Screening and Investigation of Novel Antihelminthic Drugs and Study of their Mechanism of Action

Submitted for the award of the

Degree of

Doctor of Philosophy

Submitted by

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August 2021

Candidate's Declaration

I, hereby declare that the work presented in the thesis entitled "SCREENING AND INVESTIGATION OF NOVEL ANTIHELMINTHIC DRUGS AND STUDY OF THEIR MECHANISM OF ACTION" in partial fulfillment of the requirement for the award of the Degree of Philosophy, School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of my work carried out under the supervision of Dr. Diptiman Choudhury, Assistant Professor, School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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Certificate

This is to certify that thesis entitled "**SCREENING AND INVESTIGATION OF NOVEL ANTIHELMINTHIC DRUGS AND STUDY OF THEIR MECHANISM OF ACTION**" being submitted by Vanshita Goel in the fulfillment of the requirement for the award of the Degree of Philosophy, School of Chemistry and Biochemistry, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of candidate's work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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Acknowledgment

I would like to pay high regard to Almighty God for giving me skills, wisdom, knowledge, and the ability to work hard to reach where I am today. I thank God for the blessings and support. Without his blessings and support, this thesis would have never been possible.

An acknowledgment in my thesis is just a small gesture and doesn't truly represent how much I appreciate the influence that many people have had on my life. It is a pleasant task to express my thanks to all those who contributed in many ways to my doctoral work and made it an unforgettable journey for me.

My joy knows no boundaries as I take this opportunity to express my deepest and sincere gratitude to my supervisor, Dr. Diptiman Choudhury, Assistant Professor, School of Chemistry and Biochemistry (SCBC), Thapar Institute of Engineering & Technology (TIET), Patiala, who has provided me unwavering support, and mentorship throughout this endeavor. He has always treated me like his own child and guided me with motivation, patience, scholarly inputs, and consistent encouragement in the professional as well as in the personal field. He has always been approachable to clarify my doubts despite their busy schedules. He has always poured me with exquisite knowledge and has always motivated me with constructive remarks. It has been a great experience to work under his guidance and to learn from his research expertise.

With deep regards and profound respect, I also thank Dr. Lachhman Das Singla, Head, and Professor, and Dr. Paramjeet Kaur, Assistant Professor, Department of Veterinary Parasitology, Guru Angad Dev Veterinary and Animal Sciences (GADVASU), Ludhiana. I feel privileged to be associated with them during my life.

I express my gratitude to Director, Thapar Institute of Engineering & Technology, Patiala, Dr. Rafat Siddique, Dean of Research and Sponsored Projects (RoSP), Prof. O. P. Pandey, former Dean RSP, Dr. Susheel Mittal (Head of SCBC), Dr. Amjad Ali (Former Head of SCBC), Prof. Bonamali Pal (Former Head of SCBC) for all facilities which have been immensely helpful in completing my work.

I am also very grateful to my doctoral committee members Dr. Manmohan Chhibber, Dr. Satnam Singh, and Dr. Siddharth Sharms, for their fruitful discussion and encouragement, constructive criticism, and inspiration.

Sincere thanks to Dr. Soumen Basu, Associate Professor, (SCBC), Dr. Neloy Chakroborty, Assistant Professor, (Psychology), Thapar Institute of Engineering & Technology (TIET), Patiala.

I am thankful for my labmates Pawandeep Kaur, Parmandeep Kaur, Sunidhi Sharma, Deepinder Sharda, Komal Attri, Harman, and Poulomi Chandra who all have extended their support during my research work. Special thanks to Dr. Anirudh Sharma, Dr. Iqubal Singh, Dr. Jyoti Sharma, Dr. Purnima Sharma, Dr. Amit Mishra, Dr. Ishfaq, and Dr. Rahul Hundal for always standing by my side and sharing a great relationship as compassionate friends. I will recover cherish the warmth shown by them, whose smiling faces always made me refreshing and also for unforgettable memories during my research work. I gained a lot from them, through their professional and scholarly interactions. I acknowledge the help of Mr. Mayank Sharma, office staff, and Chander Singh Thakur, Chandar Shekhar, Hemant Sharma, and Vishwanath Dass, technical staff of SCBC, T.I.E.T, Patiala and Subodh and Ankur, technical and office staff of GADVASU, Ludhiana, for their support in various aspects.

Some of the results described in this thesis would not have been possible without the help of laboratories at the institutes like SAI labs, Thapar Institute of Engineering and Technology, Patiala. I extend my thankful acknowledgment to T.I.E.T for providing me a fellowship and giving me great teaching experience through Teaching Associate.

Finally, none of this would have been possible without the constant love, support, guidance, and patience of all my family members. I can't put my words to thank my mother, Mrs. Anupam Goel, father, Mr. Parminder Kumar, brother, Mr. Dhiren Goel, husband, Mr. Aashish Goyal, and my entire family for everything they have done for me. They all have been amazing and have always been there for me. Thank you for your everlasting love and support. This is for you!

I may be short of words to pay the debt of recognition towards the man who has always been a source of strength for me, my grandfather, Late Sh. Vinod Kumar. I, in the whole of my lifetime, will not be able to compensate for his love, which nourished me and accompanied me through every step of my life and always leading from behind. It was because of him only that I was courageous enough to dream big.

Kaushika

DEDICATION

This thesis is dedicated to my beloved Grandfather

Whose support and love helped me in achieving my goals

ABBREVIATIONS

ALE	Aqueous Lansium extract
Alb	Albendazole
$AgNO_3$	Silver Nitrate
AgNPs	Silver Nanoparticle
AMA	Adult Motility Assay
ASE	Aqueous Selaginella extract
CA	Cuminaldehyde
CAT	Catalase
C. elegans	*Caenorhabditis elegans*
DCFDA	2',7' –dichlorofluorescin diacetate
DLS	Dynamic Light Scattering
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EHA	Egg Hatch Assay
FAMACHA	FAffa MAlan CHArt
FDA	Food and Drug Association
FEC	Faecal Egg Count
FTIR	Fourier Transform Infrared Spectroscopy
GSH	Reduced Glutathione
GPx	Glutathione Peroxidase
H. contortus	*Haemonchus contortus*
H and E staining	Hematoxylin and Eosin Stain
HEK	Human Embryonic Kidney cells
L	Larvae
L. parasiticum	*Lansium parasiticum*
LAgNPs	Lansium coated silver nanoparticles
LD	Lethal Dose
LMA	Larval Motility assay
NOS	Nitric Oxide Synthase
NTD	Neglected Tropical Diseases
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
S. moellendorffii	*Selaginella moellendorffii*
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
SPR	Surface Plasmon resonance
TEM	Transmission Electron Microscopy
USD	United State Dollar
WHO	World Health Organisation
WHA	World Health Assembly

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INTRODUCTION

Soil-transmitted helminthic infections are widely distributed in tropical and subtropical areas and, since they are linked to a lack of sanitation, occur wherever there is poverty [1]. Based on Subba, 2020 report, more than 880 million children are estimated to be treated for parasitic infection [2]. Nematodes are commonly known as roundworms and are considered the largest phyla in the animal kingdom [3, 4]. Despite, the true number of nematodes ranges between 100,000 and 10 million, only 20,000 – 30,000 species have been described [5]. Nematodes have a bilaterally symmetrical body with a defined head and tail [6]. The most commonly found helminths are as follows: *Ascaris lumbricoides*, *Trichuris trichiura*, *Toxocara canis*, *Taenia solium*, *Haemonchus contortus*, and *Hymenolepis nana* with prevalence: 3.36, 2.2, 2.9, 1.68, 0.9 and 2.2%, respectively [7, 8]. The highest infection rate of *Ascaris spp.* has been reported in China, Southeast Asia, coastal regions of West Africa, and Central Africa [9]. *Trichuris spp.* infection infestation is at its highest rate in Central Africa, Southern India, and Southeast Asia [10, 11]. Hookworm such as *Ancylostoma duodenale, Necator americanus,* and *H. contortus* infection is most prevalent in Sub-Saharan Africa, South China, and Southeast Asia [12]. *H. contortus* infection is mainly prevalent in temperate and tropical regions like sub-Saharan Africa, North America, and South and East Asia [13]. Countries with a hot and humid tropical climate have a high prevalence of intestinal parasites such as *Trichostrongylus spp*, which has high infection infestation in sheep than the general infected individuals in colder and dry weather countries it ranges from 4- 18 ℃ [14]. Sub-Saharan Africa and East Asia have the highest infestation of intestinal worms [15]. An estimated 4- 28% of children are infected with pinworm infection, globally [16]. The prevalence of hookworm infection is 9 % among infected individuals whereas prevalence is as high as 56% in new-born, infants and toddlers [17].

Roundworm infection- Human health and Economy:

Helminthes are some of the world's commonest parasites [18]. They belong to two major groups of animals, the flatworms or Platyhelminthes (flukes and tapeworms) and Aschelminthes, commonly known as nematodes [19, 20]. All are relatively large and some are very large, exceeding one meter in length. Their bodies have well-developed organ systems such as reproductive organs, neuronal networks, alimentary canals, etc and most helminths are active feeders [21, 22]. The bodies

of flatworms are flattened and covered by a membrane, whereas roundworms are cylindrical and covered by a tough cuticle [23].

Parasitic nematodes are multi-cellular eukaryotes with a long round body and require a living host to survive [24]. Soil-transmitted nematodes are commonly found in soil and stool in the egg or larval stage and can enter the body of the host through the skin or direct contact with the mouth [25]. Nematodes are divided into various types: giant roundworm**,** hookworm, pinworm, strongyloidiasis, whipworm, and trichinosis [26]. These worms commonly survive in moderate to warm and humid climates and are found in equatorial, tropical, subtropical, and temperate regions [27]. Exposure to infection is influenced by climate, hygiene, food preferences, and contact with vectors [12, 28]. Many potential infections are eliminated by host defenses; others become established and may persist for prolonged periods, even years [29]. Small ruminants are more susceptible to nematode infection than adult animals. Similarly, newborns, infants, and pre-school children are more prone to nematode infection than adult humans [30]. They usually cause malnutrition, anemia in children which results in poor growth. Some species may also affect the lungs, eyes, brain, and sometimes blockage in the gut which leads to a serious infection in the liver and pancreas [26, 31].

Roundworm infects Approximatelyimately 1.4 billion people in around 150 countries worldwide [32]. Infection is fatal, but due to its prevalence, it is responsible for 8000-100,000 deaths annually [33]. Infection of roundworms in the abdomen many times causes death or several deficiencies to a newborn or unborn child in the mother's womb [34, 35]. An important cause of roundworms is malnutrition, mostly in children by causing protein-energy loss and vitamin A and C deficiencies [36, 37]. Overall roundworm infection causes stunting of linear growth, which leads to both physical and mental deficits [38]. Because worms are large and often migrate through the body, they can damage the host's tissues directly by their activity or metabolism. Damage also occurs indirectly as a result of host defense mechanisms. Almost all organ systems can be affected [39-41].

Studying the epidemiology of helminths is highly important because they have a wide geographical distribution worldwide, especially in Sub-Saharan Africa, South America, Central America, Middle East, the Caribbean, and Asia is described in **Table 1**, where the global prevalence and regional distribution is detailed and a world map is explored in **Figure 1** where the helminthic infection is marked with different color distribution [42-47].

Table 1. The prevalence of different parasitic nematodes worldwide.

Helminths	**Species**	**Global Prevalence**	**Region**
Ascariasis	*Ascariasis lumbricoides*	807 million	Southeast Asia, Africa, and Latin America
Trichuriasis	*Trichuris trichiura*	604 million	Southeast Asia, Africa, and Latin America
Hookworm	*Haemonchus contortus* *Necator americanus*; *Ancylostoma duodenale*	576 million	Southeast Asia, Africa, and Latin America
Strongyloidiasis	*Strongyloides stercoralis*	30-100 million	Southeast Asia, Africa, and Latin America
Lymphatic Filariasis	*Wuchereria bancrofti* *Brugia malayi*	120 million	India and sub-continents, Southeast Asia and sub-Saharan Africa
Onchocerciasis	*Onchocerca volvulus*	20.9 million	Sub- Saharan Africa
Loiasis	*Loa loa*	14.4 million	Sub- Saharan Africa
Schistosomiasis	*Schistosoma haematobium*	779 million	Sub- Saharan Africa
	Schistosoma mansoni		Sub- Saharan Africa and Eastern Brazil
	Schistosoma japonicum		China and Southeast Asia
	Clonorchis sinensis		Southeast Asia
	Opisthorchis viverrini		Developing regions of East Asia
	Paragonimus spp		Developing regions of East Asia
	Fasciolopsis buski		Developing regions of East Asia
	Fasciola hepatica		Developing regions of East Asia
Cysticercosis	*Taenia solium*	50 million	Southeast Asia

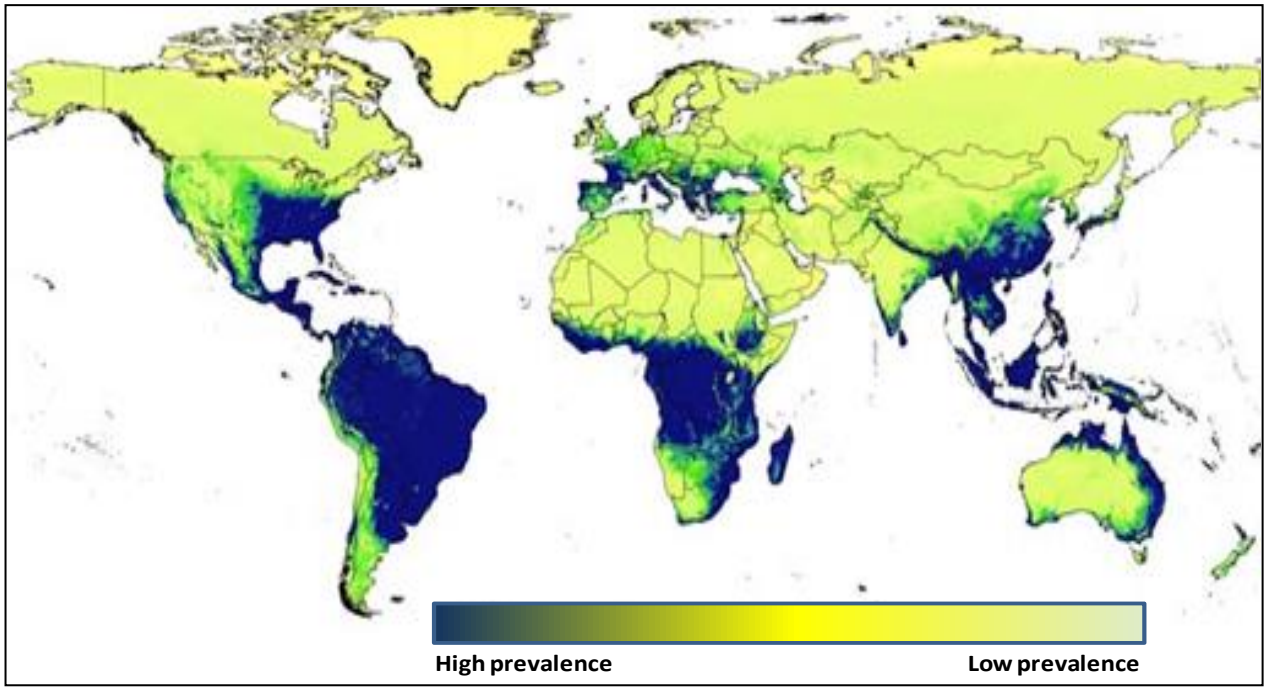


Figure 1. Prevalence of helminthic infection around the world. The dark blue regions are showing the high prevalence of infection i.e., in moderate and temperate regions and light green and white regions are describing the low prevalence of helminth infection worldwide. (McCarty, 2014, World Health Assembly, WHA)

H. contortus, a highly infectious gastrointestinal parasitic nematode responsible for acute anemia, hemorrhagic gastroenteritis, diarrhea, depression, etc in mainly and not limited to ruminants leads to the reduction of livestock production, milk, and meat production which caused huge economic loss worth 120 billion USD worldwide every year [48, 49]. *H contortus* is a blood-sucking nematode parasite, transmitted through infected soils in various species including humans [50, 51]. The *H. contortus* infection is highly prevalent in hot and humid climatic conditions and is found in moderate and temperate regions globally. The adult females are usually 18- 30 mm and males are 10- 20 mm long and easily recognized in the infected areas of the intestine due to their red and white appearance because of their blood-sucking feature [52].

The complete life cycle of *H. contortus* is divided into different stages including eggs, L1, L2, L3, L4, and adult stages. *H. contortus* eggs are usually clear and about 70- 85 µm long and 44 µm wide (**Figure 2a**). Then they develop into the L3 stage (**Figure 2b**) where they contain a defined protective cuticle and further develop into male and female adults [52] (**Figure 2c**). Where adult

male worm is usually 10- 20 mm long and has a blunt anterior end and well-defined bursa region at the end of the posterior end. Whereas adult female worms are 18- 30 mm long and have a sharp anterior end and no bursa region at the posterior end but have a vulvar flap in the middle of the body of the worm (**Figure 3a-e**).

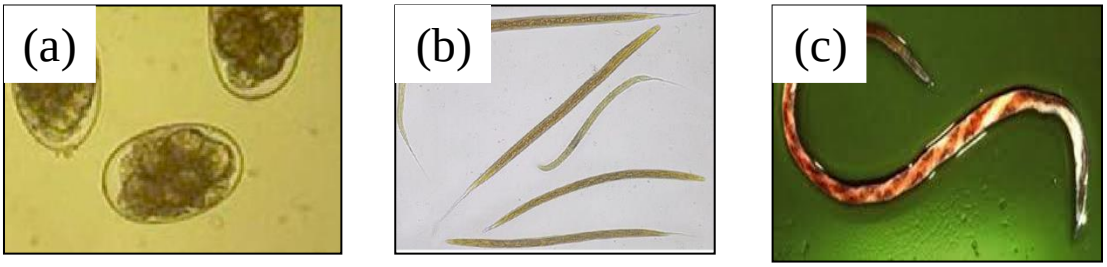


Figure 2. Different life stages of *H. contortus* are defined, (a) egg, (b) larvae or L3 stage, and (c) adult

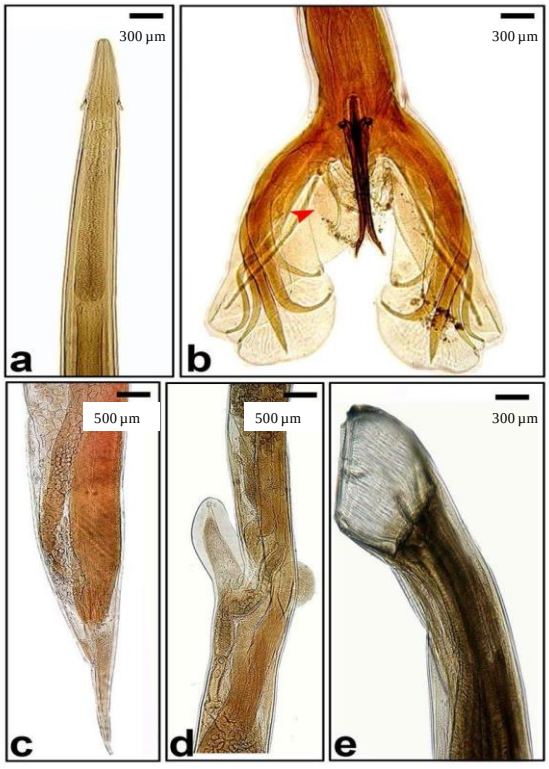


Figure 3. Morphology of *H. contortus* adult male and female worm, isolated from sheep, a. anterior end, b, bursa region of males, c, posterior end of the female worm, d, Vulvar flap, and e, capsule-shaped anterior end.

H. contortus female worms may produce a huge number of the egg of approximately 5000-15000 eggs per worm and usually prefer warm or moderate weather to develop [53]. These eggs come out of the body of the infected host through the anal route and then develop into the L1 stage and juvenile stage under 24- 29 ℃ temperature. Then during the grazing process, small ruminants, goats, and sheep get infected with L3 staged larvae. These larvae travel to the abomasum of the host and grow into L4 (preadult larvae) and further develop into adult male and female worms, feed on blood, mate, and live inside the abomasum (**Figure 4**) [51, 54].

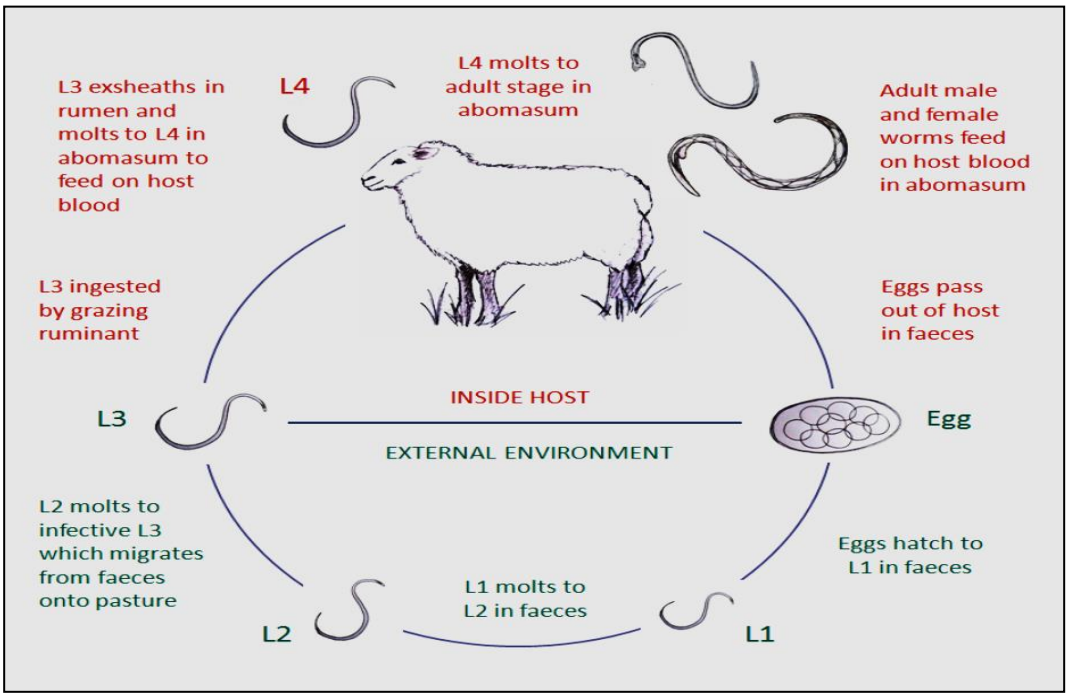


Figure 4. The life cycle of *H. contortus*, the parasitic nematode is described here, where eggs come out of the infected host through feces and develop into L2 larvae in the soil which then mature into L3 in the soil environment and further get transmitted to ruminants while grazing and develop into adult male and female worms inside the body (abomasum) of the host.

These parasites can survive in various conditions, where larval or L3 parasites can live in extreme heat, cold or inside host to develop into an adult [55]. *H. contortus* infection in blood has also been reported in many animals including goats, sheep, rabbits, humans, etc. causing inflammation and immune-modulation [56]. Very selective targeted diagnostic techniques which are used to track anemia and fecal egg count to estimate adult worm burdens infections, fecal flotation assay, and fluorescein-labeled peanut lectin staining test to specifically identify *H. contortus* from other trichostrongyle parasites [57]. The treatment of *H. contortus* infection involves the usage of various antihelminthic drugs, albendazole, ivermectin, and medical treatments such as blood transfusions and supportive care.

But resistance has been developed against all the antihelminthic drugs [58]. At present, there are only a handful of drugs available (like benzimidazole, imidazothiazole, and ivermectin) for the helminth treatment, and the development of chemoresistance is a major challenge of this field [59]. In 1964 the first report of chemo-resistance in *H. contortus* came against benzimidazole drug [60]. After that few more reports were published reporting the resistance against different antihelminthic drugs. In 1983 Cawthorne and Whitehead were reported the resistance for inhibition of microtubule

polymerization by benzimidazole in the *H. contortus* [61]. Moreover, in 1979 Sangster has reported the development of the resistance against imidazothiazole [62]. Gill, (1996) first-ever reported resistance to albendazole in parasites [63]. Further, Hoti (2009), Ram (2007) reported the resistance in presently available drugs (Albendazole, Levamisole, Fenbendazole, etc.) [63, 64]. Due to overdosing or treatment frequency of antihelminthic drugs various side effects have arisen, such as nausea, vomiting, headache, diarrhea and skin rashes etc.

Host defense can act through nonspecific mechanisms of resistance and specific immune responses. Antibody-mediated, cellular, and inflammatory mechanisms all contribute to resistance. However, many worms successfully avoid host defenses in a variety of ways and can survive in the face of otherwise effective host responses. Prevalence of infection, shortage of drugs, undesired side effects, and increasing resistance against existing drugs bring us a situation of the urgent need for new drugs.

CHAPTER 1

A literature review

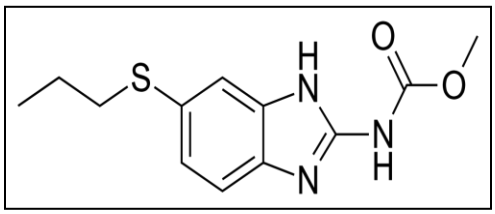
The parasitic helminthic infection is among the major Neglected Tropical Diseases (NTDs) identified by the WHO as a focus for research and control [64]. Soil-transmitted helminthiasis is caused by infections with the nematodes including *A. lumbricoides, N. americanus, A. duodenale, T. trichiura, H. contortus, T. canis*, *T. solium,* and *H. nana,* etc. An estimated 5.3 billion people are at risk, while 1.5 billion are infected with at least one of the soil-transmitted helminths [65]. Prevalence of infection is high in Asia, followed by sub-Saharan Africa and Latin America. Infected people predominantly live in poor conditions in the least developed countries, where households lack adequate facilities such as clean water, hygiene, and social awareness. Despite the huge burden large-scale control is still lacking behind the required [66, 67]. In 2001, WHO started various programs and strategies to control soil-transmitted infection in preschool and school-aged children and pregnant and lactating mothers, and in 2018, about 676 million children were treated with various antihelminthic drugs in endemic countries. Moreover, WHO has taken a mission to reduce the prevalence of moderate and heavy infections with soil-transmitted helminths in preschool and school-aged children to below 1% by 2030 [68-70]. To achieve this target school children received regular treatment of preventive chemotherapeutic drugs such as albendazole, mebendazole, levamisole, pyrantel, and ivermectin [65].

1.1 Clinically available antihelminthic drugs in practice

The recommended doses for the drugs are as follows;

- **Albendazole:** A single dose of around 400 mg is given to infected adult patients and is the same effect as mebendazole [71].
- **Mebendazole:** (100 mg for 3 days or 500 mg as a single dose). The high infection causes adverse effects which give several symptoms like an infection in the GI tract, headache, and leucopenia [72].
- **Levamisole: (**150 mg for adults and 5 mg/kg for children) is recommended safe and is effective in 77 % to 96 % of cases of *Ascaris* [73].
- **Pyrantel pamoate:** (11 mg/kg to a maximum of 1 g) is recommended as a single dose. Adverse infection effects include GI tract infection, headaches, rashes, and fever. Parasite immobilization and death are seen, although it happens slowly. Complete clearance of worms may take up to 3 days [74].
- **Ivermectin:** This medicine causes paralysis to adult worms but is not generally used [75].

1.1 .1 Albendazole



Albendazole belongs to the group of Benzimidazole, also known as 'Albendazolum' is commonly used for the treatment of intestinal infestations [76]. It was invented by Robert J. Gyurik and Vassilios J. Theodorides and handed to SmithKline Corporation for production [77]. This drug was first introduced to treat helminthic infection in sheep in 1977 and then got approved for human parasitic treatment in 1982 by FDA [78]. It is currently used for treating various diseases as well such as filariasis, hydatid disease, ascariasis, trichuriasis, and neurocysticercosis, etc [79]. Albendazole is administered orally and has a half-life in blood of around 8- 12 h [80].

The mechanism of action of albendazole involves degenerative alterations inside the body of the intestinal worms [81]. This drug binds to the β-tubulin of helminths and inhibits the polymerization into microtubules (**Figure 5**). Treatment with albendazole also disrupts cell motility and shape and intracellular transport. A single dose of albendazole is around 400 mg in adults and in children it consisting of a single dose of 10 mg/kg of bodyweight for children [79, 82]. For ruminants and cattle, 2500 mg/kg of body weight is given to the infected host but its overdose or regular use causes headaches, lethargy, anorexia, anemia, nausea, abdominal pain, bone marrow suppression, and liver inflammation [83].

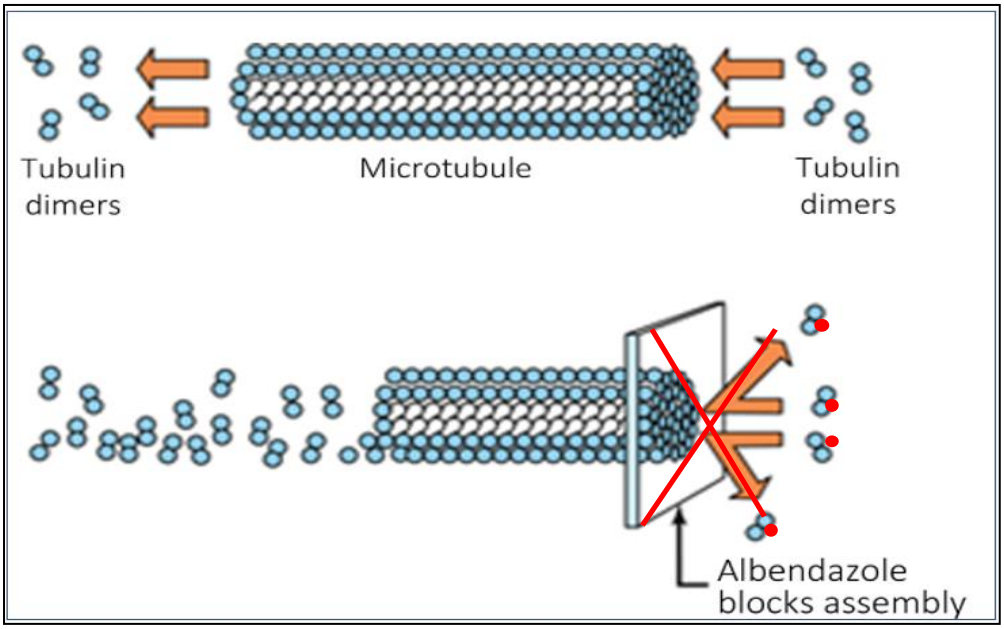


Figure 5. The mechanism of action of Albendazole is described here, where they are attached to the β tubulin and inhibit the polymerization (here red dot denotes binding of albendazole).

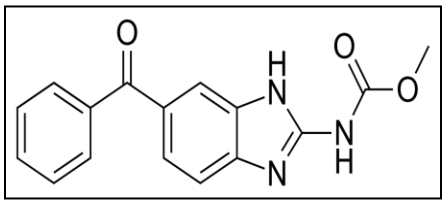
Albendazole is commonly prescribed for the treatment of parasitic infection in cattle and sheep but sometimes it is also found useful in the treatment of dogs, cats, birds, and pigs, etc. But therapeutic resistance has been reported due to intensive use of the drug. Gill in 1996 first-ever reported the albendazole resistance in sheep in India [84]. Then, Miller, 1996 showed the resistance in albendazole treatment against *H. contortus* in small ruminants [85]. Horton, 2000 explored the resistance in the efficiency of albendazole in humans to treat *T. trichuriasis* parasitic infection [79]. In 2016, Ramos reported the resistance against gastrointestinal worms in Brazil [86], and in 2018, Rashid reported the resistance in the same in Australia [87]. Moreover, Coles, 2006 detected the resistance in albendazole against *in vivo* assay of fecal egg count FEC, larval and adult stage of tapeworms using PCR technique [88]. In 2000, Jackson developed the resistance in nematodes against albendazole in sheep which further affected the non-sustainability of farming in tropical and temperate regions of the United Kingdom and Europe [89]. Taylor in 2002, developed few methods to measure the efficacy of antihelminthic resistance, FEC, larval developmental test, egg hatch assay, etc [61]. Kaplan in 2002 reported the resistance of albendazole in nematodes of horses i.e., *Strongylus vulgaris* [90]. The understanding of the resistance among the drug in parasites is crucial but a few measures listed below may be a cause of albendazole resistance.

- Treatment frequency: Where scientist suggests only 1-2 albendazole treatment per year, cattle were treated 10-15 treatments per year.
- Overdosing
- Underdosing
- Mass prophylactic
- The single-drug regime
- Transmission of resistance

In 2019, Hodgkinson showed antihelminthic resistance in the process to alleviate the threat to global food- security [91]. Ceballos in 2019 discovered egg hatch assay to be useful to measure the albendazole resistance in *Fasciola hepatica* [92]. Therefore, certain combinations with albendazole were designed such as with ivermectin, doramectin, pyrantel, etc. to overcome the problem of resistance. But these combinations also failed and resistance developed against newly synthesized drugs. Borgsteede, 2007 reported the resistance against albendazole and doramectin combination [93]. Entrocasso, 2008 reported the resistance against albendazole and ivermectin

combination drugs [94]. Moreover, Patel in 2019 showed the resistance of ivermectin and albendazole combination against *T. trichiura* in school-aged children [95]. In addition to that, Namara in 2004 reported that when albendazole is given to infected lactating mothers then 1.5 % (of the total 400 mg) of albendazole gets mixed into the breast milk which results in malnutrition and poor growth [96-98].

1.1.2 Mebendazole



Mebendazole, derived from Benzimidazole, is commonly used to treat parasites [99]. It was designed by Jansen Pharmaceuticals, Belgium, and came into use in 1971 [100]. Mebendazole is usually used to treat antihelminthic infestation, hookworm, whipworm, pinworm, guinea worm, and sometimes used as an anti-cancer drug in chemotherapy as well [101]. This drug is administered orally and has a half-life of 3- 6 h and 5- 10 % of the drug gets excreted through urine [102]. Mebendazole is usually used for a mild infection and is poorly absorbed in the bloodstream. This drug is highly effective in treating the larval and egg staged infections of the worm along with the adults. The prescribed dose of mebendazole by the FDA is 100 mg for 3days or 500mg as a single dose in adults and 10mg/kg body weight to infants [103].

Mebendazole has nearly the same mechanism of action as albendazole. It inhibits the polymerization of microtubules and the binding of β- tubulin [104]. This drug is usually well tolerated but sometimes it causes side effects that include vomiting, diarrhea, and headache, and hair loss [105].

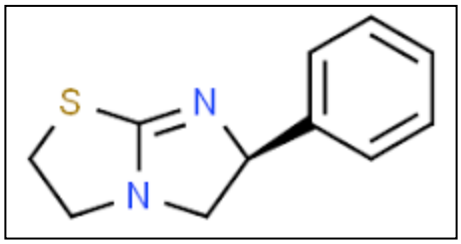
Further due to some ongoing factors a resistance has been developed against mebendazole. The hypothetical mechanism of resistance of mebendazole is due to increasing changes in β- tubulin protein, which further reduced the binding of β- tubulin with mebendazole [106]. The reasons for resistance are not exactly known but a few are listed below.

- Treatment frequency
- Overdosing
- Underdosing
- Mass prophylactic

- The single-drug regime

Due to increasing resistance, various treatment failures have been observed. Kelly in 1979 reported the resistance to treat *T. colubriformis* [107]. Clercq, 1997 reported the failure of mebendazole to treat human hookworm infestation [108]. Then Yadav in 1995 reported the multiple drug resistance in *H. contortus* of Sheep in India [109]. Bordes in 2020 showed the resistance in mebendazole combination with eprinomectin for the treatment of *H. contortus* in France [110]. Claerebout, 2020 reported the resistance in a combination of mebendazole and ivermectin in sheep in Belgium [111]. Moreover, Wang in 2020 reported the chemoresistant in T-cells for the treatment of leukemia cells [112]. Thus, to control the efficacy of parasites due to mebendazole resistance, an urgent need has arisen to design new drugs with new formulations that could easily treat the rapidly increasing helminthic infestation.

1.1.3 Levamisole

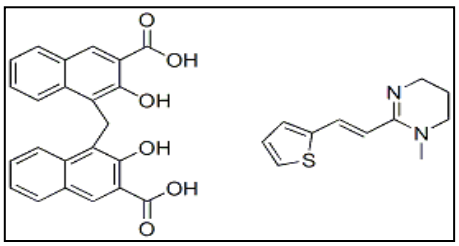


Levamisole is commonly used for helminthic infestations, usually, ascariasis and hookworm infection, and part of it have also been used to treat skin infections, leprosy, ulcers, cancer, and nephritic syndrome in children [113]. It is administered orally and has a half-life of 3-4 h and 70 % of the drug is excreted through urine only [102]. This drug was discovered by Jansen Pharmaceuticals in 1966 and got listed as an essential drug by WHO [100]. But serious side effects have been reported due to various factors against levamisole, which includes vomiting, abdominal pain, skin rashes, febrile illness, headache and sometimes causes liver toxicity. The mechanism of action of levamisole involves that it binds to the L- subtype of nicotine acetylcholine receptors in parasite muscles and further reduces the capability of males to reproduce muscles [114]. The effective dose recommended by the FDA for the treatment of parasitic infection is 150mg for adults and 5 mg/kg for children [115].

Due to various changes in animal species and humans, resistance has been developed against levamisole. This may be due to a reduction in the number of receptors that bind with levamisole. Lewis in 1980 reported the resistance in *C. elegans* [116]. Becerra-Nava in 2014 first-ever reported the resistance of levamisole in gastrointestinal nematodes in Mexico [117]. Then Barrere, 2014 showed the resistance in *H. contortus* adult and larval stages [118]. Moreover, Hu in 2009

discovered the resistance in levamisole and pyrantel combination [118]. Ruffell in 2018 and Santos in 2019 reported the resistance in *H. contortus* [119, 120].

