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Anthelmintic resistance modifying properties of extracts of *Cyperus difformis* L. (Cyperiaceae)

Francis Adu[1], Christian Agyare[1*], George Henry Sam[2], Yaw Duah Boakye[1], Vivian Etsiapa Boamah[1]

Abstract

Background: Resistance by parasites to anthelmintic drugs is known to be on the increase and little attention is paid to the subject. The aim of study was to determine the anthelmintic and helminth resistance modifying activities of methanol whole plant *Cyperus difformis* extract against the adult Indian worm *Pherethima posthuma* using albendazole, mebendazole and levamisole as reference anthelmintics.

Methods: The worms were exposed to various concentrations of the extracts and reference anthelmintics (albendazole, mebendazole and levamisole) and observed for times for paralysis and death.

Results: The extract exhibited a concentration dependent anthelmintic activity against *P. posthuma* with a significant ($p<0.001$) paralysis and death times of 66.67±1.8 and 140.7±2.3 min respectively when the extract concentration was 20 mg/mL compared to the negative control (Ringer's lactate) group with paralysis and death time above 200 min. In the presence of 1, 2 and 5 mg/mL of the extract the reference anthelmintics (albendazole, mebendazole and levamisole), showed a potentiated activity against the test organism. In the presence of 2 mg/mL of the extract the paralysis and death times of 8 mg/mL albendazole against *P. Posthuma* were reduced from 41.33±0.33 and 106.67±0.88 min respectively to 33.33±0.88 and 85.67±1.2 min, respectively. Similar results were obtained for mebendazole and levamisole.

Conclusion: The extract had anthelmintic activity against *P. posthuma* and modified the resistance of the organism to albendazole, mebendazole and levamisole.

Keywords: *Cyperus difformis; Pherethima posthuma;* anthelmintic activity; potentiation.

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Background

Helminths have enormous economic impact on animal husbandry thus affecting the livelihood of humans. Many people around the globe harbour parasitic worms to the detriment of their health. It is therefore surprising and worrying that resistance to anthelmintics has received much less attention compared to antibacterial or other anti-infective agents [1]. Helminth resistance to the various anthelmintic agents is now a worldwide problem requiring similar attention as bacterial resistance especially among farm animals. Gastrointestinal helminth parasites are important source of economic loss to sheep industry [2]. The periodic administration of single-dose anthelmintics increases the selective pressure for possible occurrence of resistance in helminths [3]. Complete deworming of farm animals is currently very difficult as parasites are now resistant to most of the medicines used against them [4]. There is a high level of resistance among human helminth infections including soil-transmitted helminths (STH), (*Ascaris lumbricoides*, hookworms (*Necator americanus* and *Ancylostoma duodenale*) and *Trichuris trichiura*). This is due to selective pressure that has resulted from periodic mass administration of anthelmintic medicines to children and other at risk groups [5]. The benzimidazoles (including albendazole and mebendazole) act by interfering with tubulin polymerization to form microtubules by binding to β-tubulin [6]. Resistance to these drugs in a number of nematode species is caused by a phenylalanine (TTT/TTC) to tyrosine (TAT/TAC) substitution at either position 167 or 200 of nematode β-tubulin. The position 200 tyrosine mutation appears to be more common in parasitic nematodes [7]. It has been recognised that not all benzimidazole resistance is associated with this specific change and that other identified and unidentified single nucleotide polymorphisms (SNP) in β-tubulin genes are associated with this phenotype [7][8]. Levamisole like pyrantel is a cholinergic anthelmintic and acts by action on the nicotinic receptors. The response of the parasite depends on the sensitivity of nicotinic acetylcholine (ACh) receptors on muscle, and the extent of depolarization and entry of Ca^{2+} that levamisole produces, together with the sensitivity of the contractile proteins to Ca^{2+}. If the parasite's muscle contractile response to levamisole or other cholinergic anthelmintic sufficiently reduced by any component in the receptor signal-transduction pathway, the sensitivity of the parasite is reduced or resistance to the drug is increased [8]. Many plants have been shown to have anthelmintic activity [9][10]. Hence the need to screen more plants for their anthelminthic activity. *Cyperus difformis* L. (Family: Cyperaceae) is an annual plant with fibrous and reddish roots; up to 100 cm tall. It is native to southern Europe, Africa, Asia and Australia. It is very common in Ghana, United States and Egypt [11]. Traditionally the leaves of *C. difformis* are used for the management of scorpion bite and malaria. Hot infusion of the roots was taken orally to enhance libido in both males and females and to treat impotence in men (personal communication). Hence the aim of this study was to investigate the anthelmintic activity of the methanol extract of *C. difformis* and its effects on the anthelmintic activities of albendazole, mebendazole and levamisole were determined.

Methods

Collection and preparation of methanol extract of plant material

Whole *Cyperus difformis* L. plants were collected from swamps and rice fields in the Ashanti region of Ghana and authenticated by Dr G.H. Sam, a botanist in the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. The collected plant material washed under running tap water and air dried at room temperature (28 to 32^{o}C) for 10 days to constant weight. The dried plant material was milled using a laboratory hammer mill into coarse powder. A hundred gram (100 g) of the powdered material was taken into a container and cold macerated with 500 mL of 70% v/v methanol (Sigma-Aldrich, London, UK) for five (5) days with continual stirring. It was then filtered using Whatman paper No.1 (Sigma-Aldrich, Michigan, USA) under reduced pressure. It was then evaporated to dryness using rotary evaporator under 38^{o}C, lyophilized and stored in a fridge at 4^{o}C.

Collection of adult Pheretima posthuma

Pheretima posthuma (common name: Adult Indian earthworms; Order- Haplotaxida) which have anatomical and physiological resemblance to the human intestinal roundworms *Ascaris lumbricoides* [12] were collected from soil on the banks of Wiwi River in the Botanical Garden at Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The earthworms measured 5.0 to 7.0 cm in length and 0.20 to 0.30 cm in width.

