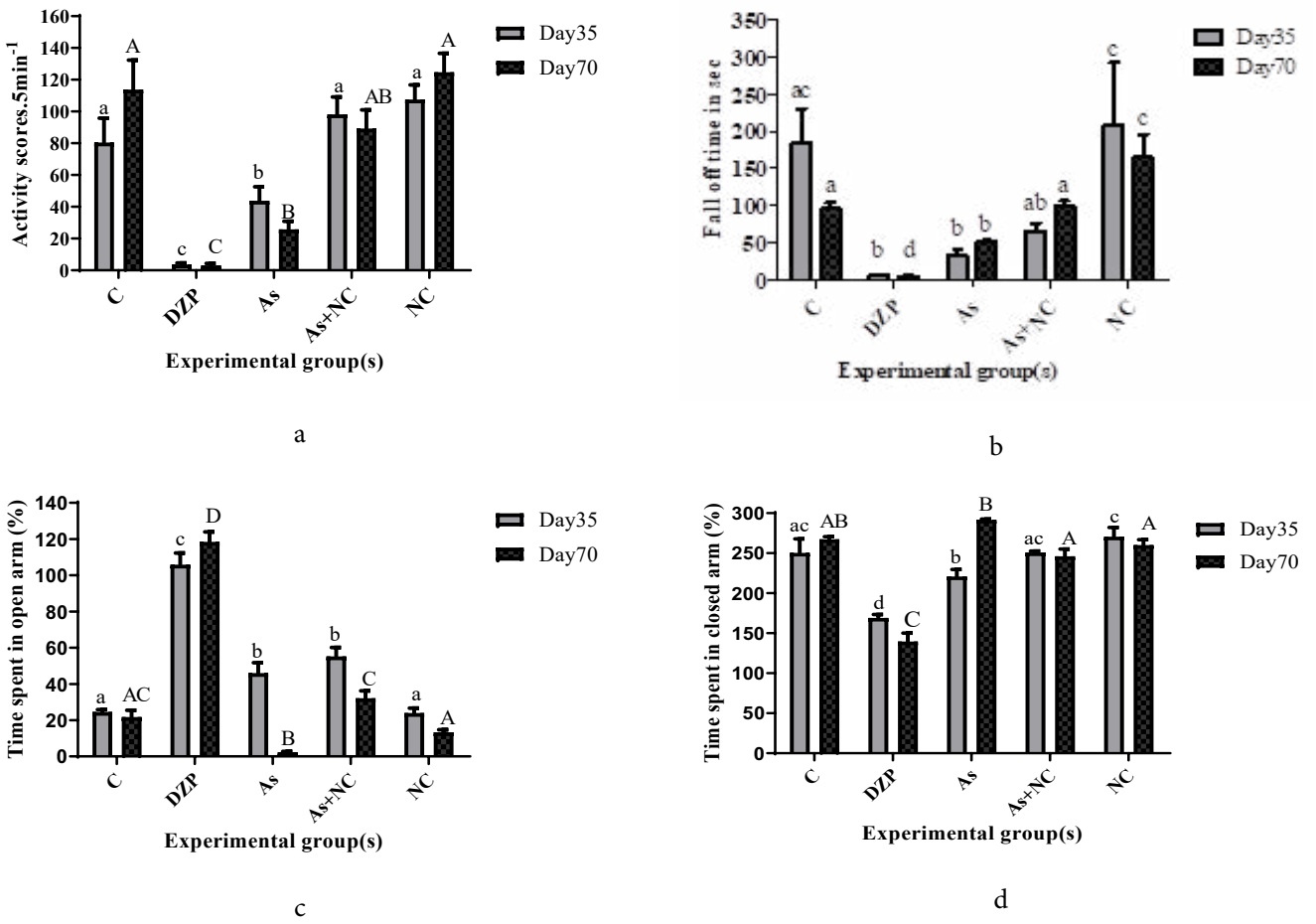


Figure 3. Neuropharmacological activities (n=6 each). **a.** Mean (±SE) voluntary locomotor activity (scores for 5 min.) measured on day 35 and day 70; **b.** Mean (±SE) forced locomotor activity (fall-off time in sec.) measured on rota rod on day 35 and day 70; **c.** Mean(±SE) time spent in open arm (%) of elevated plus maze in different groups on day 35 and day 70; **d.** Mean(±SE) time spent in closed arm (%) of elevated plus maze in different groups on day 35 and day 70.