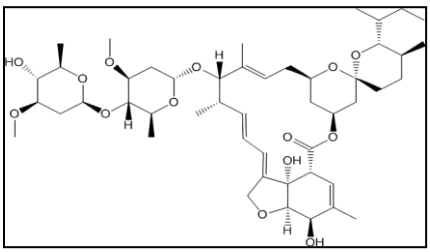
1.1.4 Pyrantel pamoate



Pyrantel pamoate is a class of drugs used to treat gastrointestinal parasitic infections [121]. It is usually used to treat pinworm, hookworm, trichostrongyliasis, and roundworm infestations [122]. Pyrantel is administered orally and has a half-life of 6- 8 h [123]. The drug was developed in 1965 and acts by depolarizing the neuromuscular [124]. The recommendation of pyrantel by the FDA is 11 mg/kg to a maximum of 1 g [125]. This drug causes paralysis in helminths and is poorly absorbed in the intestine of the host [105]. Overdose or frequent treatment causes various side effects of the drug such as dizziness, vomiting, diarrhea, abdominal cramps, nausea, loss of appetite and headache, etc [126].

Due to various factors, resistance has been developed against pyrantel and its various combinations with other drugs. Kaplan, 2002 and 2012 reported the resistance of drugs in nematodes of horses [58, 90]. In 2012, Himmelstjerna reported the resistance in equine parasites in horses [127]. In 2015, Lawson showed resistance in the treatment of parasitic infection in donkeys in the United Kingdom [128]. Moreover, in 2011 Nareaho showed resistance against the combination of ivermectin and pyrantel in strongyle infections in Finland [129]. Bodecek in 2018 discovered the resistance in avermectin and pyrantel combination against cyathostomina and ascarids in horses [130]. In 2010, Ihler reported the resistance in *Oesophagostumum spp.* in Nordic countries [131]. In 2013, Sarai reported the resistance in *H. contortus, Trichostrongylus colubriformis,* and *Teladorsagia circumcincta* worms against levamisole and pyrantel in Australia [132].

1.1.5 Ivermectin



Ivermectin is an FDA-approved, most common drug used for the treatment of helminths, heart diseases, skin, and vision problems [133]. Moreover, some scientists have also reported the use of ivermectin in treating COVID-19 [134]. It belongs

to the family avermectin and is synthesized by the mixture of two avermectin B1 derivatives [135]. Ivermectin was discovered by Campbell and started its medical use in 1981 and has a half-life of 18 h inside the body of the host [136]. This drug has the least excretion <1% in urine and has the longest absorbance than other antihelminthic drugs [137]. The mechanism of action of ivermectin involves the hyperpolarization and increased flow of chloride channels in cell membranes [138]. Ivermectin is unable to cross the blood-brain barrier due to the presence of P-glycoprotein thus it is unable to treat parasites within the brain and spinal cord [139]. According to WHO, ivermectin is the best available drug to treat onchocerciasis. Ivermectin is administered orally and a dose has 150 to 200 µg/kg body weight [140]. Due to frequent treatment resistance has developed in nematode parasites. The resistance mechanism involved mutation in P-glycoprotein and acts as a transporter in the membrane which pumps the drug inside the cell [141]. Dent in 2000 showed the resistance in *C. elegans* due to mutation in Avr-14, avr-15, and GLC-1 genes [142]. In 2004, Currie reported the *in vitro* and *in vivo* resistance in *Sarcoptes scabiei* [143]. Redman in 2012, reported the resistance in *H. contortus* population in humans [144]. Waghorn in 2016 reported the resistance in *Ostertagia ostertagi* against ivermectin in cattle in New Zealand [145] and Gall in 2018 reported resistance in *Rhipicephalus (Boophilus) microplus* in cattle [145]. Thus, due to increasing resistance in ivermectin, a need will be emerged in the future to develop a new drug for the treatment of parasitic nematodes.

1.2 Antihelminthic drugs under development

Several reports of treatment failures of human schistosomes and nematodes have been reported [106]. Partial or complete resistance has been frequently seen against drugs of benzimidazole (BZ) group because of their similar action mechanisms control of benzimidazole resistant parasites by levamisole can be expected because of its different action mode [146]. Nematodes resistant to levamisole are cross-resistant to morantel because of the similarities of their action mechanisms. The global increase in antihelminthic single or multidrug-resistant worms in humans and animals is a major health and economic concern. Despite intensive research, anthelmintic resistance mechanisms are not fully been understood yet [147]. Therefore, the development of new drugs/formulations and understanding of the mechanism of action of drugs is in prime demand to overcome the present situation.

It should first be noted that the complete cure of hookworm infection (and most other helminth infections for that matter is usually not achieved with any drug. Depending on the dosage

and the coprological method applied (with lack of standardization and control methods being a noteworthy problem), cure rates as low as 61% (400 mg) and 67% (800 mg) for albendazole, 0% (single dose) and 23% (repeated dose) for levamisole, 30% (single) and 37% (repeated) for pyrantel pamoate, 27% for thiabendazole, 19% (single) and 45% (repeated) for mebendazole have been reported [148, 149]. Thus, at least some hookworm populations and immature stage show some degree of (innate) tolerance to at least one of the drugs currently in use. The different susceptibilities of the two species *A. duodenale* and *N. americanus* are well established. Most probably, the degree of tolerance varies regionally, even locally, within a species and some of the encysted parasites (hypobiotic larvae) may not be impacted by drug use [150].

Depending on local epidemiology, availability, and cost, these drugs have been widely available in most health care systems for the curative treatment of clinical cases for many years. Also, the use of anthelmintics is now being strongly advocated in a preventive, population-based way as well [151].

1.2.1 Plant extract

The use of natural resources to treat various communicable and non-communicable diseases is a practice from pre-historical ages [152]. Various herbal formulations are derived from chamomile, nettle, shatavari, garcinia, neem, karela, pippali, ashwagandha, ajwain, fenugreek, wormwood, etc., are documented in Indian, Chinese, Vietnamese, and African traditional medicine [153, 154]. These herbal formulations are rich in various secondary metabolites, including various polyphenolic compounds like alkaloids, flavonoids, tannins, etc., which are documented to have antihelminthic activity [155]. Despite the prevalence of helminthic infections, scarcity of medication, and profound documentation of traditional medicines, only a handful of those natural resources have ever been explored for their potential in modern medicine.

Eguale et al. (2011) performed *in vitro* antihelminthic activity using hydro-alcoholic and aqueous extracts of *Coriandrum sativum* seeds for the investigation of inhibition of egg hatch and growth in the adult stage [156]. Maciel et al. (2006) used the ethanol extract of the leaf and seed of *Melia azedarach* plant on *H. contortus* larval growth inhibition and egg hatching to confirm its antihelmintic activity [157]. Pessoa et al. (2002) used *H. contortus* eggs for checking *Ocimum gratissimum* antihelminthic activity on the development of larva from eggs [158], whereas Tomar and Preet (2017) synthesized silver nanoparticles using *Azadirachta indica*, an aqueous extract, to analyze the antihelminthic activity against *H. contortus* [159]. Kumara Singha et al. (2016)

performed antihelminthic activity on *H. contortus* using various ethnomedicinal plants like *Lansium domesticum*, *Linariantha bicolor*, and *Tetracera akara* [160]. Ahmed et al. (2020) used traditional medicinal plants like *Artemisia herba-aba* and *Punica grantum* to study the antihelminthic activity of *H. contortus* worms [161]. Moreover, Zenebe et al. (2017) studied the *in vitro* antihelminthic activity of gastrointestinal worms with the crude extracts of *Cissus quadrangularis* and *Schinus molle* [162].

In India (described in the Atharva Veda) herbs are being used as a traditional medicine from 1500 BC, and in China, the use of herbs for therapeutics is as old as 2700 BC (described in Neijing Sawan). In the present day, almost 90% of the rural population depends on herbal medicines in developing countries [163]. The use of herbs in therapeutics is becoming very popular and accounts for a trade of US$ 85 billion annually worldwide which is expected to grow to U$ 117 billion by 2024. Several medicinal plant-based treatments with *Allium sativum, A. cepa,* and *Mentha arvensis* species came into consideration for the development of new drugs to control gastrointestinal parasitism [164-168]. Many seed oils such as pomegranate oil, grape oil, carvacrol, eugenol, cumin oil had been reported to show their antihelminthic activities [169-173]. But some essential oils may be toxic and highly volatile for example, *Mentha arvensis* and *Chysanthemum zawadzki* may cause even mortality to the treated livestock [174-178]. Tanaka et al., 2002 isolated different onoceranoid triterpenes and studied their toxicity against brine shrimp [179]. Ruhisha and Choudhury, 2017 showed the presence of different phytochemicals using various plant extracts [180]. Potipiranun et al., 2018 showed the isolation of different plant compounds from the ethyl acetate extract and studied its activity against maltase [181]. Moreover, Ramadhan et al., 2018 showed the antioxidant activity and α-glucosidase effect with different compounds isolated from Lansium [182].

Plant-based drugs have created a keen interest among the scientist to control gastrointestinal parasites [183]. This is due to emerging antihelminthic resistance in the nematode population and growing concern about the presence of drug residues in animal products [184]. The parasitic helminth infection is mostly found in countries with poor hygiene and low economy [185]. Therefore, antihelminthic treatment remained a challenge for companies due to low cost-effectiveness. Antihelminthic should be used in the majority of treatments where very scarce clinical support is being found. From the last couple of decades, chemoresistance has been developed among the presently available albendazole, benzimidazole, and ivermectin [186]. However, a large number of plants-based treatments are available for parasitic infections but new potential plants are highly needed to overcome the increasing efficacy of helminths. Moreover, more mechanistic studies are also required to unravel the novel mechanisms of active principles.

1.2.2 Pure compounds

Different pure compounds (natural or synthetic) have also been reported for their antihelminthic activity [163]. Fei in 2018 reported the antiparasitic activity in *Ascaris suum* and *Trichuris suis* when treated with lactone antibiotics [187]. Setoguchi, 2012 reported the antihelminthic activity of phenylureido against *Ascaris spp.* [188]. Xu in 2020 explored the antiparasitic activity of Radicicol, a lead compound, against praziquantel resistant *Schistosoma japonicum* [189]. Pereira in 2020 and Probst in 2020 showed the antischistosomal activity with aurone derivatives, a type of flavonoids, and phenylethanolamine derivatives, respectively against the larval stage and adult worms of *S. mansoni* [190, 191]. Rehman in 2020 reported the antiparasitic activity against adult *F. gigantica* worms and In 2016, Miyasaka showed the antihelminthic activity against *C. elegans* when treated with curcumin [192, 193]. Lam in 2018 explored the antihelminthic activity with Artemisinin against *Schistosoma spp.* in both humans and animals [194]. In 2014, Bah explored the antiparasitic activity with oxytetracycline against Onchocerciasis, caused by filarial nematode [195]. Nixon in 2019 showed the antihelminthic activity with antitrypanosomal diarylamidines, diminazene, and pentamidine against *H. contortus* worms [196]. In 2020, Dar explored the potential of sodium stibogluconate with ketoconazole for the treatment of leishmaniasis [197]. In 2009, Verma showed treatment of the benzimidazole and avermectins resistance in *A. suum* using neuropeptide (AF2) [189].

Tannins derived from diverse plant sources show high toxicity against anthelmintic effects. Williams in 2014 showed the treatment of *Oesophagostomum dentatum*, a parasite affecting the pigs using naturally extracted tannins [198]. Quijada in 2015 reported the treatment of *H. contortus* larval and adult stage using plant-derived tannin, prodelphinidins [199]. In 2017, Fomum showed the *in vitro* treatment of nematodes using alkaloids and tannins extracted from *Crinum macowanii, Gunnera perpensa, Nicotiana tabacum, Sarcostema viminale, Vernonia amygdalina, Zingiber officinale, and Zizyphus mucronate* [200]. Moreover, Assefa in 2017 published the report on the treatment of larval and adult stages of *H. contortus* using condensed tannins extracted from *Balanites aegyptiaca, Tamarindus indica, and Celtis toka* [201]. Despite the ongoing treatments with pure compounds, the mechanism of action is still unknown therefore, new research aims to develop drugs for the treatment of human and animal parasitic worms and study their mode of the mechanism of action.

1.2.3 Nanoparticles

According to popular belief, green synthesized nanoparticles give an advantage over chemically synthesized nanoparticles using reduction of toxicity and use of hazardous chemicals [202]. A variety of green nanoparticles with well-defined chemical composition, size, and morphology have been synthesized by different methods and their applications in many cutting-edge technological areas have been explored in recent days. All though the efficiency of nanoformulation for their antihelminthic activity has never been explored, various new reports are coming up showing potential benefits/ toxicity of nanoformulation against various pathogenic organisms and vectors such as moths, beetles, lice, mosquitoes [203, 204].

Furthermore, the recent advancement of nanotechnology in the field of healthcare came up with new hope for the management of infectious diseases like helminth infections. Rashid et al. (2016) performed antihelminthic activity using *Momordica charantia*-coated silver nanoparticles against gastrointestinal worms [205]. Shakir et al. (2015) used cadmium-coated nanoparticles and observed the antiworm for parasitic worms [206]. Kar et al. (2014) used *Nigrospora oryzae*-coated gold nanoparticles to check the antihelminthic activity of cestodes [207]. Barbosa et al. (2019) showed the nematicide activity of silver nanoparticles coated with *Duddingtonia flagrans* on the larval stage of *A. caninum* [208]. André et al. (2020) performed antihelminthic activity on the adult stage of *H. contortus* using carvacrol-coated chitosan nanoparticles [209]. Ejaz et al. (2017) used *Artemisia vulgaris* reduced silver nanoparticles to check the anti-worm effect on various nematodes [210]. Rehman et al. (2019) used silver nanoparticles coated with *Tribulus terrestris* to observe the antihelminthic activity of flukes [193]. Preet and Tomar (2017) bio-fabricated silver nanoparticles using *Ziziphus jujube* for *in vitro* adulticidal and egg hatch assay for the same worm [211].

Therefore, in this work, we would like to explore the potential of natural resources, synthetic drugs, and their nano-formulation for antihelminthic activity against parasitic worms. Based on the above research gaps and resistance to drugs against the antihelminthic activity, this project proposal aims to develop new drugs from various types of medicinal plant extracts, nano-based formulations, and various synthetic compounds and study its antihelminthic activity. The research aims to develop drugs for the treatment of human and animal parasitic worms and study their mode of the mechanism of action.

CHAPTER 2

Materials and Methods

2.1. Synthesis of Plant aqueous protected silver nanoparticles (AgNPs)

2.1.1 Materials

$AgNO_3$ was procured from Sigma Aldrich, USA. RPMI, DMEM, Antibiotics, Fetal bovine serum was purchased from Himedia Ltd., India. All other reagents were of analytical grade were purchased from Loba Chemie, India.

2.1.2. Collection and preparation of Lansium parasiticum silver nanoparticles (LAgNPs)

Fresh fruits of 'Latka' were collected during June and July from the local market of foothills of eastern-Himalaya. The fruits were washed several times in water and then the cover of the fruits, pulps, and seeds was separated from each other and was oven-dried for 8-10 days at 60 ^{o}C. Dried tissues were grounded into powder and stored at room temperature in air containers for further use.

3 gm of the dried pulp plant powder was boiled in 100 ml distilled water for 60 mins. The aqueous *L. parasiticum* extract (ALE) was then cooled and filtered using Whatman filter paper No.1 and stored at 4 ℃ for future use. LAgNPs synthesis was carried out by using ALE as a reducing and capping agent. For the preparation of nanoparticles, 240 µM $AgNO_3$ was added dropwise in 5000 µl of ALE (pH 7.0) and was kept under sharp sunlight in slow starring conditions for 30 minutes for the reduction of Ag^+ ions into Ag^0.

2.1.3. Preparation of Leaf Extract of Selaginella moellendorffii (SAgNPs)

The *Selaginella moellendorffii* plant was collected from the forests of Kasauli (H.P). The leaves were washed thoroughly with tap water and distilled water 2-3 times and dried at room temperature at 37 ℃ overnight. Nearly 3 gms of the dried leaves were suspended in 100 ml distilled water and boiled in a water bath at 60 ℃ for about 60 min or till the color of the extract changed to yellow. The extract was filtered using Whatman filter paper No.1 and a clear solution was obtained and then refrigerated at 4 ℃ for further experiments. Sterile conditions were maintained throughout the experiment against contamination.

2.1.4 Physical Characterization of AgNPs

Thus formed AgNPs were characterized using surface plasmon resonance (SPR) pattern under UV- vis spectra ranging from 300–700 nm using Specord 205 spectrophotometers. The average hydrodynamic diameter and stability of biogenic AgNPs were determined using dynamic light scattering (DLS) and Zeta potential studies by Microtrac's DLS model Nanotrac. Shape and size were examined by transmission electron microscopy (TEM) using JEOL-2100, JEM USA. Fourier Transform Infrared Spectroscopy (FTIR) analysis of AgNPs and ALE was done to find out the possible functional groups of biomolecules involved in AgNPs protection and was recorded using Agilent Resolution Pro-carry 660.

2.2 Toxicity profiling of LAgNPs and SAgNPs on normal human cells

Human embryonic kidney cancer cells HEK239 were maintained and test cultured in the DMEM (Dulbecco's Modified Eagle Medium) medium supplemented within 10% fetal bovine serum in presence of 5% CO_2 at 37 ^oC. The cells were seeded in 96 well plates and treated with LAgNPs and SAgNPs at ~70 % confluence for 24 h. After treatment 2 µM; MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution was added and incubated for 3 h at 37 ^oC. The final results were then observed by dissolving formazan crystals in DMSO (Dimethyl sulfoxide) and measuring the absorption at 570 nm [212, 213].

2.3 Collection of H. contortus

Adult *H. contortus* were isolated from the abomasa of infected ruminants. After collecting the infected abomasa from the slaughterhouses from Ludhiana, Punjab, India, they were readily washed thoroughly with running water to remove unwanted contaminating material. Further, the abomasum was dissected and the content was washed with the sieve and parasitic helminths were identified and picked with a fine brush and immediately transferred to 1X phosphate buffer saline (PBS) at pH 7.4 and identified [214].

2.3.1 Isolation of eggs of H. contortus

The eggs of *H. contortus* were retrieved from the female worms isolated from the abomasa of the infected goat from the slaughterhouse. Female worms separated based on morphological characteristics and were washed with a 1X PBS. Then centrifugation for 15 min at 11,000 x g was

done to collect the selected worms. The process was repeated twice with fresh sterile saturated salt solution and the topmost fluid containing eggs was collected. Finally, a suspension was diluted in 1ml of 1X PBS to obtain a concentration of 200 eggs/ml using the McMaster technique [215] and stored at 4 ^{o}C for further use.

2.3.2 Harvesting L-3 Larvae of H. contortus

The Larval (L-3) stage of *H. contortus* was isolated from positive fecal samples of infected ruminants after its culture over a week. The eggs isolated from the female worms of *H. contortus* were also cultured for collecting L3 larvae. In a nutshell, the fecal samples were mixed with sterile 0.8% saline and slightly homogenized to obtain adequate oxygenation to promote better hatching of the eggs. The fecal cultures were then incubated for 7 days at 25 ^{o}C temperature in dark with regular water supplementation to avoid drying. Thereafter the infective larvae containing saline water were collected from fecal effluent and stored at 4 °C for further use. The L3 larvae of *H. contortus* were cleaned before drug treatment by centrifugation by adding 0.2 % sodium hypochlorite to remove adherent bacteria. Finally, they were used in the *in vitro* bioassays for larval mortality [216].

2.3.3 Adult mortality assay (AMA)

Mortality assay of *H. contortus* adults was performed by taking 15 male and 15 female worms each, in 35 mm culture plates in presence of various concentrations of LAgNPs (15.8, 31.7, 63.5 and 158.7 nM), SAgNPs (24.7, 49.5, 123 and 247.5 nM), CA (37, 74, 185, 370 and 740 µg/ml/ 0.2, 0.5, 1, 2.5 and 5 mM) and quercetin (0.125, 0.2, 0.5, 1 and 2 mM) against RPMI media and Alb (500 µg/ml) for 24 h in 1X RPMI (Roswell Park Memorial Institute- 1640, Lot: 98H83171) media. After the incubation, direct microscopic counting (at 40X magnification) was done for mobility (for 60 secs), response to physical stimuli, and morphology to monitor the mortality of the worms. The observations were made at regular time intervals (0, 1, 3, 6, 12, and 24 h). The paralysis of worms was observed when there was no visual movement at room temperature (37 °C) after moderate agitation. Further death of worms was confirmed by observing the response to heat shock at 50 °C in 0.8% saline for 10 secs. Thereafter, Lugol's staining was performed to check the morphological changes [217]. Results were represented as an average of 3 independent experiments.

2.3.4 Larval mortality assay (L3)

In vitro mortality test of *H. contortus* larvae (L3) was performed by taking larvae (Approximately 25- 30) in 200 µl of RPMI medium in 96 well plates in presence of various concentrations of LAgNPs (15.8, 31.7, 63.5 and 158.7 nM), SAgNPs (24.7, 49.5, 123 and 247.5nM), CA (37, 74, 185, 370 and 740 µg/ml) and quercetin (0.125, 0.2, 0.5, 1 and 2mM) and Alb (500 µg/ml) for 24 h. After the incubation, direct microscopic examination (at 40X magnification) was done for mobility (for 20 secs), response to physical stimuli, and morphology to monitor the mortality of the worms. Motile and spread larvae were considered as live otherwise considered as dead. Thereafter, Lugol's staining was performed to check the morphological changes. Results were represented as an average of 3 independent experiments. The mortality percentage of larvae L3 (% ML3) was calculated by the following formula (i):

% ML3 = [(number L3 dead/ (number of L3 dead + number of live L3)]* 100 (i)

2.3.5 Egg hatch inhibition assay (EHA)

The inhibition of the hatching of eggs is very important for an effective antihelminthic compound. Egg suspension (1 ml) containing nearly 200 eggs was placed into 24 well microtiter plates, subsequently, to check the inhibition efficiency of drugs, eggs were treated at different concentrations of LAgNPs (15.8, 31.7, 63.5, and 158.7 nM), SAgNPs (24.7, 49.5, 123 and 247.5nM), CA (37, 74, 185, 370 and 740 µg/ml) and Quercetin (0.125, 0.2, 0.5, 1 and 2 mM) against two controls, one positive with 500 µg/ml of Alb and another negative with RPMI media. After the inhibition treatment, the plates were incubated at 28 °C for 48 h. After 48 h, 10 µl of Lugol's iodine drop was added for the discontinuation of the process of hatching, and the final count of the number of eggs hatched or inhibited was obtained under the microscope. Results were represented as an average of 3 independent experiments. Further, the total larvae that came out in each well were also counted and the percentage of inhibition (survival) of egg hatching (% EHI) was determined by the following formula (ii):

% EHI = [(number of larvae/eggs hatched)/ (total number of eggs)] * 100 (ii)

2.4 SEM evaluation for physical damage of H. contortus

The physical damage due to the toxic effect of AgNPs (157.8nM LAgNPs, 247.5 nM SAgNPs, 370 µg/ml CA and 1mM quercetin) on *H. contortus* was visualized using scanning electron microscopy. The helminths were treated with AgNPs and synthetic compounds and with controls for 12 h and then were washed with 1 X PBS. After washing, control and treated helminths were dehydrated using ethanol concentration gradients from 50 % to 100 %. After dehydration, the samples were coated using gold (15 µm) under a specific vacuum condition. The gold-coated specimens were then observed using an SEM (JEOL JSM 6490LV) at electron accelerating voltage at 15 (kV).

2.5 Total protein concentration measurement

The concentration of total protein in *H. contortus* was measured by using the Bradford method [218]. In short 250 mg of treated helminth tissue samples were homogenized with 500 µl of RIPA buffer (Radioimmunoprecipitation assay). Protein concentrations were estimated from the clear supernatant of the homogenized mixture after centrifuging at 10,000 rpm for 15 mins. BSA (Bovine serum albumin) standard curve was used as a standard.

2.6.1 Generation of oxidative stress by the measurement of reactive oxygen species (ROS) due to newly synthesized drug stress

The cellular response was confirmed by the stress caused by the newly synthesized drugs and observed the balance between the production of ROS (free radicals) and antioxidant defenses. *Haemonchus contortus* adults (male and female) were treated with different concentrations of LAgNPs (158.7 nM), SAgNPs (247.5 nM), CA (370 µg/ml), and quercetin (1 mM) for 3 h, and thereafter different treated parts of the parasite were identified. Sectioning of the particular identified treated parts was done and further treated with 100 nM of DCFDA (2', 7' – dichlorofluorescein diacetate) and incubated for 20 min in dark at 37 ^{o}C. After incubation samples were washed with distilled water and fixed in the slide for fluorescent imaging using a Dewinter fluorescence inverted microscope. Anterior and posterior end (differentiating the male and the female), and complete body structure was specifically observed to compare the ROS generation of treated helminths against a set of controls [213, 219, 220].

2.6.2 Generation of oxidative stress by the measurement of NOS

NOS (Nitric Oxide synthase) mediates the interactions between the reductase and the oxygenase domains, having several layers of regulation by involving enzymatic activities. Therefore, the hindrance of superoxide dismutase (SOD), reduction of glutathione, and loss of other enzymatic activities could allow the increase in reactive oxygen species, which in turn further improve the toxicity level of nitric oxide synthase. Thus, for nitric oxide generation, adult *H. contortus* were treated as before for 3 h and NOS was determined from the total protein content. Then in 0.05 ml of the above-homogenized sample, 0.1 ml of Greiss reagent was added (prepared by mixing two stocks a) N (1- naphthyl) ethylenediamine dihydrochloride in distilled water and b) 1% sulfanilamide and 5% orthophosphoric acid, mixing both a and b in 1:1 ratio). After the addition of the Greiss reagent, the test reaction was incubated at 37 ℃ and observed spectrophotometrically at 540 nm at different time points, i.e. 30 min, 6 h, 12 h, and 24 h [221].

2.7 Measurement of antioxidant enzymes activity

During normal metabolic functions, the highly generated free radicals react with the cellular molecules which induce the oxidative damage defense mechanisms which include the following: CAT, SOD, GPx, and thiol-specific antioxidants. To monitor the changes the following estimation were performed;

2.7.1 Estimation of superoxide dismutase activity

After the protein extraction, 25 mg of protein (in 1 ml 1 X PBS) was incubated with 1.4 ml aliquot of reaction mixture (50 mM phosphate buffer pH 7.4, 20 mM Methionine, 1% (v/v) Triton X-100, 10 mM Hydroxylamine hydrochloride, 50 mM EDTA) at 37 ℃ for 5 min. Then 50 µM of riboflavin was added and exposed to the 200W white fluorescent lamp for 10 min. Thereafter 1 ml Greiss reagent was added as the final step and absorbance was measured at 543nm [222]. The SOD activity was determined by using the following equation (iii)

$$\text{Enzymatic activity} = \frac{\text{Absorbance} \times \text{Vol. of assay} \times \text{Dilution factor}}{\text{Vol. of enzyme} \times \text{Time (in minutes)}} \quad \text{(iii)}$$

2.7.2 Estimation of catalase activity

Worm protein was extracted as before. Thereafter reaction mixture with 0.2 M; H_2O_2 prepared in phosphate buffer (pH 7) was added to pre-incubated helminth protein 1 ml (25 mg/ml) along with control. This was followed by the addition of dichromate acetic acid solution (v/v) after 0, 30, 60, 90 secs heating of the above mixture was done for 10 min and color change of the solutions was monitored measuring absorption at 610 nm against positive control, RPMI media solutions as negative control and 500 µg/ml Alb [223]. Total enzymatic activity was determined as before the following equation (iii).

2.7.3 Estimation of glutathione peroxidase activity

The reduction ability of glutathione peroxidase was measured and calculated using 25 mg of protein extracted from the treated helminths. Then 0.2 ml of Tris buffer, 0.2 ml EDTA and 0.1 ml of sodium azide (NaN_3) in 0.5 ml tissue were homogenates. Following this 0.2 ml glutathione and 0.1 ml of H_2O_2 and incubated at 37 ^oC for 10 min. Then 0.5 ml trichloroacetic was added and centrifuged to dissolve the pellet. The supernatant was collected and Na_2HPO_4 and DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid) were added and absorbance was observed at 412 nm against positive and negative controls [224].

2.7.4 Estimation of total glutathione concentration

The oxidative stress was measured directly proportional to the increasing concentration of test compounds. The reduction of glutathione was calculated using 1.0 ml of 10% tissue homogenate and was precipitated using 4.0 ml metaphosphoric acid. Precipitation was removed after centrifugation and 2 ml of supernatant, 2 ml Na_2HPO_4, and 1 ml of DTNB were added and absorbance was observed at 405 nm against a blank [225].

2.8 Statistical Analysis

Data were presented as the mean of at least five independent experiments. Statistical analyses of data were conducted in MS Excel. The student's t-test was performed to compare between the groups. Measurements were considered statistically significant if the corresponding p-value was <0.01.

CHAPTER 3

The antihelminthic activity of medicinal plant extracts

The use of traditional plant extracts to treat various communicable and non-communicable diseases is a practice from pre-historical ages. Various plants contain medicinal properties through which novel formulations are derived such as chamomile, nettle, shatavari, garcinia, neem, karela, pippali, ashwagandha, ajwain, fenugreek, wormwood, etc., are documented in Indian, Chinese, Vietnamese, and African traditional medicine. These herbal formulations are rich in various secondary metabolites; including various polyphenolic compounds like alkaloids, flavonoids, tannins, etc., which are documented to have antihelminthic activity. Here, *Lansium parasiticum* and *Selaginella moellendorffii*, very commonly known plants are first ever used for the treatment of helminthic parasites (**Table 2**).

Table 2. Medicinal plants and their traditional uses

Plant	**Family**	**Traditional uses**	**Secondary Metabolites**	**Ref**
Lansium parasiticum	Meliaceae	dysentery and malaria	alkaloids, flavonoids, tannins	[182]
Selaginella moellendorffii	Selaginellaceae	bleeding and chronic inflammation, such as arthritis, gonorrhea, hepatitis, and mastitis	phenolic acids, terpenoids, steroids,	[226]

3.1 Aqueous extract of *Lansium Parasiticum* fruit pulp

Lansium parasiticum also known as ‘latkan’ or ‘latko’ in West Bengal and Burmese grape worldwide [181, 182]. The tree is found wild or cultivated in the sub-Himalayan tract in eastern India from Bihar to Arunachal Pradesh and in the lower hills and valleys of Meghalaya, Assam, Nagaland, Manipur, Mizoram, Tripura, and Orissa, ascending to an altitude of 900 m, and in Andaman and Nicobar Islands, chiefly in the moist tropical forests [227] (**Figure 6**).

Latka Burmese grape has been exploited to a very small extent as far as the genotype and its physicochemical properties are concerned so until now there is very limited literature available regarding this plant. The different parts of the plant are used in various treatments: bitter seeds can be pounded and mixed with water to make a deworming and ulcer medication. The bark of the tree is used to treat dysentery and malaria; the powdered bark can also be used to treat scorpion stings. The fruit's skin is used to treat diarrhea, and in the Philippines, the dried skin is burned as a mosquito repellent. The skin, especially of the langsat variety, can be dried and burned as incense [228].

Figure 6. Fruits of *Lansium parasiticum* also known as ‘Latkan’ or ‘Latko’ are shown that grow usually in temperate regions and are known as Burmese grape worldwide.

3.1.1 Preliminary Phytochemical assay of Lansium parasiticum

Various plant extracts (hexane, chloroform, ethyl acetate, methanol, and aqueous) are tested for the presence of different phytochemicals. Following tests are performed for the phytochemical essay (**Table 3 and 4**).

Test for Alkaloids- The extract was dissolved in dilute HCl separately, filtered and filtrates were tested for the presence of alkaloids.

- *Mayer's test*- The filtrates were treated with a few drops of Mayer's reagent (2 gm I_2 + 6 gm KI in 100 ml H_2O). The formation of yellow-colored precipitates confirmed the presence of alkaloids.
- *Wagner's test*- The filtrates were treated with a few drops of Wagner's reagent (1.36 gm $HgCl_2$ + 5 gm KI in 100 ml H_2O). The formation of reddish/brown precipitates indicates the presence of alkaloids.

Test for Carbohydrates- The extract was dissolved in distilled water, filtered and filtrates were tested for the presence of carbohydrates.

- *Benedict's test*- The filtrate was treated with a few drops of benedict's reagent (10 gm Na_2CO_3 + 17.3 gm $Na_3C_6H_5O_7$+ 1.7 gm $CuSO_4$). The formation of orange-red precipitates indicates the presence of carbohydrates.

Test for Flavonoids-

- *Alkaline reagent test*- Each extract was tested with 2 ml of 1M NaOH. The formation of intense yellow color which disappears with the addition of an equal amount of acid indicates the presence of flavonoids.
- *Lead acetate test*- Each extract was treated with a few drops of lead acetate and the formation of a yellow precipitate indicates the presence of flavonoids.

Test for Glycosides-

- *Modified Borntrager's test*- The extracts were treated with 1-2 ml of $FeCl_3$ and then heated over the water bath for 5 minutes. This solution was allowed to cool and then extracted with equal volumes of benzene. The benzene layer was separated and treated with a few drops of ammonia solution. Pink coloration indicates the presence of glycoside.

Test for Saponins

- *Froth test*- The extracts were diluted with distilled water in a graduated cylinder up to 10 ml and this was shaken for 5 minutes. The formation of a foam layer of at least 1 cm indicates the presence of saponins.

• *Foam test*- 2-3 ml of extract was shaken with water in a test tube for 5 minutes. If the foam persisted for 10 minutes, it indicates the presence of saponins.

Test for Phenols-

• *Ferric Chloride test*- The extracts were treated with 1-2 ml of $FeCl_3$ solution. The formation of bluish-black precipitates indicates the presence of phenols.

Test for Tannins- The extracts were dissolved in 5 ml distilled water for the following tests and filtered.

• *Lead Acetate test*- To 1-2 ml of filtrate a few drops of 1% lead acetate were added. The formation of yellow precipitates infers the presence of tannins.

• *$FeCl_3$ test*- To 1-2 ml filtrates, a few drops of 5% $FeCl_3$ was added. The formation of a green precipitate indicates the presence of tannins.

Test for Steroids- Each extract was dissolved in 5 ml chloroform and filtered. The filtrate was analyzed for the presence of steroids.

• *Salkowaski's Test*- The filtrate was treated with a few drops of H_2SO_4, shaken for a few minutes, and allowed to stand undisturbed for some time. The appearance of a golden yellow coloration indicates the presence of steroids.

• *Libermann Burchard's test*- The filtrate was treated with a few drops of acetic anhydride. The solution was boiled and then allowed to cool. To this a few drops of conc. H_2SO_4 was added. The appearance of a brown ring at the junction indicates the presence of steroids.

Test for Proteins and Amino Acids-

• *Xanthoprotic test*- The extracts were treated with a few drops of nitric acid. The appearance of yellow color indicates the presence of proteins.

Test for Oils and Fats-

• *Filter paper test*- Different extracts were pressed between filter papers. An oil stain on the filter paper indicates the presence of oils and fats.

Table 3. Phytochemical Analysis of fruit pulp extract of *L. parasiticum*

S. No.	Test Performed	Hexane Extract	Chloroform Extract	Petroleum Ether	Ethyl Acetate Extract	Methanol Extract	Aqueous Extract
1	**Alkaloids**						
Mayer's Test		-	+	+	+	+	+
Wagner's Test		-	+	+	+	+	+
2.	**Carbohydrates**						
Benedict's Test		-	+	-	-	+	+
3.	**Flavonoids**						
Alkaline R. Test		-	+	+	-	+	-
Lead Acetate Test		-	+	+	-	+	-
4.	**Glycosides**						
Borntrager's Test		-	-	-	-	-	-
5.	**Saponins**						
Froth Test		-	-	-	-	+	+
Foam Test		-	-	-	-	+	+
6.	**Phenols**						
$FeCl_3$ Test		-	-	-	+	+	+
7.	**Tannins**						
Lead Ac. Test		-	-	-	-	+	+
$FeCl_3$ Test		-	-	-	-	+	+
8.	**Steroids**						
Salkowski Test		+	+	-	+	-	+
Liebermann's Test		+	+	-	+	-	+
9.	**Proteins**						
Xanthoprotic Test		-	-	-	-	-	+
10.	**Oils and Fats**						
Filter Paper Test		-	-	-	-	+	+

Table 4. Phytochemical composition of the aqueous fruit pulp extract of *L. parasiticum*.

Phytochemicals	**Total amount (mg)**
Alkaloids	0.64
Flavonoids	2.56
Terpenoids	0.52
Tannins	1.55

3.1.2 Experimental design for collection and treatment of *H. contortus* worms

Adult *H. contortus* worms were isolated from the abomasa of infected ruminants. The adult male and female worms were treated with an aqueous extract of *L. parasiticum* and compared against albendazole (Alb) and control (RPMI Media) for 24 h.

The detailed procedure for the paralysis and death time analysis has been discussed in Chapter 2 (2.3.3).

3.1.3 Results:

Table 5. Paralysis and Death time analysis of ALE on adult *H. contortus*. Fifteen worms (male and female) were exposed to ALE and Alb in RPMI media for 24 hr. Death and paralysis of worms

were analyzed at various time points. The table shows the number of worms affected per culture plate.

Time of Exposure (h)	Paralysis Time			Death Time		
	Male worms					
	Control	Alb	ALE	Control	Alb	ALE
0	-	-	-	-	-	-
0.5	-	-	-	-	-	-
1	-	-	-	-	-	-
3	-	-	-	-	--	-
6	-	-	5±0.5	-	-	-
12	-	-	10±0.5	-	-	-
24	1	-	12±0.5	-	-	0.7 ± 0.7
Female worms						
0	-	-	-	-	-	-
0.5	-	-	-	-	-	-
1	-	-	-	-	-	-
3	-	-	-	-	-	-
6	-	-	3±0	-	-	-
12	-	-	5±0.5	-	-	-
24	-	-	8±0.5	-	-	-

3.2 Aqueous extract of *Selaginella moellendorffii* leaf

Pteridophytes are considered as one of the oldest groups of plants that are present on the earth and they consist of a large group of vascular cryptograms [229]. They have made the group fascinating because of their position between the lower cryptograms and the higher vascular plants [229]. They are present in the Coastal and the Himalayan regions of India and they prefer moist and shady habitats with moderate temperature conditions. Out of 1,200 species of the Pteridophytes that are present in India, about 170 species are used for medicine, food, oil, dye, bio-fertilizer, flavor, fiber, and the production of biogas, etc [230].

Selaginella moellendorffii is an ancient plant with vascular lineage which formed the largest section in the world during the Carboniferous period [231]. These pteridophytes do not have true leaves and roots but have microphylla instead (**Figure 7**). Due to the unavailability of enough knowledge on this plant, very limited research has been done [232].