Determination of in vitro anthelminthic activity of methanol extract of *C. difformis*

The *in vitro* anthelmintic bio-assay was performed according to the method described by [13]. Ten grams (10 g) of the methanol whole plant extract of *C. difformis* (MWCD) was mixed in 1 mL DMSO and

diluted with Ringer's lactate solution (Amanta Healthcare Ltd, Gujarat, India) to 500 mL to produce 20 mg/mL extract solution. It was then serially diluted to produce 10, 5, 2 and 1 mg/mL solutions using Ringer's lactate solution. Albendazole (Sigma-Aldrich, London, UK) (10 mg/mL) was similarly prepared and used as positive control. A solution of DMSO (0.2% v/v) in Ringer's lactate was used as negative control.
The *P. posthuma* worms were cleaned of all debris using 0.9% w/v NaCl solution (Amanta Healthcare Ltd, Gujarat, India) and placed in separate petri dishes (3 worms per petri dish) into which the various extract solutions and reference standard solution (50 mL in each petri dish) had been added. The period taken for the various solutions to cause paralysis and death of the individual worms were recorded. Concentrations of extract that produced activity after 200 min were considered inactive. Marked decrease in wriggling movement of the worms indicated paralysis which was confirmed with slow movement of the worm after being pricked with a pin. Death was deemed to have occurred when the worms lost motility followed by a fading away of their body colour which was confirmed by shaking the worm in warm water at 50^{o}C with no response [14].

Determination of the influence of methanol extract of C. diffformis on the anthelminthic activity of albendazole, mebendazole and levamisole

The effects of MCWD on the activity of some anthelmintics were determined in the presence of 1, 2 and 5 mg/mL of the extract. Concentrations of 1, 2, and 5 mg/mL of MWCD were prepared using DMSO in Ringer's lactate solution as above. These solutions were each used to prepare albendazole to concentrations of 1, 2, 4, 8 and 16 mg/mL. Fifty millilitres of these solutions were dispensed into separate petri dishes and three worms were added to each petri dish. The time taken for the worms to paralyse and die were recorded. The study was repeated using mebendazole and levamisole (Sigma-Aldrich, London, UK).

Data analysis

Data obtained was analysed using Graph Pad Prism Version 5.0 for Windows (Graph Pad Software Inc, San Diego, CA, USA) statistical package programme. One-way ANOVA followed by Dunnett's *post hoc* test was employed in the analysis of data.

Results

Anthelminthic activity of methanol extract of C. diffformis

The extract (MWCD) exhibited weak anthelmintic activity against *P. posthuma.* At 1 and 2 mg/mL it exhibited no activity after the 200 min exposure time, which for the purpose of this study was considered to have shown no activity. At the same concentration as albendazole (10 mg/mL) the extract was approximately 4 times less active as a paralytic agent than albendazole and about 1.5 times less active as a killing agent. There were significant differences ($p<0.001$) between the effects of the negative control and the extracts at all concentrations of the extract that exhibited activity. The activity of the extract was concentration dependent giving a significant ($p<0.001$) paralysis and death times of 66.67±1.8 and 140.7±2.3 min respectively at 20 mg/mL compared to the negative control group (ringers lactate) with paralysis and death time above 720 mins (Figure 1).

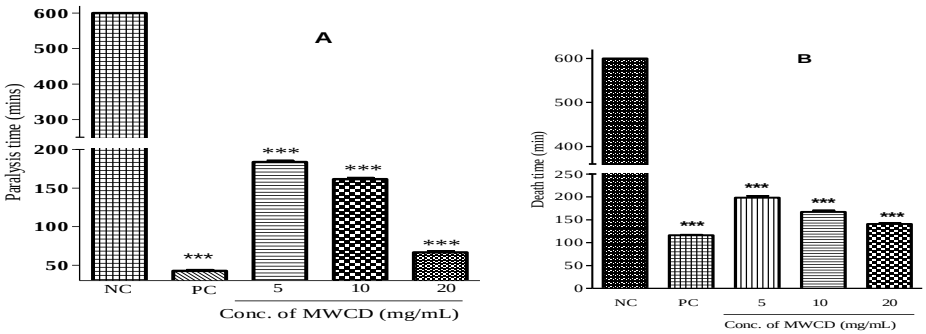

Fig.1. Anthelmintic activity of methanol whole plant extract (MWCD) of *C. diffformis*. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (Albendazole), n=3, values are mean ±SEM, ***$p<0.001$ compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

Influence of methanol extract of C. diffformis on the anthelminthic activity of albendazole, mebendazole and levamizole

At 1 mg/mL, albendazole alone was able to cause paralysis and death after 57.33±1.76 and 155.0±2.65

min of exposure, respectively. When 1 mg/mL of albendazole was combined with 1 mg/mL of methanol whole plant extract of *C. difformis*, worm paralysis and death times were significantly reduced (p<0.01) to 42.33±0.88 and 114.33±0.88 min respectively. Similar significant increment in activity with consequent reduction in paralysis and death times occurred at all the combinations of the concentrations of albendazole (2, 4, 8 and 16 mg/mL) and extract (1, 2 and 5 mg/mL) used (Figures 2 to 6). Similar results were obtained for mebendazole (Figures 7 to 11) and levamisole (Figures 12 to 16).

