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In vitro antifilarial activity of *Azadirachta indica* aqueous extract through reactive oxygen species enhancement

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ABSTRACT

Objective: To evaluate an aqueous preparation from the *Azadirachta indica* leaves (AEA) against *Setaria cervi* (*S. cervi*), a model filarial parasite. **Methods:** *In vitro* efficacy of AEA was evaluated against *S. cervi* through estimation of relative motility value, dye exclusion test and MTT assay. Visible morphological alterations were monitored using conventional microscopic techniques in microfilariae and haematoxylin–eosin stained sections of AEA-treated adults. **Results:** Enhancement of reactive oxygen species in *S. cervi* treated with AEA was established through alteration in the activity of glutathione S-transferase, superoxide dismutase, catalase, peroxidase and level of superoxide anion and reduced glutathione. **Conclusions:** *In vitro* filariocidal activity of AEA is possibly through disturbing redox homeostasis by down-regulating and altering the level of some key antioxidants and regulatory enzymes like reduced glutathione, glutathione S-transferase, superoxide dismutase, catalase and glutathione peroxidase of *S. cervi*.

1. Introduction

Lymphatic filariasis, one of the major common causes of global disability, affects an estimated 120 million people whereas 1.34 billion peoples are at risk in 81 countries according to WHO[1]. *Azadirachta indica* (*A. indica*, Meliaceae) is commonly an evergreen tree, widely available throughout the Indian subcontinent especially in India and Burma. *A. indica* is a widely diversified medicinal plant that have been used from immemorial time in the Indian subcontinent and still regarded as the village dispensary. Almost all the parts of this plant are important to cure several diseases and these properties have been well

documented[2]. Some medicinal properties of different parts used in Ayurveda had been summarized in a review by Biswas *et al*[3]. The anthelmintic properties were evident from the preparations of leaves along with flowers, fruits, twigs, seed pulp oil and so on[3].

Setaria cervi (*S. cervi*), a bovine filarial nematode parasite (family Onchocercidae and subfamily Diofilarinae), can be found in the abdominal cavity of the cattle. *S. cervi* has been used routinely as a test organism for *in vitro* screening of potential antifilarial compounds[4,5].

Evidence of plant extract induced increased generation of reactive oxygen species (ROS) was adequate in literature and also *A. indica* induced ROS enhancement leading to death was worked out in case of rat oocytes[6]. Several complex mechanisms are synergistically responsible for maintaining the delicate balance of ROS generation and elimination and any deregulation can lead to alterations of redox homeostasis either by an increase in ROS concentrations or by a decrease in the activity of one or more antioxidant systems. However, under oxidative stress, excessive ROS and RNS (reactive nitrogen species) constantly attack

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lipids, proteins, and DNA, leading to severe and irreversible oxidative damage. These oxidative modifications may lead to changes in protein function, chemical fragmentation, or increased susceptibility to proteolytic attack[7]. According to Halliwell and Gutteridge[8], relatively low concentration antioxidants are able to significantly delay or inhibit the oxidation of these substrates. This definition includes the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase as well as non-enzymatic compounds such as vitamin E, β -carotene, vitamin C and glutathione. It is well known that the roles of ROS and their regulatory antioxidant systems regarding cell survival are bifurcated. Generally, ROS at low levels act as signaling molecules where they can promote cell proliferation and cell survival but a severe increase in ROS can induce cell death[9].

There is always a scope to find a better antifilarial agent through screening of plant extracts. This has tempted us to evaluate effectiveness of *A. indica* leaves on adults and microfilariae of *S. cervi* by relative motility reduction, percentage mortality evaluation by trypan blue dye exclusion test and percentage death by MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] reduction assay. Enhancement of ROS parameters was also evaluated through detection of changes in reduced glutathione (GSH) and superoxide anion levels and by estimation of changes in the enzymatic activity of glutathione S-transferase (GST), SOD, catalase and GPx.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol and other solvents of highest purity grade were purchased from Merck, India. Double distilled autoclaved water was used for the assays. FBS (Foetal bovine serum), HEPES buffer, streptomycin, penicillin, and amphotericin-B were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RPMI-1640, MTT, NBT (nitroblue tetrazolium) and reagents required for GST and GSH assays were purchased from Hi-Media Laboratories, Mumbai, India. SOD assay kit was procured from Cayman Chemical, Ann Arbor, USA.