Figure 7. Leaves of *Selaginella moellendorffii* belonging to the family Selaginellaceae are shown above, usually grown in cold regions.

3.2.1 Preliminary Phytochemical assay of *Lansium parasiticum*

Various plant extracts (hexane, chloroform, ethyl acetate, methanol, and aqueous) are tested for the presence of different phytochemicals. Following tests are performed for the phytochemical essay (**Table 6**).

Test for Alkaloids- The extract was dissolved in dilute HCl separately, filtered and filtrates were tested for the presence of alkaloids.

- *Mayer's test*- The filtrates were treated with a few drops of Mayer's reagent (2 gm I_2 + 6 gm KI in 100 ml H_2O). The formation of yellow-colored precipitates confirmed the presence of alkaloids.
- *Wagner's test*- The filtrates were treated with a few drops of Wagner's reagent (1.36 gm $HgCl_2$ + 5 gm KI in 100 ml H_2O). The formation of reddish/brown precipitates indicates the presence of alkaloids.

Test for Flavonoids-

- *Alkaline reagent test*- Each extract was tested with 2 ml of 1M NaOH. The formation of intense yellow color which disappears with the addition of an equal amount of acid indicates the presence of flavonoids.
- *Lead acetate test*- Each extract was treated with a few drops of lead acetate and the formation of a yellow precipitate indicates the presence of flavonoids.

Test for Glycosides-

- *Modified Borntrager's test*- The extracts were treated with 1-2 ml of FeCl3 and then heated over the water bath for 5 minutes. This solution was allowed to cool and then extracted with equal volumes of benzene. The benzene layer was separated and treated with a few drops of ammonia solution. Pink coloration indicates the presence of glycoside.

Test for Saponins

- *Froth test*- The extracts were diluted with distilled water in a graduated cylinder up to 10 ml and this was shaken for 5 minutes. The formation of a foam layer of at least 1 cm indicates the presence of saponins.
- *Foam test*- 2-3 ml of extract was shaken with water in a test tube for 5 minutes. If the foam persisted for 10 minutes, it indicates the presence of saponins.

Test for Phenols-

- *Ferric Chloride test*- The extracts were treated with 1-2 ml of $FeCl_3$ solution. The formation of bluish-black precipitates indicates the presence of phenols.

Test for Tannins- The extracts were dissolved in 5 ml distilled water for the following tests and filtered.

- *Lead Acetate test*- To 1-2 ml of filtrate a few drops of 1% lead acetate were added. The formation of yellow precipitates infers the presence of tannins.
- *FeCl3 test*- To 1-2 ml filtrates, a few drops of 5% $FeCl_3$ was added. The formation of a green precipitate indicates the presence of tannins.

Test for Steroids- Each extract was dissolved in 5 ml chloroform and filtered. The filtrate was analyzed for the presence of steroids.

- *Salkowaski's Test*- The filtrate was treated with a few drops of H_2SO_4, shaken for a few minutes, and allowed to stand undisturbed for some time. The appearance of a golden yellow coloration indicates the presence of steroids.
- *Libermann Burchard's test*- The filtrate was treated with a few drops of acetic anhydride. The solution was boiled and then allowed to cool. To this a few drops of conc. $H_2SO_4$was added. The appearance of a brown ring at the junction indicates the presence of steroids.

Table 6. Phytochemical Analysis of leaf extract of *S. moellendorffii*

S. No.	**Test Performed**	**Aqueous Extract**
1	**Alkaloids**	
Mayer's Test		+
Wagner's Test		+
2.	**Flavonoids**	
Alkaline R. Test		-
Lead Acetate Test		-
3.	**Glycosides**	
Borntrager's Test		-
4.	**Saponins**	
Froth Test		+
Foam Test		+
5.	**Phenols**	
$FeCl_3$ Test		+
6.	**Tannins**	
Lead Ac. Test		+
$FeCl_3$ Test		+
7.	**Steroids**	
Salkowski Test		+
Liebermann's Test		+

3.2.2 Experimental design for collection and treatment of *H. contortus* worms

Adult *H. contortus* worms were isolated from the abomasa of infected ruminants. The adult male and female worms were treated with an aqueous extract of *L. parasiticum* against albendazole (Alb) and control (RPMI Media) for 24 h.

A detailed procedure for the paralysis and death time analysis has been discussed in Chapter 2 (2.3.3).

3.2.3 Results:

Table 7. Paralysis and Death time analysis of ASE on adult *H. contortus*. Worms were exposed to ALE and Alb in RPMI media for 24 h. Death and paralysis of worms were analyzed at various time points.

Time of Exposure (h)	Paralysis Time			Death Time		
	Male worms					
	Control	Alb	ASE	Control	Alb	ASE
0	-	-	-	-	-	-
0.5	-	-	-	-	-	-
1	-	-	-	-	-	-
3	-	-	-	-	-	-
6	-	-	-	-	-	-
12	-	-	5±0.5	-	-	-
24	1	-	9±0.5	-	-	-
Female worms						
0	-	-	-	-	-	-
0.5	-	-	-	-	-	-
1	-	-	-	-	-	-
3	-	-	-	-	-	-
6	-	-	-	-	-	-
12	-	-	-	-	-	-
24	-	-	5±0.5	-	-	-

Conclusions:

The unprecedented antihelminthic efficiency of different aqueous plant extracts showed the mild antihelminthic effect on the parasitic worms, *H. contortus*. Therefore, no significant reduction in paralysis and death time was observed after the plant extract treatment. Henceforth, the plant extracts can't stand alone as an antihelminthic drug. Therefore, further detailed antihelminthic studies have been done using silver nanoparticles of *L. parasiticum* and *S. moellendorffii* plant extracts to understand the active principle(s), efficacy, and mechanism of action.

CHAPTER 4

The antihelminthic activity of aqueous plant extracts protected silver nanoparticles (AgNPs)

Despite the efficacy of traditional plants to treat helminthic infections, scarcity of medicinal information, has been reported and only a handful of those natural resources have ever been explored for their potential in modern medicine. *L. parasiticum* and *S. moellendorffii* are well known for their therapeutic uses in dysentery, malaria, and bleeding, chronic inflammation, such as arthritis, gonorrhea, hepatitis, and mastitis but still, they are not highly useful in the wake of the increasing problem of helminthic infection. Thus, here in this section silver nanoparticles have been synthesized by coating the aqueous plant extract of the same plant to study their detailed antihelminthic studies to understand the active principle(s), efficacy, and mechanism of action.

4.1 *Lansium parasiticum* silver nanoparticles

The use of natural resources to treat various communicable and non-communicable diseases is a practice from pre-historical ages [152]. Various herbal formulations are derived from chamomile, nettle, shatavari, garcinia, neem, karela, pippali, ashwagandha, ajwain, fenugreek, wormwood, etc., are documented in Indian, Chinese, Vietnamese, and African traditional medicine [153, 154]. These herbal formulations are rich in various secondary metabolites, including various polyphenolic compounds like alkaloids, flavonoids, tannins, etc., which are documented to have antihelminthic activity [155]. Despite the prevalence of helminthic infections, scarcity of medication, and profound documentation of traditional medicines, only a handful of those natural resources have ever been explored for their potential in modern medicine.

Lansium parasiticum is a widely grown plant in the northeastern Himalayan regions of India, Bangladesh, Myanmar, etc., used mainly for eating and timber purposes [233]. The fruits of the plant grow during July and August season and are rich in polyphenolic compounds like minerals (potassium, sodium, manganese, phosphorus, etc.), organic acids (maleic acid, citric acid, and glycolic acid), and vitamins (vitamin C, vitamin A, thiamine, riboflavin, niacin, etc.) [234]. It is known for its use in deworming, ulcer medication, dysentery, and malaria in traditional medicine. Langsat contains nutritional-rich vital elements like minerals, proteins, vitamins, dietary fibers, and carbohydrates. The presence of antioxidants in Langsat inhibits the damage to cells caused by free radicals. Moreover, the pulp of the former fruits contains oleoresin that helps in the treatment of digestive tract problems. The seed extract of *L. parasiticum* is known for its use in deworming, ulcer medication, dysentery, and malaria in traditional medicine. Tanaka, 2002 isolated different onoceranoid triterpenes and studied their toxicity against brine shrimp [179].

Ruhisha and Choudhury, 2017 showed the presence of different phytochemicals using various plant extracts [180]. Potipiranun, 2018 showed the isolation of different plant compounds from the ethyl acetate extract and studied its activity against maltase [181]. Moreover, Ramadhan, 2018 showed the antioxidant activity and α-glucosidase effect with different compounds isolated from *Lansium* [182]. Kumara Singha, 2016 performed antihelminthic activity on *H. contortus* using various ethnomedicinal plants like *Lansium domesticum*, *Linariantha bicolor*, and *Tetracera akara* [160].

Here we show the novel antihelminthic activity of the fruit extract-protected silver nanoparticles (LAgNPs) for the first time, which opens the scope of exploring this plant for the development of modern medicine.

4.1.1 Experimental design for the treatment of egg hatch, larval stage, and paralysis and death of adult H. contortus worms with LAgNPs.

Adult *H. contortus* worms were isolated from abomasums of infected ruminants. The adult worms were treated with an aqueous extract of *L. parasiticum* coated silver nanoparticles (15.8, 31.7, 63.5, and 158.7 nM) compared against albendazole, Alb (9.4µM), Ag^+ (100µM), and control (RPMI Media) for 24 h to observe theirs *in vitro* mortality.

In vitro morbidity test of *H. contortus* larvae (L3) was performed by taking larvae (Approximately 25-30) in 200 µl of RPMI medium in 96 well plates in presence of LAgNPs (15.8, 31.7, 63.5 and 158.7 nM), Alb (9.4µM) and Ag^+ (100µM) for 24 h. After the incubation, a direct microscopic examination (at 40X magnification) was done for mobility (for 20 secs).

Egg suspension (1 ml) containing nearly 200 eggs was taken into 24 well microtiter plates, subsequently, to check the inhibition efficiency of drugs, eggs were treated at different concentrations of LAgNPs (15.8, 31.7, 63.5, and 158.7 nM), against two controls, one positive with Alb (9.4µM) and Ag^+ (100µM) and another negative with RPMI media. After the inhibition treatment, the plates were incubated at 28 ℃ for 48 h.

A detailed procedure for adult motility assay (2.3.3), larval motility assay (2.3.4), egg hatch assay (2.3.5), and morphological analysis, SEM (2.4), generation of oxidative stress with ROS (2.6.1), NOS (2.6.2), and catalytic activities CAT, 2.7.1, SOD, 2.7.2, GPx, 2.7.3 and GSH, 2.7.4 has been explained in Chapter 2.

4.1.2 Results:

4.1.2.1 Evaluation of ALE protected AgNPs physical properties

The formation of LAgNPs under the sunlight was visibly observed by color change (**Figure 8**) and thereafter was confirmed by observing by SPR. Upon the formation of LAgNPs, a time-dependent blue shift in the SPR peak position (absorption maximum) was observed from 450 nm. A saturation of peak intensity and the completion of the SPR shift at 435 nm were observed after 30 min of incubation (**Figure 9**).

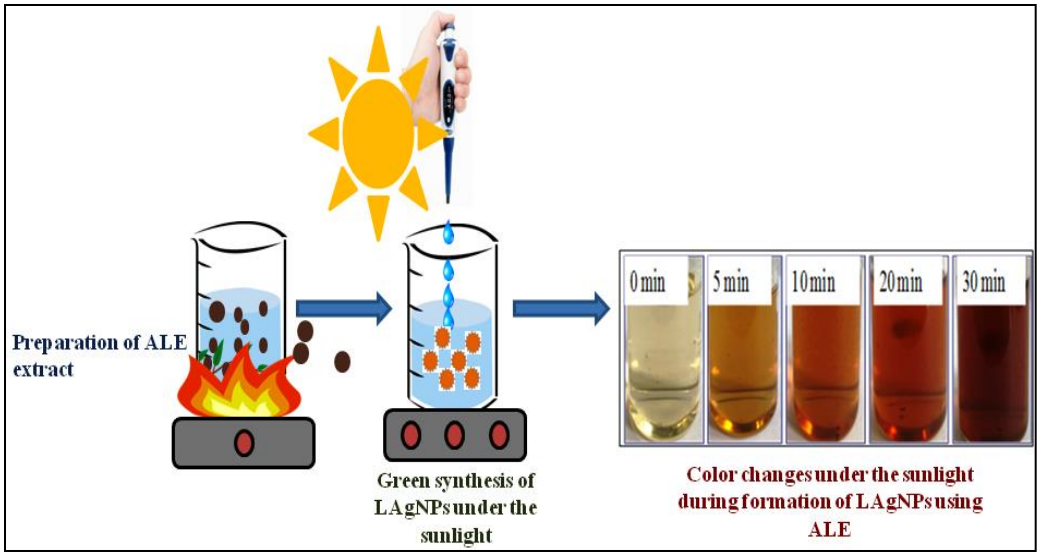


Figure 8. Schematic representation of the preparation of aqueous *Lansium parasiticum* pulp extract (ALE) and the synthesis of *L. parasiticum* protected silver nanoparticles (LAgNPs) under sunlight.

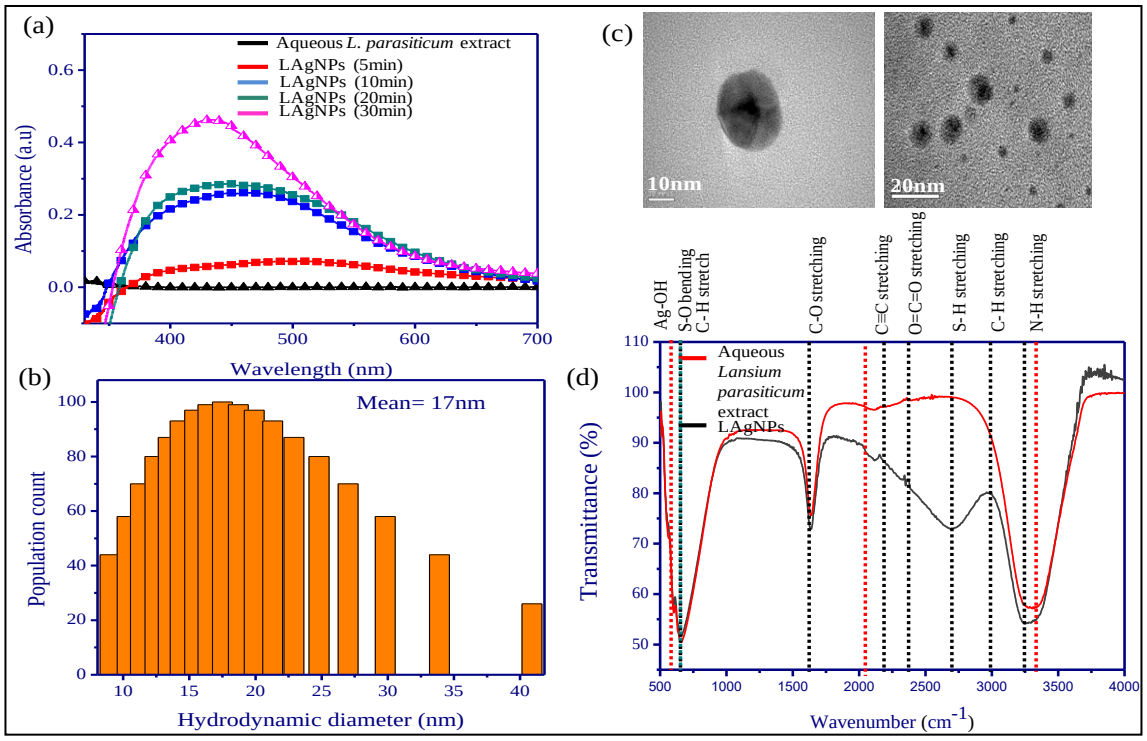


Figure 9. Characterization of silver nanoparticles (AgNPs). (a) Surface plasmon resonance spectra of photo-reduced AgNPs of different sizes. The spectra were scanned in the range of 300 nm-700 nm. (b) Dynamic light scattering study showing the distribution of hydrodynamic diameter and disparity of the LAgNPs. The hydrodynamic size obtained was 17 nM. (c) Transmission electron microscopic studies for the size and shape of the AgNPs. The inset is showing the mean values of the LAgNPs. (d) FTIR studies confirmed the changes in functional groups, including plant extract and LAgNPs.

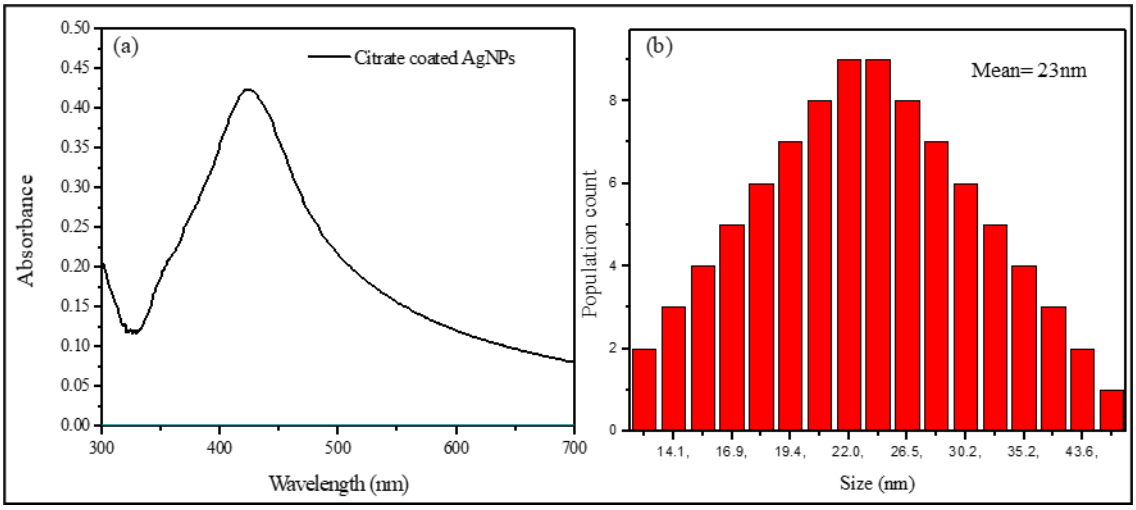


Figure 10. Physical characterization of citrate-protected AgNPs: (a) Surface plasmon resonance spectra of citrate reduced AgNPs. The spectra were scanned in the range of 300 nm-700 nm. (b) Dynamic light scattering study showing the distribution of hydrodynamic diameter and disparity of the citrate coated AgNPs. The hydrodynamic size obtained was 23 nm.

Visible color changes under the sunlight during the formation of LAgNPs at different time intervals, 0–30 min. DLS data showed the formation of monodispersed nanoparticles with an average hydrodynamic diameter of 17 ± 9 nm (**Figure 9b**). Furthermore, the zeta potential analysis of LAgNPs showed the formation of a stable particle surface charge of −21 mV. TEM studies confirmed the synthesis of spherical nanoparticles with an average diameter of ~16 ± 5 nm (**Figure 9c**). Images of LAgNPs at higher magnifications are presented in the inset. The stability and metal leaching of LAgNPs Inductively coupled plasma atomic emission spectroscopy (ICP-AES), (AGILENT, 4100) studies were performed after the aging of samples up to 6 months (**Table 8**).

Table 8. The maximum amount of around ~30% Ag^+ release was observed after 6 months of aging. LAgNPs lasted for 6 months and found a maximum of ~ 30% release of silver ions in the solution. Therefore 30% silver solution was kept as the control for most of the experimental sets.

Parameters	**Results for Ag^+ leaching for LAgNPs**			
Time of Aging	**0 Months**	**2 Months**	**4 Months**	**6 Months**
Silver ions (Ag^+) in mg/l	0.1 ± 0.1	1.27 ± 0.567 (~5 %)	3.24 ± 0.891 (~12 %)	7.72 ± 1.458 (~30 %)

In addition to these studies, to check the involvement of different functional groups of ALE in the synthesis of LAgNPs, FTIR studies were performed. The surface protection of $AgNO_3$ was observed with the formation of the Ag–OH (557 cm^{-1}) bond in LAgNPs. Along with this, the other bonds C=C and O–H shift from 2,118.5 and 3,299 cm^{-1} in ALE to 2,155 and 3,245 cm^{-1}, respectively, in LAgNPs, indicating that these functional groups may play an important role in the reduction and capping of LAgNPs (**Figure 9e**). The band positions and respective shifts in FTIR along with the references are given in **Table 9** [235-238].

Table 9. FTIR analysis for functional groups for *Lansium parasiticum* and silver nanoparticles coated with *Lansium parasiticum*

Functional Groups	**Wavenumber (cm^{-1})**		**References**
	Aqueous *Lansium parasiticum* extract	**LAgNPs**	
Ag-OH	-	557	[239]
C-Br stretching	609.8	609.8	[236]
C-H stretching	663.3	648.4	[237]
C=O stretching	1637	1637	[237]
C=C stretch	2118.5	2155	[238]
Unknown (maybe Carbonate compound)	2354	2348.4	
Unknown	-	2695	
O-H stretch	3299	3245	[235]

4.1.2.2 Toxicity of LAgNPs on the human normal kidney cells

The cell viability results showed mild toxicity of LAgNPs on HEK293 cells for 24 h exposure. At the lowest concentration (15.8 nM), there were no significant changes were observed in HEK293 viability. With an increase in LAgNPs concentration, however, the percentage of viable cells was observed (77.5 ± 5.06 %, 71.3 ± 9.8 %, and 62.1 ± 9.3 %) for 31.7, 63.5, and 158.7nM treatments respectively. Further, silver nanoparticles are known for their leaching effect, and a maximum of ~30% (70 μM) silver leaching was observed upon 6 months of storage of silver nanoparticles (**Table 8**). Therefore, we have also checked toxicity caused by the leaching effect HEK293 cells were treated with various concentrations (50-250 μM) of Ag+ for 24 h. 76.5 ± 8.8%, 72.5 ± 5.8%, and 68.4 ± 0.2% cells were found to be alive due to (50, 100 and 250 μM) of Ag+ treatment for 24 h. On the contrary, *H. contortus* cells showed high susceptibility in the presence of LAgNPs. A sharp loss of cell viability (28.2± 7.3, 32± 12.4, 58.6 ± 3.3, and 68.8 ± 16.6 % loss of cell viability was observed for (15.8, 31.7, 63.5, 158.7 nM LAgNPs treatment respectively (**Figure 11**).

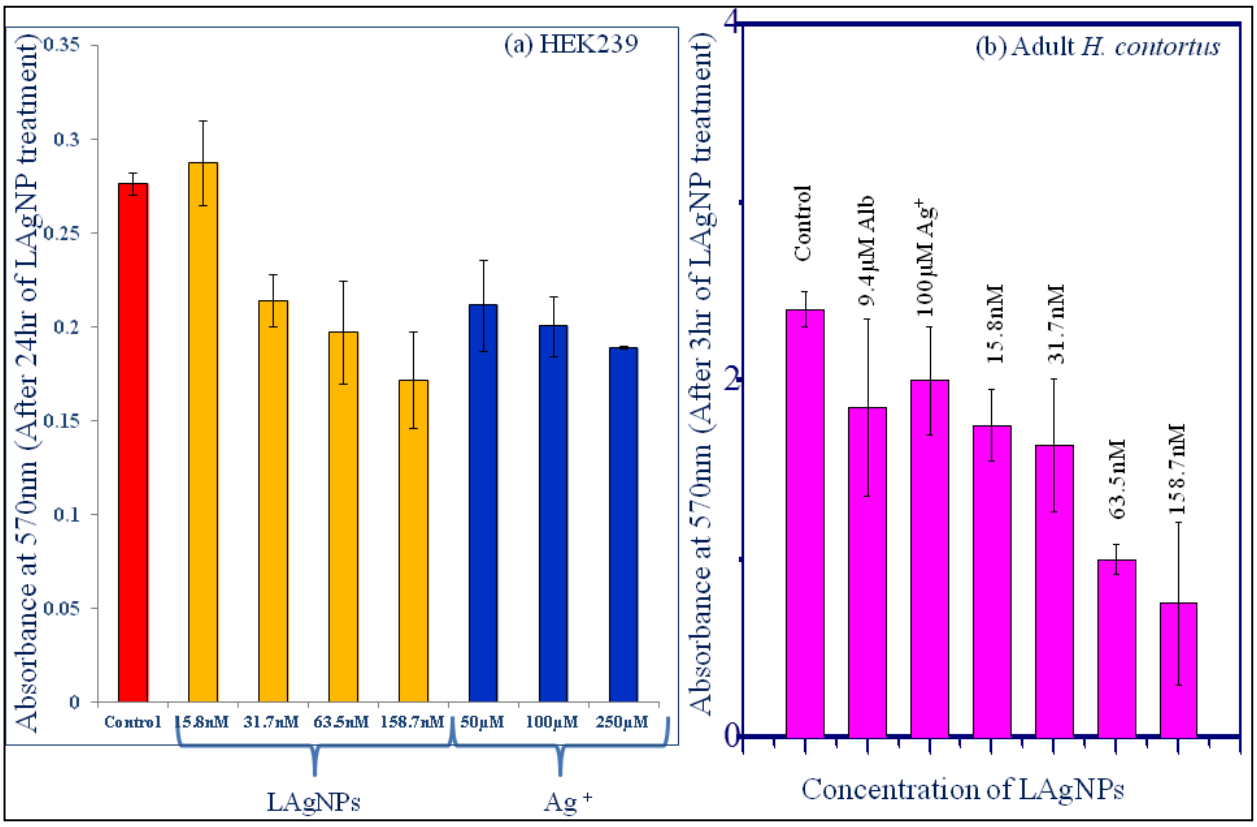


Figure 11. Cell viability study of humans and adult *H. contortus* upon exposure to LAgNPs. (a) Human embryonic kidney (HEK239) cells were treated with various concentrations of LAgNPs and different concentrations of Ag+ (100 µM) for 24 h and thereafter MTT assay was performed to determine the viability of cells. LAgNPs showed mild toxicity on HEK239 cells upon 24 h exposure. With a maximum dose of 158.7 nM, 62.1 ± 9.3% of cells died after 24 h in LAgNPs, whereas 60.5 ± 2.9 % of cells died because of 24 h treatment of 100 µM Ag+. (b) *H. contortus* were treated with various concentrations of LAgNPs (15.8, 31.7, 63.5, and 158.7 nM) for 3 h, and thereafter MTT assay was performed to determine the viability of cells. LAgNPs showed higher toxicity on worm cells upon 3 h exposure. Upon exposure to LAgNPs just for 3 h worms showed 72 ± 8.3 %, 68 ± 15.4 %, 41.6 ± 3.3 and 31.2 ± 16.6 % respectively.

4.1.2.3 Assessment of antihelminthic activities

The results for the antihelminthic activities are discussed below:

4.1.2.3.1 Adult Motility and Morbidity Assay (AMMA)

The effect of LAgNPs on the viability of *H. contortus* is concentration-dependent. Approximately 60 % and 20 % of males and female worms respectively were paralyzed in 1 h. 100% of male worms and 80% of the female worms died within 12 h of treatment with 158.7 nM LAgNPs compared against 31.2 ± 16.6 % loss of cell viability in HEK239 after 3 h treatment. The detailed death and time of paralysis are given in Table 10. The LD_{50} values were found to be 65.6 ± 32.8 and 139.6 ± 39.9 nM for adult male and female worms for 12 h treatment (**Figure 13** for male (a) and female (b) adult worms respectively).

Table 10. Paralysis and Death time analysis of AgNPs on adult *H. contortus.* Worms were exposed to LAgNPs of different doses (15.8 nM, 31.7 nM, 63.5 nM, 158.7 nM), Alb (9.4 µM) and Ag^{+} (100 µM) in RPMI media for 24 h. Death and paralysis of worms were analyzed at various time points in between. Worms were first noted for their movement. If no movement was observed then worms were exposed to a warm saline solution (50 ℃) for 1 min to stimulate movement. The counts were taken for the number based on the worm's movement. If the worm previously immobile worm starts moving upon exposure to warm saline, then considered as paralyzed otherwise considered alive.

Time of Exposure	Paralysis Time								Death Time							
	Male Worms															
	Control (RPMI)	Citrate AgNPs	Alb	Ag^{+}	15.8	31.7	63.5	158.7	Control (RPMI)	Citrate AgNPs	Alb	Ag^{+}	15.8	31.7	63.5	158.7
0 h	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-
0.5 h	-	-	-	-	-	-	-	3±0.5	-	-	-	-	-	-	-	-
1 h	-	-	-	-	-	-	6±0.5	6±0.5	-	-	-	-	-	-	-	-
3 h	-	1±0	-	-	-	-	9±0.5	12 ± 0.5	-	-	-	-	-	-	-	-
6 h	-	4± 0.5	3 ± 0.5	-	-	3±0.5	12 ± 0.5	9±0.5	-	-	-	-	-	-	-	6±0.5
12 h	-	15± 0.5	6 ± 0.5	3±0.5	6 ± 0.5	6±0.5	9±0.5	0	-	4 ± 1		-	3±0.5	7 ± 0.7	6±0.5	15±0
24 h	1	15± 0.5	0	6±0.5	6 ± 0.5	3 ± 0.5	0	0	-	15 ± 0	15±0	9±0.5	15±0	12 ± 0.5	15±0	15±0
	Female Worms															
0 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 h	-	-	-	-	-	-	-	3 ± 0.5	-	-	-	-	-	-	-	-
3 h	-	-	-	-	-	-	-	9 ± 0.5	-	-	-	-	-	-	-	-
6 h	-	-	-	-	-	-	6±0.5	15±0	-	-	-	-	-	-	-	-
12 h	-	5.7 ± 0.5	-	-	3±0.5	3±0.5	6±0.5	6±0	-	1.7 ± 1	3±0.5	-	-	3±0.5	9±0.5	9±0.5
24 h	1	10.7 ± 1.3	-	3±0.5	3±0.5	6±0.5	0	0	-	5.5 ± 1.3	6±0.5	-	3±0.5	9 ± 0.5	15±0	15±0

4.1.2.3.2 Larval Morbidity Assay (LMA)

After 24 h treatment with different concentrations of LAgNPs and respective controls as before L3 larvae looked for movement of morphological changes like shrinkage and ruptured morphology. Concentration-dependent loss of viability of L3 larvae was prominent with LAgNPs. 33.3 ± 2.6, 29.5 ± 1.7, 22.2 ± 2.5 and 14.8 ± 2.1 % survival of larvae was observed for 15.8, 31.7, 63.5 and 158.7 nM LAgNPs treatment respectively, whereas the recommended dose of Alb (9.4 μM) showed 60 ± 0.5 % and Ag+ (100 μM) showed 31 ± 0.7 % survival for 24 h treatment (**Figure 12**). For the larval mortality inhibition, LD_{50} has obtained 64.3 ± 8.5 nM for 24 h (**Figure 13** (**c**)). The p-values for the LMA for L3 of *H. contortus* worms, when treated with different concentrations of LAgNPs, are described in **Table 11.**

4.1.2.3.3 Egg Hatch Assay (EHA)

After 48 h of treatment with LAgNPs and respective controls number of eggs hatched was calculated using the equation (ii). The treatment concentration-dependent reduction of egg hatching efficiency was due to LAgNPs treatment. 32.1 ± 2.6, 45 ± 2.7, 47.2 ± 1.3, and 51.2 ± 1.9 % reduction of egg hatching were observed in comparison with control for 15.8, 31.5, 63.5, and 158.7 nM LAgNPs respectively. Whereas recommended dose of Alb (9.4 μM) showed 13.5 ± 0.5 % and Ag+ (100 μM) 33 ± 0.7 % reduction of egg hatching or larvae (L1) formation (**Figure 12**). LD_{50} for inhibition of egg hatch obtained was 144.4 ± 3.1 nM for 48 h (**Figure 13 (d)**). The p-values for the EHA for L3 of *H. contortus* worms, when treated with different concentrations of LAgNPs, are described in **Table 11.**

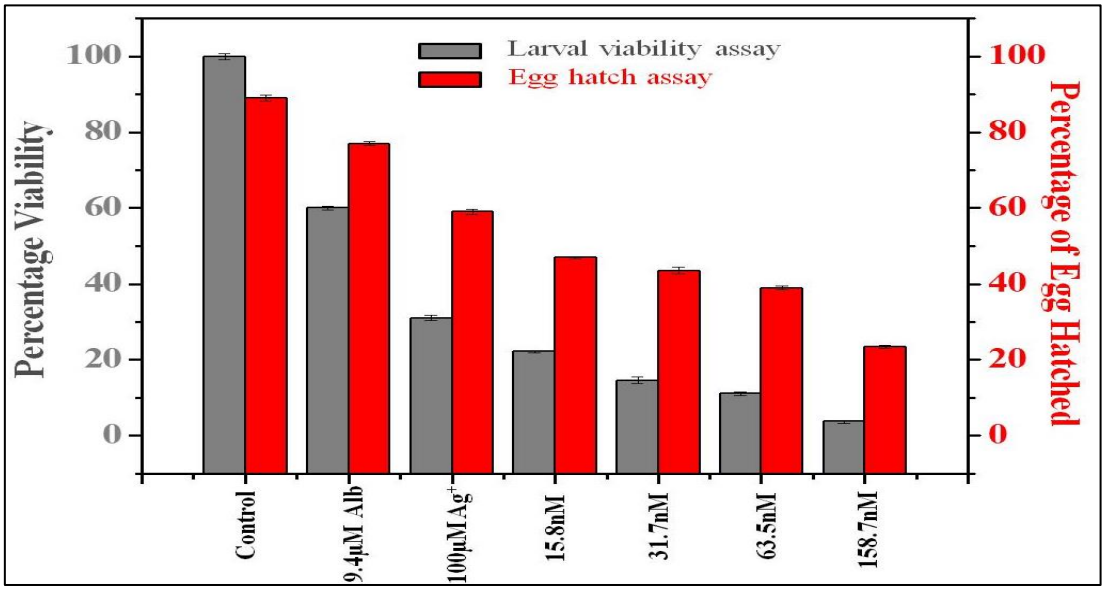


Figure 12. Percentage viability effect of *Lansium parasiticum* protected silver nanoparticles (LAgNPs) on L3 larvae survival and hatching of *Haemonchus contortus* eggs. (Gray) Viability (survival) percentage of the larval stage L3 after 24 h of treatments with different concentrations of LAgNPs along with controls. (Red) Comparative graph showing the influence of LAgNPs on egg hatching assay. For 48 h, the treatment was performed using LAgNPs of different concentrations along with controls. In each well, around 200 eggs were taken, and the results of three independent experiments were presented as an average.

Table 11. The p-values for the Egg Hatch Assay and Larval Motility Assay (L-3) of *H. contortus* worms when treated with different concentrations of LAgNPs (15.8 nM, 31.7 nM, 63.5 nM, and 158.7 nM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

	15.8 nM	**31.7 nM**	**63.5 nM**	**158.7 nM**
Egg Hatch Assay	1.38355E-11	2.18651E-11	1.13407E-10	6.27186E-10
Larval Motility Assay	5.50957E-13	2.37401E-12	9.97562E-12	7.93434E-11

The half-minimal inhibitory concentration (LD_{50}) was measured against the death percentage of adult *H. contortus*, (L3) larvae, and egg hatch assay.

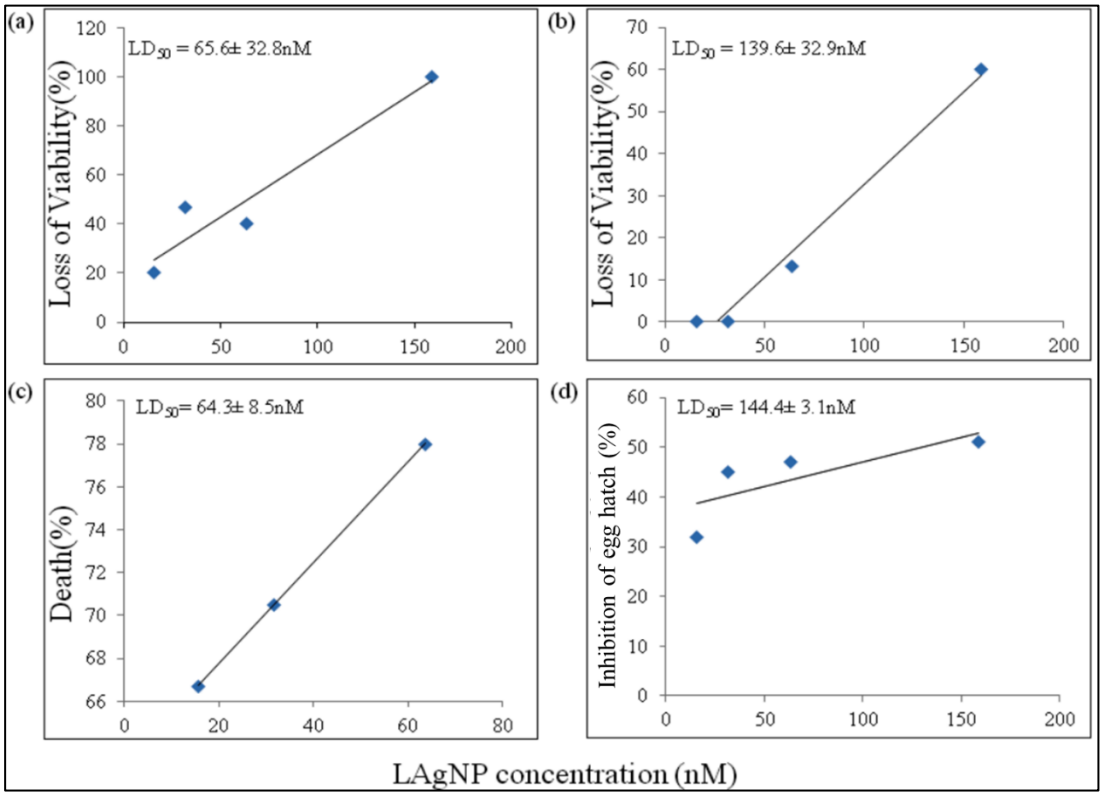


Figure 13. LD_{50} values of LAgNPs against various forms of *H. contortus*: In (a) Male death percentage in 12 h (b) Female death percentage (c) Larval Motility assay in 24 h and (c) Inhibition of egg hatch percentage in 48 h.