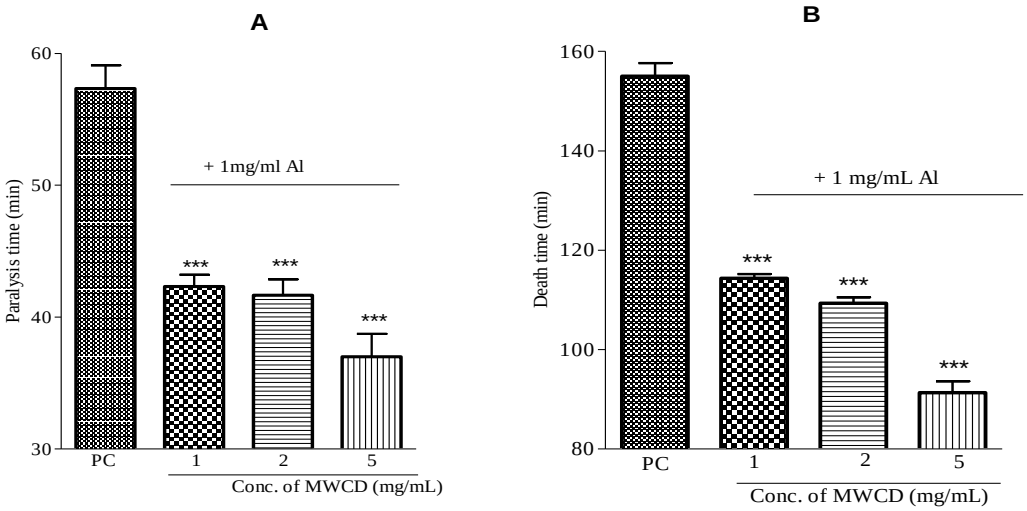

Fig.2. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 1 mg/mL albendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (albendazole), n=3, values are mean ±SEM, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test)

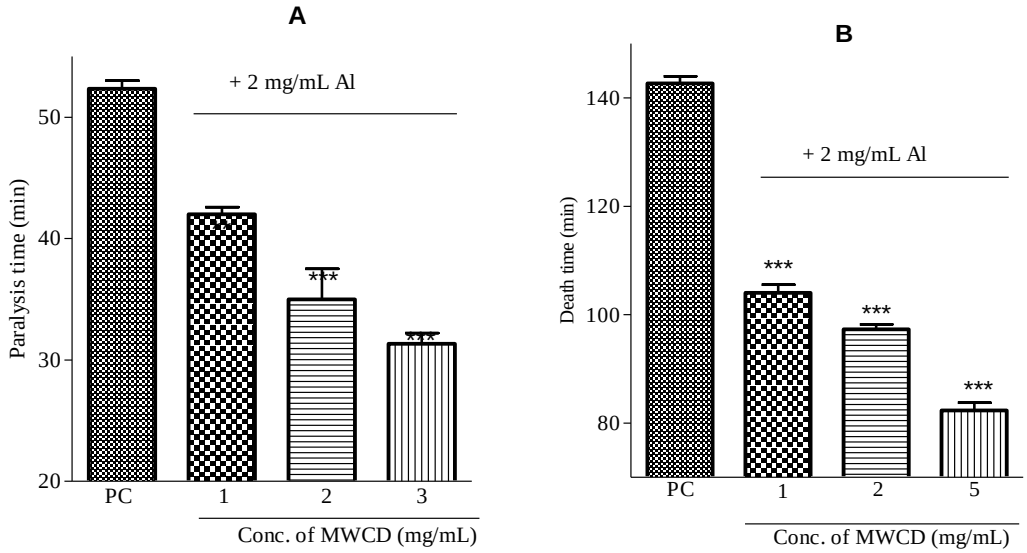

Fig.3. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 2 mg/mL albendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (albendazole), n=3, values are mean ±SEM, **p<0.01, ***p<0.001 compared to control (One-wayANOVA followed by Dunnett's *post hoc* test).

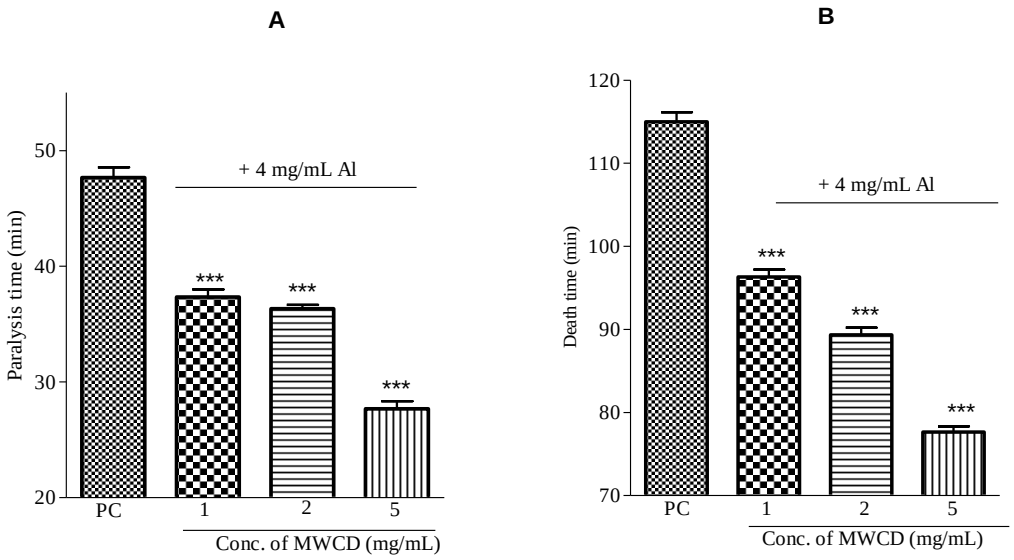

Fig.4. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 4 mg/mL albendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (albendazole), n=3, values are mean ±SEM, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