2.2. Preparation of aqueous extract of *A. indica* leaves (AEA)

Fresh matured leaves of *A. indica* were collected locally during May–June. A voucher specimen has been deposited at the herbarium (Zoo/VB/SPS-52) at the Department of

Botany, Visva-Bharati University, Santiniketan, West Bengal, India for future studies. Accurately weighed 100 g of leaves were crushed using a mortar–pestle with 100 mL of autoclaved double distilled H₂O. The thick green paste was further left for 2 h for complete extraction. The thick fine slurry thus obtained was further centrifuged at 3 000 r/min for 5 min and at 8 000 r/min for 15 min, respectively at 4 °C. The light green coloured aqueous extract was finally stored at 4 °C for further use.

2.3. Collection and treatment of parasites

Adult male and female *S. cervi* were collected locally from the freshly slaughtered cattle (*Bovis indica* Linn.) and were taken to the laboratory in a vacuum flask containing Kreb's Ringer bicarbonate buffer (Sigma) at 37 °C. In the laboratory, the worms were repeatedly washed to make them free of any extraneous materials. Microfilariae were dissected out from the gravid females and kept in Ringer's solution at 37 °C until use.

Adults worms (one male and one female or two females) were incubated in 10 mL of culture media, RPMI-1640 supplemented with 25 mmol/L HEPES buffer, 2 mmol/L glutamine, 100 U/mL streptomycin, 100 μ g/mL penicillin, 0.25 μ g/mL of amphotericin B, and 10% (v/v) FBS alone and in combination with AEA at different volumes, *ie.*, 5, 10, 25 and 50 μ L/mL in Petri plates (60 mm diameter, Tarson, India). Microfilariae ($n=1.0\times 10^4$) were incubated in 2 000 μ L of culture media alone and in culture media with AEA at the same volumetric concentrations, in a 24-well flat-bottomed culture plate (Tarson, India). Both the cultures were maintained for 24 h at 37 °C in a humidified atmosphere of 5% (v/v) CO₂[10]. The cultures were carried out in duplicate for adults and in triplicate for microfilariae and repeated at least 4 times.

2.4. Relative motility (RM) value assessment

RM of both adults and microfilariae of *S. cervi* was assessed according to the method of Zaridah *et al*[11] with modifications. Both adults and microfilariae of filarial nematodes was categorized into several phases of motility and activeness, *ie.*, 0 (dead); 1 (slightly active); 2 (slightly active and motile); 3 (moderately active and motile); and 4 (highly active and motile). Effectiveness of AEA against the filarial worm was primarily studied in accordance with the alteration of RM value in respect to untreated control. The mathematical formulae used to calculate the RM value is

noted as:

$$\text{Motility index (MI)} = \frac{\sum_{i=0}^4 (iN_i)}{\sum N} \quad (1)$$

Where, i =Index of motility score (0, 1, 2, 3 or 4); N_i =Number of parasites with the score i ; $\sum N$ =Total parasite number for a particular observation.

$$RM = MI_{\text{sample}}/MI_{\text{control}} \times 100. \quad (2)$$

Where, RM value of 100=Filaricidal activity is least, and RM value of 0=The strongest filaricidal activity.

2.5. Trypan blue dye exclusion test

Viability of *S. cervi* microfilariae was checked primarily by trypan blue, as it can stain dead microfilariae blue whereas live ones remain translucent because they can exclude the dye. The microfilariae viability (%) was determined by dividing the number of live ones by the total number of microfilariae.