Note: Bars with dissimilar letters on day 35 and day 70 vary significantly $p< 0.05$; [C = Control, DZP = Diazepam HCl, As = Arsenic, As+NC = Arsenic + Nanocurcumin, NC=Nanocurcumin].

3.4 Biochemical Parameters

3.4.1 Protein Concentration

The protein content in the brain of 'As' exposed rats was significantly reduced when compared to untreated control. Administration of NC significantly ($p<0.05$) increased the protein level (Figure 4a).

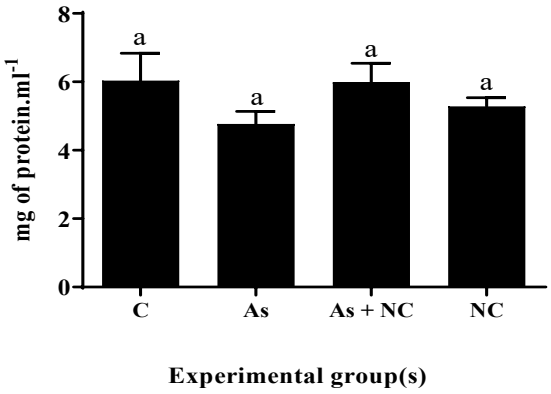
3.4.2 $O_2^{\bullet}$ Radical

The mean(±SE) $O_2^{\bullet}$ generation measured as pmol of NBT reduced.min^{-1}.mg^{-1}protein indicated a significant ($p<0.05$) increase in levels of $O_2^{\bullet}$ radical in 'As' treated rats (1504.53±162.89) as compared to untreated control (858.56±134.23). Administration of NC significantly ($p<0.05$) reduced the $O_2^{\bullet}$ generation in 'As' exposed rats (876.83±69.53) (Figure 4b).

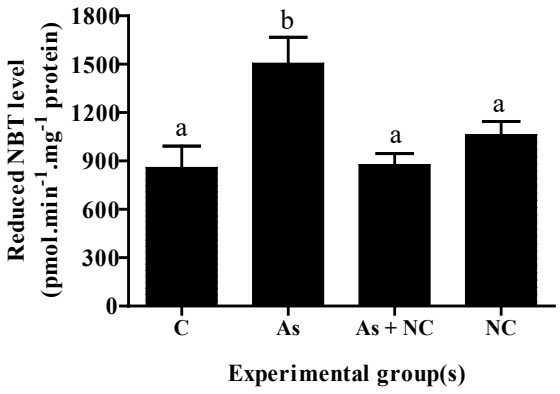
3.5 Enzymatic Antioxidants

The mean(±SE) SOD activity (U.mg^{-1}protein) in the brain tissue of rats belonging to control and 'As' treated group were 0.34±0.05 and 0.19±0.02, respectively and these values differed significantly ($p<0.05$). Administration of NC significantly ($p<0.05$) improved the SOD activity (0.32±0.03). However, NC alone treated rats showed non-significant difference in enzyme activity (0.30±0.01) when compared to untreated control group (Figure 5a).

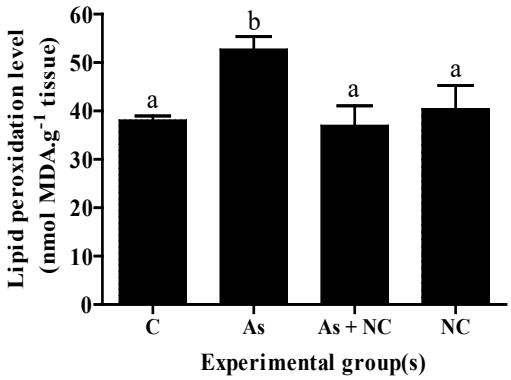
The CAT activity (mmol H_2O_2 utilized.min^{-1}.mg^{-1} protein) has been presented in Figure 5b. The mean (±SE)



a



b



c

Figure 4. Biochemical parameters (n=6 each). **a.** Mean(±SE) protein content (mg.g^{-1}) in brain tissue; **b.** Mean(±SE) superoxide radical activity (pmol NBT reduced.min^{-1}. mg^{-1} protein); **c.** Mean(±SE) levels of LPO formed (nmol.g^{-1} tissue).

Note: Bars bearing dissimilar alphabets vary significantly; $p< 0.05$; [NBT = nitroblue tetrazolium, LPO = Lipid peroxidation, C = Control, As = Arsenic, As+NC = Arsenic + Nanocurcumin, NC = Nanocurcumin].

