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SAFFRON EXTRACT'S PROTECTIVE EFFECTS AGAINST ARSENIC INDUCED EXCITOTOXICITY AND LEARNING DISABILITIES IN MALE WISTAR RATS

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Abstract: The present study reports the protective efficiency of saffron extract against arsenic induced learning impairments and excitotoxicity. The efficacy of saffron was studied using open field, maze learning and estimating the levels of glutamate and aspartate. The experimental rats received 100 mg/kg bw arsenic through drinking water and the protectant saffron extract at dosage 100 mg/kg bw was given through gavage needle. The group receiving arsenic showed severe weight loss, learning dysfunction and increased levels of glutamate and aspartate in hippocampus and cerebral cortex. Whereas the saffron receiving rats have shown reduced toxic effects of arsenic, which was observed as reversal of the learning ability, levels of glutamate and aspartate in both hippocampus and cortex region of brain as an indication of recovery. Therefore it is evident that saffron improvises the behavioural impairment, glutamate and aspartate profile indicating its potential to be a good protective agent against arsenic induced excitotoxicity in rats.

Key words: Arsenic, Saffron, Excitotoxicity, Learning and Cognition dysfunction

INTRODUCTION

Arsenic (Ar) is one of the most abundant pollutants in the earth's crust. Humans and animals are exposed to Arsenic through variety of sources, including diet, homoepathy, ayurveda drugs, sea food, drinking water, pesticides, herbicides, insecticides (Monosodium methyl arsenate (MSMA), Disodium methyl arsenate (DSMA) etc. Exposure to insecticidal sprays used for agricultural practice is sometimes resulted in brain damage among those working with the sprayers (Hughes *et al.,* 2011: Alam *et al.,* 2002). Epidemiological studies have suggested a correlation between chronic consumption of drinking water contaminated with arsenic and the incidence of all leading causes of mortality (Ratnaike 2003). On chronic induction arsenic accumulates in soft tissues such as liver, kidneys, heart, lungs, brain and residual arsenic is also found in keratin-rich tissues like nails, hair, and skin (Ratnaike 2003). Arsenic effects surfaces weeks after initial exposure as both central and peripheral neuropathy. The earlier studies on chronic exposure of Arsenic were focused on the central neuropathy and ailments in neurological functions due to Arsenic intoxication such as learning inability, short-term memory, memory and concentration were severely impaired on chronic exposure of arsenic and also showed signs of delirium and encephalopathy (Wilson *et al.,* 1987; Hall *et al.,* 2002). Inorganic arsenic at high and low doses is a known cause, neuropsychological dysfunction, neuro-developmental and neurocognitive impacts in foetal brain (Chattopadhyay *et al.,* 2002). From a neuropathological aspect, arsenic exposure has been associated with formation β-amyloids, hyperphosphorylation of tau protein, oxidative stress, neuroinflammation, endothelial cell dysfunction and angiogenesis, all of which have been linked to proposed mechanisms underlying Alzheimer's disease (O'Bryant *et al.,* 2011).

Arsenic mode of action is through the formation of ROS and RNS, In animal models, arsenic exposure has been shown to cause morphological and neurochemical alterations in the hippocampus and other memory-related neuronal structures and expected learning and memory deficits have been noted. The excessive accumulation of glutamate in hippocampus of rat in Arsenic exposure resulted in excitotoxicity (Chavez *et al.,* 2015; Huo *et al.,* 2014; Mehta *et al.,* 2012). However, the direct link between chronic low-level arsenic exposure and detailed neuropsychological status is still unclear.