4.1.2.3.4 Ultra morphological analysis for tissue damage due to LAgNPs exposure

The extent of physical damage of *H. contortus* was monitored using scanning electron microscopy. A complete comparative study for the changes in the morphology of tissue damage is shown in **Figure 14**, comparing control (RPMI Media), Alb (9.4 μM), Ag+ (100 μM), and 158.7 nM LAgNPs after 3 h of treatment. The complete body region of the adult worm is depicted in **Figures 14a, d, g, j.** The untreated *H. contortus* worm with a smooth cuticle and having a well-developed body region of an adult can be seen in **Figures 14a, d, g,** whereas in **Figure 14j, a** complete distortion of the outer morphology along with prominent shrinkage of the worm body can be observed. **Figures 14b, e, h, k** shows the adult helminths' anterior region. Almost no distortions were observed in the control (**Figure 14b**). Partial and complete disruptions were observed in Ag+, Alb, and LAgNPs treated (158.7 nM) worms, respectively (**Figures 14c, h, k**). Similar observations were prominent in the posterior ends of the control and treated worms as well (**Figures 14c, f, i, l**).

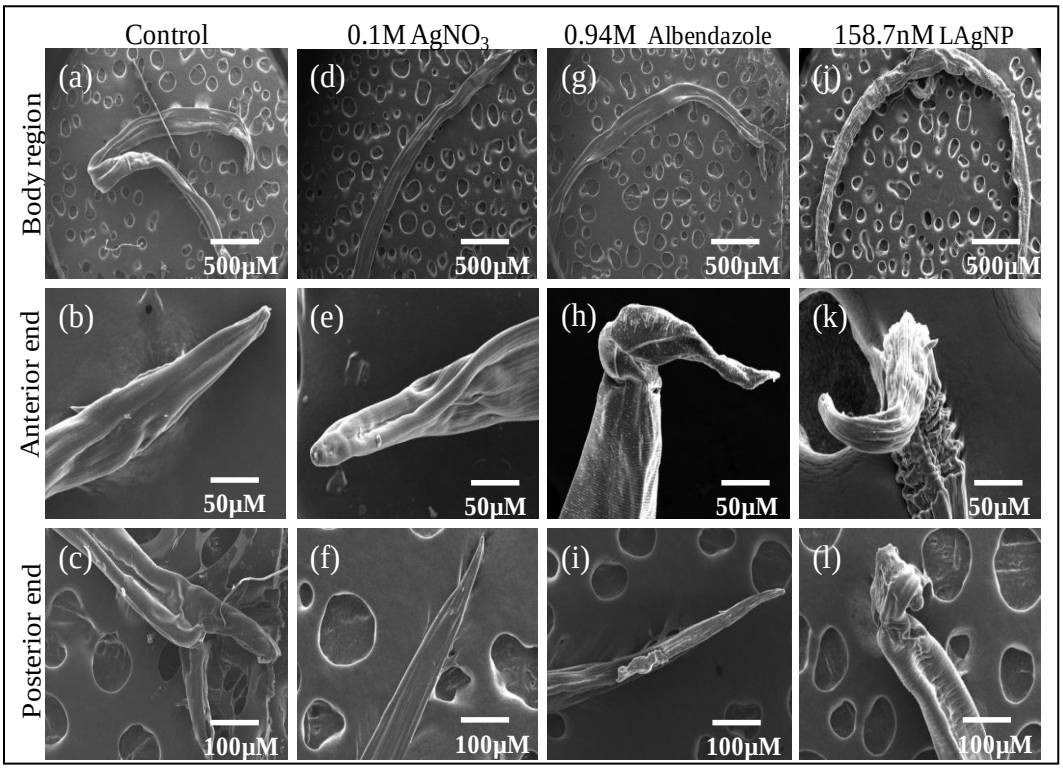


Figure 14. Study of ultramicroscopic morphological damage in an adult *H. contortus* under the influence of *L. parasiticum* protected silver nanoparticles (LAgNPs). The helminth was treated for 12 h, and after treatment, the helminths were dehydrated and gold-coated. Thereafter, the ultra-structures were observed using SEM at 15 (kV). (**a, b, c)** treatment with control, RPMI media; (d**, e, f**) Ag^+ (100 µM), and (**g, h, i**) 9.4 µM Alb treated adult worms. (**j, k, l**) Adult worms after treatment with 158.7 nM LAgNPs. (**a, d, g, and j**) Whole-body of the control and Ag^+-treated, Alb treated, and LAgNPs treated worms, respectively. (**b, e, h, and k**) The anterior end of the control and Ag^+ treated, Alb-treated, and LAgNPs treated worms, respectively. (**c, e, f, and l**) Posterior ends of the control and Ag^+ treated, Alb-treated, and LAgNPs treated worms, respectively. LAgNPs treated worms showed profound damages to tissue architecture and loss of tissue integrity, with disorganization of the bursa regions.

4.1.2.3.5 Generation of Reactive oxygen species (ROS) stress due to LAgNPs exposure

Massive cellular stress response for ROS generation had been observed due to LAgNPs (158.7 nM) treatment within 3 h. A complete comparative study for the changes in ROS generation of the stress response is shown in **Figure 15**, comparing control (RPMI media), Alb (9.4 µM), Ag^+

(100 µM), and 158.7 nM LAgNPs after 3 h of treatment. In the control tissue (**Figure 15**), the low level of ROS generation showed a moderate level of ROS that was observed using DCFDA fluorescence. **Figures 15a, d, g, j** showed the complete body region of LAgNPs treated adult *H. contortus*, where **a, d**, and **g** showed a very less or partial generation of ROS, respectively, and showed a high generation of ROS. The generation of ROS was prominent in the anterior (**Figures 15b, e, h**) and the posterior ends (**Figures 15 c, f, i**) when treated with control (RPMI media), Ag^{+}, and Alb, but the high generation of oxygenated species was observed in **k** (anterior) and **l** (posterior) ends when treated with LAgNPs.

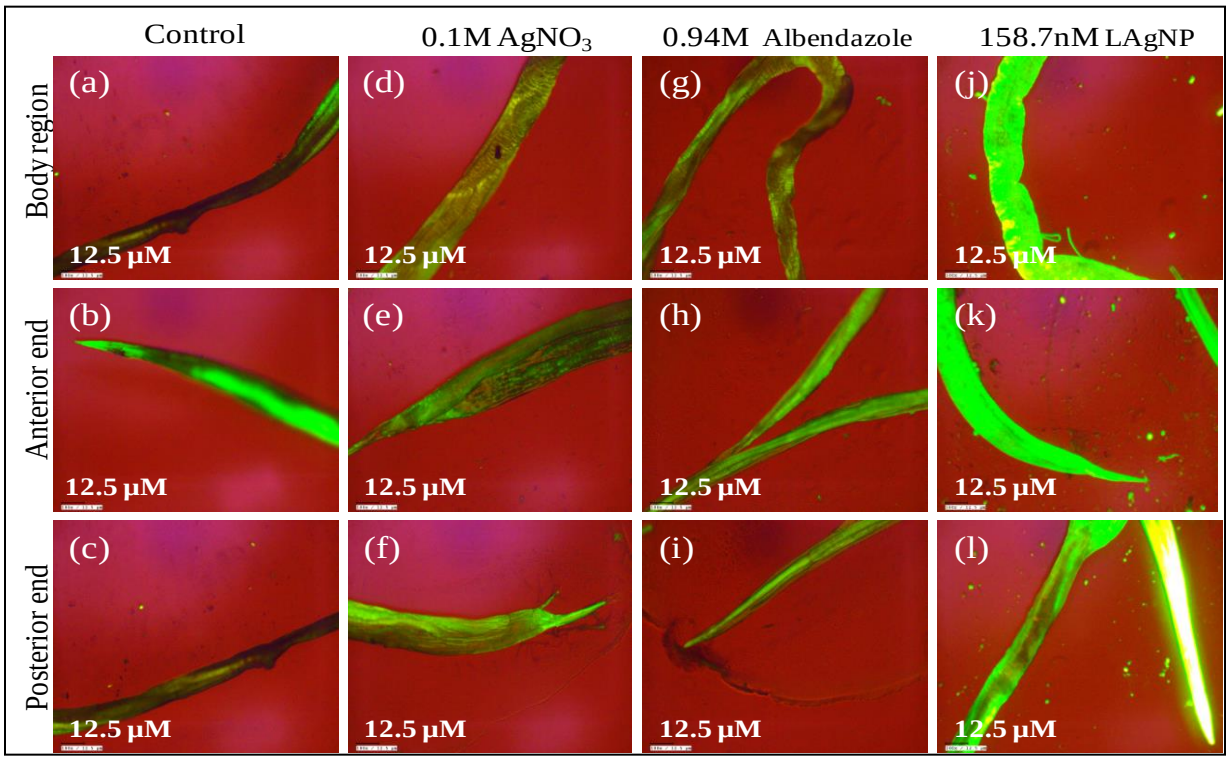


Figure 15. Generation of oxidative stress in adult worms due to exposure of *L. parasiticum* protected silver nanoparticles (LAgNPs). Adult *H. contortus* were treated for 3 h with LAgNPs and controls and processed for reactive oxygen species (ROS) [staining with 2′,7′-dichlorofluorescein diacetate (DCFDA)]. (**a, b, c**) amount of ROS in the body, anterior and posterior regions, respectively, of the adult helminth in the control, RPMI media. (**d, e, f)** Amount of ROS in the body and the anterior and posterior regions, respectively, of the adult helminth in 100 µM Ag^{+}. **(g, h, i**) Amount of ROS in the body and in the anterior and posterior regions, respectively, of the adult helminth, when treated with 9.4 µM Alb. (**j, k, l**) Amount of ROS when treated with the

highest concentration of LAgNPs (158.7 nM). After the desired treatment, the worms were incubated with 100 nM DCFDA in the dark for 20 min. Thereafter, the images were taken using an inverted fluorescence microscope by Dewinter, Italy.

4.1.2.3.6 Determination of nitric oxide synthase

The generation of NOS with the stress response in the worm due to LAgNPs treatment was monitored using the Greiss reagent. The results showed a dose-dependent and subsequently time-dependent increase in the NOS level in the worms. The NOS level has increased from 0.0462 ± 0.028 to 0.081 ± 0.01 within 24 h for the lowest dose (15.8 nM) of LAgNPs, whereas the NOS response becomes further significantly prominent and increases from 0.07767 ± 0.01 and 0.351 ± 0.01 (within 24 h) due to 158.7 nM LAgNPs treatment (**Figure 16**). The change of NOS level in response to Ag+ and Alb was moderate and not of high significance (**Figure 16**). The p-values for the NOS for adult *H. contortus* worms, when treated with different concentrations of LAgNPs, are described in **Table 12.**

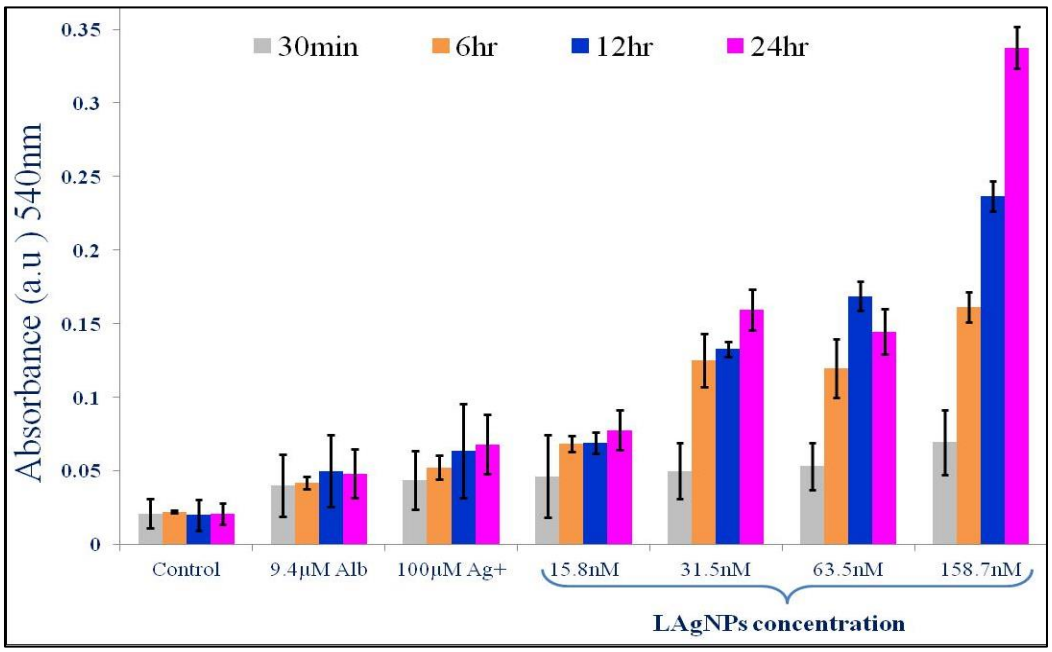


Figure 16. Alteration of activity of nitric oxide synthase (NOS) enzyme due to LAgNPs stress. A comparative graph showing the generation of nitric oxide free radicals as a result of NOS activity in *H. contortus* worms after treating with LAgNPs different concentrations (158.7 nM) along with controls for 30 mins, 6 h, 12 h, and 24 h.

Table 12. The p-values for nitric oxide synthase (NOS) activity of *H. contortus* worms when treated with different concentrations of LAgNPs (15.8 nM, 31.7 nM, 63.5 nM, and 158.7 nM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

	15.8 nM	**31.7 nM**	**63.5 nM**	**158.7 nM**
6 h	8.21842E-10	6.22619E-10	4.13012E-10	1.76271E-10
12 h	9.49E-10	8.7E-10	8.13E-10	4.35E-10
24 h	1.74E-09	1.64E-09	1.7E-09	1.62E-09

4.1.2.3.7 Alteration of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity in response to LAgNPs induced oxidative stress

The increase of reactive oxygen and nitrogen species due to LAgNPs treatment generated huge oxidative stress within the worm's physiological system, which may alter the change in the activity of metabolic and stress-responsive enzymes. A steady enhancement in CAT activity with an increase of LAgNPs concentration, increase in stress response, had been observed. In different concentrations of LAgNPs (15.8, 31.7, 63.5, and 158.7 nM), the amount of CAT enzyme was calculated as 4.75 ± 0.17, 5.35 ± 0.36, 7.9 ± 0.37, and 8.2 ± 0.4 U/mg proteins, respectively, after 3 h of treatment, in comparison with the 0.22 ± 0.05, 4.1 ± 0.37, and 0.52 ± 0.05 U/mg proteins for the untreated control, Alb (9.4 μM), and Ag^{+} (100 μM), respectively **Table 13**. A consistent SOD activity increment with an increase in stress response had also been observed. The SOD enzyme activity after the treatment was found to be 5.70 ± 0.4, 5.85 ± 0.17, 6.11 ± 0.26, and 6.60 ± 0.54 U/mg protein for LAgNPs 15.8, 31.7, 63.5, and 158.7 nM, respectively, in relation to the control where 4.8 ± 0.22 Alb and 0.56 ± 0.07 Ag+ were used. The untreated worms showed a SOD activity of 0.21 ± 0.018 U/mg of worm protein (**Table 13**). In contrast to other antioxidant activities, the activity of GPx also increases when the worms were exposed to LAgNPs' increasing concentrations. At 340 nm, spectrophotometrically, the amount of GPx was observed. The activity of the GPx enzyme was calculated using Equation (3). The GPx enzymes' active amount was 2.5 ± 0.15, 2.6 ± 0.16, 2.73 ± 0.15, and 2.9 ± 0.16 U/mg protein for different concentrations of LAgNPs (15.8, 31.7, 63.5, and 158.7 nM, respectively), in comparison with the control where the amount

was 2.6 ± 0.25 U/mg proteins in Alb and 0.58 ± 0.058 U/mg proteins in Ag^{+}. In the untreated worms, it was found to be 0.21 ± 0.071 U/mg proteins (**Table 13)**. The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of LAgNPs are described in **Table 14**.

4.1.2.3.8 ROS enhances cellular combat by increasing the concentration of reduced glutathione (GSH)

In combat to ROS, cellular response induced by GSH causes oxidative stress in the worms when exposed to LAgNPs. A significant enhancement in the amount of GSH produced was measured in LAgNPs treated worms. The increase in GSH was observed (0.28 ± 0.05 µM/ mg for untreated) along with the increase of the dose of LAgNPs (15.8, 31.7, 63.5 and 158.7 nM) from 1.105 ± 0.025, 1.24 ± 0.06, 1.3 ± 0.141 and 1.41 ± 0.1078 µM/ mg protein respectively. Treatment of Alb also induced a significant increment in the GSH concentration (1.75 ± 0.050 µM/ mg protein) and Ag+ causes 0.59 ± 0.009 µM/ mg protein accumulation of GSH (**Table 13)**.

Table 13. Oxidative stress-responsive elements in *H. contortus* to counter the toxic effect of LAgNPs on infectious helminths. Due to the generation of oxide synthase-related stress helminth counteracts conditions through alteration of its metabolic functions. The toxic effect of LAgNPs on the body of the helminth reacts with the cellular molecules as normal metabolic functions and increases the regeneration of free radicals induced by oxidative damage defense mechanisms to include the following: enzymatic factors such as CAT, SOD, GPx, and GSH. The enzymatic activity was measured as the enzyme unit per mg of protein (U/ mg protein) for CAT, SOD, and GPx and (µM/ mg protein) in GSH.

Treatment	**U/mg protein**			**µM/mg protein**
	CAT	**SOD**	**GPx**	**GSH**
Concentrations				
Control	0.22± 0.05	0.21± 0.018	0.25± 0.07	0.28± 0.01
9.4µM Alb	4.1± 0.37	4.8± 0.2	2.6± 0.25	1.4± 0.05
100µM Ag^{+}	0.56± 0.05	0.52± 0.07	0.58± 0.057	0.59± 0.009
15.8 nM LAgNP	7.9± 0.37	6.11± 0.26	2.73± 0.15	1.3± 0.14
31.7 nM LAgNP	8.2± 0.41	6.6± 0.54	2.9± 0.16	1.41± 0.10
63.5 nM LAgNP	8.36± 0.1	6.83± 0.20	3.2± 0.25	1.59± 0.11
158.7 nM LAgNP	10.7± 0.37	7.74± 0.89	3.3± 0.18	1.75± 0.05

Table 14. The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of LAgNPs (15.8 nM, 31.7 nM, 63.5 nM, and 158.7 nM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

LAgNPs concentration	**Superoxide Dismutase (SOD)**	**Catalase (CAT)**	**Glutathione Peroxidase (GPx)**	**Glutathione (GSH)**
15.8nM	3.62694E-13	2.74722E-11	5.2248E-13	1.62E-11
31.7nM	2.5069E-12	4.51635E-11	2.57836E-12	3.17E-11
63.5nM	1.65888E-11	5.05788E-11	1.22419E-11	8.49E-11
158.7nM	1.41278E-10	4.43158E-10	8.71562E-10	3.84E-10

Conclusion:

Due to the shortage of drug availability and the development of resistance against existing drugs, there is an utmost necessity to develop new drugs for the treatment and management of parasitic worm infections. This is the first report showing the antihelminthic activity of *L. parasiticum* aqueous extract-protected silver nanoparticles. Here biocompatible, stable, and eco-friendly LAgNPs were synthesized using the green synthesis technique under the sunlight. *L. parasiticum* is a relatively less studied plant for medical purposes, although the use of seed extracts as antihelminthic and anti-ulcer medication treatment had been reported in Vietnamese traditional medicine [181]. Further use of bark extract had been reported for dysentery and malarial treatment [240]. Traditionally, the fruit extract of the same has also been reported for diarrhea treatment [227]. Despite the abundance of production, the use of this plant for modern medication has not been explored to its true potential. *H. contortus* is resistant to albendazole, a widely used antihelminthic drug of the benzimidazole group. Treatment of *H. contortus* with LAgNPs showed a rapid elevation of (within 3 h) ROS-dependent stress in the worms' bodies. Due to the generation of stress, the worms undergo metabolic changes which were also evident by observing the change in the activity of ROS-neutralizing enzymes in the worm's body due to LAgNPs treatment. As a control experiment, citrate-protected AgNPs showed limited antihelminthic activity in comparison with LAgNPs, suggesting a significant role of the active principal component(s) of ALE. Although

the active principal component(s) of ALE has not been identified yet, these findings open hope for the development of next-generation antihelminthic drugs (**Figure 17**).

Henceforth, the findings are highly useful in the wake of the increasing problem of drug resistance to the commercially available antihelminthic, an increase of life quality, and reduction of healthcare cost, but further detailed studies are required to understand the active principal component(s), efficacy, and toxicity of the system in animal models. In addition to that, the utility of the formulations against other parasitic infections, including helminthic infections, needs to be evaluated in the future.

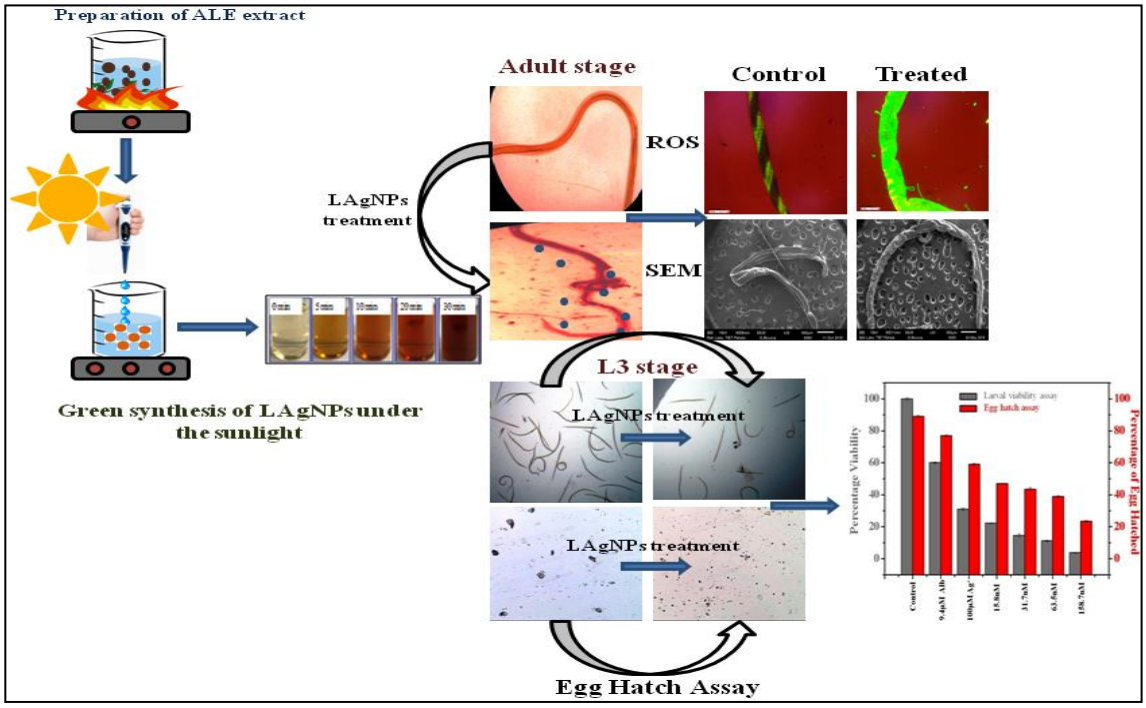


Figure 17. Scheme showing the synthesis of LAgNPs under direct sunlight, then treatment of adult *H. contortus* worms, L3 stage, and egg hatch assay with LAgNPs at different concentrations. After that, changes in morphology were analyzed using SEM and then the generation of ROS was observed.

4.2 *Selaginella moellendorffii* silver nanoparticles

Green Nanotechnology is also referred to as the photo biological approach that uses the plants and the plant extracts as reducing and capping agents for AgNPs synthesis [241]. In this context, the AgNPs are biosynthesized using the *Selaginella moellendorffii* plant species extract. Pteridophytes are considered as one of the oldest groups of plants that are present on the earth and they consist of a large group of vascular cryptograms [229]. They have made the group fascinating because of their position between the lower cryptograms and the higher vascular plants. They are present in the Coastal and the Himalayan regions of India and they prefer moist and shady habitats with moderate temperature conditions [229]. Out of 1,200 species of the Pteridophytes that are present in India, about 170 species are used for medicine, food, oil, dye, bio-fertilizer, flavor, fiber, and the production of biogas, etc. *Selaginella moellendorffii* is much unexplored virgin plant species which is creating great interest among the scientist for its various uses in industries, medicine, and therapeutic uses.

Selaginella moellendorffii Hieron. is a traditional Chinese folk herb and belongs to the family Selaginellaceae, and with various useful resources [226]. Shi in 2008 showed the traditional uses for the treatment of chronic inflammation, like hepatitis, gonorrhea, and bleeding with *Selaginella moellendorffii Hieron* [242, 243]. Cao, 2010 showed the isolation of the new flavones from the herb *Selaginella moellendorffii Hieron* and studied their cytotoxic activity against Hep G2.2.15 cell line[244]. These flavones also showed inhibitory activity on the hepatitis B virus. Almeida in 2013 showed the presence of flavonoids [245] and Cao in 2010 showed the presence of phenols in *Selaginella doederleinii* [245]. Thus, making *Selaginella* ideal for hyperuricemia and gouty arthritis. Moreover, the presence of flavonoids and phenols also makes it a perfect model for antioxidant and anti-inflammatory studies.

4.2.1 Experimental design for the treatment of egg hatch, larval stage, and paralysis and death of adult H. contortus worms with SAgNPs.

Adult *H. contortus* worms were isolated from abomasums of infected ruminants. The adult worms were treated with aqueous extract of *S. moellendorffii* coated silver nanoparticles (24.7, 49.5, 123, and 247.5 nM) compared against albendazole, Alb (9.4µM), Ag^{+} (100 µM), and control (RPMI Media) for 24 h to observe theirs *in vitro* mortality.

In vitro morbidity test of *H. contortus* larvae (L3) was performed by taking larvae (Approximately 25-30) in 200 µl of RPMI medium in 96 well plates in presence of SAgNPs (24.7, 49.5, 123 and 247.5 nM), Alb (9.4 µM) and Ag^{+} (100 µM) for 24 h. After the incubation (37 °C), a direct microscopic examination (at 40X magnification) was done for mobility (for 20 secs).

Egg suspension (1 ml) containing nearly 200 eggs was taken into 24 well microtiter plates, subsequently, to check the inhibition efficiency of drugs, eggs were treated at different concentrations of SAgNPs (24.7, 49.5, 123, and 247.5 nM), against two controls, one positive with Alb (9.4 µM) and Ag^{+} (100 µM) and another negative with RPMI media. After the inhibition treatment, the plates were incubated at 28 °C for 48 h.

A detailed procedure for adult motility assay (2.3.3), larval motility assay (2.3.4), egg hatch assay (2.3.5), and morphological analysis, SEM (2.4), generation of oxidative stress with ROS (2.6.1), NOS (2.6.2), and catalytic activities CAT, 2.7.1, SOD, 2.7.2, GPx, 2.7.3 and GSH, 2.7.4 has been explained in Chapter 2.

4.2.2 Results:

4.2.2.1 Evaluation of APE protected AgNPs physical properties

The synthesis of SAgNPs under the sunlight (**Figure 18)** was confirmed visibly by monitoring change of color (**Figure 19)** and thereafter was confirmed by observing surface plasmon resonance (SPR) with an extinction maximum at 435 nm (**Figure 20a**). Dynamic light scattering (DLS) data showed the formation of monodispersed highly stable SAgNPs with an average hydrodynamic diameter of 91 nm (**Figure 20b**) surface charge of -24 mV (Zeta potential analysis). Transmission electron microscopic (TEM) studies further confirmed sheet nanoparticles formation, (**Figure 20c**) a further amplified picture of single SAgNPs has been presented in the inset. Elemental analysis using EDS showed the involvement of 16.21% silver by weight in the SAgNPs. As a typical absorption peak of metal silver, synthesis showed an optical absorption peak at 3 keV (shown in **Figure 21**). To confirm the interaction of reducing agents of the ASE with AgNPs, FTIR studies were performed. The formation of the Ag-OH bond along with the involvement of C-Br, C-H, C=O, C=C, O-H has been observed (**Figure 20d**). The band positions and respective shifts are given in **Table 15**.

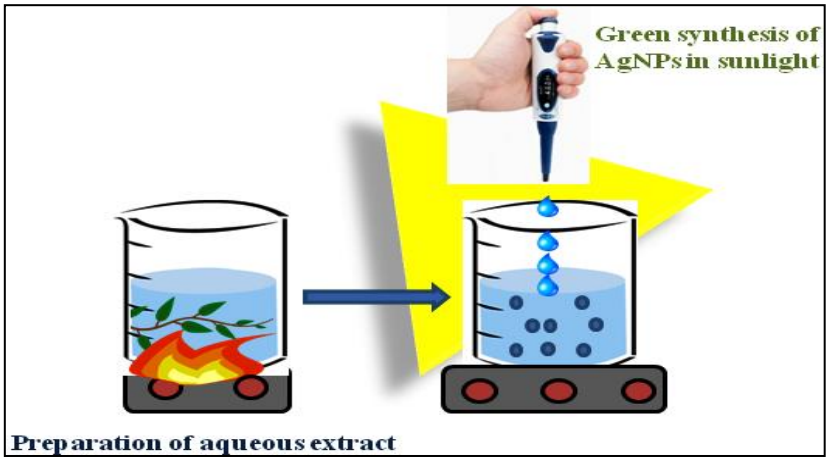


Figure 18. Schematic representation of the synthesis of silver nanoparticles (AgNPs) using aqueous plant extract of *Selaginella moellendorffii* under direct sunlight.

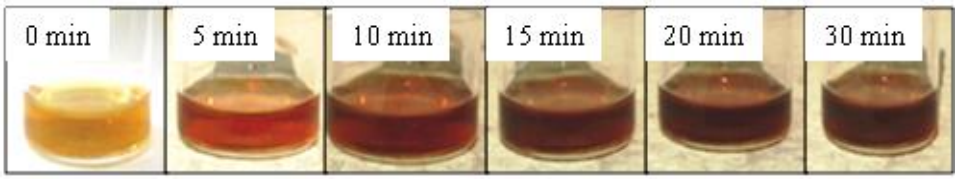


Figure 19. Color changes under the sunlight during the formation of SAgNPs using aqueous *S. moellendorffii* extract (ASE) as a reducing agent. The pictures were taken at different time intervals 0-30 min

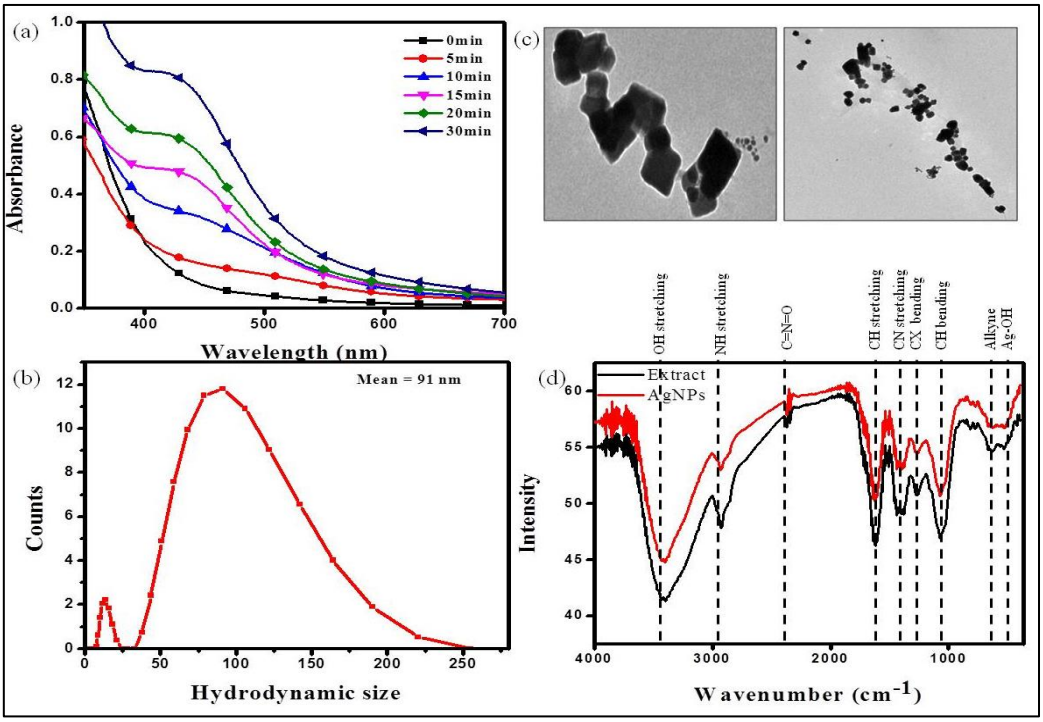


Figure 20. Characterization of silver nanoparticles (AgNPs). (a) Surface plasmon resonance spectra of photo-reduced AgNPs of different sizes. The spectra were scanned in the range of 300 nm-700 nm. (b) Dynamic light scattering study showing the distribution of hydrodynamic diameter and disparity of the SAgNPs. The hydrodynamic size obtained was 91 nM. (c) Transmission electron microscopic studies for the size and shape of the AgNPs. The inset is showing the mean values of the SAgNPs. (d) FTIR studies confirmed the changes in functional groups, including plant extract and SAgNPs.

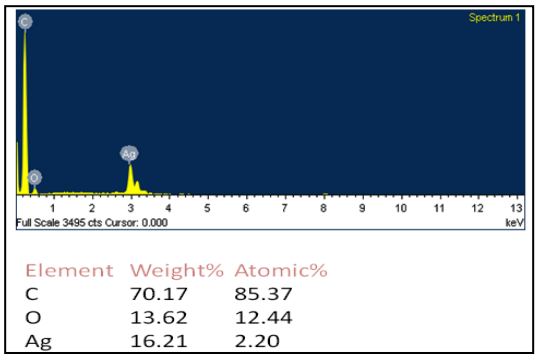


Figure 21. Below EDS microanalysis confirmed the presence of silver. The qualitative analysis showed a 16 % weight percentage of silver.

Table 15. FTIR analysis for functional groups for *S. moellendorffii* and silver nanoparticles coated with aqueous *S. moellendorffii* extract.

Functional Groups	**Wavenumber (cm^{-1})**		
	Aqueous *S. moellendorffii* extract	**SAgNPs**	**References**
Ag-OH	-	526	[246]
Alkyne	630	630	[247]
C-H bending	777	777	[247]
C-X stretching	817	817	[248]
C-N stretching	1266	1266	[248]
C-H stretching	1619	1619	[249]
C=N=O	2377	2377	[249]
N-H stretching	2932	2932	[250]
O-H stretching	3425	3425	[251]

4.2.2.2 Toxicity of SAgNPs on the human normal kidney cells

The cell viability results showed mild toxicity of SAgNPs on HEK293 cells for 24 h exposure. At the lowest concentration (247.5 nM), there were no significant changes were observed in HEK293 viability. With an increase in SAgNPs concentration, however, the percentage of viable cells was observed (71.15 ± 8.4 %, 60.63 ± 4.09 %, and 51.13 ± 0.8 %). Further, silver nanoparticles are known for their leaching effect, and a maximum of ~30 % (70 μM) silver leaching was observed upon 6 months of storage of silver nanoparticles. Therefore, we have also checked toxicity caused by the leaching effect HEK293 cells were treated with various concentrations (50-250 μM) of Ag+ for 24 h. 76.5 ± 8.8 %, 72.5 ± 5.8 %, and 68.4 ± 0.2 % cells were found to be alive following (50, 100 and 250 μM) of Ag^{+} treatment for 24 h (**Figure 22a**). On the contrary, *H. contortus* cells showed high susceptibility in the presence of SAgNPs. A sharp loss of cell viability absorbance (1.84± 0.26, 1.21± 0.11, 1 ± 0.07, and 0.82 ± 0.19 loss of cell viability was observed for (24.7 nM, 49.5 nM, 123 nM and 247.5 nM) SAgNPs treatment respectively (**Figure 22b**).

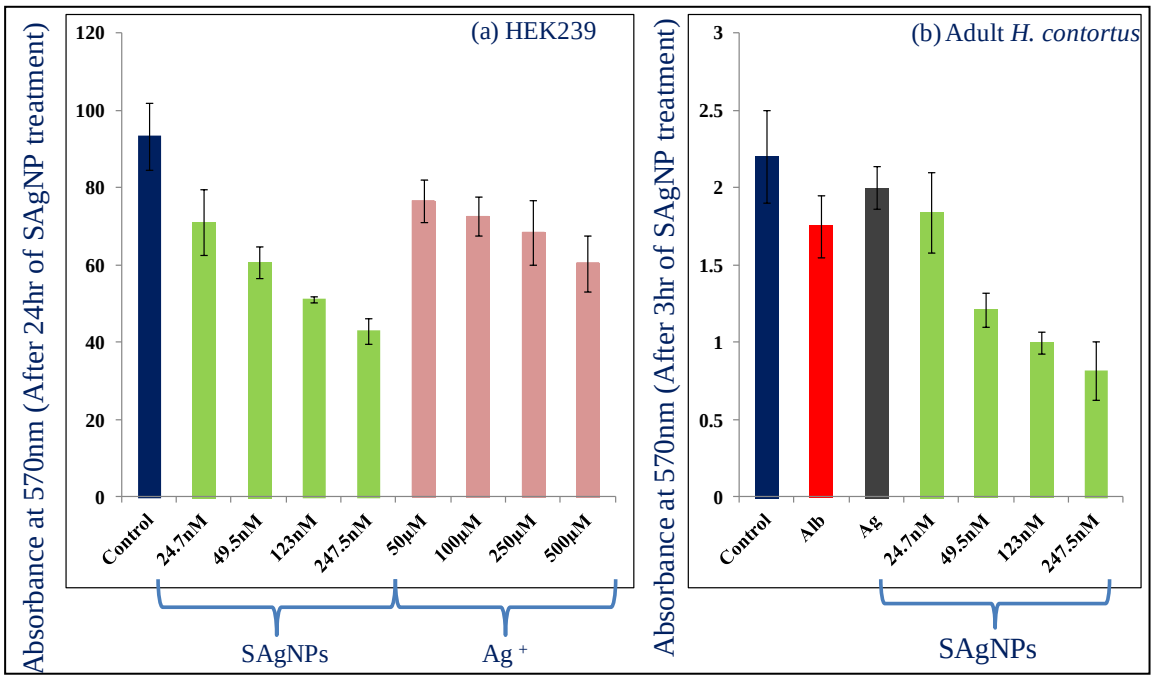


Figure 22. Cell viability study of humans and adult *H. contortus* upon exposure to SAgNPs. (a) Human embryonic kidney (HEK239) cells were treated with various concentrations of SAgNPs and different concentrations of Ag^+ (100 µM) for 24 h and thereafter MTT assay was performed to determine the viability of cells. SAgNPs showed mild toxicity on HEK239 cells upon 24 h exposure.