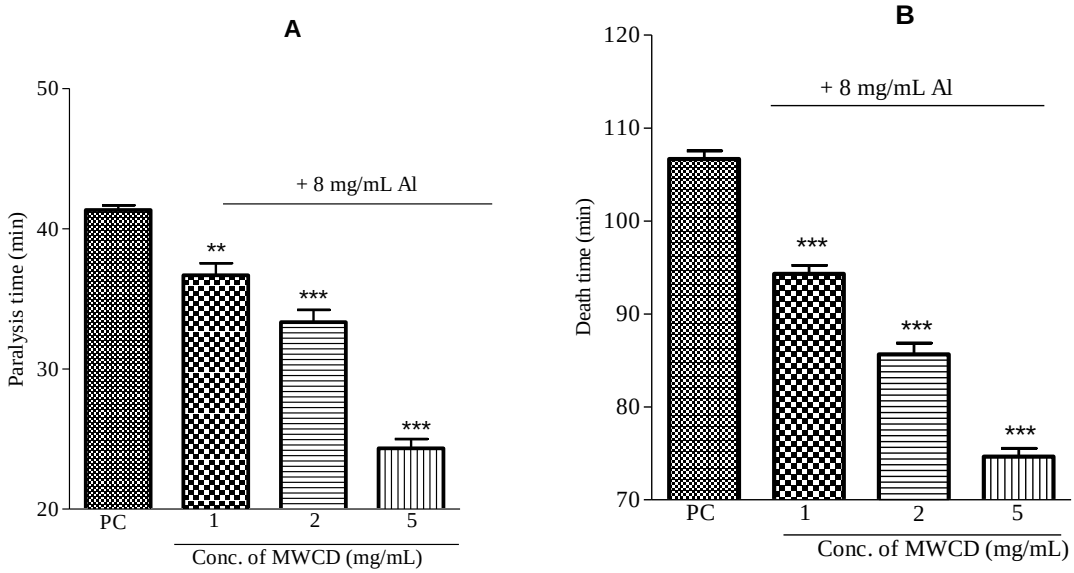

Fig.5. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 8 mg/mL albendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (albendazole), n=3, values are mean ±SEM, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

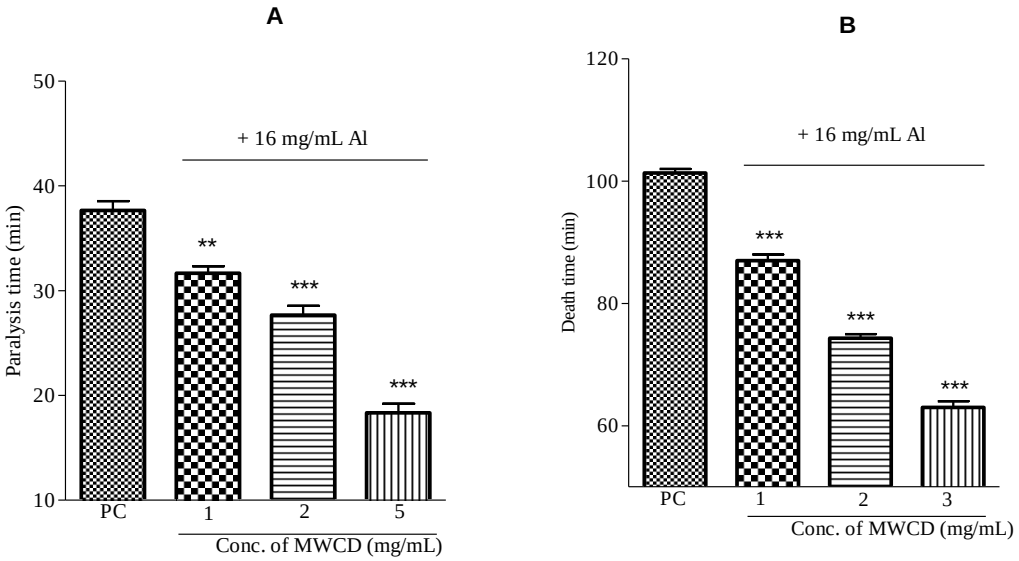

Fig.6. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 16 mg/mL albendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (albendazole), n=3, values are mean ±SEM, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's*post hoc* test).

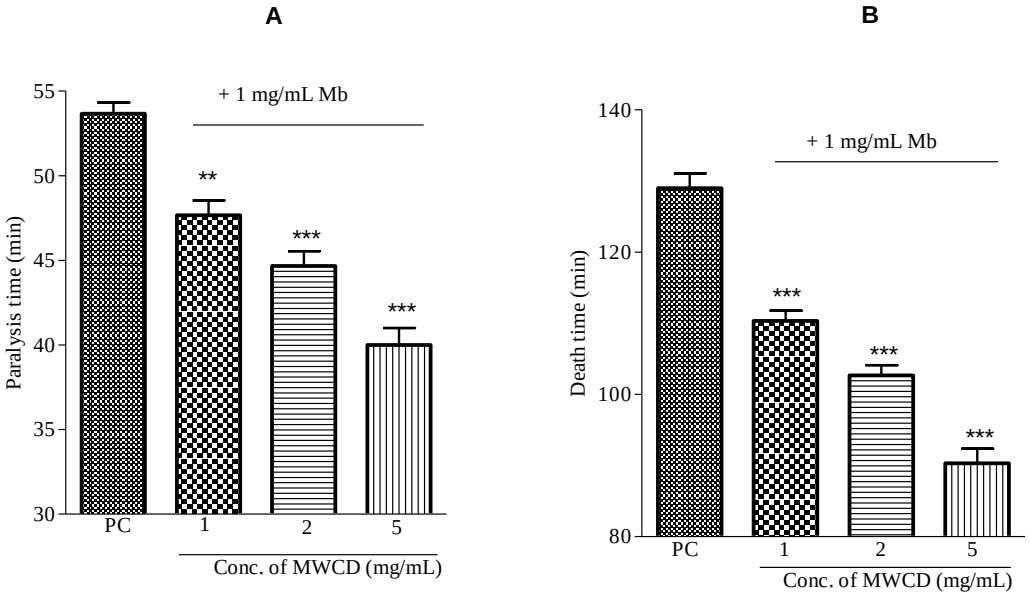

Fig.7. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 1 mg/mL mebendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (mebendazole), n=3, values are mean ±SEM, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