Crocus sativus, commonly known as the saffron crocus, is the source of saffron, a known condiment used in food and Ayurvedic, Unani and folk medicine to treat various ailments (Darshan and Doreswamy, 2004). Saffron extract consists of more than 150 active volatile, non-volatile, aroma-yielding compounds, such as zeaxanthin, lycopene, α- and β-carotenes (Razavi and Hosseinzadeh 2015). Saffron has a long medicinal history as part of traditional healing; several modern pharmacological research studies have hinted about the broad spectrum activities of saffron i.e. anticonvulsant (Hosseinzadeh and Talebzadeh 2005), antidepressants and anxiolytics (Ghasemi *et al.,* 2014; Hassani *et al.,* 2014), antinociceptive and anti-inflammatory (Hosseinzadeh and Younesi 2002),

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memory enhancers (Ghadrdoost *et al.,* 2011), anti-carcinogenic (cancer-suppressing), anti-mutagenic (mutation-preventing), immune-modulating, anti-nephropathy, anti-arrhythymic, genotoxicity, anti-nephrolithiasis and antioxidant-like properties (Premkumar *et al.,* 2003; Razavi and Hossein 2015; Bahareh *et al.,* 2015). Crocins are basically hydrophilic carotenoids (monoglycosyl or diglycosylpolyene) also known as esters of crocetin (Razavi and Hosseinzadeh 2015). This crocin is an trans-crocetin di-(β-D-gentiobiosyl) ester responsible for saffron's colour, aroma and bitter taste.

Other protective agents employed in ameliorative studies against arsenic induced toxicity are Resveratrol (Zhang *et al.,* 2014), Curcumin (Yadav *et al.,* 2010), Neurotrophin 4/5 (Lou *et al.,* 1999), Arjunolic acid (Sinha and Manna 2008), Vitamin E, Methylamine and Benzyl alcohol (Lee and Ho, 1994). There are only few reports available on natural products used as neuroprotective agents against arsenic induced neurodegeneration and there is no evidence of reversal of neurodegeneration effects etc.

In this study we proposed the protective role of saffron extract in arsenic induced neurotoxicity. The main objective is to investigate the Arsenic effect on rat brain and mitigation by saffron extract. The study has provided evidence on reversal of arsenic induced Behavioral dysfunction, excitotoxicity and neurotoxic effects on administration of saffron extract.

MATERIALS AND METHODS

Weanling wistar Albino male rats aged between 60-90days old and weighing 125±10gms were selected for this study. The animals obtained from National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition (NIN), Jamia Osmania, Hyderabad-500007, India. The protocols of experiment were approved by the departmental ethical committee, CPCSEA No: 383/01/a/CPCSE. The rats were divided into three groups (n=6/group/day or 30 rats per group) i.e. Control (Group-I), Arsenic (Group-II) and Arsenic + Saffron (Group-III) and Group-II animals received 100 mg/kg.bw arsenic through drinking water (sodium arsenate dissolved in distilled water) and Group-III received 100mg/kg.bw of Arsenic ad libitum and 1ml of 100mg/kg.bw of saffron through gavage (commercially available, Sigma chemicals). The animals were maintained in standard laboratory conditions. Polypropylene cages were used to house 2 rats per cage with 12/12hr: day/night cycle and 20-22°C room temperature was maintained. The animals received water, food (Standard pellet diet, NIN, Hyderabad) provided *ad libitum*.

All the animals were sacrificed by cervical dislocation at regular intervals (i.e. 1, 7, 14, 21, 30 days). The experimentation duration was one month, on the day of sacrifice 6 rats per group were sacrificed, and brain tissues were dissected out and were used for biochemical and histology parameters. Behavior studies were performed at 21st and 30th day of experimentation.

The Body weight, Brain weight and the total proteins in whole brain were analyzed. Behavior studies such as maze learning, open field test were performed. Glutamate and Aspartate levels were analyzed on spectrophotometer on 1st, 7th, 14th, 21stand 30th day in both control and experimental group.

Preparation of saffron extract

Dried stigmas of *Crocus sativus* were procured from regional markets of Telangana. 25 g of stigmas were boiled in 400ml of distilled water, and were further heated at 60–70°C to a concentrated solution (~50ml). The extract was lyophilized into powder. The lyophilized powder was administered to rats (100mg/kg/bw) through gavage

Behavioral Tests

All Behavior tests performed after acclimatization and adaptation (2-3 days) of animal prior to experiment.