CAT activity was significantly ($p<0.05$) reduced in brain tissue of 'As' treated rats (49.10±15.77) when compared to untreated control (459.86±52.00). NC administration to 'As' exposed rats showed a significant ($p<0.05$) increase in CAT activity (348.85±50.94).

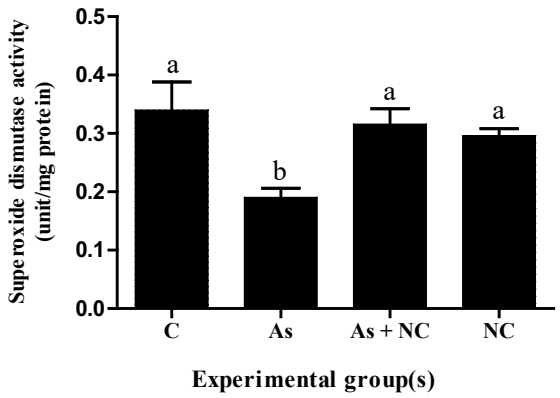
In the case of GPx activity (µmol of NADPH oxidized.min^{-1}.mg^{-1} protein) in 'As' treated rats (0.06±0.01) was significantly ($p<0.05$) reduced when compared to untreated control (0.11±0.02). However, NC administration to 'As' treated rats showed a marked recovery in GPx activity (0.10±0.01) in the brain tissue (Figure 5c).

Figure 5d illustrates activity of GR (µmol of NADPH oxidized.min^{-1}.mg^{-1} protein) in the brain of 'As' exposed and untreated control group of rats were 6868.01±272.05 and 13394.58±3938.62, respectively. Statistical comparison between these two groups indicated that 'As' induced significant ($p<0.05$) decline in GR activity. However, NC administration to rats receiving 'As' showed a non-significant ($p>0.05$) marginal recovery in GR activity (8467.91±125.26).

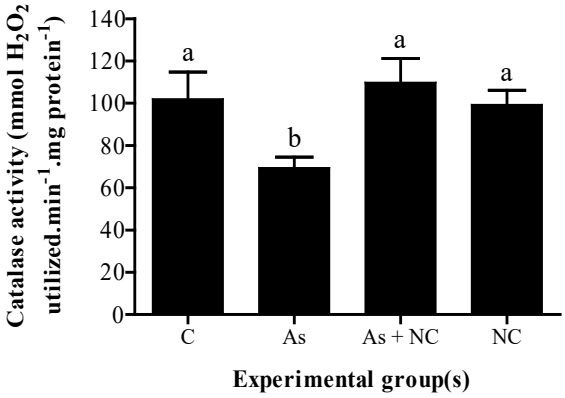
3.6 Non-enzymatic Antioxidant Activity

3.6.1 Reduced Glutathione (GSH)

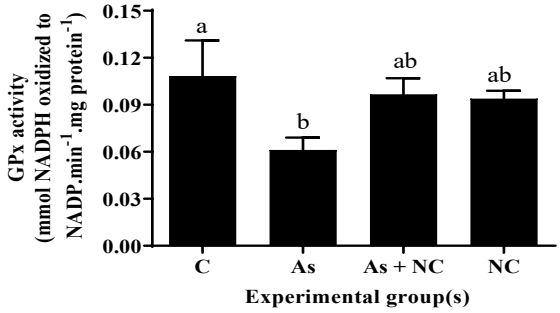
The GSH content (mmol.g^{-1} tissue) in brain tissue of untreated control and 'As' exposed rats were 0.11±0.01 and 0.07±0.01, respectively and were differ significantly ($p<0.05$). The 'As' induced depletion of reduced glutathione levels in brain were significantly ($p<0.05$) elevated (0.10±0.01) when treated with NC (Figure 5e).



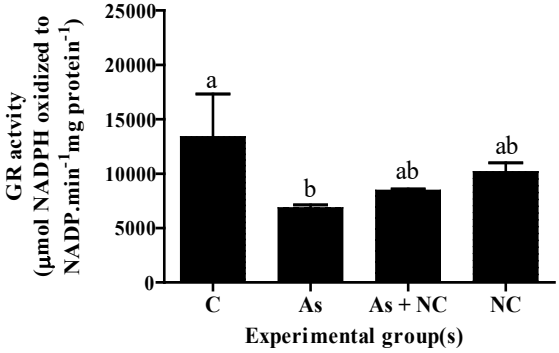
a



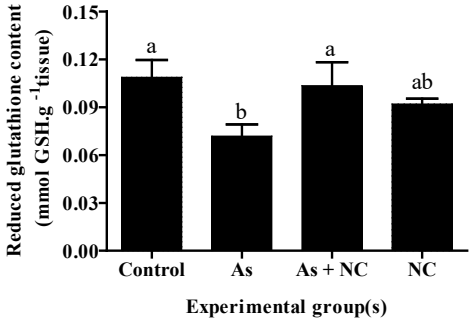
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d