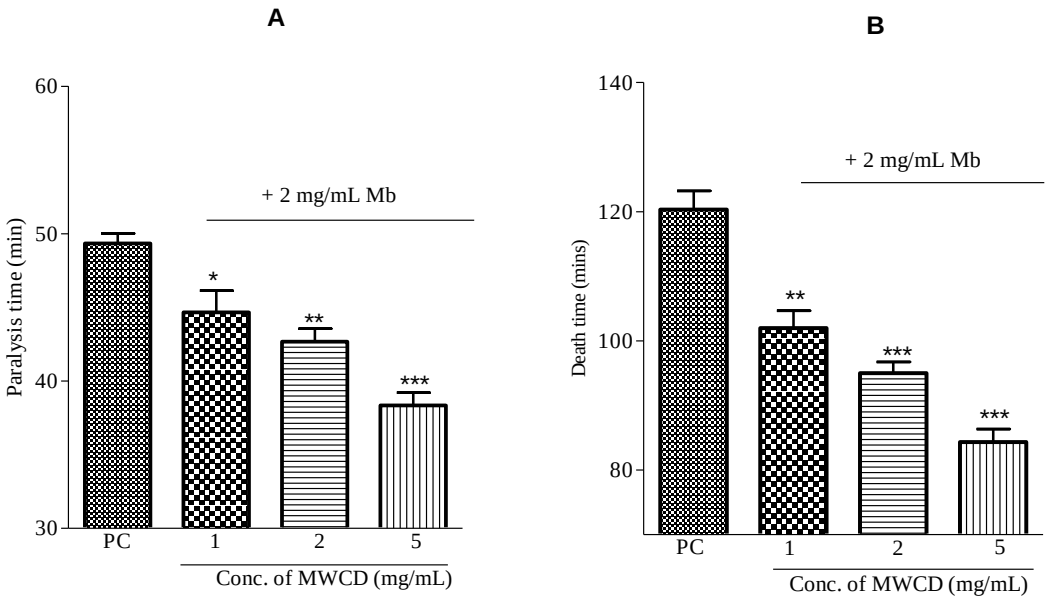

Fig.8. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 2 mg/mL mebendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (mebendazole), n=3, values are mean ±SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

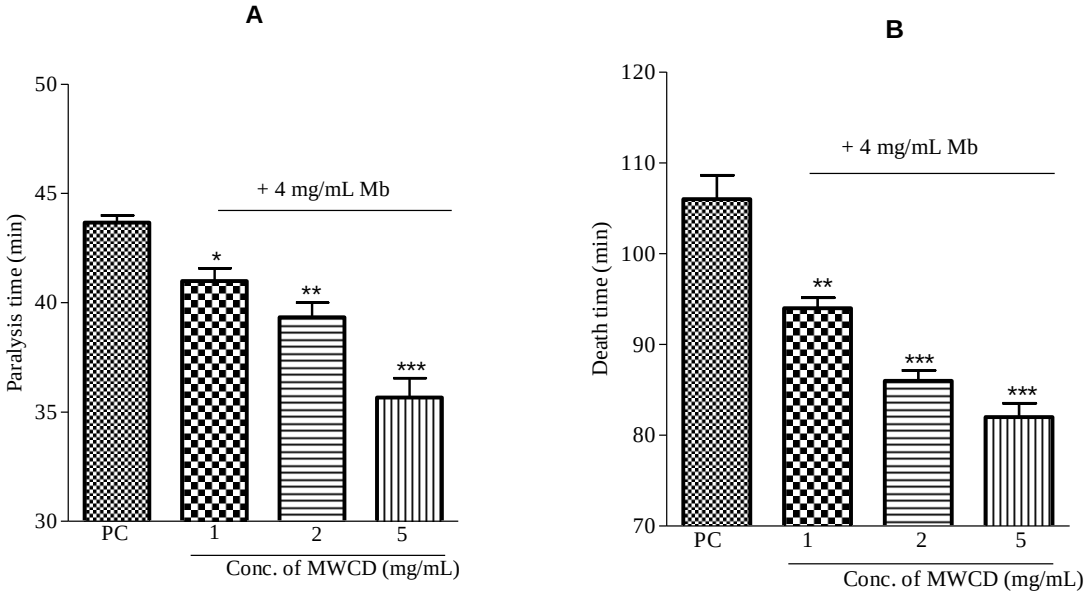

Fig.9. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 4 mg/mL mebendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (mebendazole), n=3, values are mean ±SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