Open field test: The open field test was designed to measure behavioral responses such as locomotory activity, hyperactivity and exploratory Behaviors (emotional reactivity/anxiety and locomotory activity). Open field was performed at 21st, 30th day in both control and experimental groups (Netto *et al.,* 1986).

Maze learning test: Maze learning is the process of learning a route through a maze in order to obtain reinforcement. This process is a popular experiment in behavioral laboratory and it is the main method of studying spatial learning. Maze learning performed at an age of 21 and 30 day old. Rats were allowed to learn the board and reach the goal (food), the time the each rat is taking to reach the goal is noted and time is expressed in minutes (Hull, 1932; Morris, 1984; Stewart, 1975), and for the experiment the same animals were used which were given training.

Protein Estimation

Protein estimation was done by using modified method of Lowry *et al.,* 1951.

Estimation of neurotransmitters

Glutamate: Estimation of glutamate was done by using modified methods of Waelsch, 1957 and

Joseph 1957. The samples were read at 420nm on UV-Visible spectrophotometer.

Aspartate: Estimation of glutamate was done by using modified methods of Joseph, 1957 and Samuel, 1957. The optical densities of samples were read at 570 nm on UV-Visible spectrophotometer.

RESULTS

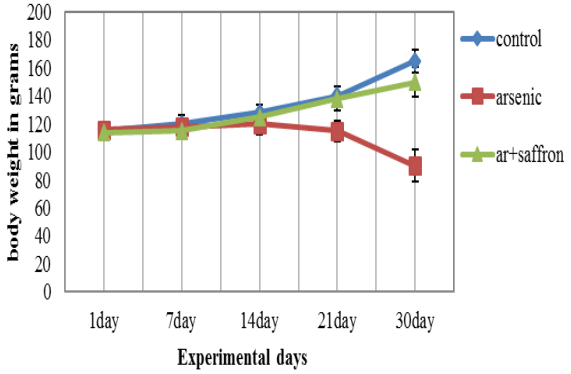

Figure 1: Effect of saffron treatment of body weights of rats exposed to arsenic

The average body weights (n=6) were measured before scarifying the rats on regular intervals during the experimentation. Based on the observations, it is evident that the control rats showed normal body weights and the rats exposed with 100mg/kg.bw arsenic showed loss in body weight gradually. Whereas the rats receiving 100mg/kg.bw Saffron in combination with 100mg/kg.bw Arsenic showed gradual increase in body weight but the weights were less than the controls and more in comparison to the rats which received only arsenic during the whole experiment. It is apparent from the observation that Saffron extract prevents the loss of body weight ($p>0.05$).

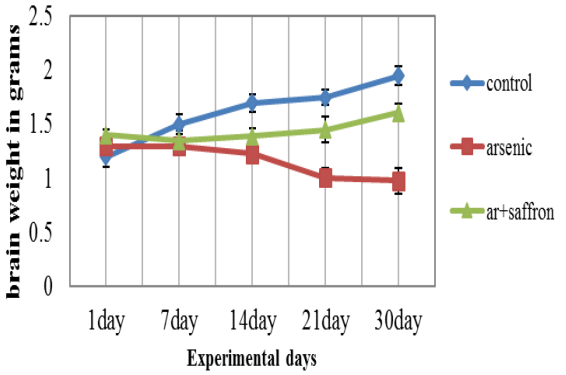

Figure 2: Effect of saffron on arsenic toxicity and brain weights

The average brain weights were measured from the scarified rats (n=6) on every scarifying day. The whole brain weights, in case of control rats showed normal and gradual increase and the rats exposed with 100 mg/kg bw arsenic showed less brain weights in comparison to the control group and the group receiving the saffron. The rats receiving Saffron extract in combination with 100mg/ kg bw Arsenic showed more brain weights than the rats which received only arsenic but weights were less when compared to the control rats. It is evident that saffron provides protection and helps the rats to retain their brain size and weights.