4.2.2.3 Assessment of antihelminthic activities

The results for the antihelminthic activities are discussed below:

4.2.2.3.1 Adult Motility and Morbidity Assay (AMMA)

The effect of SAgNPs on the viability of *H. contortus* is concentration-dependent. Approximately 13% and 6% of males and female worms respectively were paralyzed in 1 h and 26% of male worms and 40% of the female worms died within 12 h of treatment with 247.5 nM SAgNPs. The detailed death and time of paralysis are given in **Table 16**.

Table 16. Paralysis and Death time analysis of AgNPs on adult *H. contortus.* Worms were exposed to SAgNPs of different doses (24.7nM, 49.5nM, 123nM and 247.5nM), Alb (9.4µM) and Ag^{+} (100µM) in RPMI media for 24 h. Death and paralysis of worms were analyzed at various time points in between. Worms were first noted for their movement. If no movement was observed then worms were exposed to a warm saline solution (50 ℃) for 1 min to stimulate movement. The counts were taken for the number based on the worm's movement. If the worm previously immobile worm starts moving upon exposure to warm saline, then considered as paralyzed otherwise considered alive.

Time of Exposure	**Paralysis Time**							**Death Time**						
	Male Worms													
	Control	**Alb**	**Ag^{+}**	**24.7**	**49.5**	**123**	**247.5**	**Control**	**Alb**	**Ag^{+}**	**24.7**	**49.5**	**123**	**247.5**
0 h	-	-	-	-	-	-	-	-	-		-	-	-	-
0.5 h	-	-	-	-	-	-	**1**±0	-	-	-	-	-	-	-
1 h	-	-	-	-	-	4±0.7	2±0.7	-	-	-	-	-	-	-
3 h	-	-	-	-	-	6±0.5	2±0.7	-	-	-	-	-	-	-
6 h	-	3±0.5	-	-	1±0	1±0	3±0.5	-	-	-	-	-	-	4±0.7
12 h	-	6±0.5	3±0.5	3±0.5	2±0.7	1±0	0	-		-	2±0.7	2±0.7	4±0.7	4 ±0.7
24 h	1	0	6±0.5	3±0.5	2±0.7	0	0	-	15±0	9±0.5	12±0.5	9±0.5	15±0	15±0
	Female Worms													
0 h	-	-		-	-	-	-	-	-	-	-	-	-	-
0.5 h	-	-		-	-	-	-	-	-	-	-	-	-	-
1 h	-	-		-	-	-	1±0	-	-	-	-	-	-	-
3 h	-	-		-	-	-	4±0.5	-	-	-	-	-	-	-
6 h	-	-		-	-	4±0.7	4±0.7	-	-	-	-	-	-	-
12 h	-	-		2±0.7	1±0	6±0.5	3±0.5	-	3±0.5	-	-	3±0.5	4±0.7	6±0.5
24 h	1	0	3±0.5	1±0	3±0.5	0	0	-	6±0.5	-	3±0.5	9±0.5	9±0.5	12±0.5

4.2.2.3.2 Larval Morbidity Assay (LMA)

After 24 h treatment with different concentrations of SAgNPs and respective controls as before L3 larvae looked for movement of morphological changes like shrinkage and ruptured morphology. Concentration-dependent loss of viability of L3 larvae was prominent with SAgNPs. 42 ± 2.6, 26 ± 1.7, 19 ± 2.5 and 7 ± 2.1 % survival of larvae was observed for 24.7 nM, 49.5 nM, 123 nM and 247.5 nM SAgNPs treatment respectively, whereas the recommended dose of Alb (9.4 µM) showed 52 ± 0.5 % and Ag+ (100 µM) showed 61 ± 0.7 % survival for 24 h treatment (**Figure 23**). The p-values for the Larval Motility Assay (L-3) of *H. contortus* worms, when treated with different concentrations of SAgNPs, are described in **Table 17**.

4.2.2.3.3 Egg Hatch Assay (EHA)

After 48 h of treatment with SAgNPs and respective controls number of eggs hatched was calculated using the equation (ii). The treatment concentration-dependent reduction of egg hatching efficiency was due to SAgNPs treatment. 63 ± 2.6, 34 ± 2.7, 10 ± 1.3, and 3.5 ± 1.9 % reduction of egg hatching were observed in comparison with control for 24.7nM, 49.5nM, 123nM and 247.5nM SAgNPs respectively. Whereas recommended dose of Alb (9.4 µM) showed 68 ± 0.5 % and Ag+ (100 µM) 72 ± 0.7 % reduction of egg hatching or larvae (L1) formation (**Figure 23**). The p-values for the Egg Hatch Assay of *H. contortus* worms, when treated with different concentrations of SAgNPs, are described in **Table 17.**

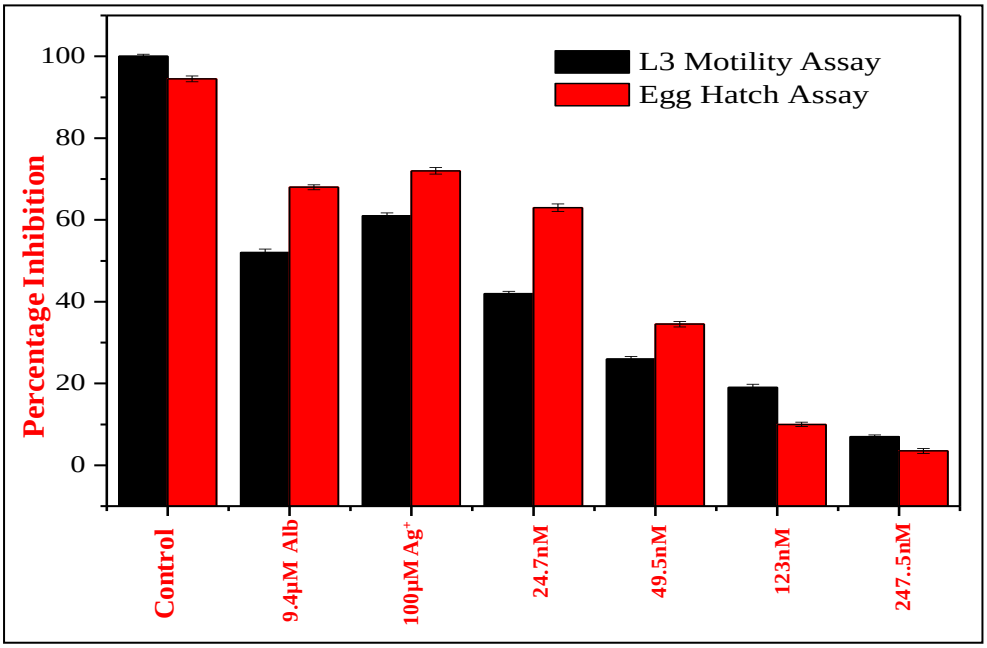


Figure 23. Percentage viability effect of SAgNPs on L-3 Larvae survival and hatching of *H. contortus* eggs (Black) viability (survival) percentage of the larval stage L3, after 24 h treatments with SAgNPs different concentrations along with controls. (Red) Comparative graph showing SAgNPs the influence of on egg hatch. For 48 h the treatment was performed using the SAgNPs different concentrations along with the controls. In each well around 200 eggs were taken and the results of 3 independent experiments were represented as an average.

Table 17. The p-values for the Egg Hatch Assay and Larval Motility Assay (L-3) of *H. contortus* worms when treated with different concentrations of SAgNPs (24.7 nM, 49.5 nM, 123 nM, and 247.5 nM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

	24.7 nM	**49.5 nM**	**123 nM**	**247.5 nM**
Egg Hatch Assay	1.37641E-10	1.69269E-10	8.17911E-11	1.03611E-10
Larval Motility Assay	2.12529E-13	2.92879E-13	4.16119E-12	1.63234E-10

4.2.2.3.4 Ultra morphological analysis for tissue damage due to SAgNPs exposure

The extent of physical damage of *H. contortus* was monitored using scanning electron microscopy. In **Figure 24**, between the sets of the control's comparative studies (RPMI media), and SAgNPs (247.5 nM) treated *H. contortus* are shown. A complete comparative study for the changes in the morphology of tissue damage is shown in **Figure 24**, comparing control (RPMI Media), Alb (9.4 µM), Ag+ (100 µM), and 247.5 nM SAgNPs after 3 h of treatment. The complete body region of the adult worm is depicted in **Figure 24a- c**. The untreated *H. contortus* worm with a smooth cuticle having a well-developed body region of an adult was seen in **Figure 24a**. Whereas in **Figure 24j- l, a** complete distortion of outer morphology along with prominent shrinkage worm body was observed when treated with SAgNPs. **Figure 24e, h,** and **k** showed the adult helminths anterior region. Almost no distortions were observed in the control (**Figure 24b**). Complete disruptions were observed in SAgNPs (247.5 nM) treated worms (**Figure 24k**). Similar

observations were prominent in the posterior ends of the control and treated worms as well (**Figure 24c, f, i,** and **l**).

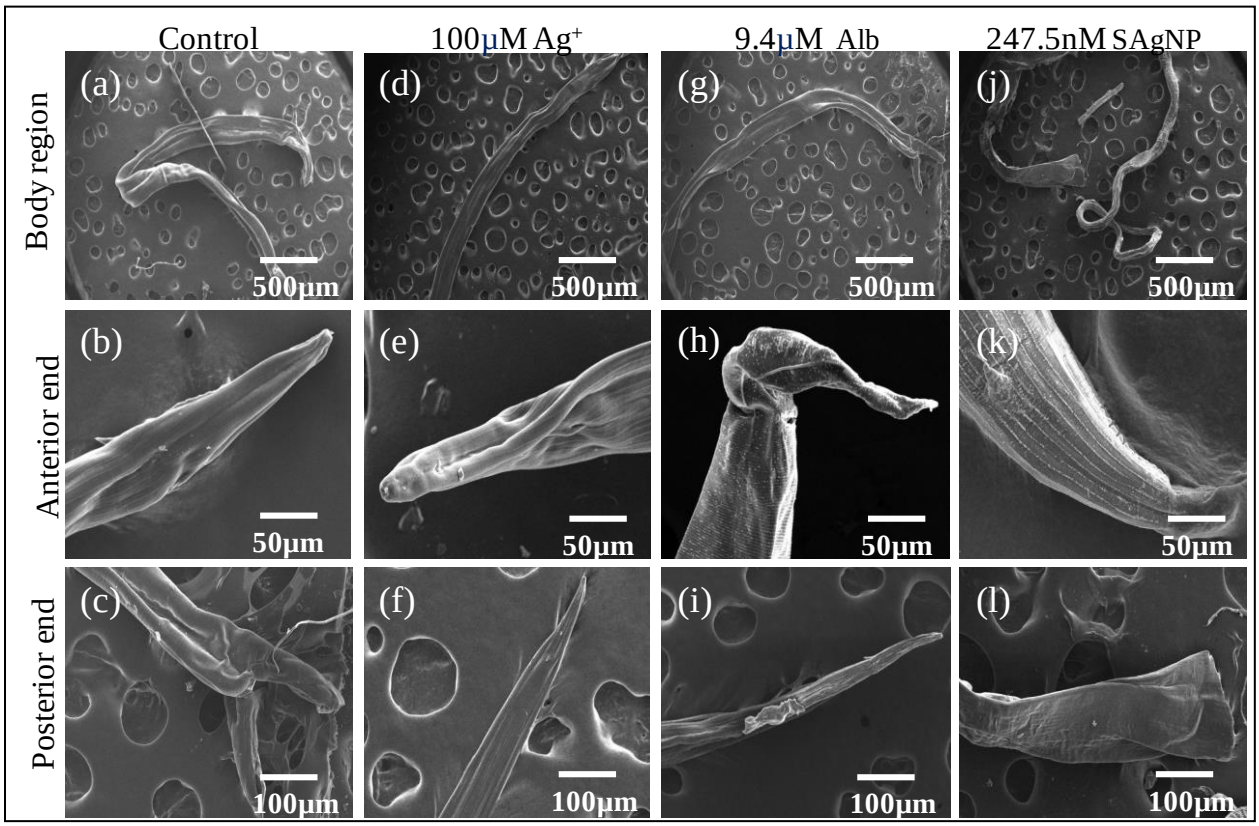


Figure 24. Study of ultramicroscopic morphological damage in *H. contortus* adults under the SAgNPs influence. For 3 h helminths were treated and after that, the helminths were dehydrated and coated with gold, and then using SEM at 15 (kV) ultra-structures was observed. (**a, b,** and **c**) images show the treatment with control (RPMI media), (**d, e,** and **f**) shows adult worms after treatment with 100µM Ag^{+}, (g, h and i) showed treatment with 9.4µM Alb and (**j, k**, and **l)** showed treatment with 247.5 nM SAgNPs. a, d, g, and j are describing the whole body of the controls, and worms treated with SAgNPs respectively. b, e, h, and k are showing the anterior end of the controls, and worms treated with SAgNPs respectively. **c, f, i** and **l** are showing controls posterior ends, and worms treated with SAgNPs respectively. SAgNPs treated worms showed profound tissue architecture damages and tissue integrity loss and bursa region's disorganization.

4.2.2.3.5 Generation of Reactive oxygen species (ROS) stress due to SAgNPs exposure

A complete comparative study for the changes in ROS generation of the stress response is shown in **Figure 25**, comparing control (RPMI Media), Alb (9.4 µM), Ag+ (100 µM), and 247.5 nM SAgNPs after 3 h of treatment. In control tissue (**Figure 25**) low level of ROS generation showed a moderate level of ROS was observed using DCFDA fluorescence. Massive cellular stress response for ROS generation had been observed due to SAgNPs (247.5 nM) treatment within 3 h. **Figure 25 a, d, g, and j** showed a complete body region of SAgNPs treated adult *H. contortus*. The high generation of ROS was prominent in both anterior (**Figure 25 k**) and posterior ends (**Figure 25 l**).

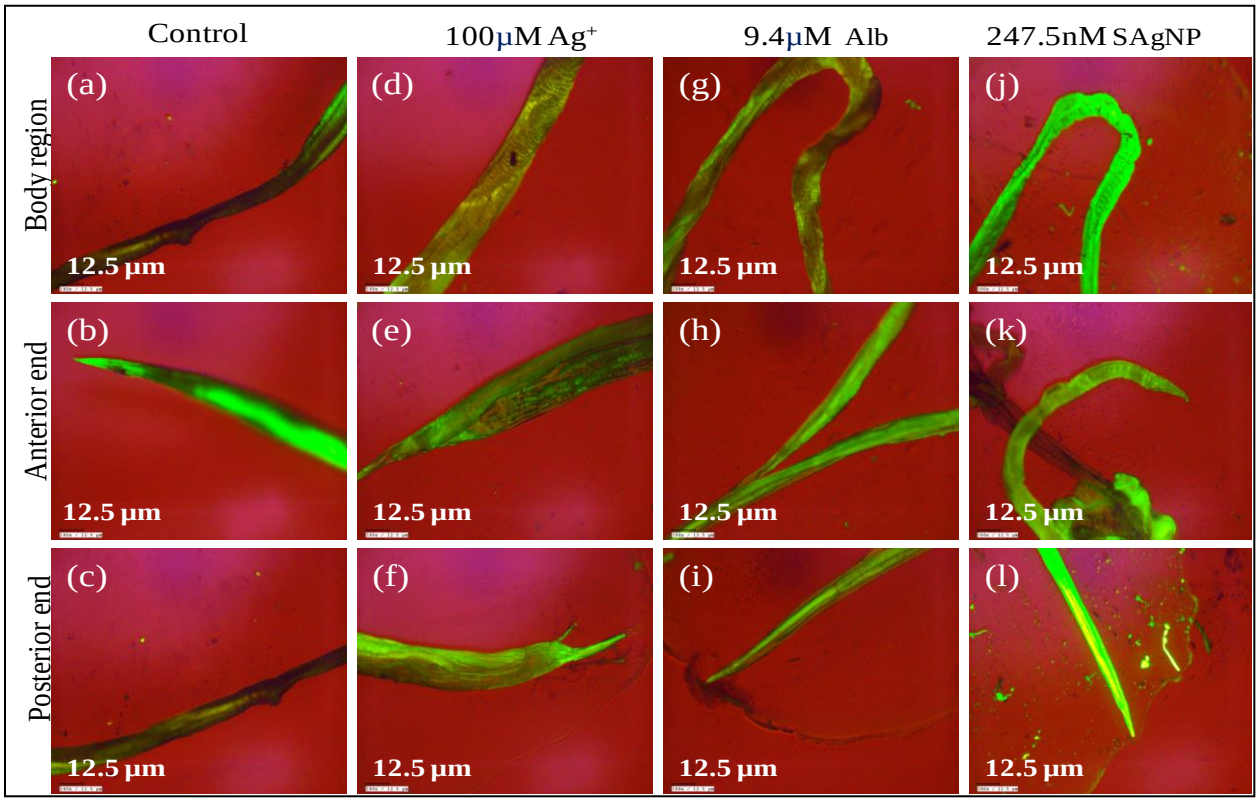


Figure 25. Oxidative stress generation in the adult worms due to SAgNPs exposure in *H. contortus* adults was treated with SAgNPs for 3 h and controls and then processed for ROS (DCFDA staining). (**a, b**, and **c**) images show the treatment with control (RPMI media), (**d, e**, and **f**) shows adult worms after treatment with 100µM Ag^{+}, (**g, h** and **i**) showed treatment with 9.4µM Alb and (**j, k**, and **l**) showed treatment with 247.5 nM SAgNPs**. a, d, g,** and **j** are describing the whole body of the controls, and worms treated with SAgNPs respectively. **b, c, h**, and **k** are showing the anterior end of the controls, and worms treated with SAgNPs respectively. **c, f, i** and **l** are showing

controls posterior ends, and worms treated with SAgNPs respectively. After the desired treatment of the worms were incubated for 20 mins with DCFDA (100 nM) in dark. Thereafter inverted fluorescence microscope by Dewinter, Italy was used for taking the images.

4.2.2.3.6 Determination of nitric oxide synthase

The generation of NOS with the stress response in the worm due to SAgNPs treatment was monitored using the Greiss reagent. The results showed a dose-dependent and subsequent time and increase in the NOS level in the worms. The level NOS has increased from 0.057 ± 0.016 to 0.074 ± 0.05 within 24 h for the lowest dose (24.7 nM) of SAgNPs, whereas the NOS response become further significantly prominent and increase from 0.084 ± 0.03 and 0.62 ± 0.01 (within 24 h) due to 247.5 nM SAgNPs treatment (**Figure 26**). Whereas the change of level of NOS in response to Ag^{+} and Alb was moderate and not of high significance.

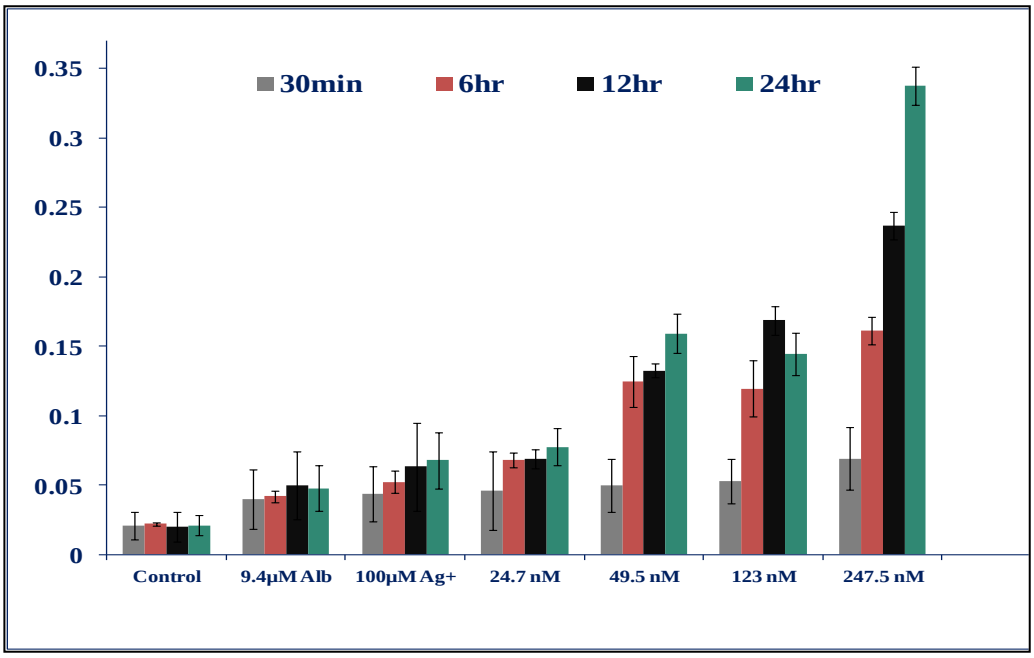


Figure 26. Alteration of activity of nitric oxide synthase (NOS) enzyme due to SAgNPs stress. A comparative graph showing the generation of nitric oxide free radicals as a result of NOS activity in *H. contortus* worms after treating with SAgNPs different concentrations (247.5 nM) along with controls for 30 mins, 6 h, 12 h, and 24 h.

4.2.2.3.7 Alteration of catalase, superoxide dismutase, and glutathione peroxidase activity in response to SAgNPs induced oxidative stress

The increase of reactive oxygen and nitrogen species due to SAgNPs treatment generated huge oxidative stress within the worm's physiological system which may alter the change in the activity of metabolic and stress-responsive enzymes. A steady enhancement in CAT activity with an increase of SAgNPs concentration results in an increase in stress response had been observed. In different concentration of SAgNPs (24.7nM, 49.5nM, 123nM and 247.5nM) the amount of CAT enzyme was calculated as 2.36 ± 0.15, 4.62 ± 0.17, 5.63 ± 0.24 and 6.22 ± 0.18 U/mg proteins respectively after 3h of treatment in comparison with the 0.22 ± 0.05, 4.1 ± 0.37 and 0.52 ± 0.05 U/mg proteins for untreated control, Alb (9.4 μM) and Ag+ (100 μM) respectively **Table 18.** A consistent SOD activity increment with an increase in stress response had also been observed. The SOD enzyme activity after the treatment was found to be 2.21 ± 0.7, 2.55 ± 0.15, 3.01 ± 0.18 and 3.19 ± 0.25 U/mg protein for SAgNPs 24.7nM, 49.5nM, 123nM and 247.5nM respectively in relation to the control where 4.8 ± 0.22 Alb and 0.56 ± 0.07 Ag^+ amount was used. Untreated worms showed SOD activity of 0.21 ± 0.018 U/mg of worm protein **Table 18**. In contrast to other antioxidant activities, the activity of GPx also increases when worms are exposed to SAgNPs' increasing concentrations. At 340 nm spectrophotometrically the amount of GPx was observed. The activity of the GPx enzyme was calculated using the (iii) equation. The GPx enzymes active amount was 2.22 ± 0.057, 2.67 ± 0.21, 3.21 ± 0.08 and 3.4 ± 0.15 U/mg protein for SAgNPs different concentrations (24.7nM, 49.5nM, 123nM and 247.5nM) respectively in comparison with the control where the amount was 2.6 ± 0.25 U/mg proteins in Alb and 0.58 ± 0.058 U/mg proteins in Ag^+. Untreated was found to be 0.21 ± 0.071 U/mg proteins (**Table 18**). The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of SAgNPs are described in **Table 19.**

4.2.2.3.8 ROS enhances cellular combat by increasing the concentration of reduced glutathione

In combat to ROS, cellular response induced by GSH causes oxidative stress in the worms when exposed to SAgNPs. A significant enhancement in the amount of GSH produced was measured in SAgNPs treated worms. The increase in GSH was observed (0.28 ± 0.05 μM/ mg for untreated) along with the increase of the dose of SAgNPs (24.7nM, 49.5nM, 123nM and 247.5nM) from 2.23 ± 0.15, 3.49 ± 0.24, 4.02 ± 0.7 and 4.21 ± 0.057 μM/ mg protein respectively. Treatment

of Alb also induced a significant increment in the GSH concentration (1.75 ± 0.050 µM/ mg protein) and Ag+ causes 0.59 ± 0.009 µM/ mg protein accumulation of GSH (**Table 18)**. The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of SAgNPs are described in **Table 19.**

Table 18. Oxidative stress-responsive elements in *H. contortus* to counter the toxic effect of SAgNPs on infectious helminths. Due to the generation of oxide synthase-related stress helminth counteracts conditions through alteration of its metabolic functions. The toxic effect of SAgNPs on the body of the helminth reacts with the cellular molecules as normal metabolic functions and increases the regeneration of free radicals induced by oxidative damage defense mechanisms to include the following: enzymatic factors such as CAT, SOD, GPx, and GSH. The enzymatic activity was measured as the enzyme unit per mg of protein (U/ mg protein) for CAT, SOD, and GPx and (µM/ mg protein) in GSH.

Treatment	U/mg protein			µM/mg protein
	CAT	**SOD**	**GPx**	**GSH**
Concentrations				
Control	0.22 ± 0.05	0.21 ± 0.018	0.25 ± 0.07	0.28 ± 0.01
9.4µM Alb	4.1 ± 0.37	4.8 ± 0.2	2.6 ± 0.25	1.4 ± 0.05
100µM Ag^{+}	0.56 ± 0.05	0.52 ± 0.07	0.58 ± 0.057	0.59 ± 0.009
24.7 nM SAgNP	2.35 ± 0.15	2.21 ± 0.07	2.22 ± 0.15	2.23 ± 0.15
49.5 nM SAgNP	4.62 ± 0.17	2.55 ± 0.15	2.67 ± 0.16	3.49 ± 0.24
123 nM SAgNP	5.63 ± 0.24	3.01 ± 0.18	3.21 ± 0.08	4.02 ± 0.07
247.5 nM SAgNP	6.22 ± 0.18	3.19 ± 0.25	3.48 ± 0.15	4.21 ± 0.057

Table 19. The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of SAgNPs (24.7, 49.5, 123, and 247.5 nM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

SAgNP concentration	Superoxide Dismutase (SOD)	Catalase (CAT)	Glutathione Peroxidase (GPx)	Glutathione (GSH)
24.7nM	6.69354E-10	7.72E-11	1.28E-10	2.63E-10
49.5nM	4.13047E-10	2.87E-10	3.76E-10	1.94E-10
123nM	1.69716E-09	1.1E-09	1.47E-09	8.56E-10
247.5nM	2.49973E-08	2.5E-08	1.254-09	3.84E-10

Conclusions:

The Silver Nanoparticles (AgNPs) were synthesized successfully from the bio-reduction of $AgNO_3$ solution using the plant leaf extracts of the *Selaginella moellendorffii*. The biosynthesized silver nanoparticles were appropriately characterized using UV-vis spectroscopy, FTIR analysis, DLS, and Zeta Potential. The UV-vis analysis showed that the absorption spectra of the silver nanoparticles synthesized in the reaction media have absorption maxima in the range of 400 - 475 nm because of the surface plasmon resonance of the silver nanoparticles. From the DLS study, we came to know about the hydrodynamic size that was 91 nm in counts and the stability of the nanoparticles in the solution, whereas the chemical composition of the aqueous extract and biosynthesized AgNPs were analyzed by FTIR. The qualitative analysis was also performed that showed the phytochemical constituents such as the alkaloids, flavonoids, tannins, saponins, phenolic compounds, steroids, and glycosides are present in the *Selaginella moellendorffii* plant species and which may possess various medical and pharmaceutical values. Therefore, plant species also possessed some antihelminthic activities. Significant percentage inhibition of viability of larvae and egg hatch assay showed the toxic nature of pteridophytes. Thus, reduction in the growth of larvae and adults and egg hatching proves efficient antihelminthic nature of pteridophyte plants that are easily available in hilly regions and are pocket friendly and environment friendly as well.

This is the first report of the unprecedented antihelminthic efficiency of *S. moellendorffii* aqueous extract-protected AgNPs by increasing both ROS and NOS and thereby causing alteration

of metabolic activity and physical damage in worm tissue. *H. contortus* shows high resistivity against albendazole, which was also consistent in our findings, whereas SAgNPs not only killed adult worms but also showed a significant effect of reducing larval viability and egg hatching (**Figure 27**).

Henceforth, the findings are highly useful in the wake of the increasing problem of drug resistance to the commercially available antihelminthic, an increase of life quality, and reduction of healthcare cost, but further detailed studies are required to understand the active principal component(s), efficacy, and toxicity of the system in animal models. In addition to that, the utility of the formulations against other parasitic infections, including helminthic infections, needs to be evaluated in the future.

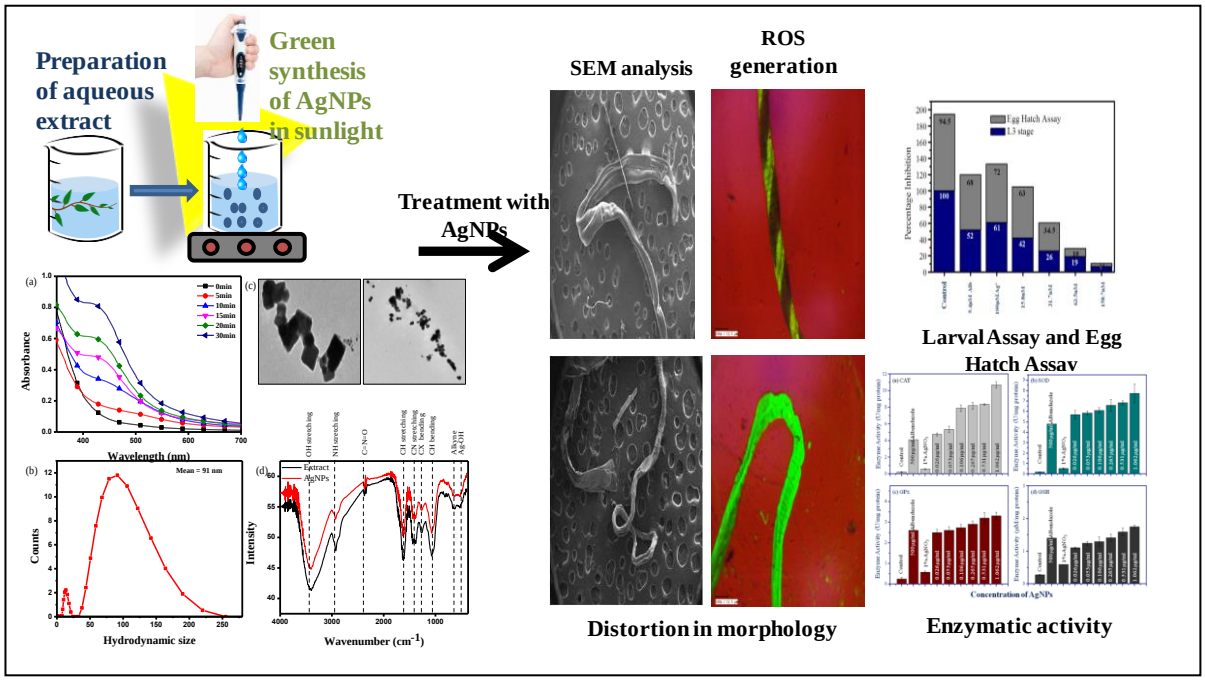


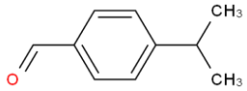
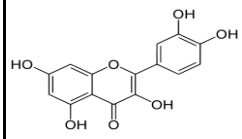
Figure 27. Schematic figure showing the synthesis of SAgNPs under direct sunlight, then treatment of adult *H. contortus* worms, L3 stage, and egg hatch assay with SAgNPs at different concentrations. After that, changes in morphology were analyzed using SEM and then a generation of ROS was observed.

CHAPTER 5

The antihelminthic activity of pure compounds

Since all the herbs in the world have been used for therapeutic purposes. In India, traditional medicine is used since 1500 BC, and in China, the use of herbs for therapeutics is as old as 2700 BC. In the present day, almost 90% of the rural population depends on herbal medicines in developing countries [163]. Several medicinal plant-based treatments with *Allium sativum, A. cepa*, and *Mentha arvensis* species came into consideration for the development of new drugs to control gastrointestinal parasitism [164-168]. Many seed oils such as pomegranate oil, grape oil, carvacrol, eugenol, cumin oil had been reported to show their antihelminthic activities [169-173]. Here, plant-based synthetic compounds, cuminaldehyde, and quercetin have been used to analyze the antihelminthic effect against *H. contortus* and studied the novel mechanism of action of the same (**Table 18**).

Table 18. Pure compounds and their traditional uses

Compound	Structure	Source	Uses	References
Cuminaldehyde		eucalyptus, myrrh, cassia, cumin	diarrhea, colic, bowel spasms, and gas	[252, 253]
Quercetin		apples, berries, Brassica vegetables, capers, grapes, onions, shallots, tea, and tomatoes	Arthritis, bladder infections, and diabetes.	[254]

5.1 Cuminaldehyde

Cumin, a novel monoterpenoid belonging to the family Apiaceae is a common aromatic herb growing annually [178]. Though Cumin is indigenous to India, it is a widely used spice cultivated in India, Iran, Egypt but later refined widely in areas such as Asia, North Africa, southern Russia, and southern America [255, 256]. Cumin contains fixed oil (about 10%), volatile oil (about 1.5%), protein, sugar and phenolic constituents [257, 258]. Various pharmacological potentials of cumin such as digestive conditions, toothache, epilepsy, antitumor, anti-bacterial, anti-fungal, anticonvulsant, anti-inflammatory, antihelminthic (anti-tapeworm), and antioxidant have been reported.

(Madhavan and Tharakan, 2017) had reported the antihelminthic activity of *C. cyminum* aqueous extract against Indian common earthworm *Pheretima posthuma* [259] The seed extract of *Nigella sativa* (black cumin) has extensively been used in Indian and African traditional medicine because of its therapeutic potential and possesses a wide spectrum of activities, namely, diuretic, antihypertensive, antidiabetic, anticancer, immune-modulatory, antimicrobial, anthelmintic, analgesic and anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepato-protective, and renal protective properties [260]. Mnif et al. 2015 showed the biological activities, phytochemical profile, and pharmacological uses of the *Cuminum cyminum L* [257].

Cuminaldehyde (CA), is a natural monoterpenoid with an aldehyde group abundant in cumin oil [261]. CA has been reported to show beneficial effects against various diseases such as skin diseases, fever, vomiting, nausea, anticancer, antidiabetic, neuroprotection, and anti-inflammation [262, 263]. Monteiro-Neto et al. 2020 explored the antimicrobial activity against *S. aureus* and *E. coli* induced infections in comparison to ciprofloxacin [264]. Moreover, nephroprotective, gastroprotective, and neuroprotective activities along with nutraceutical effects have been studied by Srinivasan et al. 2018 [265]. Wongkattiya et al. 2019 showed the antibacterial activity against food-borne pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella Typhi* using cuminaldehyde [266].

Here, in this work, we have reported the antihelminthic effect of CA for the first time against *H. contortus* and studied the novel mechanism of action of the same. We found CA induces oxidative stress to various stages of *H. contortus* causing a change in metabolism, physical damage, and death of the worms. Thus, CA could be evaluated for its clinical use against gastro-intestinal parasitic infection with special emphasis on *Haemonchus contortus*.

5.1.1 Experimental design for the treatment of egg hatch, larval stage and paralysis, and death of adult H. contortus worms with CA.

Adult *H. contortus* worms were isolated from the abomasa of infected ruminants. The adult worms were treated with CA (37, 74, 185, 370, and 740 µg/ml) and compared to albendazole, (500 µg/ml), and control (RPMI Media) for 24 h to observe theirs *in vitro* mortality.

In vitro morbidity test of *H. contortus* larvae (L3) was performed by taking larvae (Approximately 25-30) in 200 µl of RPMI medium in 96 well plates in presence of CA (37, 74, 185, 370, and 740 µg/ml), and Alb (500 µg/ml) for 24 h. After the incubation, a direct microscopic examination (at 40X magnification) was done for mobility (for 20 secs).

Egg suspension (1 ml) containing nearly 200 eggs was placed into 24 well microtiter plates, subsequently, to check the inhibition efficiency of drugs, eggs were treated at different concentrations of CA (37, 74, 185, 370, and 740 µg/ml), against two controls, one positive with Alb (500 µg/ml) and another negative with RPMI media. After the inhibition treatment, the plates were incubated at 28 °C for 48 h.

A detailed procedure for adult motility assay (2.3.3), larval motility assay (2.3.4), egg hatch assay (2.3.5), and morphological analysis, SEM (2.4), generation of oxidative stress with ROS (2.6.1), NOS (2.6.2), and catalytic activities CAT, 2.7.1, SOD, 2.7.2, GPx, 2.7.3 and GSH, 2.7.4 has been explained in Chapter 2.

5.1.2 Results:

5.1.2.1 Assessment of death time and paralysis time H. contortus due to the treatment of CA

In male worms, CA was most active at 740 µg/ml concentration, where Approximately 73% of the worms were paralyzed in 1 h and nearly 80% of the worms died within 6 h of CA treatment. Further, in female worms also, CA was most active in 740 µg/ml concentration, where Approximately 80% of the worms were paralyzed in 3 h and nearly 60% of the worms died within 12 h of CA treatment. Whereas CA at 37 µg/ml at 12 h of treatment, showed only 20% of the female worms were paralyzed and 20% died in 24 h of CA treatment. The LD_{50} values of 184.5 ±

12.1, 127.3 ± 7.5µg/ml for an adult female, adult male worms at 12 h of CA exposure (**Figure 30a-b**). The dose-dependent death and paralysis time of adult worms are represented in **Table 19**.

Table 19. Paralysis and Death time analysis of CA on adult *H. contortus*. Worms were exposed to CA of different doses (37, 74, 185, 370, and 740 µg/ml), Alb (500 µg/ml) in RPMI media for 24 h. Death and paralysis of worms were analyzed at various time points in between. Worms were first noted for their movement. If no movement was observed then worms were exposed to a warm saline solution (50 ^{o}C) for 1 min to stimulate movement. The counts were taken for the number based on the worm's movement. If the worm previously immobile worm starts moving upon exposure to warm saline, then considered as paralyzed otherwise considered dead.