e

Figure 5. Antioxidant parameters (n=6 each). **a.** Mean(±SE) superoxide dismutase (SOD) activity (U.mg^{-1} protein); **b.** Mean(±SE) catalase (CAT) activity (mmol of H_2O_2 utilized.min^{-1}.mg^{-1} protein); **c.** Mean(±SE) glutathione peroxidase (GPx) activity (µmol of NADPH oxidized.mg^{-1}.min^{-1}); **d.** Mean(±SE) glutathione reductase (GR) activity (µmol of NADPH oxidized. mg^{-1}.min^{-1}); **e.** Mean(±SE) glutathione (GSH; mmol.g^{-1} tissue).

Note: Bars bearing dissimilar letters vary significantly; $p<0.05$; [C = Control, As = Arsenic, As+NC = Arsenic + Nanocurcumin, NC = Nanocurcumin].

3.7 Assessment of Oxidative Damage

3.7.1 Lipid Peroxidation

Effects of 'As' on LPO (nmol of MDA.g^{-1} tissue) in rat brain tissue have been presented in Figure. 4c. Sub-chronic exposure to 'As' significantly (p<0.05) elevated the LPO levels (52.80±2.60) when compared to the levels in untreated control group of rats (38.10±0.85). Administration of NC to 'As' exposed rats showed a significant (p<0.05) reduction in LPO level (37.04±4.06) when compared to 'As' treated rats. Further, the LPO levels in rats which received NC alone did not influence the basal level observed in rats belonging to untreated control (40.47±4.80) group.

3.7.2 Inflammatory Cytokines- and Mediators

Effects on the levels of pro-inflammatory cytokines, *i.e.*, IL-1β, IL6 and TNF-α (pg.mg^{-1} protein) are summarized in Figure 6a, 6b and 6c, respectively. The IL-1β levels in brain tissue were significantly (p<0.05) elevated following sub-chronic exposure to 'As' (520.00±81.00) when compared to untreated control group of rats (342.99±41.30). However, NC co-administration significantly (p<0.05) reduced (362.02±33.74) its production down to levels similar to the untreated control group of rats. However, NC treatment alone did not significantly (p<0.05) influence (374.60±25.67) its production (Figure 6a).

The IL-6 levels in brain tissue were significantly (p<0.05) elevated following sub-chronic exposure to 'As' (302.78±9.92) when compared to untreated control rats (179.12±10.00). On the contrary, NC co- administration significantly (p<0.05) inhibited 'As' induced surge (178.42±13.12) in IL-6 production and reduced to the level similar to untreated control group of rats (Figure 6b).

The TNF-α levels in brain tissue were significantly (p<0.05) elevated following sub-chronic exposure to 'As' (80.44±3.43), while NC co- administration inhibited the