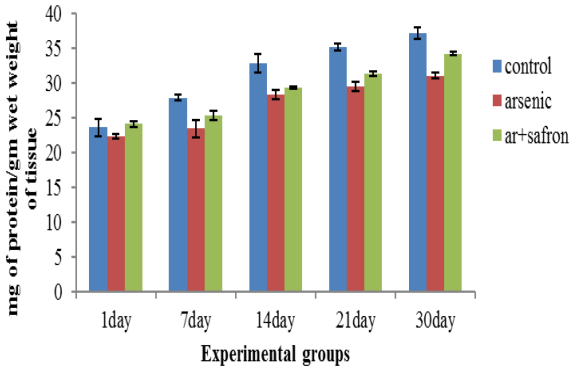

Figure 3: Effect of arsenic and saffron on total proteins in whole brain

The spectrometric estimation of whole brain lysates revealed that the levels of proteins in case of rats receiving the 100mg/kg.bw, Arsenic (Ar) showed decrease in comparison to the control and 100mg/kg.bw Saffron extract receiving rats. The protein levels in rats receiving saffron extract were recovered compared to Arsenic receiving rats.

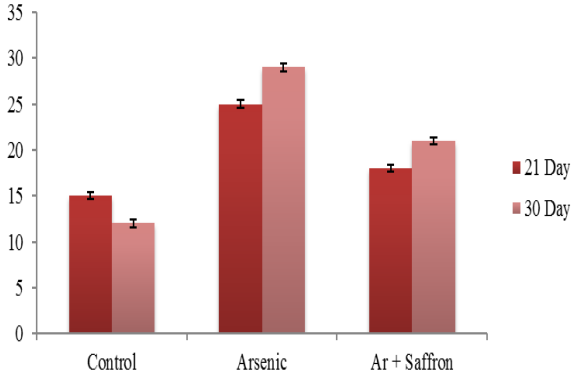

Figure 4: The memory and cognition ability in rats receiving the arsenic and saffron treatment.

The rats exposed to arsenic showed decreased ability to learn and guide themselves through the maze and it took more time in comparison to control and protectant receiving rats. In case of the protectant receiving group showed recovery in learning ability and reached the target in less time comparison to group receiving only arsenic.

Table 1: Effect of Arsenic and Saffron on animals subjected to the Open field test

	Control	100 mg/kg Arsenic	Saffron-Arsenic
Head Elevation (no./30 sec)	14.9 ± 0.8	14.41 ± 1.0 *	14.7 ± 1.1*
Hind limb elevation (no./30 sec)	11.5 ± 0.8	10.0 ± 1.2*	10.8 ± 1.6*
Rearing (no. of rear/60 sec.)	7.42 ± 1.2	6.35 ± 2.1*	7.13 ± 1.6*
Faecal boluses (No.)	46.5 ± 1.1	44.8 ± 1.4*	45.8 ± 1.0*
Urination (no. of pools of Urine)	73.5 ± 0.6	71.8 ± 1.0*	72.5 ± 0.9*
Grooming	1.7 ± 0.5	1.5 ± 0.3*	1.6 ± 0.2*
Sniffing	1.8 ± 0.5	1.6 ± 0.7*	1.7 ± 1.2*
Biting and licking	0.4 ± 0.2	0.2 ± 0.5*	0.3 ± 0.4*

From the above table1, the observations made from the open field test, showed alterations in locomotion Behavior such as head elevation, hind limb elevation, sniffing, grooming, auditory startle, pivoting scores on arsenic exposure and on concomitant treatment with saffron. Rearing data stated that arsenic does cause depressed Behavior pattern in weanling rats in comparison to that of controls. The protectant group showed intermittent scores in between controls and treated groups