Time of Exposure	**Paralysis Time**							**Death Time**						
	Male Worms													
	Control	**Alb** (500)	**37**	**74**	**185**	**347**	**740**	**Control**	**Alb** (500)	**37**	**74**	**185**	**370**	**740**
0 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5 h	-	-	-	-	-	-	9±0	-	-	-	-	-	-	-
1 h	-	-	-	-	-	6±0	12±0.5	-	-	-	-	-	-	-
3 h	-	-	-	3±0.5	9±0	12±0.5	15±0	-	-	-	-	-	-	-
6 h	-	3±0.5	-	6±0	12±0.5	9±0	3±0	-	-	-	-	3±0.5	6±0	12±0.5
12 h	-	6±0.5	6±0.5	9±0	9±0	3±0.5	0±0	-		-	-	6±0.5	12±0.5	15±0
24 h	1±0	0±0	6±0.5	12±0.5	0±0	0±0	0±0	-	15±0	3±0.5	3±0.5	15±0	15±0	15±0
	Female Worms													
0 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 h	-	-	-	-	-	3±0.5	6±0.5	-	-	-	-	-	-	-
3 h	-	-	-	-	3±0	6±0.5	12±10	-	-	-	-	-	-	-
6 h	-	-	-	3±0.5	9±0.5	12±0	12±0.5	-	-	-	-	-	-	3±0.5
12 h	-	-	3±0	9±0	15±0	0±0	6±0.5	-	3±0	-	-	0±0	9±0	9±0.5
24 h	1±0	0±0	3±0.5	9±0.5	0±0	0±0	0±0	-	6±0	3±0	6±0.5	15±0	15±0	15±0

The morphological modifications after treatment were observed visually in **Figure 28(a-c)** under an optical microscope at 40X magnification. The control *H. contortus* contained a smooth cuticle along with well-developed anterior and posterior ends and a smooth body with well-organized morphological features. But after treatment with CA any dose (370 µg/ml shown in **Figure 28c**), the cuticle of the worm got ruptured; disorganization of the complete body morphology along with loss of anterior ends was observed. Similar damages were also been found for albendazole (500 µg/ml) treated worms [267, 268].

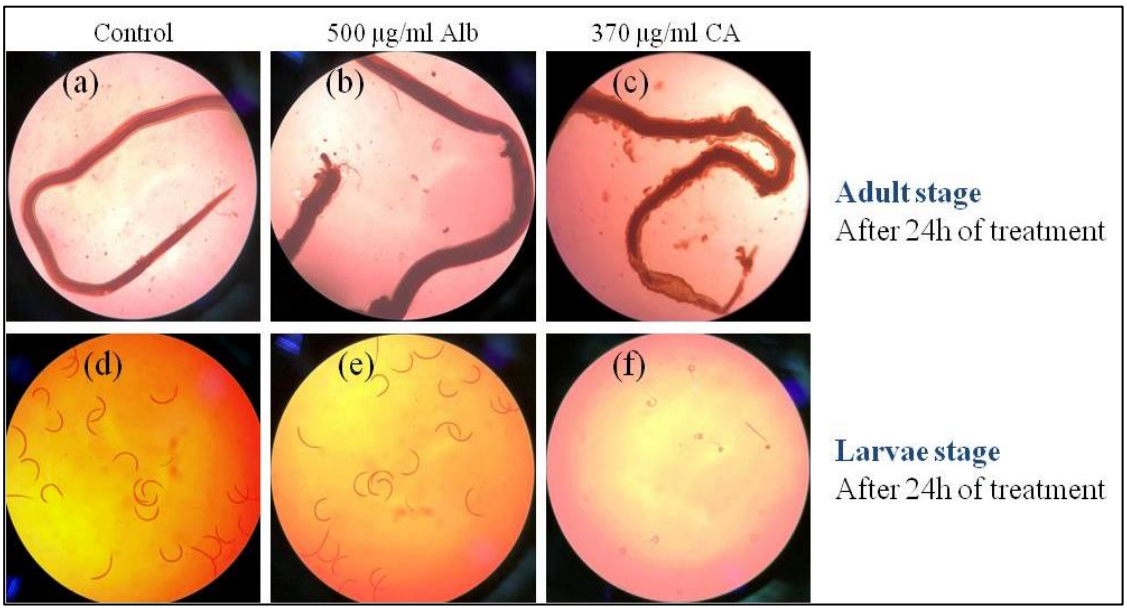


Figure 28. Optical microscopic images of control and treated adult and L-3 larval stages of *H. contortus.* The worms were isolated from the abomasums of the ruminants, washed, and identified. Thereafter they were treated with CA and Alb along with control. After treatment, the worms were stained with Lacto phenol cotton blue (LPCB) for at least 15- 20 min, before taking a picture under an optical microscope at 40X magnification. (**a**) shows of adult *H. contortus* treated with RPMI media for 24 h, as a negative control (b) shows of adult *H. contortus* treated with 500 µg/ml Alb, as a positive control (**c**) shows of adult *H. contortus* treated with CA at 24 h of treatment at concentration, 370 µg/ml. (**d**)) shows of L-3 larvae *H. contortus* treated with RPMI media for 24 h, as a negative control (**e**) shows of L-3 larvae *H. contortus* treated with 500 µg/ml Alb, as a positive control (**f**) shows of L-3 larvae *H. contortus* treated with CA at 24 h of treatment at concentration, 370 µg/ml.

5.1.2.2 Assessment of larval motility assay (LMA)

The L3 larvae extracted from the infected fecal matter or eggs isolated from adult female worms (after culture) were treated with CA and control (0.8 percent normal saline) for 24 h. Further morphological details were observed under an optical microscope. The response to CA treatment (morphological changes) against the control set of worms is shown in **Figure 28 (d-f).** Spindling, shrinkage, and ruptured morphology of L-3 larvae were observed in the case of CA (370 µg/ml) treatment whereas no such changes were observed in control as well as Alb (500 µg/ml) treat samples. Moreover, the LMA inhibition percentage was calculated after 24 h with the equation mentioned above in methods. The effect of CA on larval motility was concentration depended. The percentage of larval survival were obtained 68%, 48.1%, 33.3%, 18.5%, 7.4% and 7.4%, respectively for (0-740 µg/ml) of CA treatment respectively in compared to 60.2% for widely used antihelminthic drug, Alb (500µg/ml) **Figure 29a**. LD_{50} for inhibition of larva mortality obtained was 104.1 ± 7.9 µg/ml for 24 h **(Figure 30c**). The p-values for the Larval Motility Assay (L-3) of *H. contortus* worms, when treated with different concentrations of CA, are described in **Table 20**.

5.1.2.3 Assessment of egg hatch in response to CA treatment

The EHA inhibition percentage was calculated after 48 h with the equation mentioned above in methods using the Mc Master slide. The effect of CA on egg hatching was found to be dose-dependent. The percentage of hatched eggs into larvae were obtained 89%, 57%, 48%, 36%, 28.5%, and 14.5% respectively for (0-740 µg/ml) of CA treatment respectively in compared to 77% for widely used antihelminthic drug, Alb (500µg/ml) **Figure 29b.** Over 50% inhibition of egg hatching was observed at as low as 142.4 ± 11.4 µg/ml CA concentrations at 48 h treatment (**Figure 30d**). The results depict that the CA is highly effective in inhibiting the egg hatching of *H. contortus*. The p-values for the Egg Hatch Assay of *H. contortus* worms, when treated with different concentrations of CA, are described in **Table 20.**

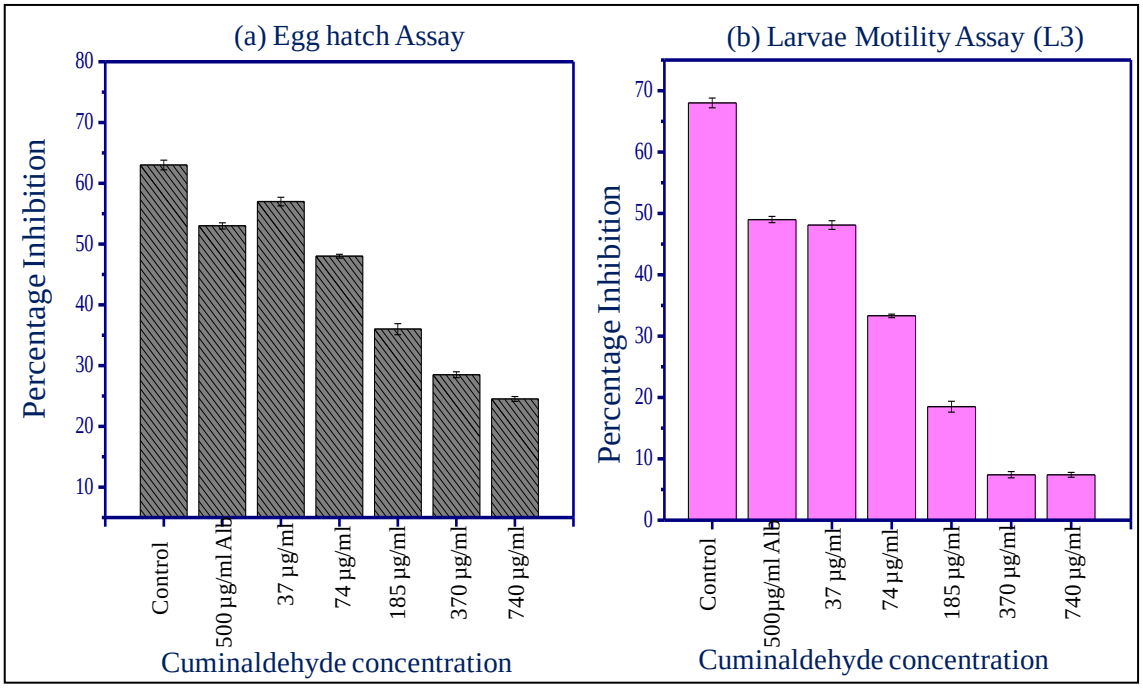


Figure 29. Inhibitory effect of CA on L-3 Larvae survival and egg hatching of *H. contortus*. (a) Percentage of inhibition (survival) of the L3, larval stage after treatment with different concentrations of CA for 24 h along with controls. (b) Comparative graph showing the influence of CA on egg hatch. The treatment was performed for 48 h in the presence of different concentrations of CA along with controls. Around 200 eggs were taken in each well and results were represented as an average of 3 independent experiments.

Table 20. Table showing the p-values for the Egg Hatch Assay and Larval Motility Assay (L-3) of *H. contortus* worms when treated with different concentrations of Cuminaldehyde (37, 74, 185, 370, and 740 µg/ml). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

	37 µg/ml	**74 µg/ml**	**185 µg/ml**	**370 µg/ml**	**740 µg/ml**
Egg Hatch Assay	7.2909E-09	2.45E-09	1.0865E-09	2.63E-11	3.2635E-09
Larval Motility Assay	4.1768E-09	3.986E-09	1.4565E-09	3.552E-08	2.5523E-08

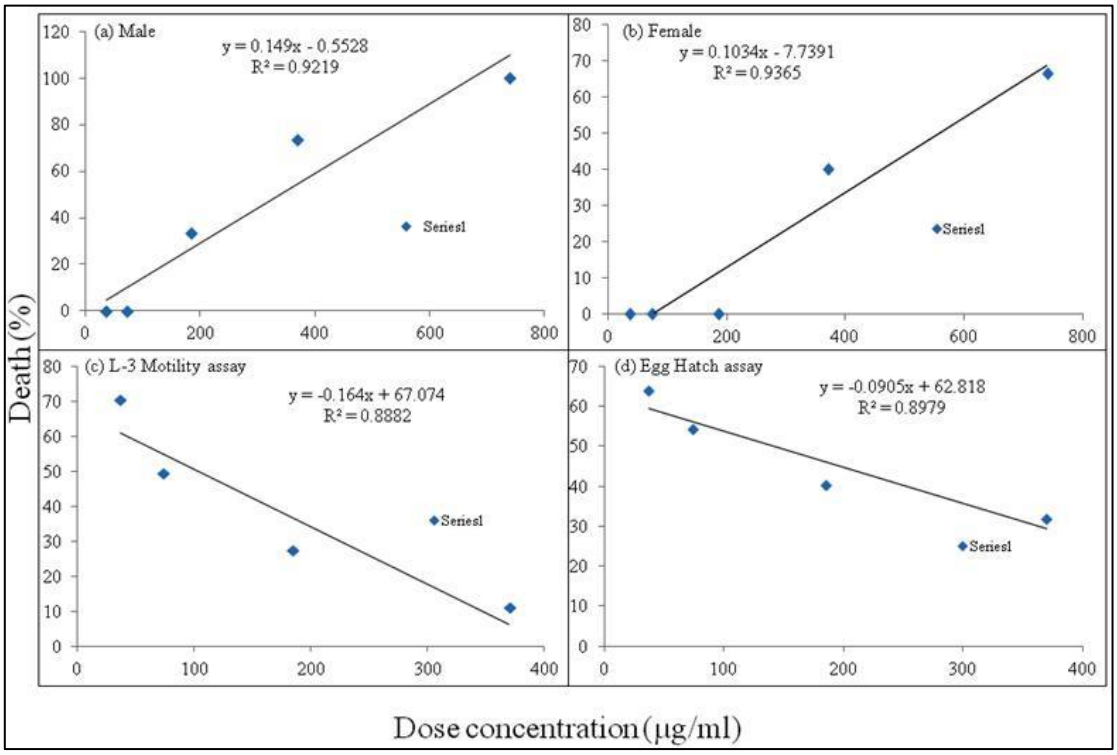


Figure 30. The half-minimal inhibitory concentration (LD_{50}) was measured against the death percentage of adult *H. contortus*, (L- 3) larvae, and egg hatch assay. In (a) Male death percentage in 12 h (b) Female death percentage (c) Larval Motility assay in 24 h and (d) Egg hatch assay in 48 h.

5.1.2.4 Damage as seen from the ultra-structural features due to CA treatment

To check the extent of damage in ultra-microscopic studies were done for control and treated samples. In **Figure 31**, comparative studies between a set of controls (RPMI media), Alb (500 µg/ml) and CA treated (370 µg/ml) *H. contortus* are shown. **Figure 31a-c** shows the whole body region of the adult helminth **Figure 31a** depicts the complete intact cuticle of the body region of adult *H. contortus* where united parts can be identified when treated with RPMI media **Figure 31b** partial disorganizations of the cuticle when Alb treated helminths where analyzed, where limited shrinkage of the entire body was observed and **Figure 31c** complete rupturing and disorganizations of the complete body region are observed when treated with high dosage concentration of CA, shrinkage of the entire body, and rupturing of the cuticle can be seen. Further, **Figure 31d-f** shows the anterior region of the adult helminth **Figure 31d** when treated with media, the complete sharp anterior end can be visualized where no disruptions and intact bloodsucking

mouth region are observed **Figure 31e** partial shrinkage and folds on the anterior end can be visualized when treated with Alb but the blood-sucking mouth region remains intact **Figure 31f** after the treatment with CA complete rupturing and shrinkage of the anterior end with complete loss of blood-sucking mouth region was observed. Further, **Figure 31g-i** shows the posterior region of the adult helminth **Figure 31g** well-defined bursa region of the male adult helminth can be identified when treated with media, **Figure 31h** partial loss of the bursa region and shrinkage can be visualized when treated with Alb **Figure 31i** loss of complete bursa region of the male and shrinkage of the helminth along with rupture of cuticle was observed when treated with CA.

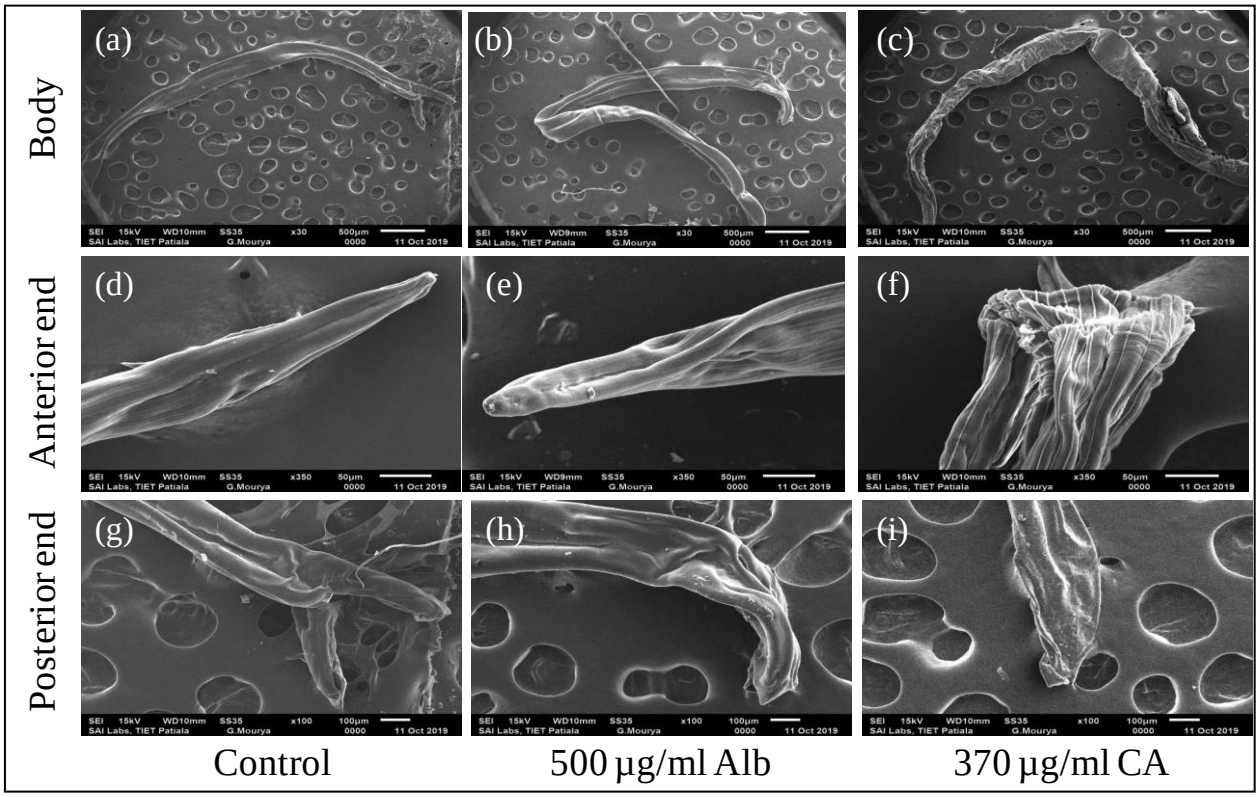


Figure 31. Study of morphological damage in adults *H. contortus* under the influence of CA. Scanning electron microscopic images of helminth treated with the highest concentration of cumin aldehyde, 370 µg/ml. The helminth was treated for 12 h and after treatment, the helminths were processed, coated and the ultra-structures were observed using SEM at 15 (kV). (**a, d, and g**) images show the treatment with negative control, RPMI media, (**b, e, and h**) are showing Alb (500 µg/ml) and (**c, f and i**) are showing 370 µg/ml CA treated adult male worms. **a, d and g** are anterior regions of the control, Alb treated and CA treated worms respectively. **b, e and h** are showing the whole body of the control, Alb treated and CA treated worms respectively. **c, f and i**

are showing posterior ends of the control, Alb treated and CA treated worms respectively. CA treated worms showed profound damages to tissue architecture and loss of tissue integrity and disorganization of bursa regions.

5.1.2.5 Reactive Oxygen Species dependent stress due to exposure to CA

Different concentrations of CA triggered the typical concentration free radical response, i.e. higher the concentration of CA higher was the stress response. Massive cellular stress response was seen in the anterior and posterior ends of the helminths after the treatment with CA, whereas a moderate ROS response was seen in the body region. In control helminths noticeable sharp ends were visible but after the treatment with CA, the rupturing of the anterior end was observed. The finding suggests that CA penetrates deep into the parasite body and causes rupturing and disorganizations. **Figure 32a-h** exhibits the amount of ROS in **Figure 32a** anterior end and **Figure 32c** posterior ends and Fig. 4b body in the negative control, where no generation of ROS was seen, **Figure 32d-f** exhibited the Alb treated helminths, where a minimal generation was seen and in (**Figure 32g, h and i**) depicted the high generation of ROS when treated with CA (highest concentration, 370 µg/ml.

5.1.2.6 Determination of NOS when treated with CA

NOS generation with the response to CA treatment was monitored using Greiss reagent. The experimental results exhibited a subsequent increase in NOS generation from 0.04467± 0.02 in lowest concentration 37µg/ml (at 30 min) to 0.31567± 0.016 in 370 µg/ml (at 24 h), represented in **Figure 32j**. The results were recorded spectrophotometrically at 540 nm at different times, i.e. 30min, 6 h, 12 h, and 24 h for all the different concentrations. Therefore, as a result of the nitric response, the generation of oxide species increased with an increase in concentration and time whereas in the negative control, no generation of oxide species was observed but a slight increase in oxide species generation with an increase in time was analyzed in positive control.

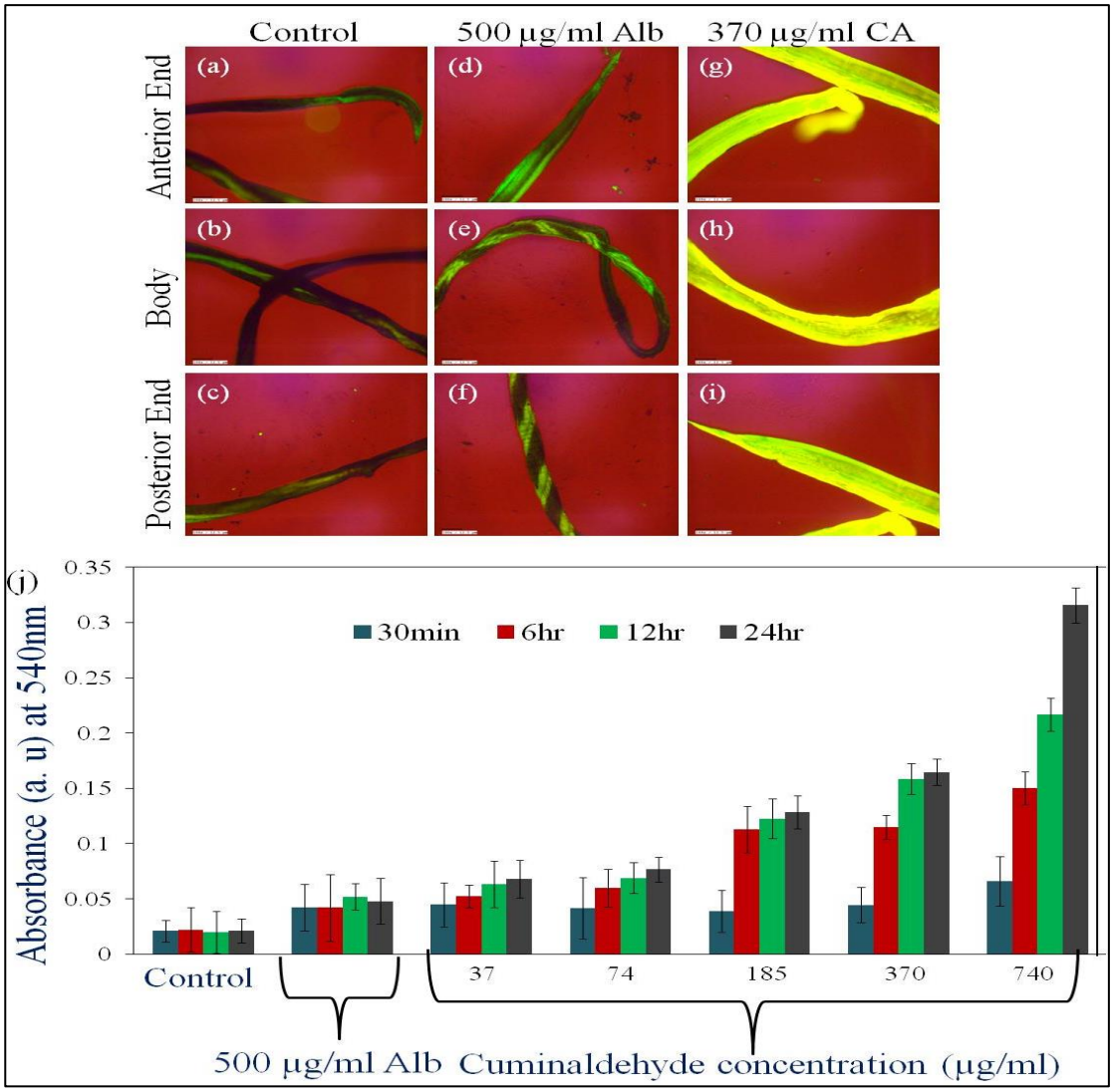


Figure 32. Generation of oxidative stress in adult worms due to exposure of CA adult *H. contortus* were treated for 3 h with CA and positive as well negative controls and processed for ROS (staining with DCFDA) or NOS (using Greiss reagent). (**a-c**) are showing the amount of ROS in anterior, intestinal, and posterior regions respectively of the adult helminth in the negative control, RPMI media. (**d-f**) are showing the amount of ROS in anterior, intestinal, and posterior regions respectively of the adult helminth in the positive control, 500 µg/ml Alb. (**g- i**) is showing the amount of ROS in anterior, intestinal, and posterior regions respectively of the adult helminth when treated with the highest concentration of CA (370 µg/ml). After the desired treatment the worms were incubated with 100 nM of DCFDA in dark for 20 mins. Thereafter the images were taken using an inverted fluorescence microscope by Dewinter, Italy. (**j**) A comparative graph showing the result of nitric oxide free radicals' generations in *H. contortus* adult worms after

treatment with different concentrations (37-740 µg/ml) of CA along with controls for 30 mins, 6 h, 12 h, and 24 h.

Table 21. Table showing the p-values for nitric oxide synthase (NOS) activity of *H. contortus* worms when treated with different concentrations of Cuminaldehyde (37, 74, 185, 370, and 740 µg/ml). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

	37 µg/ml	**74 µg/ml**	**185 µg/ml**	**370 µg/ml**	**740 µg/ml**
6h	1.24729E-09	1.41995E-10	6.14231E-09	1.25952E-08	2.62196E-11
12h	1.77E-09	1.76E-09	1.83E-09	1.32E-09	4.34E-09
24h	3.22E-09	3.16E-09	3.64E-09	4.45E-09	1.07E-08

5.1.2.7.1 Measurement of Superoxide Dismutase overexpression as a response to ROS induced stress

The helminths showed increased stress conditions when treated with CA. The activity of SOD was measured spectrophotometrically at 543nm, the higher the concentration of CA higher was the stress-induced. A steady increase of SOD activity with an increase of stress response had been observed and the amount of active SOD enzyme in different concentrations of CA (37, 74, 185, 370, and 740 µg/ml) was found to be 2.15± 0.1, 2.92± 0.2, 4.12± 0.5, 4.81± 0.20 and 5.59± 0.1 U/mg protein, respectively in comparison with the control where the amount was 4.8± 0.22. In **Figure 33a** the average value of active SOD enzyme per mg of protein of different concentrations of CA in comparison to positive and negative controls is displayed.

5.1.2.7.2 Activation of catalase to combat with ROS generated stress

The enzymatic activity was calculated using equation (iii). A steady increase in CAT activity with an increase in stress response with an increase of CA concentration had been observed. The amount of CAT enzyme in different concentrations of CA (37, 74, 185, 370 and 740 µg/ml) was calculated as 5.08± 0.4, 9.43± 0.8, 12.71± 0.4, 14.14± 0.1, and 17.63± 0.1 U/mg protein respectively in comparison with the control where the amount was 4.1± 0.01. The average value of active CAT enzyme per mg of protein of different concentrations of CA in comparison to positive and negative controls is represented in **Figure 33b**.

5.1.2.7.3 The increase of glutathione peroxidase activity due to CA stress

In contrary to other antioxidant activities, GPx also shows an increase in activity when exposed to CA of increasing concentrations. After following the protocol OD was taken at 340 nm. The activity of the enzyme GPx was calculated using equation 1. The amount of active GPx enzymes were 8.03± 0.2, 8.58± 0.1, 9.19± 0.4, 10.15± 0.5 and 10.73± 0.04 U/mg protein for different concentration of CA (37, 74, 185, 370 and 740 µg/ml) respectively in comparison with the amount 2.6± 0.008 in negative control **Figure 33c.**

5.1.2.7.4 Cellular combat with ROS by increasing the reduced glutathione concentration

In combat to ROS, GSH also induces cellular response which causes oxidative stress in helminths when exposed to CA. We found a significant increase in GSH content in CA-treated helminths. The amount of GSH increased from 23.08± 0.6, 24.5± 0.5, 26.6± 0.7, 40± 0.1 µM/ mg protein to 41.5± 0.030 µM/ mg protein for different concentration of CA (37, 74, 185, 370 and 740 µg/ml) respectively **Figure 33d.**

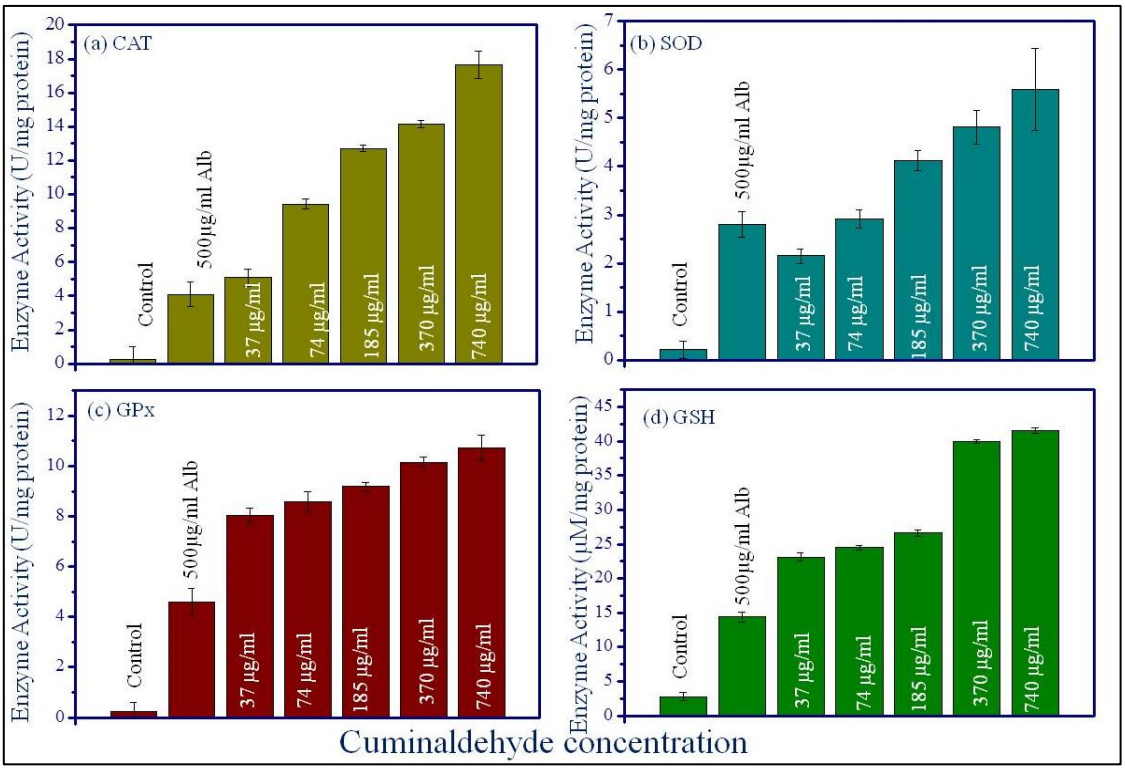


Figure 33. Oxidative stress-responsive elements in *H. contortus* to counter the toxic effect of CA on infectious helminths. Due to the generation of oxide synthase-related stress helminth counteracts conditions through alteration of its metabolic functions. The toxic effect of CA on the

body of the helminth reacts with the cellular molecules as normal metabolic functions and increases the regeneration of free radicals induced by oxidative damage defense mechanisms to include the following: enzymatic factors such as (**a**) CAT (**b**) SOD (**c**) GPx and (**d**) GSH. The enzymatic activity was measured as the enzyme unit per mg of protein (U/ mg protein) for CAT, SOD, and GPx and (µM/ mg protein) in GSH.

Table 22. Table showing the p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of Cuminaldehyde (37, 74, 185, 370, and 740 µg/ml). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

Cuminaldehyde concentration	**Superoxide Dismutase (SOD)**	**Catalase (CAT)**	**Glutathione Peroxidase (GPx)**	**Glutathione (GSH)**
37 µg/ml	5.83121E-10	7.54E-10	1.5E-11	2.677E-10
74 µg/ml	1.0478E-09	6.71E-10	1.2591E-10	1.2584E-09
185 µg/ml	2.70251E-09	1.76E-09	1.67562E-09	6.3855E-09
370 µg/ml	2.53101E-08	2.51E-08	2.52652E-08	2.5752E-08
740 µg/ml	9.52201E-09	1.06E-08	9.31654E-09	8.2722E-09

Conclusion:

Helminth infection is predominantly present in countries with a low economy and poor hygiene therefore the cost-effectiveness of the antihelminthic remained a challenge for companies to engage in antihelminthic research. Furthermore, for tropical medicine, antihelminthic must be used in mass chemotherapy programs in regions where clinical support is sparse and therefore drugs need to be very well tolerated in animals. For the last couple of decades, albendazole, benzimidazole, and ivermectin have been used for both veterinary and human use. But prolonged use of antihelminthic drugs resulted in the development of chemoresistance. The first report of chemoresistance in *H. contortus* came from Drudge et al., 1964 against benzimidazole [269]. Soon after many more reports got published reporting resistance against various antihelminthic drugs

such as Cawthorne and Whitehead, 1983; reported the resistance in microtubule inhibition of polymerizations by Benzimidazole in *H. contortus* [270]. Sangster et al., 1979 reported resistance development against imidazothiazole [271]. This pattern very common in different temperate regions including In India, such as benzimidazole, imidazothiazole, and ivermectin derivatives was reported on multiple occasions in various reports like Das et al., 2015; Dixit et al., 2017; Easwaran et al., 2009; Rajagopal et al. 2017 [272-275]. But due to the progressive development of resistance against the existing drugs the mankind is on the verge of urgent need of novel, inexpensive and effective drugs [267]. This is the first report of cuminaldehyde, an abundant compound present in widely used herb cumin to have a potential inhibitory effect against the egg, larvae, and adult stages of *H. contortus*, one of the most economically important parasitic nematodes which cost a loss of 120 billion of USD every year worldwide [94, 268, 276]. In this work, the antihelminthic effect of CA against *H. contortus* has been evaluated by novel mechanisms. The potential of CA has been found higher than the widely used, antihelminthic drug albendazole. It has been found that CA exerts quenching of free oxygen radicals on lipid peroxidation of several compounds and enhances the activity of endogenous antioxidant enzymes [277] and induces oxidative stress to various stages of *H. contortus* causing a change in metabolism, physical damage, and death of the worms. In the antimicrobial activity of cumin, the phenolic components constituted by antioxidant properties are responsible for membrane permeabilizes and increased membrane permeability leads to proton, phosphate, and potassium leakage, which further affects pH homeostasis and equilibrium of inorganic ions [278]. Moreover, according to Juglal et al. 2002; the mechanism of antimicrobial action of spices involves the bonding of phenolic compounds to membrane proteins causing membrane disruption and destruction of electron transport systems [279]. These *in vitro* studies are highly useful in the wake of the increasing problem of drug resistance to commercially available antihelminthic. The exact mechanism of action for its antihelminthic activity is not clear as most components in Cuminaldehyde have several targets. Predictions about the mode of action of crude cumin require thorough investigations of their constituents, target sites, and their interactions with the surrounding environment. Further CA could be evaluated by *in vivo* models for its use against gastro-intestinal parasitic infection with special emphasis on *Haemonchus contortus* (**Figure 34**). Therefore, these findings have opened a new window of the pharmaceutical application of CA for the treatment of gastrointestinal helminth infection.

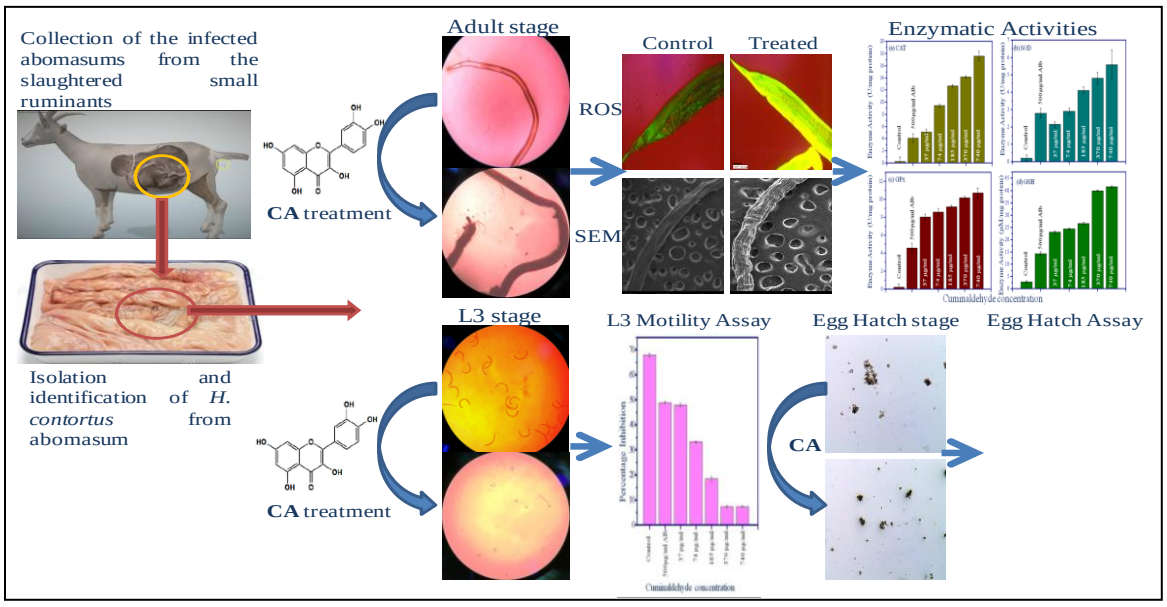


Figure 34. Scheme showing the effect of CA on various stages of *H. contortus* worms. CA causes oxidative stress-mediated damage in the parasitic worm.