2.6. MTT assay

Comparative assay of viability of adults and microfilariae were assessed by the MTT reduction assay as described by Comley *et al*[12], with slight modifications as described previously[13]. Twelve adult worms and microfilariae ($n=1.3 \times 10^3$) were used for each treatment group. The cultures were carried out in duplicate for adult worms and in quadruplicate for microfilariae and repeated at least 3 times.

2.7. Histology and tissue staining

After 24 h, control and AEA-treated adult worms were fixed in 40 g/L paraformaldehyde at 4 °C overnight, embedded in paraffin, and cut into 3– μ m thick sections. The treated and control sections were stained with conventional haematoxylin–eosin (HE) staining to observe morphological alterations, if any.

2.8. Measurement of superoxide anion

We measured superoxide anion in control and treated worms following the method as described elsewhere[5].

2.9. Parasite homogenate preparation

Control and treated adult worms were washed repeatedly with phosphate buffered saline (PBS, pH 7.2) and eventually parasite homogenate was obtained using the method as described previously[5]. Protein content was determined by the method of Bradford *et al*[14]. The clear supernatant thus

obtained was used for enzyme assays.

2.10. Enzyme assays

SOD in the worm homogenate of control and treated parasites were estimated using SOD kit (Cayman Chemical, USA). The detailed method had been mentioned previously[15]. GSH level in control and treated parasites was determined following the method which had been reported elsewhere[15]. We assessed GST activity of fresh worm extract spectrophotometrically according to the method essentially described elsewhere[5].

We measured catalase activity in fresh adult worm extract using the method described by Aebi[16]. Briefly, a parasite homogenate (200 mg/mL) was prepared in 0.067 mol/L phosphate buffer (pH 7.0) and then centrifuged at 10 000 r/min for 20 min at 4 °C. Subsequently, 40 μ L of parasite supernatant was rapidly added and mixed with 3 mL H₂O₂ phosphate buffer (2 mmol/L H₂O₂ in phosphate buffer) in a cuvette. Absorbance was monitored in 240 nm using an UV–VIS spectrophotometer (Shimadzu, UV 1601, Japan). Enzyme activity was expressed as U/mg of protein.

We also estimated GPx activity of fresh worm extract spectrophotometrically following the method of Castro *et al*[17], with brief modifications. In detail, 1 mL OPD (Ortho-phenylenediamine) in phosphate citrate buffer (pH 5.0) was added with 100 μ L worm supernatant, mixed with 0.9 mL H₂O₂ (0.013%, v/v) properly, and incubated at room temperature for 30 min. Absorbance was measured at 492 nm. For comparison, 100 μ L extra OPD solution was used instead of worm supernatant as the blank. The enzymatic activity was expressed in units. One unit produced an increase in absorbance at 492 nm of 1.0 under the assay conditions.

2.11. Statistical analysis

All experiments were repeated at least 3 times and data expressed as the mean \pm SEM. The results were analyzed by Student's t -test and a P value <0.05 was considered as statistically significant.

3. Results

3.1. Reducing effect of AEA on *S. cervi* RM

Reduction in movement of adults and microfilariae of *S. cervi* treated with AEA is presented in Figure 1. Control panel (adults and microfilariae) gave RM value of 100 after 24 h of incubation, showing that worm was not affected. However, AEA produced a significant decreased pattern of

RM value in a dose-dependent manner. In the adult worms, AEA gave RM values of 75.2, 62.5 and 25.0 for the doses 5, 10 and 25 μ L/mL, respectively, after 24 h of incubation. The adult worms were found to have lost all its movement when exposed to AEA at 50 μ L/mL (RM=0) (Figure 1A). In microfilariae, AEA at 5 μ L/mL gave significant ($P<0.05$) RM value of about 73.1 after 24 h of incubation, which is considered as minimum effective concentration in this panel. At higher concentrations, *ie.*, 10, 25 and 50 μ L/mL, AEA reduced RM values to 60.1, 30.6 and 5.63, respectively (Figure 1B).