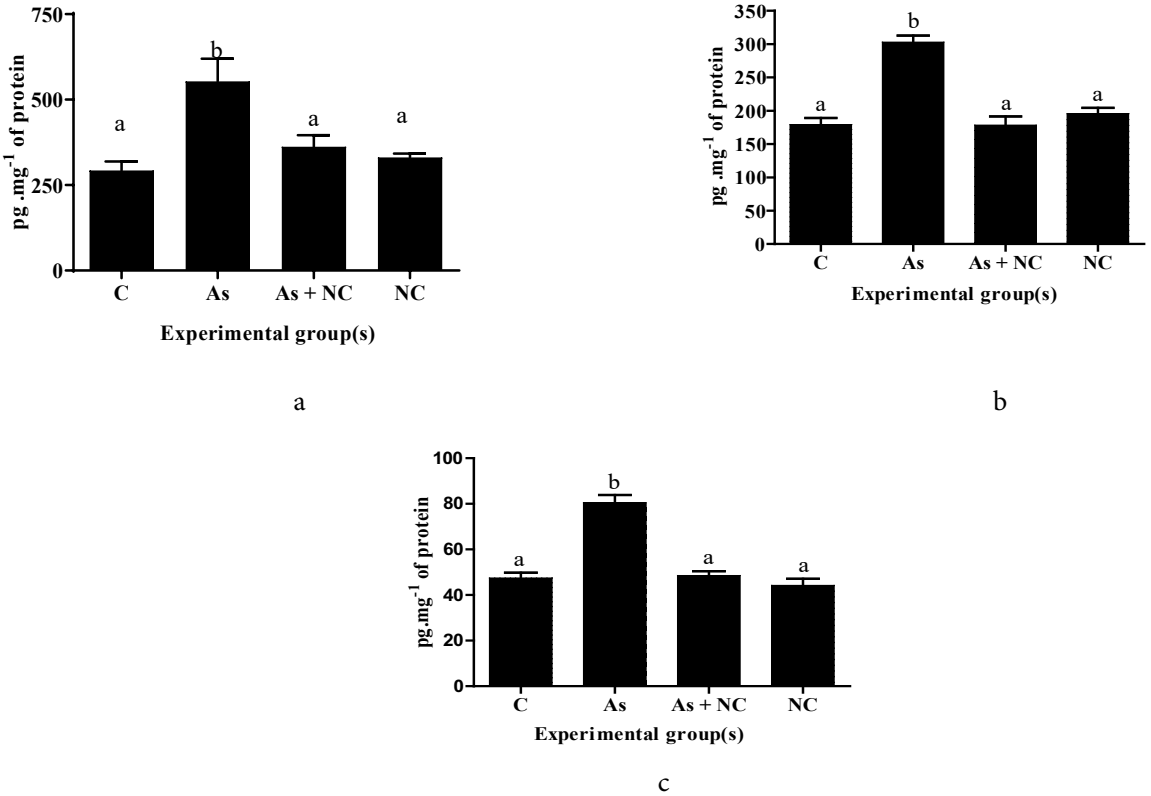


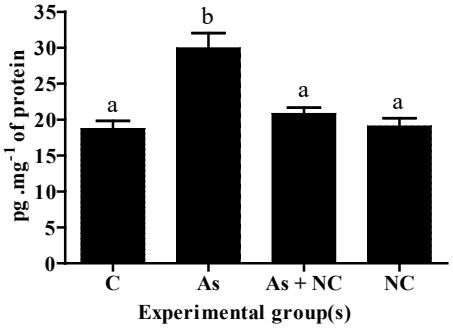
Figure 6. Inflammatory cytokine levels in brain tissue (n=6 each). **a.** IL-1β; **b.** IL-6; **c**. TNF-α

Note: Data are expressed as Mean(±SE) pg.mg^{-1}protein; Bars with dissimilar letters vary significantly; p<0.05; [C = Control, As = Arsenic, As+NC = Arsenic + Nanocurcumin, NC = Nanocurcumin].

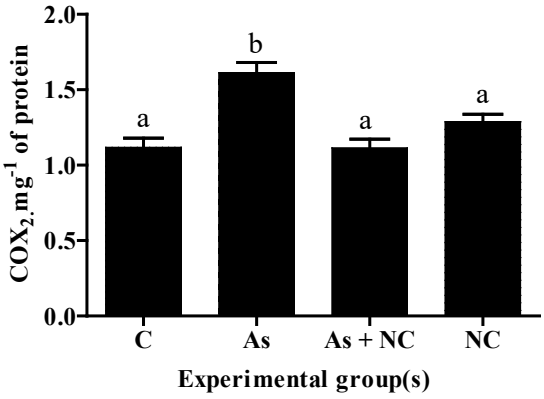
'As' induced surge in TNF-α production (48.58±1.89). However, TNF-α level in control (47.44±2.35) or NC alone treated (44.19±2.92) group of rats were comparable (Figure 6c).

Figure 7a presents the effects on PGE_2 level (pg.mg^{-1} protein). In the control group, its level was 18.74±1.08. 'As'significantly (p<0.05) increased its level to 29.97±2.06. However, in the arsenic-exposed animals, NC significantly reduced the level to 20.84±0.81, which was comparable to control rats belonging to control group.