5.2 Quercetin

Nematodes are considered the largest phyla in the animal kingdom [3, 4]. Despite, the true number of nematodes ranges between 100,000 and 10 million, only 20,000 – 30,000 species have been described [5]. In particular, *Caenorhabditis elegans* (*C. elegans*) is the most studied nematode, which is used as a popular genetic model system across biological disciplines to study complex behavior, drug responses, microbiome, and pathophysiology of human diseases [280-284] [285-289]. The nervous system of *C. elegans* has been studied extensively and only for *C. elegans*, the complete system-wide neural-connectome is known [290-293]. Nevertheless, the overall designs of the nervous system in nematodes are found to be largely similar across the phylum [294]. The overall structure displays the invariant features of the nerve ring as the central nervous system at the anterior part of the body, the ganglia, the ventral cord that runs longitudinally throughout the body, and the commissural connections. Besides, the similarity in the total number of neurons and their anatomic consonance are remarkable [294]. These similarities have helped us immensely in understanding the functioning of the neural circuits and drug-related toxicity responses in the nematodes [295-299]. For example, several antihelminthic drugs, such as avermectins, nicotinic antagonists, benzimidazole, latrotoxin, are reported to show the common lethal effect on the neuromuscular systems of various parasitic nematodes, like *Ascaris lumbricoides, Pristionchus pacificus, H. contortus,* and *Hymenolepis nana* [300, 301]. Though the evidence of direct targeting is lacking in these studies, however these results are extremely important from the standpoints of clinical applications of antihelminthic drugs, especially at the face increasing developments of drug-resistance in the parasitic nematodes.

Among the intestinal parasitic helminths, *H. contortus* is a highly pathogenic candidate, causing gastrointestinal infections, primarily and not restricted to small ruminants, with global distribution [13, 302]. Due to its blood-feeding behavior and the potential for the rapid development of large burdens, it is a frequent cause of mortalities in sheep, cattle, goats, and other ruminants, as well as is the most important parasite of the livestock in warm climatic regions, and arguably on a global basis [217]. *H. contortus* causes anemia and infirmity, which further is responsible for severe economic losses in sheep and goat breeding globally [303, 304]. Importantly, multiple reports of human infections from different continents, with *H. contortus*, have raised serious medical concerns while considering the development of resistance in this parasite against the available antihelminthic drugs [305]. Antihelminthic resistance is now a

widespread problem, especially in *H. contortus*, due to its enormous genetic diversity, which possibly is allowing the antihelminthic resistance alleles to be rapidly selected [306, 307]. At present, there are only a handful of drugs available (like benzimidazole, imidazothiazole, and ivermectin) for the treatment of helminths, albeit the development of chemoresistance is posing a massive therapeutic challenge [58, 273]. In 1983, Cawthorne and Whitehead have reported the development of resistance against benzimidazole in the *H. contortus* [270]. Other studies showed the mechanism of benzimidazole resistance due to point mutations in the β-tubulin gene; the target site of the drug [106, 308]. *In vitro* development of resistance in *H. contortus* against thiabendazole has highlighted the significance of point mutation (from glutamate to alanine) at codon 198 of the β-tubulin gene which expressed *in vivo* relevance for sheep and goats [309, 310]. Besides, detection of a point mutation at codon 167 of the isotype 1 β-tubulin gene was also shown to be associated with benzimidazole resistance in *H. contortus* [311]. Moreover, resistance was also observed against other antihelminthic drugs, ivermectin, mebendazole, Pyrantel, and Praziquantel in *H. contortus*, *Nectar americans*, *Ancylostoma duodenale*, and *Schistosoma mansoniparasites* [108, 312, 313]. *Haemonchus placei*, which is one of the most threatening parasitic nematodes of cattle in the tropical areas, also developed molecular resistance mechanisms against ivermectin, which includes the efflux of the drug from the cells and the eventual ineffectiveness of drug action [314-316]. Due to the shortage of available antihelminthic drugs along with the developments of resistance against the existing drugs, there exists an utmost urgency to develop new drugs for the effective treatment and management of the parasitic worm infections.

To cope up with the worldwide problem of the development of resistance in *H. contortus* against the antihelminthic drugs, researchers are actively engaged in discovering alternative plant-based antihelminthic [317]. Screening of phytochemicals with antihelminthic properties against *Haemonchus contortus* although has identified multiple candidates, however, their use as potential therapeutics can be hindered due to lack of knowledge on their mechanisms of action [158, 318-321]. Thus, a great demand exists for better antihelminthic remedies that can target a specific system to cause quick mortality in the parasitic worms.

To this end, we have designed our study to investigate the antihelminthic activity of quercetin, a phytochemical, against *H. contortus*. Quercetin is a naturally occurring flavonoid, present in onions, green tea, apples, berries, garlic, etc [322, 323] and is well recognized for its

anti-cancer, anti-inflammatory, and antioxidant properties [324, 325]. Quercetin has multiple intracellular molecular targets with the potential to reverse treatment resistance [326, 327]. In a recent publication, it has been shown that quercetin in combination with ivermectin is capable of causing mortality in the ivermectin-resistant larvae of *H. contortus* but not the adults and importantly quercetin alone showed no antihelminthic activity [328]. Previously, another study on *H. placei* was also found quercetin to be ineffective against the ivermectin-resistant field isolates of *H. placei* when used in combination with ivermectin [329]. Therefore, the role of quercetin as an effective antihelminthic agent remains questionable. In the present work, we found, for the first time, that quercetin, acts as an effective antihelminthic remedy and targets the central nervous system (CNS) of the parasitic nematode, *H. contortus*. We studied the novel mechanism of action of the compound in which quercetin inflicted tissue-damage and eventual death of the adult worms through oxidative-stress. The generation of reactive oxygen species in the CNS of *H. contortus* and eventual paralysis and mortality of the adult worms are extremely interesting findings keeping in mind that quercetin has characterized roles, in the CNS of animal models, as neuroprotective (inhibits oxidative stress mediated neuronal damage), anti-neuroinflammatory (suppresses of NF-κB sinagling, clearance of β-amyloid peptide and hyperphosphorylated tau) and neurogenesis agent (increases neuronal longevity by modulating signaling pathways mediated by NGF, BDNF and various kinases) [330], as well as contrastingly it can exert oxidative stress under certain conditions [331]. We have discussed the results from the perspectives of repurposing an old drug, quercetin, for a novel function and its potential to be used in clinical studies as a drug target for the nervous system of the parasitic nematodes.

5.2.1 Experimental design for the treatment of egg hatch, larval stage and paralysis, and death of adult H. contortus worms with Quercetin.

Adult *H. contortus* worms were isolated from abomasums of infected ruminants. The adult worms were treated with quercetin (0.125, 0.2, 0.5, 1, and 2mM) against albendazole, Alb (0.2mM), and control (RPMI Media) for 24 h to observe theirs *in vitro* mortality.

In vitro morbidity test of *H. contortus* larvae (L3) was performed by taking larvae (Approximately 25-30) in 200 µl of RPMI medium in 96 well plates in presence of quercetin (0.125, 0.2, 0.5, 1 and 2mM) for 24 h. After the incubation, a direct microscopic examination (at 40X magnification) was done for mobility (for 20 secs).

Egg suspension (1 ml) containing nearly 200 eggs was taken into 24 well microtiter plates, subsequently, to check the inhibition efficiency of drugs, eggs were treated at different concentrations of quercetin (0.125, 0.2, 0.5, 1, and 2mM), against two controls, one positive with Alb (0.2mM) and another negative with RPMI media. After the inhibition treatment, the plates were incubated at 28 °C for 48 h.

A detailed procedure for adult motility assay (2.3.3), larval motility assay (2.3.4), egg hatch assay (2.3.5), and morphological analysis, SEM (2.4), generation of oxidative stress with ROS (2.6.1), NOS (2.6.2), and catalytic activities CAT, 2.7.1, SOD, 2.7.2, GPx, 2.7.3 and GSH, 2.7.4 has been explained in Chapter 2.

5.2.1.1 Histopathology of H. contortus

H&E stain's simplicity and ability to elucidate the tissue's basic morphology by staining nuclei and cytoplasm in different colors. For H&E (Hematoxylin & Eosin) staining, female worm samples were treated with toxic quercetin (1mM) extract compared against control (RPMI Media), and Alb (0.2mM) was performed at room temperature. Worms were stored in 99.9% ethanol (MERCK). Ascending dehydrations of the worm tissue were done overnight. Wax blocks were prepared by embedding the worm tissue inside. Blocks were stored at -20 °C, to become even harder for sectioning. Sections were done in Microtome (0.2 mm) and readily transferred to 50 °C hot water, to remove the extra wax present on the tissue. Then the tissue was procured with a glass slide and kept at 65 °C on a hot plate, such as to allow the extra wax to melt for the fixation of the worm on the slide. Then a staining procedure (Thomson, 1986) was followed [291]. The slides were kept for 2 min in all the alcohol concentrations and 1 min each in H&E stains. Further, microscopic examinations were done to identify morphological changes.

5.2.2 Results

5.2.2.1 Adult Motility Assay (AMA)

In male adult worms, quercetin was found to be most active at the concentration of 1mM, at which 40% of the worms were paralyzed after 1 h and 80% were paralyzed after 3 h of exposure. At 6 hrs, 40% were dead and after 12 h, we found 80% mortality in the adult worms (**Table 23**). Furthermore, in adult males, quercetin at 1mM concentration caused 100 % mortality

within 12 h after the treatment. In the case of the adult females, quercetin showed a slower effect than the males as 40% paralysis of the adults took 3 h, and only at 6 h, 80% of worms were paralyzed. Mortality in the females was only visible at 12 h (60%) and 100% mortality was found after 24 h of quercetin treatment (**Table 23**). The LD_{50} values respectively for the adult male and female worms were 1.25 ± 0.23 mM and 1.56 ± 0.14 mM at 12 h of quercetin exposure.

Table 23: Paralysis and Death time analysis of quercetin on adult *H. contortus* worm. Adult *H. contortus* were exposed to quercetin of different doses (0.125, 0.25, 0.5, 1 and 2mM), Alb (0.2mM) in RPMI media for 24 h. Paralysis and death time was analyzed at different time points after the treatment with quercetin. The viability of the worms was observed after the treatment. If no movement was observed then they have exposed to a warm saline solution (50 ^{o}C) for 1 min to stimulate movement. The counts were taken for the number based on the worm's movement. If the worm previously immobile worm starts moving upon exposure to warm saline then considered as paralyzed otherwise considered dead.

Time of Exposure	Paralysis Time							Death Time						
	Male Worms													
	Control	Alb	0.125	0.25	0.5	1	2	Control	Alb	0.125	0.25	0.5	1	2
0 h	-	-	-	-	-	-		-	-	-	-	-	-	-
0.5 h	-	-	-	-	-	-	9±0	-	-	-	-	-	-	-
1 h	-	-	-	-	-	6±0	12±0.5	-	-	-	-	-	-	-
3 h	-	-	-	-	9±0	12±0.5	0	-	-	-	-	-	-	12±0.5
6 h	-	3±0.5	-	-	12±0.5	9±0	0	-	-	-	-	3±0.5	6±0	15±0
12 h	-	6±0.5	3±0	6±0.5	9±0	3±0.5	0	-		-	-	6±0.5	12±0.5	15±0
24 h	1±0	0±0	9±0.5	12±0.5	0±0	0±0	0	-	15±0	-	3±0	15±0	15±0	15±0
	Female Worms													
0 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.5 h	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1 h	-	-	-	-	-	3±0.5	6±0	-	-	-	-	-	-	-
3 h	-	-	-	-	3±0	6±0.5	12±0.5	-	-	-	-	-	-	3±0
6 h	-	-	-	-	9±0.5	12±0	0	-	-	-	-	-	-	15±0
12 h	-	-	-	3±0	15±0	6±0	0	-	3±0	-	-	0±0	9±0	15±0
24 h	1±0	0±0	3±0	3±0.5	0±0	0±0	0	-	6±0	-	3±0	15±0	15±0	15±0

5.2.2.2 Larval Motility Assay (LMA)

Spindling shrinkage and ruptured morphology of L-3 larvae were observed in the case of quercetin treatment, whereas no such changes were found in the albendazole-treated samples (**Figure 36 grey**). The survival of larvae was measured after 24 h of quercetin treatment using equation (i). The percentages of survival were respectively 66.6± 0.6%, 33.3± 0.7%, 18.5 ± 0.5, 7.4± 0.9%, 0 and 0 respectively for 0.125, 0.25, 0.50, 1, 2 and 5 mM. In contrast, albendazole (Alb) showed 81.4± 0.5% survival of the L-3 larvae compared to the control. We measured the LD_{50} and p-value of 0.310 ± 0.24mM for the larva mortality for 24 h of quercetin treatment (**Figure 35, Table 24**).

5.2.2.3 Egg Hatch Assay (EHA)

For egg hatch assay, the percentages of EHA inhibition were measured after 48 h of quercetin treatment and quantified using equation (ii). The results for the toxic effect of quercetin showed respectively 74± 0.6%, 34.5± 0.7%, 29± 0.7%, 19.5± 0.6%, 16± 0.7% and 10± 0.8% of egg hatching for the concentrations of 0.125, 0.25, 0.50, 1, 2 and 5mM. Contrastingly, albendazole (0.2 mM) showed 75±0.5% eggs hatched (presence of larvae L1). Thus, we found a concentration-dependent effect for quercetin in which nearly 80% inhibition of egg hatching was measured at 1mM. LD_{50} and p-value were measured for the inhibition of egg hatching was 0.43 ± 0.071 mM for 48 h (**Figure 35 blue, Table 24**).

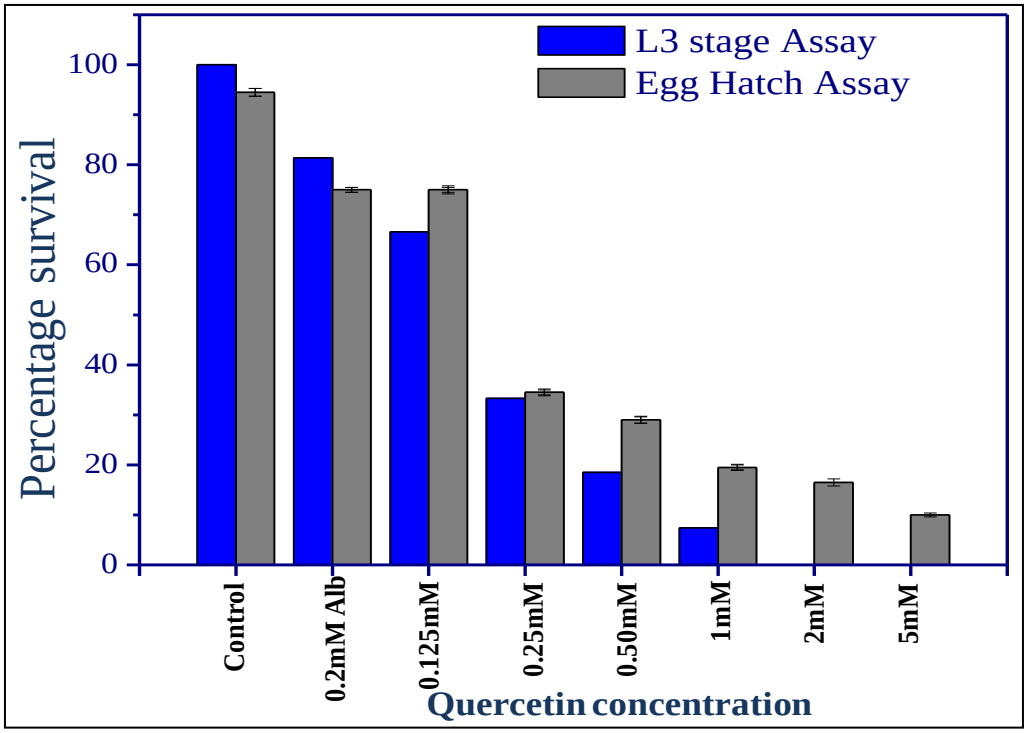


Figure 35. Inhibitory effect of quercetin on L-3 Larvae survival and egg hatching of *H. contortus*. (Grey) Percentage of inhibition (survival) of the L3, larval stage after treatment with different concentrations of quercetin for 24 h along with controls. (Blue) Comparative graph showing the influence of quercetin on egg hatching assay. The treatment was performed for 48 h in the presence of different concentrations of quercetin along with controls. Around 200 eggs were taken in each well and results were represented as an average of 3 independent experiments.

Table 24. The p-values for the Egg Hatch Assay and Larval Motility Assay (L-3) of *H. contortus* worms were treated with different concentrations of Quercetin (0.25, 0.5, and 1 µM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

	0.25	**0.5**	**1**
Egg Hatch Assay	4.91E-09	9.74E-09	2.11E-08
Larval Motility Assay	3.21803E-09	9.69793E-09	2.11146E-08

5.2.2.4 Physical damage due to quercetin exposure

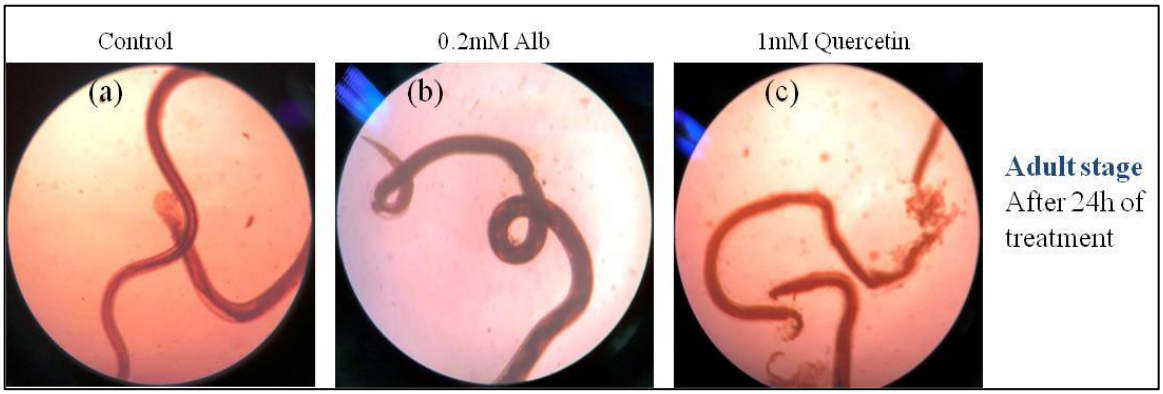


Figure 36. Optical microscopic images of control and adult treated *H. contortus* worm. After treatment, the worms were stained with Lacto phenol cotton blue (LPCB) for at least 15- 20 min, before taking a picture under an optical microscope at 40X magnification. (**a**) Adult *H. contortus* treated with RPMI media for 24 h, as a negative control (**b**) Adult *H. contortus* treated with 0.2 mM Alb, as a positive control (**c**) Adult *H. contortus* treated with 1mM quercetin at 24 h of treatment at 1mM concentration.

The physical damage after the treatment with 1mM quercetin was observed visually in **Figure 36(a-c)** under an optical microscope at 40X magnification in the control group, treated with RPMI Media, smooth cuticles with well-developed body regions (**Figure 37a)** were seen in the adult female worm. Intact sharp end and blood-sucking mouth region were identified, with no disruption, in the anterior part (**Figure 37b**) as well as sharp posterior end having intact tail region was identified (**Figure 37c)**. In the case of albendazole-treated worms (0.2 mM for 3 h), folds, partial shrinkage, and disorganization of the cuticle were observed throughout the entire body (**Figure 37d**) as well as at the anterior (**Figure 37e**) and posterior (**Figure 37f**) ends of the worms. **Figure 37g** is showing complete disorganization, disruption of the body regions, and loss of cuticle in the body region after the treatment with 1 mM quercetin for 3 hrs. Shrinkage of the ends, loss of blood-sucking anterior mouth region, and rupturing of the cuticle (of the tail) were observed at the anterior and posterior parts (**37h** and **37i**).

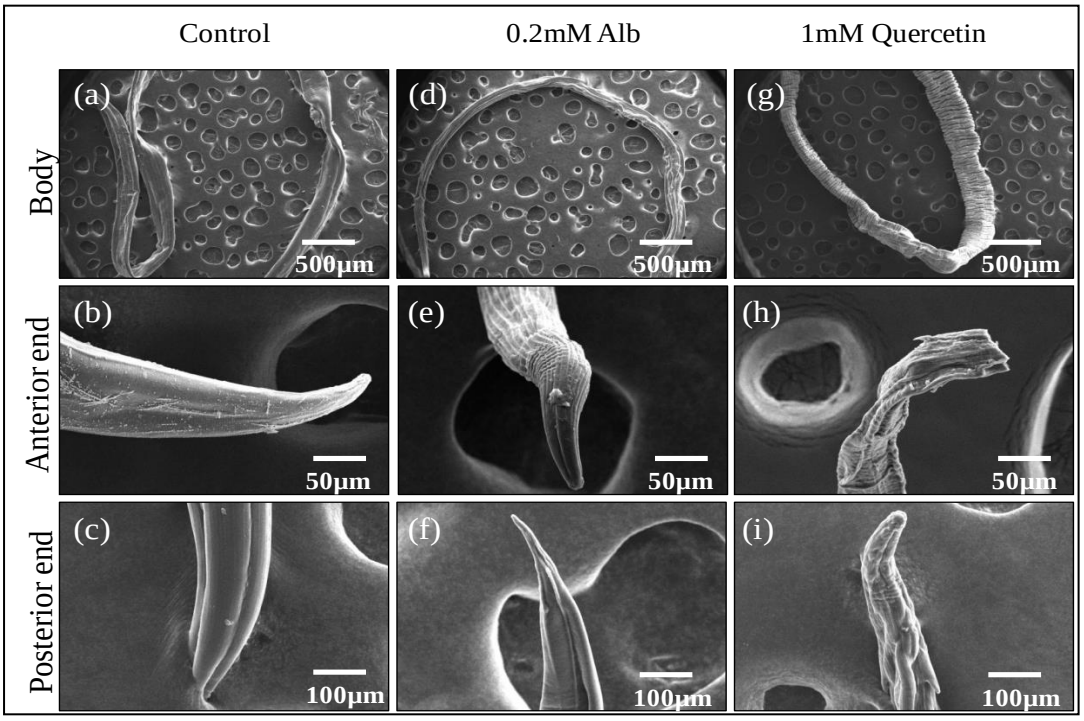


Figure 37. Study of morphological damage in adults *H. contortus* under the influence of quercetin. Scanning electron microscopic images of helminth treated with the highest concentration of quercetin, 1mM. The helminth was treated for 3 h and after treatment, the helminths were processed, coated and the ultra-structures were observed using SEM at 15 (kV). (**a, b, and c**) images show the treatment with negative control, RPMI media, (**d, e, and f**) are showing Alb (0.2 mM) and (**g, h, and i**) are showing 1mM quercetin treated adult male worms. **a, d and g** are showing the whole body of the control, Alb treated and quercetin treated worms respectively. **b, e, and h** are showing anterior regions of the control, Alb treated and quercetin treated worms, and **c, f and i** are showing posterior ends of the control, Alb treated and quercetin treated worms respectively. Quercetin-treated worms showed profound damages to tissue architecture and loss of tissue integrity and with disorganization of bursa regions.

5.2.2.5 Disruption in morphology caused by the quercetin treatment

Massive changes in morphology, caused by quercetin, were observed in the 40X and 400X magnifications of the light microscope (**Figure 38c**) whereas Alb (**Figure 38b)** and control (**Figure 38a**) showed little and no change in the morphology. In the control, complete

intact anterior and posterior ends were visible along with (ai) isthmus, (aii) Brut, (aiii) pseudocoele, (aiv) globular leukocytes (surrounded by small-sized cells), (av) muscle cells, (avi) intestinal epithelial region, (avii) ovum and growth zone of the ovary and (aviii) intact skin tissue. Mild disruptions of isthmus and brut in this group were observed due to the procedure. In the Alb-treated worm, disruptions were visible at the isthmus and pseudocoele along with partially-punctured muscle cells, a ruptured ovary, and globular leukocytes with a smaller number of surrounding cells. However, quercetin treatment has caused complete disruption of the anterior end, a near-total disruption of the ovary and loss of eggs, splitting of the pseudocoele, and presence of punctured muscle cells and damaged globular leukocytes with limited counts of surrounding cells.

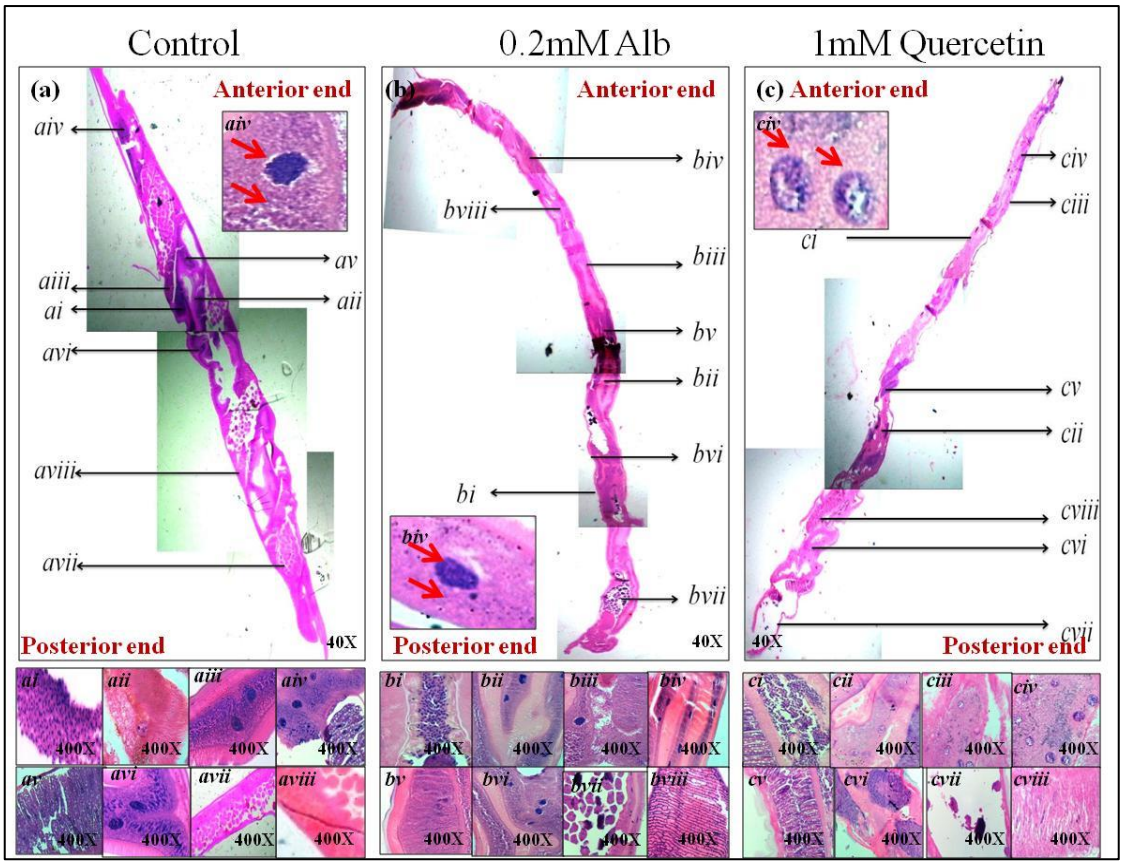


Figure 38. The H & E staining of complete body region showing, anterior and posterior regions of the *H. contortus* worm after the treatment with (**a**) control, (**b**) 0.2 mM Alb and (**c**) 1 mM quercetin. a, showed control worm, with intact anterior and posterior end along with **ai, bi and ci** isthmus, **aii, bii and cii** showed Brut, **aiii, biii and ciii**- pseudocoele, **aiv, biv and civ**- showed

globular leukocytes (surrounded with small cells), **av**, **bv, and cv**- showed muscle cells, **avi, bvi, and cvi**- intestinal epithelium region, **avii, bvii and cvii**- ovum and growth zone of the ovary, and **aviii, bviii and cviii** skin tissue of the worm. **b** showed Alb treated worm with partial stress caused by a resistant drug and **c** showed a high generation of stress after treatment with a high dosage of quercetin. **c** showed completely disrupted anterior end with different other distorted sections, ruptured ovary growth zone along with split pseudocoele. **ci**, showed the presence of some completely punctured muscle cells, **civ,** and **cvi,** ovum. Moreover, **civ**, completely wrecked globular leukocytes with a limited count of surrounding cells.

5.2.2.6 Generation of reactive oxygen species due to quercetin treatment

Reactive oxygen species (ROS) was found to be generated in the adult worm when treated with both Alb (0.2mM) and quercetin (1mM) for 3 h (**Figure 39b** and **39c**). Compared to the RPMI (media)-treated control (**Figure 39a**), higher ROS was generated in the anterior and posterior parts of the worms in the two drug-treated groups. However, quercetin treatment produced more ROS than Alb as well as disruptions in different body parts viz. anterior and posterior ends and body regions. **Figure 40d, e,** and **f** are respectively showing the generation of ROS induced by quercetin in the nerve ring (at the anterior end), commissural connections (in the middle of body region) and ventral cord, and tail ganglia (at the posterior end) at 400 X magnification.

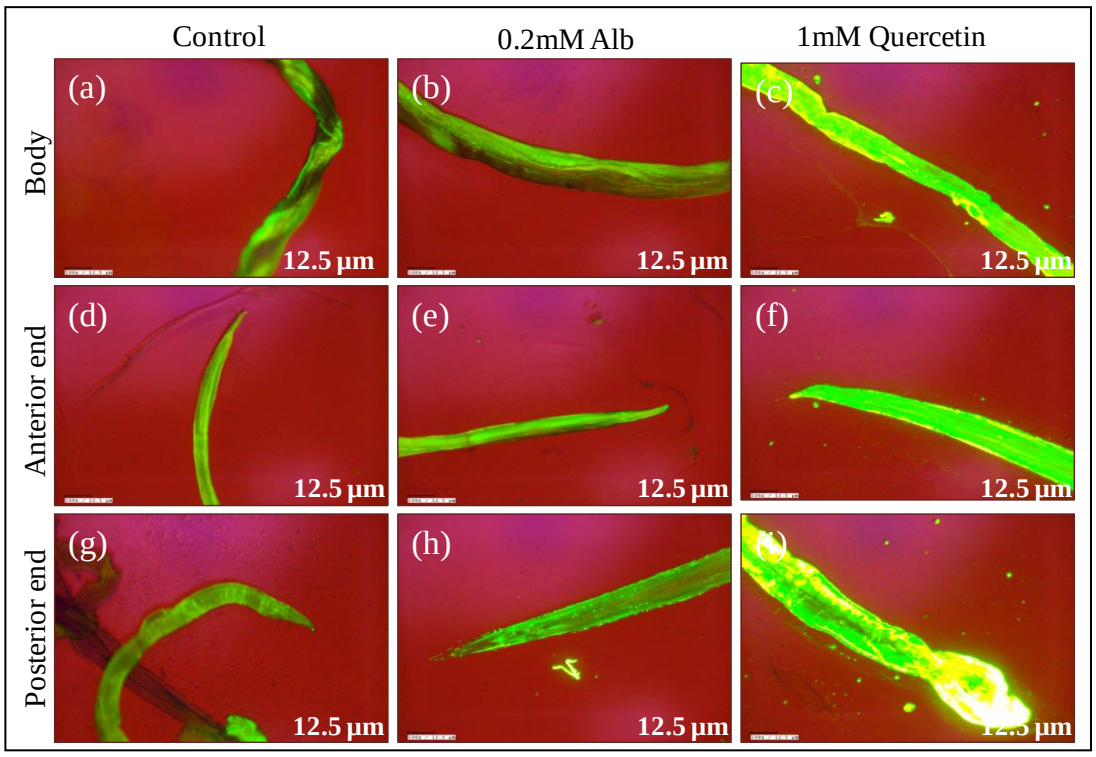


Figure 39. Generation of oxidative stress in adult worms due to exposure of Quercetin adult *H. contortus* were treated for 3 h with quercetin and positive as well negative controls and processed for ROS (staining with DCFDA) or NOS (using Griess reagent). (**a, d, g**) are showing the amount of ROS in body anterior, and posterior regions respectively of the adult helminth in the negative control, RPMI media. (**b, e, h**) are showing the amount of ROS in the body, anterior and posterior regions respectively of the adult helminth in the positive control, 0.2mM Alb. (**c, f, i**) are showing the amount of ROS in body, anterior, and posterior regions respectively of the adult helminth when treated with the highest concentration of quercetin (1 mM). After the desired treatment the worms were incubated with 100 nM of DCFDA in dark for 20 mins. Thereafter the images were taken using an inverted fluorescence microscope by Dewinter, Italy.

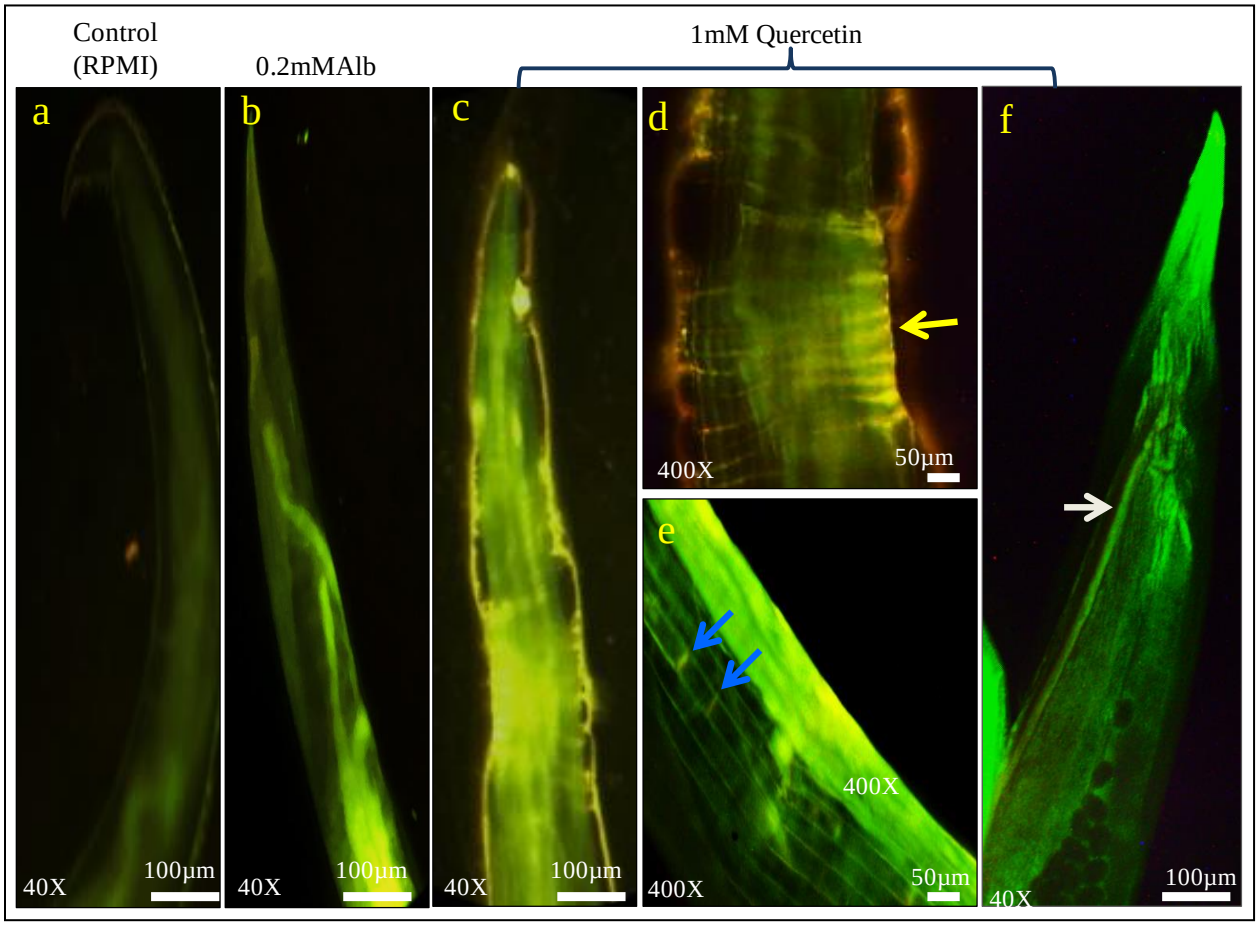


Figure 40. The stress caused by quercetin treatment on the central nervous system is shown by the generation of ROS. In **a** and **b**., the microscopic fluorescent images (40X) after the treatment with RPMI media and standard 0.2mM Alb are shown, where the partial generation of ROS and no differential parts of the nervous system were identified. **c**, quercetin (1mM) treated worms showed a high amount of ROS as compared to control and standard available drug. In **d** the yellow arrows are showing the nerve rings (400X). Further, **e**. shows the presence of commissures (400X) and in **f**, the white arrow shows the tail ganglion.