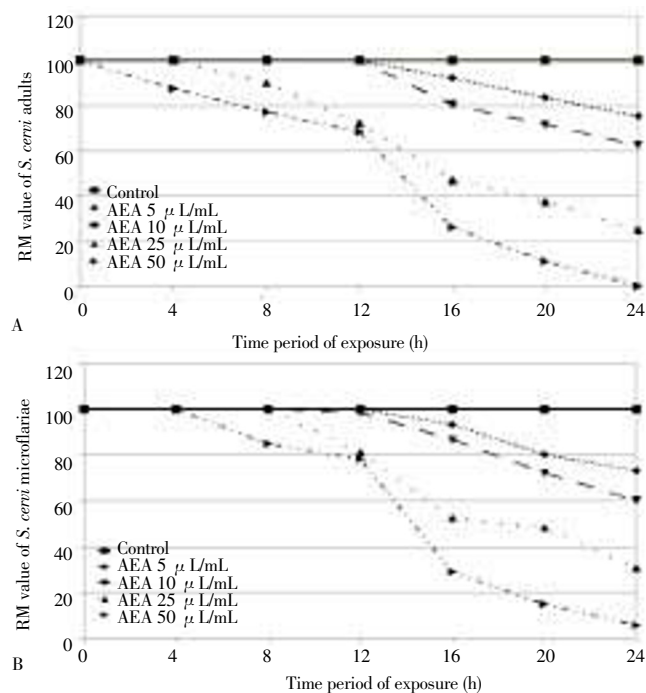


Figure 1. Reduction in RM value of *S. cervi* as a result of exposure to AEA.

(A) AEA caused a dose-dependent decrease in the RM value of *S. cervi* adults. (B) *S. cervi* microfilariae also showed reduction in RM value in a dose-dependent manner. In all cases, concentrations of AEA were 5, 10, 25 and 50 μ L/mL, and RM value was evaluated up to 24 h at regular intervals. Each point is the mean of three replicate assays and repeated 4 times.

3.2. Decreasing effect of AEA on mortality of *S. cervi* at a primary level

Mortality in control and treated microfilariae of *S. cervi* was detected primarily by trypan blue dye exclusion test. Microfilariae (treated) viability was decreased significantly in a dose-dependent manner (Figure 2A) showing viability of just 1.87% after 24 h of incubation at the highest dose of AEA (50 μ L/mL). Data were expressed as viable microfilariae percentage (Figure 2A). At 5 μ L/mL, AEA was found to be effective, showing viability of 67.07%. At higher doses, *ie.*, 10 and 25 μ L/mL, viability was reduced to 48.08% and 18.07%,

respectively (Figure 2A).

3.3. Decreasing effect of AEA on mortality of *S. cervi* at a cellular level

MTT assay is considered as a convenient method for estimating mortality of adults and microfilariae at a cellular level and is used frequently^[5,13,15]. Effects of AEA on viability of both adults and microfilariae had been summarized in Figure 2B. AEA at different concentrations (5, 10, 25 and 50 μ L/mL) showed significant reduction in worm viability in a dose-dependent manner. Viability of adult worms treated with AEA at 5, 10, 25 and 50 μ L/mL was significantly decreased ($P<0.05$) to 94.5%, 65.2%, 46.7% and 30.09%, respectively. AEA at 5, 10, 25 and 50 μ L/mL produced similar effect on microfilariae showing viability of 87.33%, 58.17%, 41.06% and 29.19%, respectively. Viability percentage was calculated in respect to control. LC₅₀ values of AEA for both adults and microfilariae were estimated (following 24 h exposure) using OriginPro6.1 software and were found to be 22.22 and 17.00 μ L/mL, respectively. Thus, AEA produced a strong micro- as well as macrofilaricidal effects on *S. cervi*.