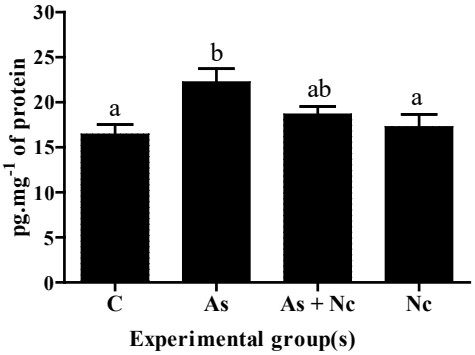
The COX_2 concentration in plasma (ng.ml^{-1} protein) is shown in Figure 7b. Its concentration was significantly (p<0.05) elevated following sub-chronic exposure to 'As' (1.61±0.07) when compared to untreated control group of rats (1.12±0.06). However, NC co-administration significantly (p<0.05) brought its activity down (1.11±0.06) to the level similar to that of untreated control group of rats.



a



b



c

Figure 7. Inflammatory mediators levels in brain tissue (n=6 each). **a.** Prostaglandin-E_2 (PGE_2) levels (pg.mg^{-1}protein) in brain tissue; **b.** Cyclooxygenase-2 (COX_2) levels (ng.ml^{-1} protein) in brain tissue **c**. Angiotensin-II levels (pg. mg^{-1} protein) in plasma.

Note: PGE_2 (pg.mg^{-1}protein), COX_2 (pg.mg^{-1} protein) and Angiotensin II (pg.ml^{-1} protein) were measured with ELISA and recorded at absorbance 450nm and data are expressed as Mean(±SE); Bars bearing dissimilar alphabets vary significantly; p<0.05; [C = Control, As = Arsenic, As+NC= Arsenic + Nanocurcumin, NC = Nanocurcumin].

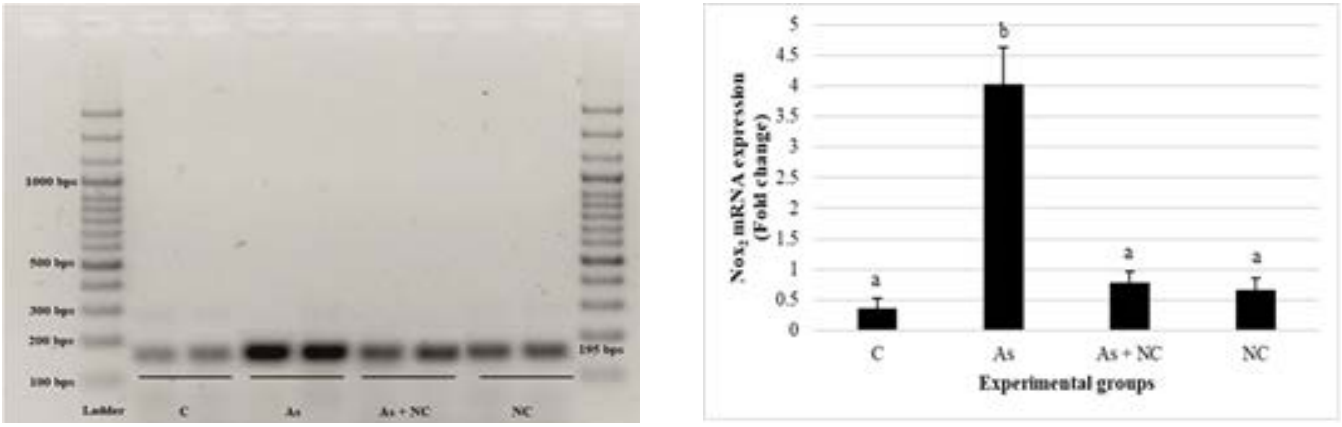
The Ang-II level was significantly (p< 0.05) increased in plasma of 'As' exposed rats (22.25±1.50 pg.ml^{-1}) when compared to control group (16.47±1.05 pg.ml^{-1}). However, NC co-administration to 'As' exposed rats showed a non-significant (p>0.05) marginal decrease in Ang-II levels (18.70±0.85 pg.ml^{-1}) in plasma (Figure 7c).

The qRT-PCR studies revealed that *Nox*$_2$ mRNA expression in the brain tissue was significantly (p<0.05) up-regulated (>3.5-fold) upon 'As' exposure (RQ: 4.03±0.60). Administration of NC in 'As' exposed rats significantly (p<0.05) down-regulated (> 5-fold) *Nox*$_2$ mRNA (RQ: 0.79±0.18) expression (Figure 8a, 8b and Table 2).

