Antifilarial activity of azadirachtin fuelled through reactive oxygen species induced apoptosis: a thorough molecular study on *Setaria cervi*

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Supplementary material & method

SDS-page profile of extracted proteins from adult and mf

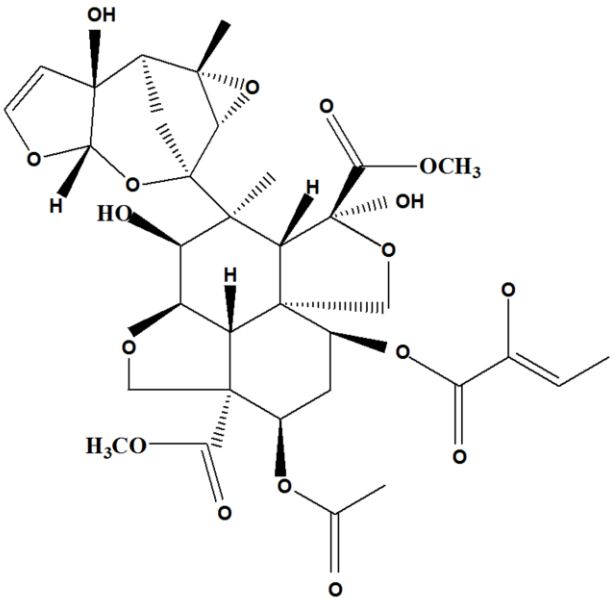
Extracted proteins (60 µg for adult and 120 µg for mf) from control and treated parasites (adult and mf) were first resolved by 12.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and for visualization of different protein bands, the resolved PAGE was processed through conventional silver-staining. Briefly, resolved gels were first fixed in fixative 1 (50% methanol and 12% acetic acid) for 8 h then kept at fixative 2 (10% ethanol and 5% acetic acid) for 30 min and finally at fixative 3 (10% ethanol) for 15 min. The gels were then washed 3 times with double distilled autoclaved water. Washed gels then kept at solution A (100 ml total volume having 50 µl formaldehyde, 42 µl Sodium thiosulphate from 0.43 g/ml stock) for 4 min and washed very promptly in double distilled autoclaved water thrice. The gels were then submerged in solution B (100 ml total volume having 50 µl formaldehyde and 0.5 g $AgNO_3$) for 8 min and washed in double distilled autoclaved water twice with mild shaking. The gels then were put in solution 3 (100 ml total volume having 50 µl formaldehyde, 2 µl $Na_2S_2O_3$ from 0.43 g/ml stock and 6 g Na_2CO_3) up to the considerable development of protein bands and lastly stop solution (5% acetic acid) was added. The gels were then visualized under gel documentation system and photographed.

Supplementary results

The result of the SDS-PAGE profiling of extracted proteins

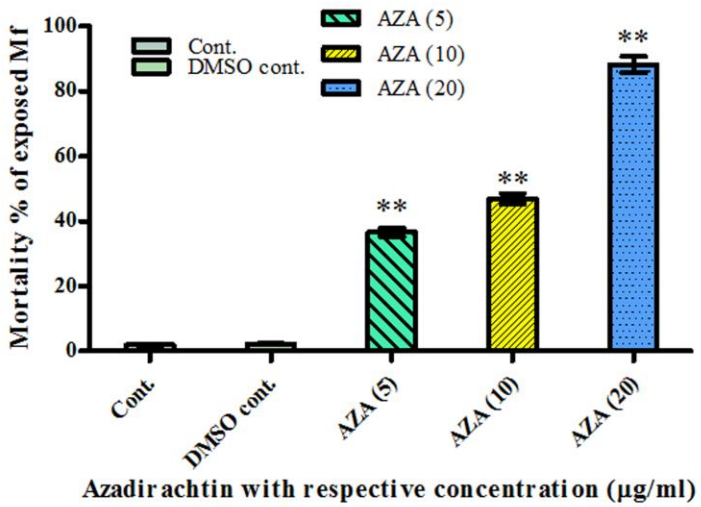
Resolved proteins, both from adults and mf in SDS-PAGE after silver staining depicted existence of proteins having different molecular weights (See supplementary figure 2B) and interestingly, protein profiles varied in respect to mf and adults. As the objective is to identify the altered expressions of different apoptogenic proteins, bands between 100-23 kDa were

immunoblotted with various nematode specific apoptogenic primary and housekeeping antibodies.