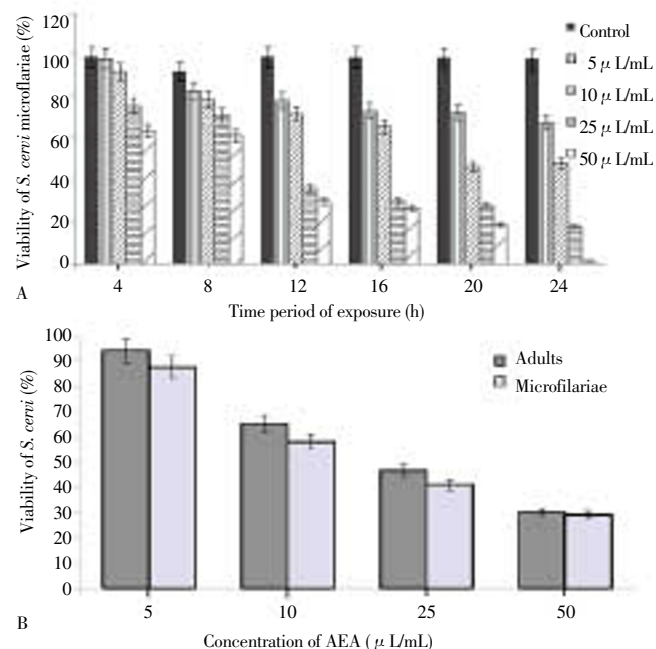


Figure 2. Mortality of *S. cervi* as a result of exposure to AEA.

(A) Trypan blue dye exclusion test. Mortality of *S. cervi* microfilariae was evaluated at 4 h intervals at the doses of 5, 10, 25 and 50 μ L/mL of AEA up to 24 h. $P<0.05$. (B) Effect of AEA on parasite mortality was evaluated by MTT reduction assay. Mortality of both adults and microfilariae of *S. cervi* was expressed at increasing concentrations of AEA as mentioned earlier after 24 h exposure and was evaluated in comparison to untreated control. $P<0.05$.

3.4. Morphological alteration of *S. cervi* after exposure to AEA

Prominent alterations were found in AEA treated adults and microfilariae. In case of treated adult sections, changes in epithelial lining and muscle layers (longitudinal and hypodermal) were visible; in case of sections with higher magnification in epicuticle layer, alterations were demonstrated with presence of syncytial hypodermis in treated sections. Higher haematoxylin staining in treated sections indicated disintegration in chromatin structure (Figure 3).

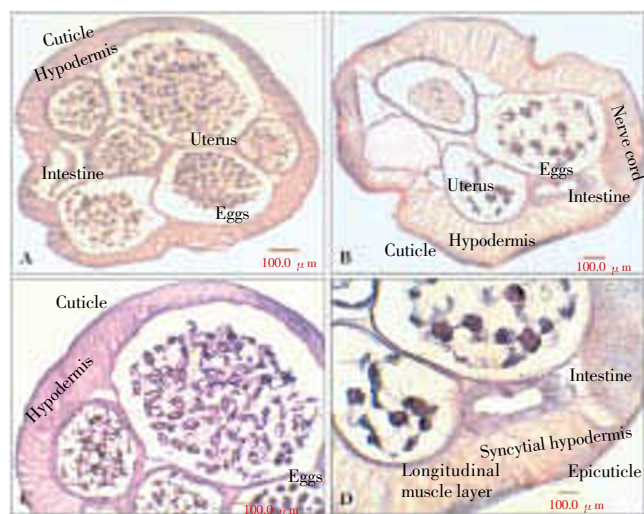


Figure 3. Haematoxylin–eosin stained light photographs of *S. cervi* adult female sections.

Control sections (A and C) showed no significant morphological damage, whereas several morphological alterations were visible in treated (50 μ L/mL aqueous extract of *A. indica* leaves) sections (B and D) particularly at the region of cuticle with disintegrated epicuticle lining. Longitudinal muscle layer, intestine and hypodermis also got morphologically altered with the presence of syncytial hypodermis. Longitudinal muscle layer was also deformed after AEA exposure. Photographs were representative of three separate examinations and scale bar was given using Dewinter Biowizard 4.2 software (Scale bar=100.0 μ m).