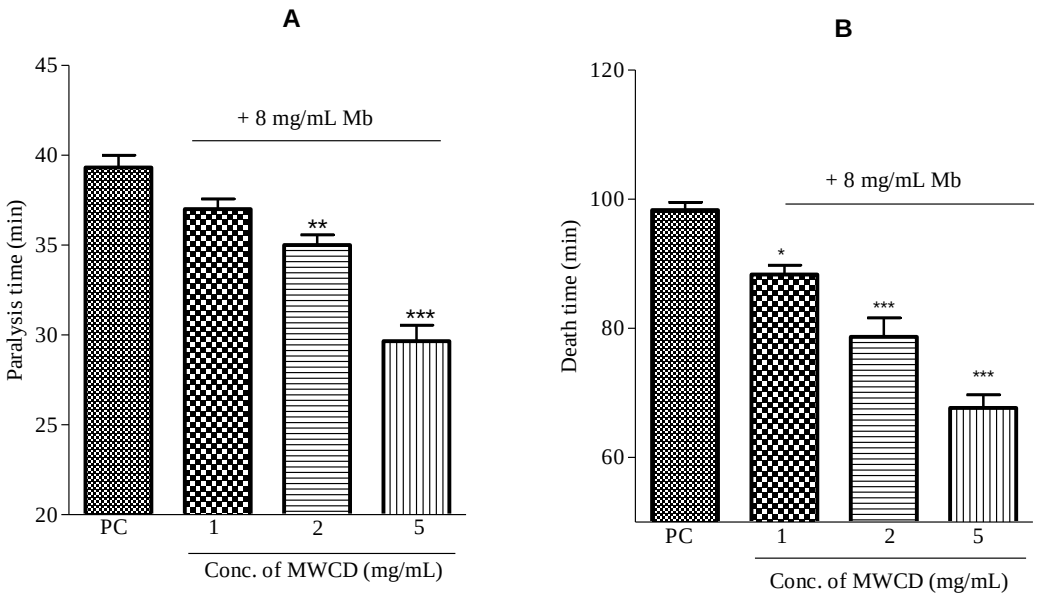

Fig.10. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 8 mg/mL mebendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (mebendazole), n=3, values are mean ±SEM, *p<0.05, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's*post hoc* test).

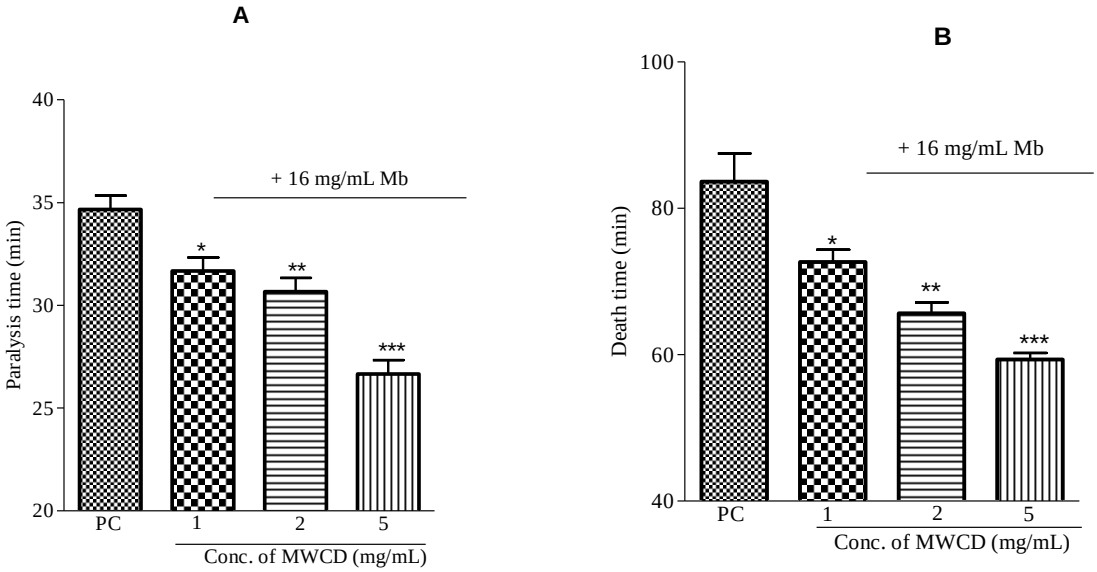

Fig.11. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 1 mg/mL mebendazole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (mebendazole), n=3, values are mean ±SEM, *p<0.05, ** p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

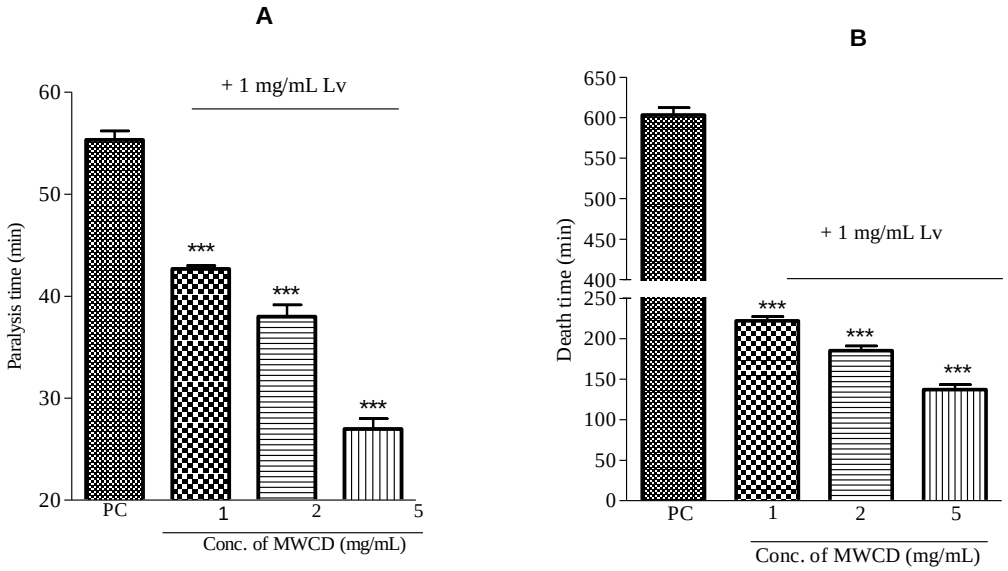

Fig.12. Influence of methanol whole plant extract (MWCD) of *C. difformis*on anthelmintic activity of 1 mg/mL levamisole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (levamisole), n=3, values are mean ±SEM, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