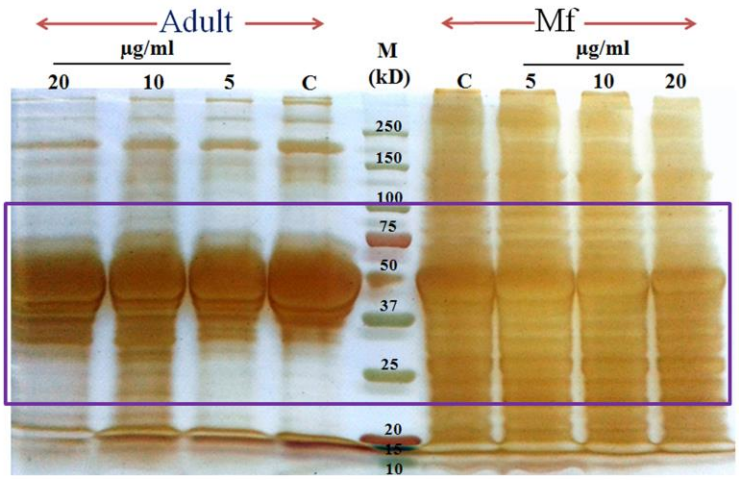


Structure of Azadirachtin ($C_{35}H_{44}O_{16}$; molecular weight 720.7)

Supplementary fig. S1. Structure of azadirachtin

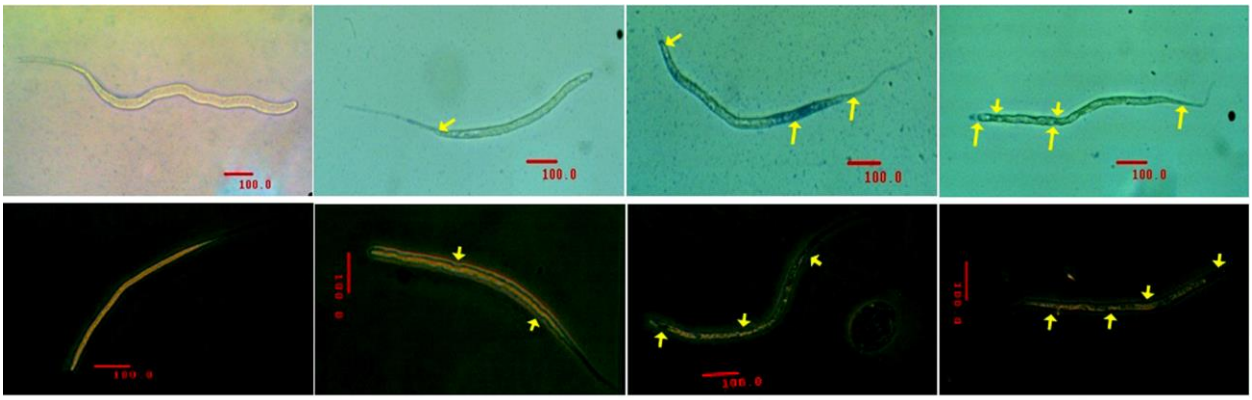


A.