In case of microfilariae as compared to control (Figure 4A), presence of dead cells became more in treated microfilariae in a dose–dependent manner when observed under phase contrast or light microscope (Dewinter, Italy). As shown in Figure 4B, AEA at 5 μ L/mL caused death to that proportion of cells that could be hardly visible, whereas at a higher dose (50 μ L/mL), almost all the cells were dead (Figure 4E). For the other two doses, *ie.*, 10 and 25 μ L/mL, death of cells was recorded in between (Figure 4C and 4D).

3.5. Changes in the redox parameters

Alterations in the enzymatic parameters related with the regulation of ROS status were studied to elucidate its role in AEA mediated death of *S. cervi* with reference to untreated ones. For this purpose, changes in the enzymatic regulators were calculated and expressed.

3.5.1. Changes in the superoxide anion level redox parameters

A colorimetric NBT assay was done to measure superoxide anion production in the worms after 24 h exposure to the indicated concentrations of AEA. Results have been shown in Figure 5A. Significant enhancement of (2.29%, 7.84%, 14.18% and 20.26%) superoxide anion production was recorded in the parasites treated with AEA (5, 10, 25 and 50 μ L/mL) over the control worms.

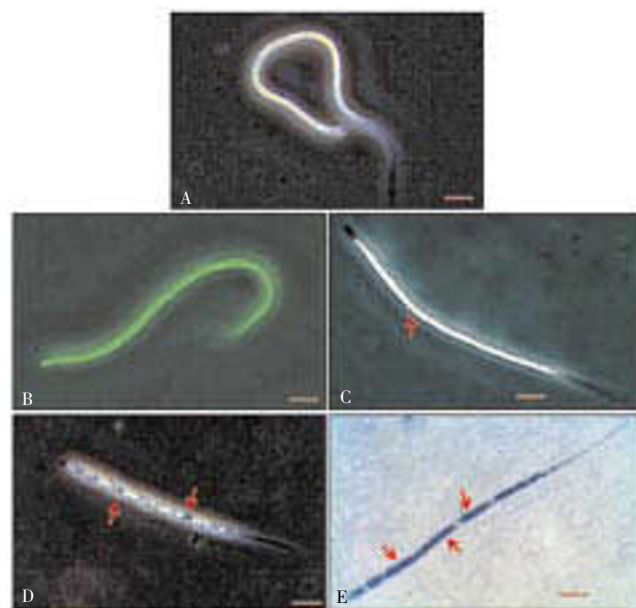


Figure 4. Bright field and phase contrast photographs of *S. cervi* microfilariae.

(A) Control microfilariae of *S. cervi*. (B–E) Microfilariae of *S. cervi* treated with aqueous extract of *A. indica* leaves (5–50 μ L/mL) showed presence of dead blue coloured cell clusters (Arrows) that increased in a dose–dependent manner and was shrunk inside the outer sheath. Photographs were taken after 24 h of exposure and were representative of three independent experiments (Scale bar=100.0 μ m).

3.5.2. Altered SOD activity

As shown in Figure 5B, AEA caused a significant increase of SOD level in a dose–dependent manner causing enhancement of 1.33, 1.41, 1.47 and 1.57 folds for the corresponding doses of 5, 10, 25 and 50 μ L/mL, respectively. The results indicate an alteration in internal

redox in the treated parasites.

3.5.3. Changes in the GSH level

Alteration in the level of GSH indicates misbalance of internal redox as it is the primary internal redox regulator and a known antioxidant. We found a decrease in GSH level in AEA treated adult worms in comparison to that in control when examined after 24 h (Figure 5C). At 5, 10, 25 and 50 μ L/mL, AEA reduced GSH level by 12.81%, 20.79%, 28.80% and 35.70%, respectively.