Table 2. Relative expression levels of *Nox*$_2$ gene of *m*RNA isolates of rats belonging to various experimental groups

Housekeeping gene-GAPDH					**Gene of interest-** Nox$_2$							
Sample	**CT1**	**CT2**	**CT3**	**Avg CT**	**Sample**	**CT1**	**CT2**	**CT3**	**Avg CT**	**ΔCT**	**ΔΔCT**	**RQ (2^-ΔΔCT)**
C	25.69	24.25	24.75	24.90	C	31.84	26.99	26.59	29.42	4.52	1.61	0.36*± 0.16[a]
As	29.57	29.19	27.53	28.76	As	30.13	30.16	29.21	29.67	0.92	-1.99	4.03 *± 0.60[b]
As + NC	27.55	27.21	27.37	27.38	As + NC	30.92	30.59	30.08	30.67	3.29	0.38	0.79 *± 0.18[a]
NC	25.52	25.14	25.79	25.48	NC	29.23	28.99	28.84	29.13	3.65	0.66	0.66 *± 0.20[a]

Note: Data were analyzed by One-way ANOVA followed by Duncan's multiple comparison *post hoc* test; Values bearing dissimilar letters within the column vary significantly; $p<0.05$; [C = Control; As + NC = Arsenic + Nanocurcumin; NC = Nanocurcumin]; RQ= Relative Quantification; GAPDH= Glyceraldehyde 3-phosphate dehydrogenase; *Nox*$_2$= NADPH Oxidase 2; CT= Threshold cycle; **Δ=** Average



a b

Figure 8. Quantitative RT-PCR: *Nox2m*RNA expression (n=6 each). **a.** Agarose gel image of PCR amplicons of *Nox*$_2$ gene of *m*RNA; **b.** Relative expression of *Nox*$_2$ gene of *m*RNA

Note: Ladder = DNA ladder (1500 bp); C= Control; As = Arsenic; As+NC = Arsenic + Nanocurcumin; NC = Nanocurcumin.

4. Discussion

Curcuminiods are known for their antioxidant and anti-inflammatory activity but the major drawback of curcumin is bioavailability in biological systems[23,24,40]. Therefore, in order to increase the bioavailability of curcumin, we employed anti solvent precipitation method to produce NC with increased solubility. The hydrodynamic radius and particle size of the NC synthesized in the present study was ~76 nm and 95 nm, respectively, which are in good agreement with Basniwal *et al.*[41] and Abirami *et al.*[42].

Sub-chronic exposure to 'As' in experimental rats resulted in significant ($p<0.05$) decrease in b.wt. in terms of body weight gain when compared to control group of rats. The decreased body weight indicates the general toxic effect of the arsenic and also associated with decreased food consumption and water intake[43]. Further, co-treatment with NC in the present study markedly prevented 'As' induced changes in body weight in experimental rats.

Experimental rats exposed (sub-chronic) to 'As' showed a significant ($p<0.05$) decrease in both voluntary- and involuntary motor activity on both time points tested

i.e. 35 and 70 days when compared to control. Dopamine plays an important role in central motor activity due to the direct actions on several regions of the brain *viz.*, basal ganglia, frontal cortex, corpus striatum and hippocampus[44]. Thus, the disturbances in motor activities observed in the current study could be attributed to the alterations in the synthesis or turnover of dopamine levels in central nervous system. However, dopamine level in the various experimental groups of rats in the current study was not measured. Elevated plus maze - a widely used tool for the investigation of psychological and neurochemical basis for anxiety. Arsenic exposed rats showed significant increase in anxiety levels when rats were subjected to test on day-70. The increase in anxiety may be due to disturbances in the distribution of neurotransmitters like serotonin, glutamate or γ-amino butyric acid (GABA) in adult rats[45]. Anti-depressant activity of curcumin attributed to modulation in the release of serotonin and dopamine in central nervous system[46]. Thus, NC neutralizes 'As' induced perturbations through modulation in release of neurotransmitters in the central nervous system.

At the molecular level 'As' causes cellular toxicity *via* multiple mechanisms including production of ROS and alterations in the activity of key anti-oxidant enzymes[47]. The brain possesses relatively poor antioxidant defence system[48], contains large amounts of polyunsaturated fatty acids, and 20% of the body's oxygen consumed by brain[49]. In the current study, sub-chronic exposure to 'As' induced oxidative stress in rat brain as evidenced by perturbations in various antioxidative biomarkers examined. Enhanced tissue LPO, a key characteristic feature of 'As' induced toxicity[50]. In the present study, LPO levels in 'As' exposed rats (brain) was significantly (p<0.05) higher than control. Oral treatment with NC significantly (p<0.05) reduced LPO in brain tissue. Arsenic significantly increased $O_2^{\bullet-}$ radical generation due to its interference with mitochondrial electron transport. Due to high ATP demand, brain consumes more oxygen compared to other tissues. Thus, 'As' mediated interference in mitochondrial function may lead to increase in $O_2^{\bullet-}$ production. These results are in agreement with Santra *et al.*[51] who reported that 'As' increases the rate of formation of ROS including $O_2^{\bullet-}$ radical. NC administration significantly (p<0.05) reduced the ROS production which can be attributed to its antioxidant nature. These results are in line with Yadav *et al.*[52]. Curcumin can counteract free radicals by quenching $O_2^{\bullet-}$ radicals, neutralize oxidative cascade and there by maintain the integrity and function of membrane and inhibit peroxidation of lipids. Additionally, curcumin can interact with StAR protein in relatively at the same magnitude when compared to cholesterol through *vander* Waals interactions. From this it is clear that curcumin is transported into mitochondria and thereby decreases the oxidative stress induced due to 'As' induced deregulation of oxidative phosphorylation and electron transport.