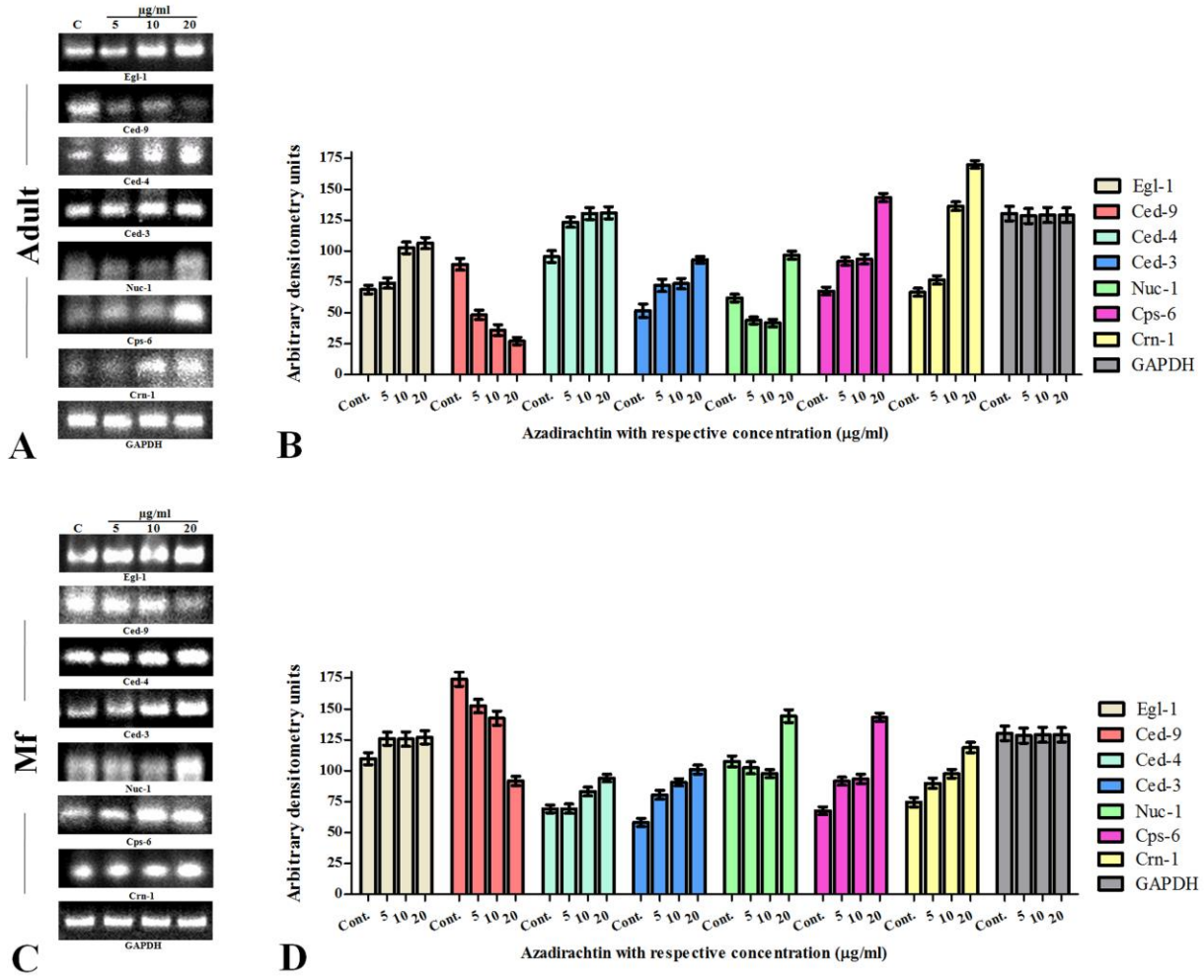


B.

Supplementary fig. S2. (A) Trypan Blue dye exclusion test. Mortality of mf of *S. cervi* was evaluated at 5, 10 and 20 µg/ml of azadirachtin after 24 h of exposure. (B) SDS-PAGE profile of extracted proteins (60 µg for adult and 120 µg for mf) from control and treated parasites after silver staining showed the presence of different protein bands.



Supplementary fig. S3. (Upper panel) Bright field micrographs of *S. cervi* mf. Control and 5, 10 and 20 µg/ml of azadirachtin treated mf of *S. cervi* after 24 h showed the presence of dead blue colored cell clusters and was shrunk inside the outer sheath. (Lower panel) Phase contrast micrographs of *S. cervi* mf. The luminous area denoted live cells and dark area for the dead parts.



Supplementary fig. S4. Alterations in the expression of anti- and proapoptotic genes in the treated parasites. RT-PCR of different apoptogenic genes in control and treated (A) adult *S. cervi* (n=2, gravid females for each set) and (C) control and treated mf (n=1×10^5/treatment set) of *S. cervi*. Azadirachtin caused a dose-dependent elevation in the expression of proapoptotic egl-1, ced-4, and ced-3, whereas expression of antiapoptotic ced-9 became downregulated with increasing concentration of this compound at the transcriptional level. Expression of nuc-1, cps-6, and crn-1 of the DNA degradation pathway was also up-regulated. GAPDH served as loading control. (B) Densitometric analyses of the bands of adult and (D) mf. Results were representative from three separate experiments.