3.5.4. Altered GST activity

GST, a phase II enzyme of detoxification cascade, catalyzes GSH dependent conjugation during redox regulation by the organism. Our experimental evidence showed that AEA at 5, 10 and 25 μ L/mL caused up regulation in GST activity in the treated worms by 6.19%, 11.49% and 13.89%, respectively. Interestingly, an elevated level of GST (24.49%) was recorded at the highest dose applied (50 μ L/mL) as compared to that in control parasites (Figure 5D).

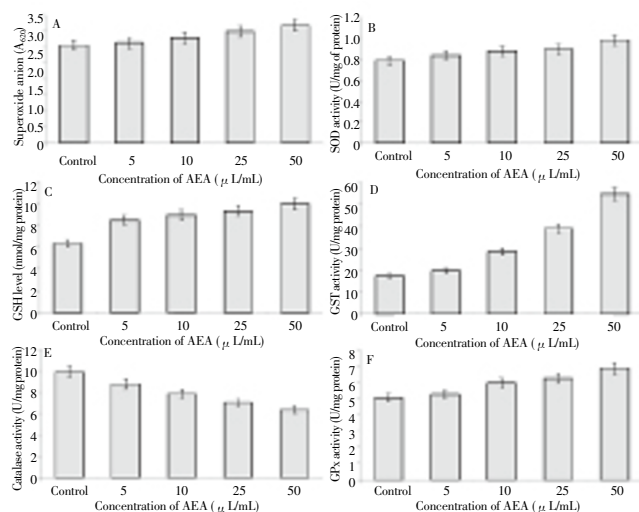


Figure 5. Alteration in the redox regulation parameters in *S. cervi* adults after 24 h exposure to AEA.

(A) Superoxide anion production got enhanced dose-dependently. (B) A significant dose dependent increase of SOD activity was tabulated. (C) We had found a decrease in GSH level in AEA treated adult worms in comparison to that in control. (D) AEA dose-dependently caused up regulation in GST activity in the treated worms. (E) A dose-dependent elevation of catalase activity was recorded in the treated worms. (F) A dose-dependent elevation of GPx activity in the parasites treated with AEA at 5, 10, 25 and 50 μ L/mL was marked.

3.5.5. Alteration in catalase activity

Catalase catalyzes the decomposition of hydrogen peroxide into oxygen and water and protects the organisms from the obnoxious effects of hydrogen peroxide. A dose-dependent

elevation of catalase activity was recorded in the treated worms as compared to that in control. At 5, 10, 25 and 50 μ L/mL, AEA increased catalase activity by 1.13, 1.64, 2.25 and 3.15 folds, respectively (Figure 5E).

3.5.6. Alteration in GPx activity

GPx protects the organisms by reducing free hydrogen peroxides into water and lipid hydroperoxides to their corresponding alcohols. We noticed a dose-dependent elevation of GPx activity in the parasites treated with AEA for 24 h (Figure 5F). At 5, 10, 25 and 50 μ L/mL, AEA increased GPx activity by 1.04, 1.18, 1.24 and 1.36 folds, respectively.

4. Discussion

It is of great significance that even in this modern era of medicine about 80% of the total world population still relies on traditional medicine which has been around for centuries for their day-to-day medicinal needs. Even many of the modern medicines that are effective against infectious agents are derivatives of natural products or from structures suggested by them. With the recent emphasis of the WHO on the development of antifilarial drugs from natural products, we are engaged in the screening of a large number of extracts obtained from terrestrial plants used in traditional medicine. Accumulative data from research with medicinal plants has brought out into the basic assumption that any plant bearing clinical efficacy must contain an active compound that can completely replace the plant extract is not always true^[18]. This assumption has to be changed in the light of the observations that there are, in many cases, adjuvant substances in the plant that increase the activity of the components actually responsible for the effectiveness^[19].

Primarily reduction of worm motility, MTT assay and dye exclusion test indicate usefulness of both micro- and macrofilaricidal potential of *A. indica* leaves. Prominent morpho-physiological alterations were also detected in both microfilariae and adults of *S. cervi* treated with AEA. Recently, Tripathi *et al*^[6] have shown that treatment with the extract of *A. indica* leaves caused death in rat oocytes through generation of ROS. This has prompted us to evaluate whether AEA-induced death is due to alteration in the parasitic redox regulation. Generation of ROS, namely, hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals ($OH\cdot$), is common during cellular metabolism.