The SOD prevents harmful effects of $O_2^{\bullet-}$ radical by hastening its dismutation into reasonably less toxic hydrogen peroxide (H_2O_2)[53]. NC acts as an effective chelating agent[54], thus such actions may be responsible for antioxidant actions in terms of protection against 'As' induced alterations. The decrease in antioxidant enzyme activities of SOD, CAT, GP_X and GR can be attributed to the increased accumulation of H_2O_2 due to deactivation of CAT and GPx by $O_2^{\bullet-}$. Thus, the removal of H_2O_2 formed during the reaction catalysed by SOD failed due to decreased CAT activity[55], obviously on account of increased $O_2^{\bullet-}$ [56]. GPx, a chief antioxidant enzyme has an important role in the decline in lipid hydroperoxides within the biological membranes and lipoproteins in the presence of GSH[57]. Biochemically, GPx removes the accumulated H_2O_2 through GSH dependent mechanism, decrease in the activity of GPx can be attributed to 'As' induced LPO which in turn decreases the levels of GSH[58]. The increase in LPO can be attributed to decreased activity of GPx following 'As' treatment indicates depletion in GSH content which may be due to the simultaneous decrease in the activity of the GSH regenerating enzyme GR[59]. Antioxidant activity of NC and their ability to enter into mitochondria may decreased ROS generated due to 'As' assault, thus reducing the ROS and also in maintaining adequate levels of GSH[60].

The elevation in IL-1β, IL-6 and TNF-α is due to activation of signalling pathways and expression pro-inflammatory genes like extracellular signal-regulated *kinase* (ERK*)*, c-*Jun* N-terminal *kinase* (JNK), *p38* mitogen-activated protein *kinase* (*p*38 MAPK) or nuclear factor-*kappa* β (NF-*κβ*). It has been reported that curcumin can prevent inflammatory response due to its ability to activate *Nrf2* signalling[61]. Exposure to 'As' causes a significant increase in COX-2 expression in an *IKKβ/NFκB*–dependent manner due to oxidative stress[62]. The elevation in PGE_2 could be due to increased production of ROS as a result of up-regulation of genes associated with inflammatory mediators[63,64] and activation of *NF*-κB pathway, which direct the expression of various

pro-inflammatory mediators[65]. NC co-administration with 'As' showed significant decrease in PGE_2, COX-2, IL-1β, IL-6, TNF-α and *Ang*-II levels due to its antioxidant activity. Scavenging of ROS generated due to irregularities in mitochondria by NC, results in deactivation of inflammatory pathways. Significant increase in Nox_2 *m*RNA expression in 'As' exposed rats can be attributed to increase in serum inflammatory cytokines and also significant increase in $O_2^{\bullet}$ radicals. Down-regulation of Nox_2 gene in NC co-administered group can be attributed to antioxidant and anti-inflammatory ability of curcumin by inhibiting protein kinase C, an activator of NADPH oxidase[66]. From the results of this study, it is evident that NC synthesized in the present study effectively scavenges free radicals and also protect mitochondria from 'As' assault, thereby ameliorates 'As' induced perturbations in the brain.

5. Conclusion

To conclude, the experimental study revealed that sub-chronic exposure to sodium (meta) arsenite induced LPO, thus leading to redox signaling and pro-inflammatory cascade and disturbed neuropharmacological activities in Wistar rats. However, concomitant administration of NC ameliorated the 'As' induced perturbations in redox homeostasis, significantly restricted the pro-inflammatory cascade, down regulated Nox_2 *m*RNA expression and restored the neuronal activities in Wistar rats. Thus, the results of the present study explicitly confirm the neutraceutical ability of synthesized NC to overcome 'As' induced perturbations in the central nervous system.

6. Acknowledgement

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7. Competing Interests

The authors declare that they have no conflicts of interest.

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