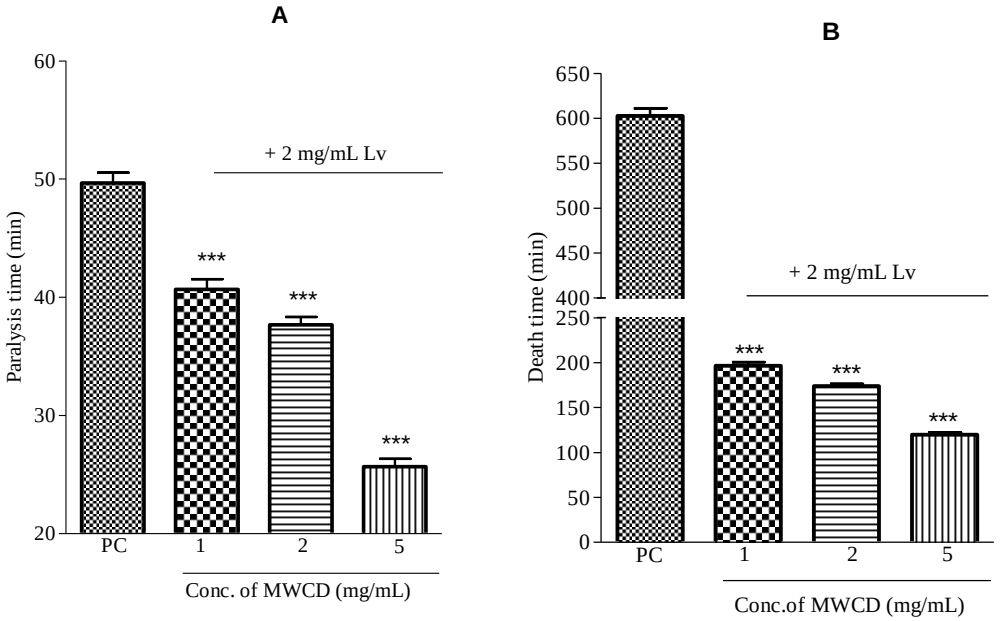

Fig.13. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 2 mg/mL levamisole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (levamisole), n=3, values are mean ±SEM, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

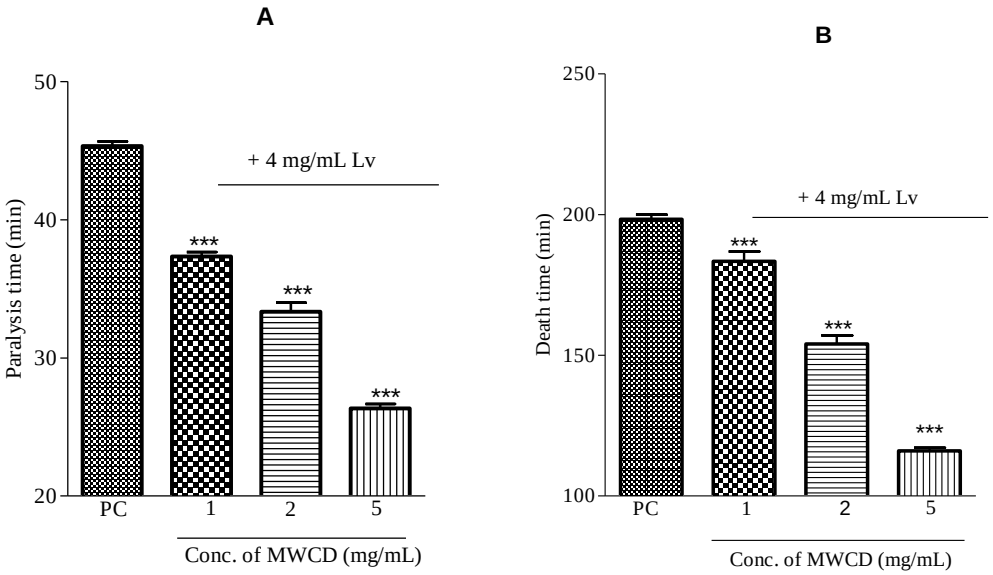

Fig.14. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 4 mg/mL levamisole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (levamisole), n=3, values are mean ±SEM, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

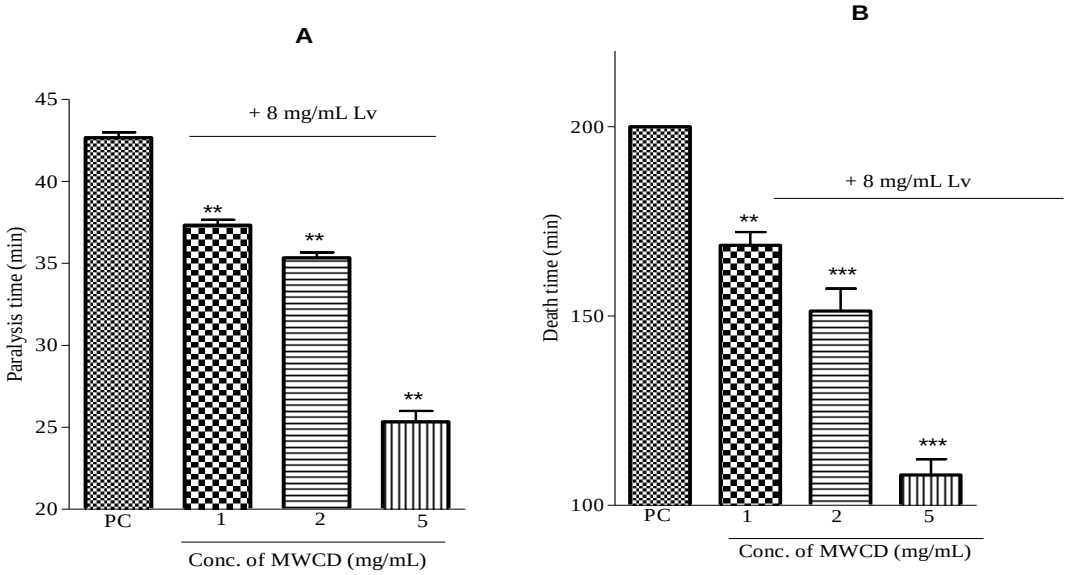

Fig.15. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 8 mg/mL levamisole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (levamisole), n=3, values are mean ±SEM, **p<0.01, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