Superoxide anions are generated when diatomic O_2 simultaneously gets two electrons and with H_2O_2 generates highly reactive hydroxyl radicals ($OH\cdot$) and cause oxidative damage[20]. A number of intracellular enzymes like catalase, SOD and GST are responsible for controlling the generated ROS[8]. An altered activity in the enzymatic antioxidant status is the marker of the altered ROS production in the concerned organism and a defect in the inherent ability to counteract the production of ROS generates a state commonly referred as oxidative stress. Potentially damaging alterations in bio-macromolecules (proteins, nucleic acids and lipids) are common during ROS exposure[21]. The ability of parasite antioxidant system to combat and neutralize ROS is essential for their survivability during altered metabolism[22] particularly for parasitic nematodes that are long lived and involved in chronic infections. Filarial parasites are furnished with marked supply of antioxidant enzymes and non-enzymatic small antioxidant molecules. Presence of SOD, catalase and GPx in different filarial parasites including *S. cervi*[23] is well established. GST, a phase II detoxification enzyme that also help in developing resistance in parasites against anthelmintics, antibiotics and drugs[24] was also reported in *S. cervi*[25]. GSH, an ubiquitous tripeptide and metabolic intermediate involved in antioxidant defense and xenobiotics detoxification has been reported in *S. cervi*[26]. In our study, we found a dose-dependent increase of superoxide anion in adult *S. cervi* treated with AEA, which was at par with our previous findings with curcumin[15] and ferulic acid[5] and provides indication of upregulation in ROS generation. An increased level of SOD was also recorded in AEA-treated parasites. Involvement of SOD in neutralizing superoxide anion was previously reported in case of parasites[27]. GST along with glutathione reductase are redox regulating enzymes to detoxify ROS by decreasing peroxide levels or by maintaining a steady supply of metabolic intermediates like glutathione to primary defense enzymes[28]. AEA caused a dose-dependent depletion of GSH level in the treated worms as compared to that in control. Therefore, it is a marker that possibly indicates the inhibition of enzymes involved in the synthesis of GSH and metabolism, and it is probably depriving the parasites of its major defensive molecule against oxidative stress. GST, an enzyme class present at different locations, catalyzes the conjugation of GSH via sulphhydryl group to electrophilic centers of a number of substrates[29] and thus comprises of very important member

of redox regulation cascade. We observed up-regulation of GST in a dose dependent way of AEA treatment. Several observations suggested that an increased level of GST during onset of apoptosis is associated with the alteration of oxidative stress and measured a strategy of protection by the cells undergoing programmed cell death[30]. It is worthy of mention that catalase along with GPx is known to attenuate the generation of ROS by removing potential oxidants or by transforming ROS into stable compounds[31]. Our observations revealed a dose-dependent increase in catalase and GPx levels as compared to control, confirming a change in the oxidative stress response of parasite exposed to AEA. Thus, in totality, antioxidant proteins have the ability to directly interact with cellular signaling molecules and can control their activity, and ROS can also trigger apoptosis and proliferation and influence immune response[32]. Any alteration in the total ROS measurement and enzymatic profile in redox regulation cascade can lead to all these detrimental effects.

In summary, the results obtained from the present study demonstrate that aqueous formulation of *A. indica* leaves possesses strong macro- and microfilaricidal activity against *S. cervi*. This antifilarial activity generated by AEA is likely due to an increase in the level of ROS. In future, it would be interesting to evaluate the antifilarial property of AEA against human filarial worms, find out the lead compound and further dissect the molecular mechanism involved in mediating the antifilarial activity against filarial worms. It will help us evolve a new strategy for the management of human filariasis.

Conflict of interest statement

The authors declare no conflicts of interest related to this work.

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