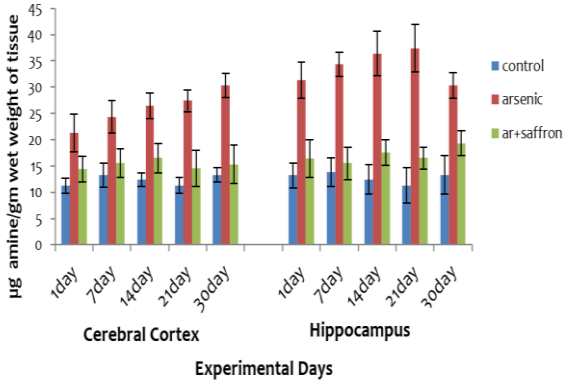

Figure 5: Effects of Arsenic and saffron extract on levels of aspartate in cortex and hippocampus region

From the above graphical representation, the levels of aspartate increased in rats when exposed to arsenic and the levels of aspartate were reduced significantly ($p<0.05$) on saffron extract administration. The levels of aspartate in saffron administered were near to that of controls. The saffron extract works as good anti-excitatory agents as it was able to reduce the levels of aspartate.

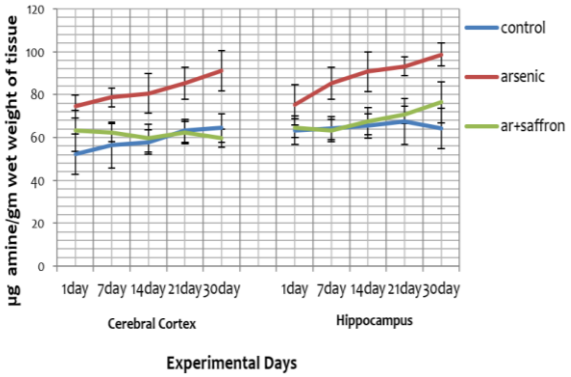

Figure 6: Effects of Arsenic (Ar) and saffron extract on levels of glutamate in cortex and hippocampus region

From the above graphical representation, the level of glutamate increased in rats on exposure of arsenic and the levels of glutamate decreased significantly ($p<0.05$) on administration of saffron extract. The levels of glutamate in protectant group were near to that of control group. It is evident that saffron extract works as good anti-excitatory agent in arsenic intoxicated rats.

DISCUSSION

Arsenic compounds react with cellular coenzymes and other metabolites with less effort and increases the cellular oxidative stress causing depletion of antioxidants in cell environment. Prolonged excitotoxicity on arsenic exposure could affect the brain tissue and cause alterations in modulator neurotransmitters, monoamino oxidases levels leading to Behavior defects. Excitotoxicity is a process where the excitatory amino acid receptors are activated for long period and results in toxic effects in the cell or organ. The excitotoxic pathway is triggered in presence of excess neuronal glutamate in the central nervous system. Glutamate is the most prominent neurotransmitter available (<50%) in the brain and it plays a pivotal role in long-term potentiation, which is important for cognition and memory (Chen *et al.*, 2009). The glutamate concentration in extracellular is lower than intracellular and it's mostly inactive. The glutamate released from post-synaptic neuron is collected by astroglia and stored in glia in glutamine form (i.e. glutamine synthetase pathway). The lower concentration of glutamate extracellular helps maintain the normal function brain (Dan, 2012; Yu *et al.*, 2008).