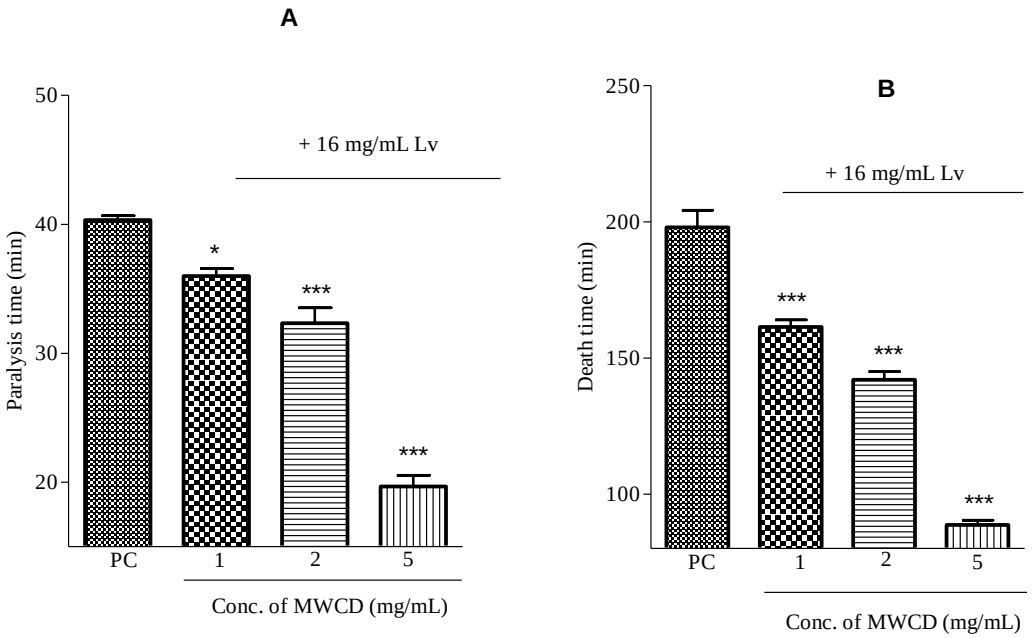

Fig.16. Influence of methanol whole plant extract (MWCD) of *C. difformis* on anthelmintic activity of 16 mg/mL levamisole. A = Paralysis time, B = Death time, NC = Negative control, PC = Positive Control (levamisole), n=3, values are mean ±SEM, *p<0.05, ***p<0.001 compared to control (One-way ANOVA followed by Dunnett's *post hoc* test).

Discussion

MWCD exhibited anthelmintic activity causing both paralysis and death of the Indian adult worm *P. posthuma*. Low concentrations (1 and 2mg/mL) had no activity. The duration of paralysis and death times at 5, 10 and 20 mg/mL of the extract compared with albendazole showed that the extract had a weak anthelmintic activity. It also showed that there was a concentration dependent activity. These findings support the report by [15] which indicated that genus *Cyperus* (*C. tegetum*) showed concentration dependent anthelmintic activity. Albendazole, mebendazole and levamisole are used for the management of helminthic infections [16]. Albendazole and mebendazole are benzimidazoles and act by interfering with tubulin polymerization to form microtubules by binding to β-tubulin [7]. The effects of these drugs result in death of the nematodes [17]. Albendazole and mebendazole exhibited paralytic effect on the test worms at all the concentrations used. Mebendazole was consistently more active than albendazole, showing smaller paralysis and death times than albendazole. This is consistent with a report by [3] where mebendazole was found to be more active than albendazole against *Ascaris lumbricoides* and *Trichuris trichiura* in children between 6 and 12 years. The enhanced activities of albendazole and mebendazole in the presence of sub-anthelmintic concentrations of MWCD (1 and 2 mg/mL) could be attributed to the presence of secondary metabolites that made the drugs more available at their sites of action thus potentiating anthelmintic drugs [18].

Levamisole alone produced characteristic paralytic activity and in the presence of MWCD, its activity was enhanced against the *P. posthuma*. From the mechanism of action of levamisole it may be due to the extract making the acetylcholine gated channels more open to levamisole enhancing the effects of the latter, allowed more depolarization and entry of Ca^{+2} or enhancing the sensitivity of the contractile proteins to Ca^{2+}. It may also contain some cholinergic agonists that help amplify the effects of levamisole [8]. The results show that at sub-activity concentrations of MWCD, the activities of albendazole, mebendazole and levamisole were enhanced indicating a resistance modulation activity of the extract. It also shows that if *C.difformis* is used as animal feed it may enhance the activities of anthelmintic agents. There is a need to isolate and characterize the bioactive agents or principles from the extract of *C. difformis* the responsible for the anthelminthic activity.

Conclusions

The methanol whole plant extract of *C. difformis* (MWCD) had anthelmintic activity against *P. posthuma* and exhibited resistance modulation activity on *P. Posthuma* in the presence of the reference anthelmintic agents.

Declaration

We confirm that all materials and data obtained from this study/project are available in laboratory notebooks and data base of the Department and can freely be accessed.

Authors' Contribution

FA participated in the design of the study, carried out laboratory analysis and drafted the manuscript. *CA* conceived the concept, design and coordinated the study, supervised the study and revised the manuscript. *GHS* participated in the design of the study and revised the manuscript. *YDB* participated in the laboratory studies and data analysis. *VEB* also participated in data analysis and revision of manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no competing interest.

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