The excitatory neurotransmitters such as glutamate and aspartate functions by communicating with a series of specific receptors, either ionotropic (NMDA, AMPA) or Metabotropic receptors (G-protein-operated). NMDA glutamate receptors participate in learning and memory and overstimulation of one of these receptors could activate a chain of intracellular signaling molecules which might lead to cell death (Chavez, 2015). As a consequence of excitotoxicity, the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxidation (LPO) and depletion of antioxidants in brain and other soft tissues on exposure to neurotoxins, both *in vitro* and *in vivo* (Chattopadhyay *et al.*, 2002). The excitotoxic cascades triggers the elevation of ROS and RNS (Nitrates or Nitrites or iNOS) which could play a role in microglia activation and release of excess glutamates and aspartates resulting in cell death. Generation of free radicals is initiated by the excitatory amino acids (glutamate, aspartate, cysteine, cysteic acid, homocysteine etc.) and intensifies the excitotoxicity

process (Farooqui and Farooqui, 2009; Xiao-xia *et al.,* 2009).

The present study has provided evidence on chronic exposure of Arsenic leading to morphological, biochemical and Behavioral alterations. From the figure 1&2 it is evident that the rats exposed to arsenic have gained less body and brain weights compared to that of controls and the decrease of body and brain weights was controlled with administration of saffron extract ($p>0.05$). Figure 3, indicates the gradual depletion of total proteins as a result of chronic arsenic exposure and the depletion of proteins was significantly ($p>0.05$) controlled on administration of saffron extract and the levels were near to that of controls. The rats receiving the arsenic exhibited cognition and memory dysfunction disabilities and the concomitant administration of Arsenic-Saffron extract showed improved cognition and memory. The time to finish the task by the rats receiving saffron extract was intermittent between the controls and arsenic treated groups (Control>Arsenic-Saffron>Arsenic) (figure 4). The scores from the maze learning and task finishing activities were significant ($p>0.05$).

Table-1 represents the activities screening which detected significant alterations in Behavioral pattern in arsenic receiving rats on open field test, such as head elevation, hind limb elevation, sniffing, grooming, urination, fecal boluses, biting and licking. The activities improved in rats receiving the protective simultaneously with Arsenic, indicating that the saffron extract is efficiently mitigating the arsenic induced Behavioral changes in rats and the scores were near to that of controls. Rearing data further stated that arsenic does depress the exploratory Behaviors in weanling rats when related to the control rats and saffron extract administered rats showing palliative or alleviating Behavioral pattern proving the efficiency of saffron against arsenic toxic effects. The levels of glutamate and aspartate showed significant fluctuations on chronic exposure of Arsenic. From the figure 5 we could deduce the effect of arsenic on aspartate profile in presence and absence of protective agent and it is evident that the saffron was expeditiously controlling the aspartate levels from increasing in cortex and hippocampus regions of rat brain and on comparison with controls and arsenic treated group the levels of aspartate were intermittent and significant ($p>0.05$). The over-excitation of neurons in presence of excess aspartate is reduced by saffron and in turn helps the brain to function normally. Figure 6, represents the profile of glutamate in cortex and hippocampus regions of rat brain when treated with Arsenic, Arsenic-saffron extract. The controls showed normal levels of glutamate, whereas arsenic receiving rats showed significant rise in glutamate levels in both cortex and hippocampus of the brain resulting in cognition and memory deficits and loss of neurons due to over-excitation in presence of glutamate in excess. The glutamate status in Arsenic-saffron receiving rats was significantly ($p>0.05$) suppressed from elevating and the levels were very much near to that of controls. The saffron extract expeditiously mitigating the arsenic induced excitotoxicity in the brain and helping the animal to maintain its normal learning and memory functions etc.

From the review of literature, it is understood that the saffron extract and its compounds have been used as a protective agent in various toxicity studies and the results were promising. The results from our experimental studies showed that saffron extract works as good anti-excitatory and ameliorative agent in maintaining the animal body and brain weights intact and also controls the Behavioral fluctuations when exposed to arsenic.

Conclusion

Saffron extracts work as a good protective agent as it able to maintain the body, brain weights and learning Behavior. In presence of the protectant the profile of glutamate and aspartate is maintained which is near to the profile as of control. Observations made during the experiment reveals that saffron exerts protective role against arsenic induced excitotoxicity in weanling male rats.

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