5.2.2.7 Alterations in activities of catalase, superoxide dismutase, and glutathione peroxidase after quercetin treatment

Next, we have tested whether high ROS generation due to quercetin may have increased the oxidative stress within the worm's body, which in turn altered the activity levels of the enzymes involved in reducing oxidative stress, such as catalase (CAT), superoxide dismutase (SOD), and

glutathione peroxidase (GPx). An increase in catalase activity with an increase in the quercetin concentration was observed (**Table 25**). The activity levels of CAT calculated were 5.2± 0.5, 5.9± 0.1, 7.2 ± 0.52, 16.1± 0.18, and 16.6.2± 0.2 U/mg proteins respectively at the concentrations of quercetin, 0.125 mM, 0.2 mM, 0.5 mM, 1.0 mM and 2.0 mM, after 3hrs of treatment. Increased catalase activity was also observed after Alb treatment compared to the control (**Table 25**). A similar increase in the activity levels of the enzyme, superoxide dismutase (SOD) was also found with the increase in quercetin concentration (**Table 25**). The activity levels of SOD calculated were 2.13± 0.56, 3.09± 0.47, 4.02± 0.56, 6.68± 0.21, and 7.61± 0.34 U/mg protein respectively at the concentrations of quercetin, 0.125 mM, 0.2 mM, 0.5 mM, 1.0 mM and 2.0 mM, after 3hrs of treatment. We also found the increasing activity of glutathione peroxidase, with increasing concentration of quercetin. The activity levels of GPx were found to be 6.32± 0.78, 9.39± 0.51,15.05± 0.31, 17.28± 0.56, and 18.47 ± 0.2 U/mg protein respectively at the concentrations of quercetin, 0.125 mM, 0.2 mM, 0.5 mM, 1.0 mM and 2.0 mM (**Table 25**). The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of Quercetin are described in **Table 26.**

5.2.2.8 Increase in concentrations of the reduced glutathione due to quercetin exposure

To combat the oxidative stress generated by ROS, cellular glutathione gets reduced (GSH). We found a significant increase in the amount of GSH in the quercetin-treated worms. The activities of GSH quantified, along with the increase in the dose of quercetin were 18.75± 0.58, 24± 0.38, 40.25± 0.61,62.6 ± 0.49, and 68.41 ± 0.68 µM/mg protein (**Table 25**). Treatment of Alb also induced a substantial increase in the GSH concentration (68.41± 0.030 µM/ mg protein) (**Table 25**). The p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of Quercetin are described in **Table 26.**

Table 25. Oxidative stress-responsive elements in *H. contortus* to counter the toxic effect of Quercetin on infectious helminths. Due to the generation of oxide synthase-related stress helminth counteracts conditions through alteration of its metabolic functions. The toxic effect of Quercetin on the body of the helminth reacts with the cellular molecules as normal metabolic functions and increases the regeneration of free radicals induced by oxidative damage defense mechanisms to

include the following: enzymatic factors such as CAT, SOD, GPx, and GSH. The enzymatic activity was measured as the enzyme unit per mg of protein (U/ mg protein) for CAT, SOD, and GPx and (µM/ mg protein) in GSH.

Treatment	**U/mg protein**			**µM/mg protein**
	CAT	**SOD**	**GPx**	**GSH**
Concentration				
Control	0.2± 0.8	0.25± 0.18	0.22± 0.34	2.2± 0.65
0.2mM Alb	4.1± 0.7	2.8± 0.27	4.6± 0.54	20.4± 0.74
0.125mM	5.2± 0.5	2.13± 0.56	6.32± 0.78	18.7± 0.58
0.25 mM	5.9± 0.1	3.09± 0.47	9.39± 0.51	24± 0.38
0.50 mM	7.2± 0.5	4.02± 0.56	15.05± 0.31	40± 0.61
1 mM	16.1± 0.18	6.68± 0.21	17.28± 0.56	62.66± 0.49
2 mM	16.6± 0.2	7.61± 0.34	18.47± 0.2	68.41± 0.68

Table 26. Table showing the p-values for the enzymatic activity of different catalysts SOD, CAT, GPx, GSH of adult *H. contortus* worms when treated with different concentrations of Quercetin (0.25, 0.5, and 1 µM). Statistical analysis of data was conducted by a student's t-test, by using MS Excel, and two measurements were statistically significant if the corresponding p-value was <0.01.

Quercetin concentration	**Superoxide Dismutase (SOD)**	**Catalase (CAT)**	**Glutathione Peroxidase (GPx)**	**Glutathione (GSH)**
0.25	5.71352E-09	5.7812E-09	3.46E-09	4.98469E-09
0.5	9.93177E-09	1.2206E-08	3.29E-09	8.19435E-09
1	2.52471E-08	2.4999E-08	2.5E-08	2.50034E-08

Conclusion:

The choice of quercetin was particularly based on its therapeutic properties. It acts as strong reducing agent for hydrogen and inhibits lipid peroxidation, which inhibits or reduces free radical toxicity and enhances antioxidant properties in the body. The present study provides strong evidence that quercetin has a significant impact on adult *H. contortus*, examined in terms of paralysis and death time analysis at different intervals and different concentration gradients.

Moreover, the egg hatch assay and the larval assay were also observed to test the toxic nature of quercetin among the nematodes. This is the first report presenting the neuronal target and histopathology of *H. contortus* through ROS generation and H and E staining, respectively on the evaluation of the antihelminthic activity of quercetin. In male worms, quercetin was most active in 1 mM, where Approximately 80 % of the worms were paralyzed in 12 h and nearly 100 % of the worms died within 24 h of quercetin treatment. Whereas, quercetin in 1 mM till 12 h of treatment, only 60 % of the female worms got paralyzed and 100 % died in 24 h of quercetin treatment. The larval stage (24 h) and egg hatch assay (48 h) were also studied to confirm the toxicity of quercetin on various stages of the worm, and its lethal dosage was calculated as 0.310 ± 0.24mM and 0.43 ± 0.071mM respectively. Thus, huge changes in metabolism and morphology were observed after the treatment with quercetin as compared to control (RPMI media) and albendazole. The morphological damage was further confirmed by SEM and fluorescence imaging for oxidative stress. CAT, SOD, and GPx further showed that oxidative stress was induced by free radicals expressed during defense mechanisms (**Figure 41**).

Based on these results, quercetin showed a significant dose-dependent free radical scavenging and *in vitro* antihelminthic activity against *H. contortus*. Thus these *in vitro* antihelminthic studies provide a way against the increasing problem of drug resistance to commercially available drugs. Therefore, this work has paved the new way for the treatment of quercetin for GIN helminthic infection.

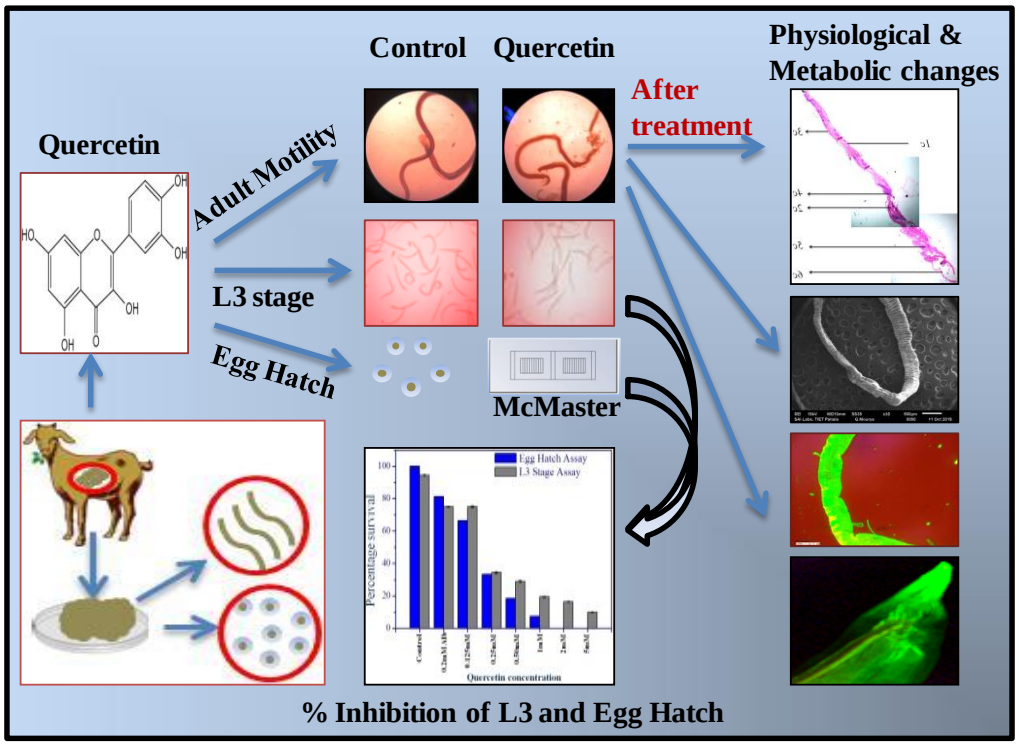


Figure 41. Scheme showing treatment of adult *H. contortus* worms, L3 stage, and egg hatch assay with Quercetin at different concentrations. After that, changes in morphology were analyzed using SEM and then the generation of ROS was observed. Moreover, neuronal targeting with Quercetin was studied in this part of the research.

Conclusion and Future prospective:

Helminth infection is predominantly present in countries with a low economy and poor hygiene therefore the cost-effectiveness of the antihelminthic remained a challenge for companies to engage in antihelminthic research. Furthermore, for tropical medicine, antihelminthic must be used in mass chemotherapy programs in regions where clinical support is sparse and therefore drugs need to be biocompatible, stable, effective, and side-effect-free in animals and humans. For the last couple of decades, albendazole, benzimidazole, and ivermectin have been used for both veterinary and human use. But prolonged use of antihelminthic drugs resulted in the development of chemoresistance [332-334]. Thus, resistance to one particular compound may be accompanied by resistance to other members of the group [217, 335]. Despite their immunogenicity, many helminths survive for extended periods in the bodies of their hosts [336]. Some of the reasons have already been mentioned (size, motility), but we now know that worms employ many sophisticated devices to render host defenses ineffective [337]. Therefore, an urgent requirement has risen to synthesize new drug formulation for the treatment of *H. contortus*, parasitic nematode, and control the rapidly increasing infection rate among ruminants and humans.

The unprecedented antihelminthic efficiency of different aqueous plant extracts, silver nanoparticles AgNPs by increasing reactive oxygen species thereby causes alteration of metabolic activity and physical damage in the worm tissue. Plant extracts and synthetic compounds are present in ample amounts widely in nature and have a promising inhibitory effect against the adult, larval, and egg stages of *H. contortus*. The antihelminthic potential of AgNPs and synthetic compounds have been found far effective than the presently available drugs. It has been observed that AgNPs, cuminaldehyde, and quercetin induce antioxidant stress against various stages of the worm, which causes a predominant change in metabolism, physical damage, and death of worms. *H. contortus* shows high resistivity against Albendazole which was also consistent in our findings, whereas AgNPs, and synthetic compounds not only killed adult worms but also showed a significant reduction of larval viability and egg hatching. Henceforth, the *in vitro* studies with quercetin are extremely useful for increasing the efficacy against the worms resistant to commercially available drugs. Moreover, quercetin is analyzed to target the neuronal system and for the very first time already available anticancer drug is reposed used alone for *in vitro* antihelminthic studies. Further quercetin should be analyzed by *in vivo* models for the treatment

against GIN parasitic infection. But further detailed studies are required to understand the active principle(s), efficacy, and toxicity of the system in animal models.

In addition to the utility of the formulations against other parasitic infections including helminthic infections need to be evaluated in the future. Moreover, pre-clinical and clinical studies need to be carried out to overcome gastrointestinal parasitic infection. Dose formations and clinical trials may lead to the manifestation of helminthic infection to some extent. Moreover, most helminthic species have the same morphology and physiology, thus these newly synthesized formulations can be used to treat the same.

List of Publications:

Published articles:

- **Goel, V**., Kaur, P., Singla, L. D., & Choudhury, D. (2020). Biomedical evaluation of *Lansium parasiticum* extract protected silver nanoparticles against *Haemonchus contortus*, a parasitic worm. *Frontiers in Molecular Biosciences*, *7*, 396. doi: 10.3389/fmolb.2020.595646 (Impact Factor 4.188)

- **Goel, V**., Singla, L.D., and Choudhury, D. (2020). Cuminaldehyde induces oxidative stress-mediated physical damage and death of *Haemonchus contortus*. *Biomedicine & Pharmacotherapy*. 130, 110411. doi: 10.1016/j.biopha.2020.110411 (Impact Factor: 4.545)

Other Publications:

1. Dutt, S., **Goel, V.,** Garg, N., Choudhury, D., Mallick, D., & Tyagi, V. (2020). Biocatalytic Aza-Michael Addition of Aromatic Amines to Enone Using α-Amylase in Water. *Advanced Synthesis & Catalysis*, *362*(4), 858-866. doi: 10.1002/adsc.20191254. (Impact factor 5.451)

- Kaur, P., Sharma, A. K., Nag, D., Das, A., Datta, S., Ganguli, A., **Goel, V**., ... & Choudhury, D. (2019). Novel nano-insulin formulation modulates cytokine secretion and remodeling to accelerate diabetic wound healing. *Nanomedicine: Nanotechnology, Biology and Medicine*, 15(1), 47-57. doi: 10.1016/j.nano.2018.08.013 (Impact factor 6.5)

Articles under preparation / communicated:

- Goel, V., Singla, L.D., Chakroborty, N., and Choudhury, D. Targeting Central Nervous System of Parasitic worm, *Haemonchus contortus*.

- Goel, V., Kaur, P., Singla, L. D., & Choudhury, D. Antihelminthic activity of Silver nanoparticles protected with *Selaginella moellendorffii*, a Himalayan pteridophyte extract.

- Goel, V., Kaur, P., Sharma, S., & Choudhury, D. Silver nanoparticles Cause Oxidative Stress-Mediated Physical Damage and Death in Free-Living Soil Organism *Eisenia fetida*.

- Goel, V., Kaur, P., Bau, S., & Choudhury, D. Eco-friendly synthesis of highly stable monodispersed silver nanoparticles using an aqueous extract of *Ocimum sanctum* bark for its enhanced catalytic activity.

- Goel, V., Kaur, P., Singla, L. D., & Choudhury, D. Evaluation of antihelminthic activity against *Haemonchus contortus*, a parasitic worm using silver nanoparticles coated with *Chenopodium quinoa* extract.

- **International Conference:**

- "Harnessing Engineering, Technology & Innovation for Sustainable Growth, (HETIS) at Panjab University, Chandigarh, (Sep 30 - Oct 1, 2016)
- International Conference on Nano for Energy and Water (NEW) 2017 and Indo French Workshop on Water Networking", University of Petroleum and Energy Studies (UPES), Dehradun, (Feb 22- 24, 2017)
- International Conference on Advanced Nanomaterials and Nanotechnology, ICANN-2017, December 18-21, 2017, Indian Institute of Technology, Guwahati.

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Summary

Introduction

Helminthic infections are the world's most prominent gastrointestinal (GIN) parasitic neglected tropical infections prevalent allover in the animal kingdom and around 24% of the human population is infected with parasites (Gutierrez et al. 2000). These infections are highly common in tropical and subtropical areas, including sub-Saharan Africa, the Americas, China, and East Asia (De Silva et al., 2003; Feng et al., 2019). *Haemonchus contortus*, a highly pathogenic, economically important, and most infectious nematode parasite that infects domestic animals especially small and large ruminants causing acute anemia, hemorrhagic gastroenteritis, diarrhea, depression, etc contributing to the reduction of milk and meat production leading to great economic loss (Adam, 2019; Pal and Chakravarty, 2019). *H contortus* is transmitted through infected soils in various species, causing infection including in humans (Vercruysse et al., 2011; Sinnathamby et al., 2018). There is only a handful of drugs (like benzimidazole, imidazothiazole, and ivermectin) are available for the treatment of ruminant and the recent development of resistance against those has resulted in an urgent need for alternatives (Kaplan et al. 2012; Das et al. 2015; Furgasa et al. 2018).

In 1964, the first report of chemo-resistance in *H. contortus* came against a benzimidazole drug (Cawthorne et al. 1983). After that, a few more reports were published on the resistance against different antihelminthic drugs. Furthermore, Hoti et al. (2009) and Ram et al. (2007) reported the resistance in presently available drugs (albendazole, levamisole, fenbendazole, etc.). The prevalence of infection, shortage of drugs, and increasing resistance against existing drugs bring us a situation of the urgent need for new drugs.

The use of natural resources to treat various communicable and non-communicable diseases is a practice from pre-historical ages (Chintamunnee and Mahomoodally et al. 2012). Various herbal formulations derived from chamomile, nettle, Shatavari, garcinia, neem, karela, pippali, ashwagandha, ajwain, fenugreek, wormwood, etc., are documented in Indian, Chinese, Vietnamese, and African traditional medicine (Croizier et al. 1968; Borins et al. 1987; Sofowora et al. 1996). Despite the prevalence of helminthic infections, scarcity of medication, and profound documentation of traditional medicines, only a handful of those natural resources have ever been explored for their potential in modern medicine. (Tomar and Preet et al. 2017) synthesized silver nanoparticles using *Azadirachta indica*, an aqueous extract, to analyze the antihelminthic activity

against *H. contortus*. (Goel et al. 2020) used an aldehyde derivative of cumin and performed *in vitro* antihelminthic activity to examine the inhibition of egg hatch and growth in the larval and adult stage, along with the generation of reactive oxygen species and enzymatic activities, to confirm the mechanism of action. (Ahmed et al. 2020) used traditional medicinal plants like *Artemisia herba-aba* and *Punica grantum* to study the antihelminthic activity of *H. contortus* worms.

Furthermore, the recent advancement of nanotechnology in the field of healthcare came up with new hope for the management of infectious diseases like helminth infections. (Rashid et al. 2016) performed antihelminthic activity using *Momordica charantia*-coated silver nanoparticles against gastrointestinal worms. (Shakir et al. 2015) synthesized cadmium coated nanoparticles and observed the antiworm for parasitic worms.

Based on the above research gaps and resistance to drugs against the antihelminthic activity, this project proposal aims to develop new drugs from various types of medicinal plant extracts, nano-based formulations, and various synthetic compounds and examine their mechanism of action.

Keeping the above points in view, the following objectives have been designed

OBJECTIVES

1. Screening of Antiworm activity using various resources such as;

a) Medicinal plant extracts and natural products.

b) Nano-particle based formulations.

c) Synthetic compounds

2. Development of novel formulation(s) for the treatment of parasitic worms.

3. Study of Mechanism of Antiworm activity of newly developed formulation(s).

Chapter 2: Antihelminthic activity of Plant extract

Table 1. Medicinal plants and their traditional uses

Plant	Family	Traditional uses	Secondary Metabolites	Ref
Lansium parasiticum	Meliaceae	dysentery and malaria	alkaloids, flavonoids, tannins	Ramadhan et al. (2019)
Selaginella moellendorffii	Selaginellaceae	bleeding and chronic inflammation, such as arthritis, gonorrhea, hepatitis, and mastitis	phenolic acids, flavonoids, terpenoids, steroids,	Zhao et al. (2017)

Preparation of aqueous plant extract: Different plant extracts were prepared in an aqueous medium by boiling different parts, for example, dried fruit pulp of *L. parasiticum*, and leaves of *S. moellendorffii*. The boiling was done at 100 ℃ till the color changes from colorless to yellow to dark brown.

Fig 1. A schematic representation for the preparation of aqueous plant extracts.

Table 2. Physiochemical analysis of aqueous plant extracts.

Test Performed	*L. parasiticum*	*S. moellendorffii*
Alkaloids	+	+
Flavonoids	+	-
Tannins	+	+
Phenols	+	+
Terpenoids	-	+
Steroids	-	+

Table 3. Paralysis time and death time analysis of different plant aqueous extracts used to treat *H. contortus*. Worms were analyzed for paralysis and death at different time intervals. The data represents the mean and SD values of 3 independent experiments.

Time of Exposure (h)	Paralysis Time				Death Time			
	Male worms							
	Control	Alb	APE	ALE	Control	Alb	APE	ALE
0	-		-	-	-		-	
0.5	-		-	-	-		-	
1	-		-	-	-		-	
3	-		-	-	-		-	
6	-		3±0	5±0.5	-		-	
12	-		7±0.5	10±0.5	-		-	
24	-		9±0.5	12±0.5	-		-	
Female worms								
0							-	
0.5							-	
1							-	
3							-	
6				3±0			-	
12			2±0.5	5±0.5			-	
24			7±0.5	8±0.5			-	

Conclusion: The unprecedented antihelminthic efficiency of different aqueous plant extracts did not show much toxic effect on the helminthic worms. Thus, *H. contortus* shows high resistivity against plant extracts and no significant reduction in paralysis and death time. Henceforth, the findings are not highly useful in the wake of the increasing problem of helminthic infection. But further detailed studies are required to understand the active principle(s), efficacy, and toxicity of the system.

Chapter 2: Antihelminthic activity of Nanoparticles (AgNPs)

Synthesis and characterization of AgNPs: Synthesis of silver nanoparticles was done by incubating under the sunlight in the presence of silver nitrate solution 1.25 mM to synthesize biogenic AgNPs. The particles were synthesized with slow stirring to obtain homogenous AgNPs.

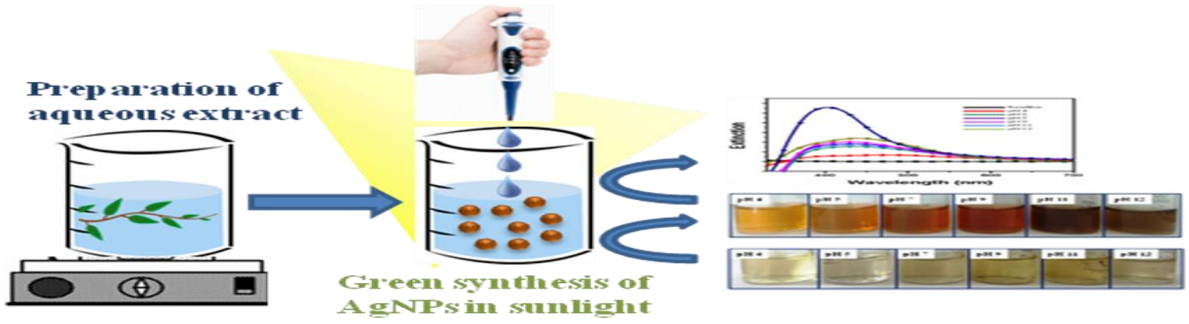


Fig 2: A schematic representation for the synthesis of plant extract protected AgNPs under sunlight.

Characterization of silver nanoparticles: The complete reduction of AgNO3 and formation of silver nanoparticles (AgNPs) was confirmed by monitoring color changes visually and the change in Surface Plasmon Resonance (SPR) peak under UV-Vis spectroscopy. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) studies were performed to determine the particle shape, particle size and hydrodynamic diameter, and disparity of the particles. Further, the structural changes were analyzed using Fourier transform infrared spectroscopy (FTIR).

2.1 *Lansium Parasiticum* silver nanoparticles (LAgNPs)

Synthesis of LAgNPs

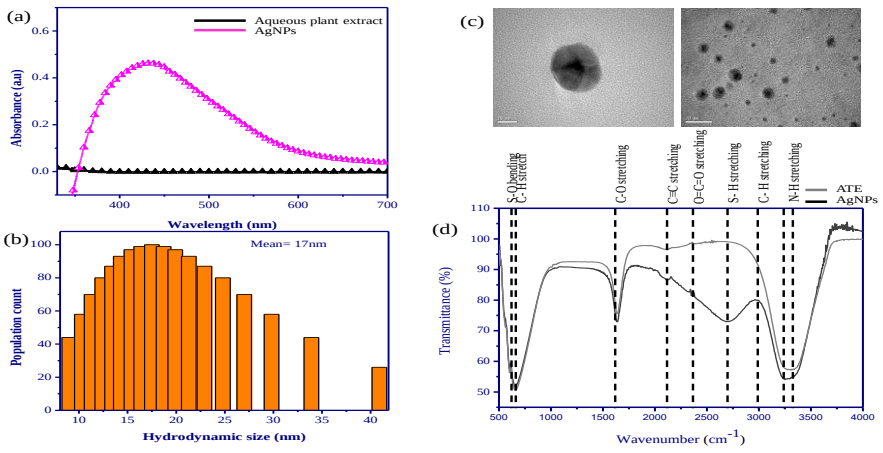


Fig 3: Characterization of *L. parasiticum* coated AgNPs (a) UV- vis spectroscopy, (b) DLS, (c) TEM analysis and (d) FTIR study.

Larval Morbidity Assay (LMA)

33.3 ± 2.6, 29.5 ± 1.7, 22.2 ± 2.5 and 14.8 ± 2.1 % survival of larvae was observed for 15.8, 31.7, 63.5 and 158.7 nM LAgNPs treatment respectively, whereas the recommended dose of Alb (9.4 μM) showed 60 ± 0.5 % and Ag+ (100 μM) showed 31 ± 0.7 % survival for 24 h treatment (Figure 2). For the larval mortality inhibition, LD50 has obtained 64.3 ± 8.5 nM for 24 h.

Egg Hatch Assay (EHA)

32.1 ± 2.6, 45 ± 2.7, 47.2 ± 1.3, and 51.2 ± 1.9 % reduction of egg hatching were observed in comparison with control for 15.8, 31.5, 63.5, and 158.7 nM LAgNPs respectively. Whereas recommended dose of Alb (9.4 μM) showed 13.5 ± 0.5 % and Ag+ (100 μM) 33 ± 0.7 % reduction of egg hatching or larvae (L1) formation. LD50 for inhibition of egg hatch obtained was 144.4 ± 3.1 nM for 48 h.

Alteration of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase(GPx) activity in response to LAgNPs induced oxidative stress

A steady enhancement in CAT activity with an increase of LAgNPs concentration results in an increase in stress response had been observed. In different concentration of LAgNPs (15.8, 31.7, 63.5 and 158.7 nM) the amount of CAT enzyme was calculated as 4.75 ± 0.17, 5.35 ± 0.36, 7.9 ± 0.37 and 8.2 ± 0.4 U/mg proteins respectively after 3h of treatment in comparison with the 0.22 ± 0.05, 4.1 ± 0.37 and 0.52 ± 0.05 U/mg proteins for untreated control, Alb (9.4 μM) and Ag+ (100 μM) respectively. A consistent SOD activity increment with an increase in stress response had also been observed. The SOD enzyme activity after the treatment was found to be 5.70 ± 0.4, 5.85 ± 0.17, 6.11 ± 0.26 and 6.60 ± 0.54 U/mg protein for LAgNPs 15.8, 31.7, 63.5 and 158.7 nM respectively in relation to the control where 4.8 ± 0.22 Alb and 0.56 ± 0.07 Ag+ amount was used. Untreated worms showed SOD activity of 0.21 ± 0.018 U/mg of worm protein Table 2. In contrast to other antioxidant activities, the activity of GPx also increases when worms are exposed to LAgNPs' increasing concentrations. At 340 nm spectrophotometrically the amount of GPx was observed. The activity of the GPx enzyme was calculated using the (iii) equation. The GPx

enzymes active amount was 2.5 ± 0.15, 2.6 ± 0.16, 2.73 ± 0.15 and 2.9 ± 0.16 U/mg protein for LAgNPs different concentrations (15.8, 31.7, 63.5 and 158.7 nM) respectively in comparison with the control where the amount was 2.6 ± 0.25 U/mg proteins in Alb and 0.58 ± 0.058 U/mg proteins in Ag+. Untreated was found to be 0.21 ± 0.071 U/mg proteins.

Reactive Oxygen Species (ROS) enhances cellular combat by increasing the concentration of reduced glutathione

The increase in GSH was observed (0.28 ± 0.05 µM/ mg for untreated) along with the increase of the dose of LAgNPs (15.8, 31.7, 63.5 and 158.7 nM) from 1.105 ± 0.025, 1.24 ± 0.06, 1.3 ± 0.141 and 1.41 ± 0.1078 µM/ mg protein respectively. Treatment of Alb also induced a significant increment in the glutathione (GSH) concentration (1.75 ± 0.050 µM/ mg protein) and Ag+ causes 0.59 ± 0.009 µM/ mg protein accumulation of GSH.

3.2 *Selaginella moellendorffii* silver nanoparticles (SAgNPs)

Synthesis of SAgNPs

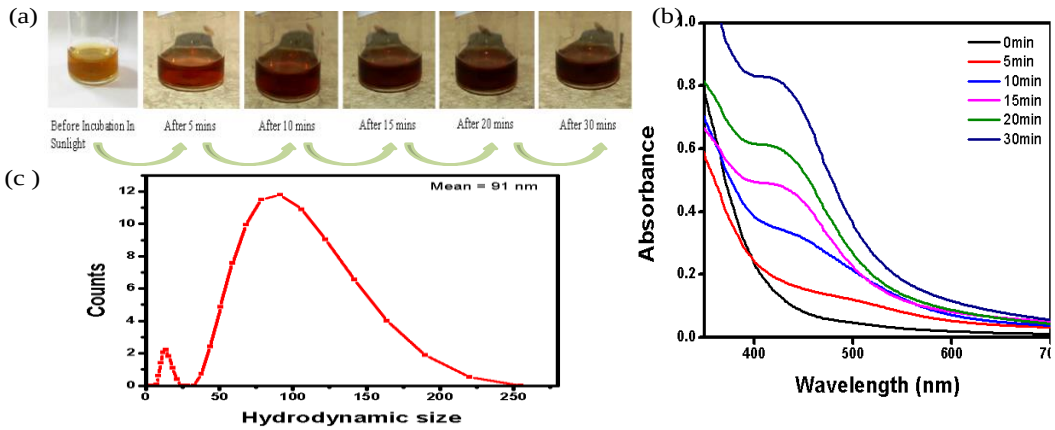


Fig 4: Characterisation of S. moellendorffii silver nanoparticles (a) visual screening at different intervals of time, (b) UV- vis spectroscopy, and (c) DLS analysis.

Larval Morbidity Assay (LMA)

42 ± 2.6, 26 ± 1.7, 19 ± 2.5 and 7 ± 2.1 % survival of larvae was observed for 24.7, 49.5, 123 and 247.5 nM SAgNPs treatment respectively, whereas the recommended dose of Alb (9.4 µM) showed 52 ± 0.5 % and Ag+ (100 µM) showed 61 ± 0.7 % survival for 24 h treatment.

Egg Hatch Assay (EHA)

63 ± 2.6, 34 ± 2.7, 10 ± 1.3, and 3.5 ± 1.9 % reduction of egg hatching were observed in comparison with control for 24.7, 49.5, 123 and 247.5 nM SAgNPs respectively. Whereas recommended dose of Alb (9.4 µM) showed 68 ± 0.5 % and Ag+ (100 µM) 72 ± 0.7 % reduction of egg hatching or larvae (L1) formation.

Alteration of catalase, superoxide dismutase, and glutathione peroxidase activity in response to PAgNPs induced oxidative stress

In different concentration of SAgNPs (24.7, 49.5, 123 and 247.5 nM) the amount of CAT enzyme was calculated as 2.36 ± 0.15, 4.62 ± 0.17, 5.63 ± 0.24 and 6.22 ± 0.18 U/mg proteins respectively after 3h of treatment in comparison with the 0.22 ± 0.05, 4.1 ± 0.37 and 0.52 ± 0.05 U/mg proteins

for untreated control, Alb (9.4 μM) and Ag+ (100 μM) respectively. A consistent SOD activity increment with an increase in stress response had also been observed. The SOD enzyme activity after the treatment was found to be 2.21 ± 0.7, 2.55 ± 0.15, 3.01 ± 0.18 and 3.19 ± 0.25 U/mg protein for SAgNPs 24.7, 49.5, 123 and 247.5 nM respectively in relation to the control where 4.8 ± 0.22 Alb and 0.56 ± 0.07 Ag+ amount was used. Untreated worms showed SOD activity of 0.21 ± 0.018 U/mg of worm protein. In contrast to other antioxidant activities, the activity of GPx also increases when worms are exposed to SAgNPs' increasing concentrations. At 340 nm spectrophotometrically the amount of GPx was observed. The activity of the GPx enzyme was calculated using the (iii) equation. The GPx enzymes active amount was 2.22 ± 0.057, 2.67 ± 0.21, 3.21 ± 0.08 and 3.4 ± 0.15 U/mg protein for SAgNPs different concentrations (24.7, 49.5, 123 and 247.5 nM) respectively in comparison with the control where the amount was 2.6 ± 0.25 U/mg proteins in Alb and 0.58 ± 0.058 U/mg proteins in Ag+. Untreated was found to be 0.21 ± 0.071 U/mg proteins.

ROS enhances cellular combat by increasing the concentration of reduced glutathione

The increase in GSH was observed (0.28 ± 0.05 μM/ mg for untreated) along with the increase of the dose of SAgNPs (24.7, 49.5, 123 and 247.5 nM) from 2.23 ± 0.15, 3.49 ± 0.24, 4.02 ± 0.7 and 4.21 ± 0.057 μM/ mg protein respectively. Treatment of Alb also induced a significant increment in the GSH concentration (1.75 ± 0.050 μM/ mg protein) and Ag+ causes 0.59 ± 0.009 μM/ mg protein accumulation of GSH.

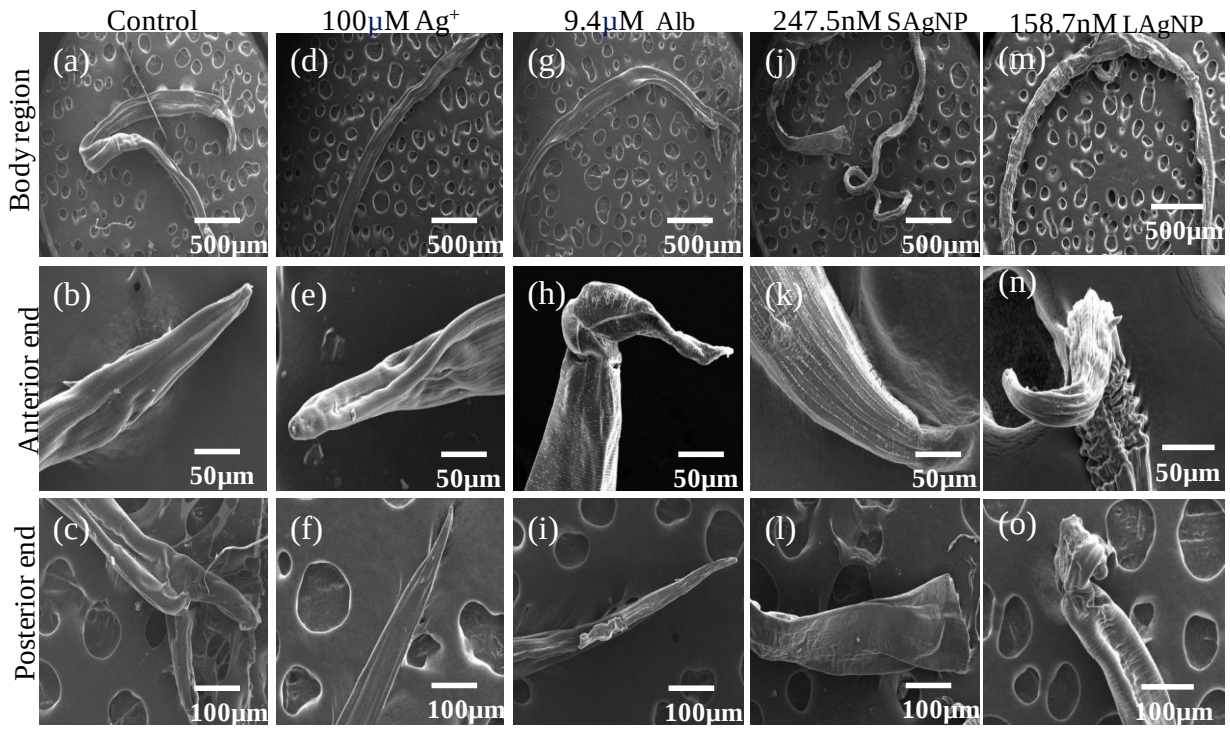


Fig 5: Study of ultramicroscopic (SEM) morphological damage in adults *H. contortus* under the SAgNPs and LAgNPs influence.

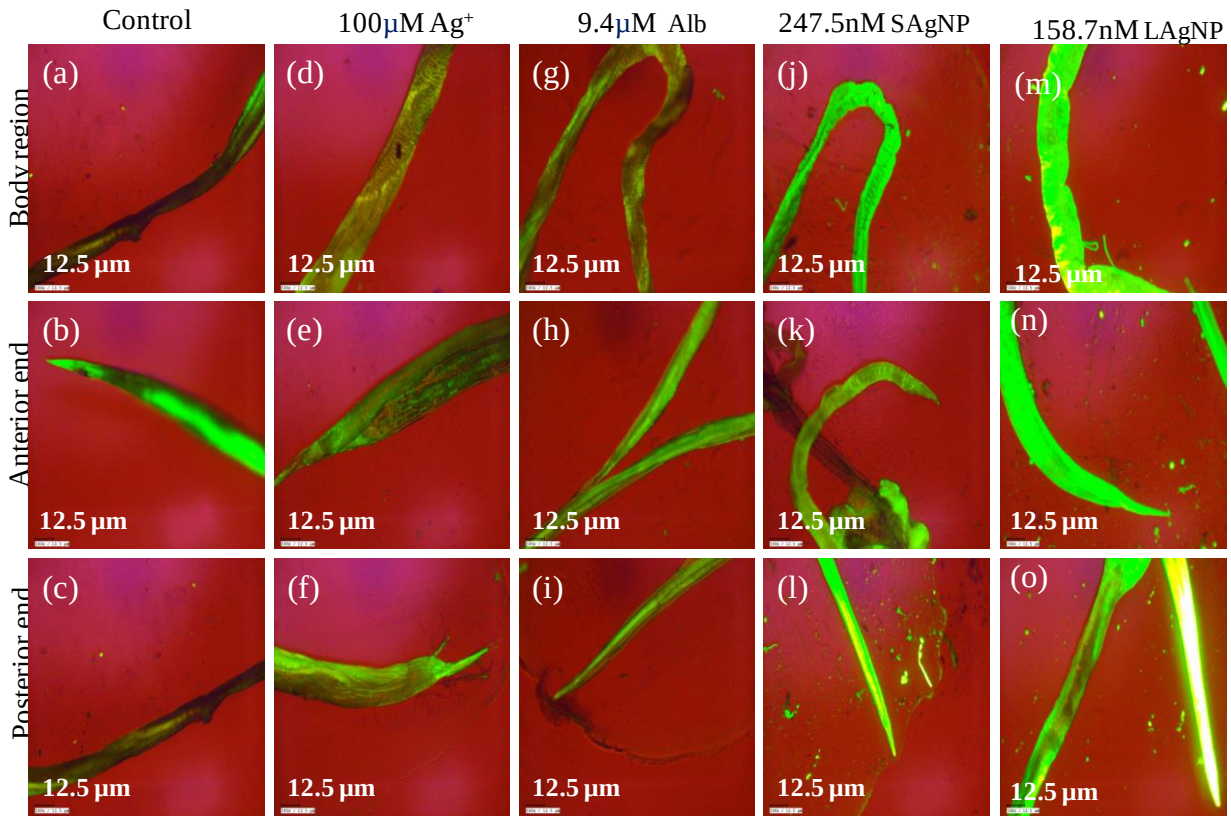
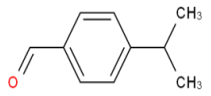
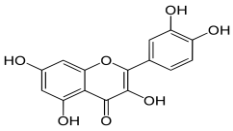


Fig 6: Study of ROS by using DCFDA morphological damage in *H. contortus* adults under the SAgNPs and LAgNPs influence.

Conclusion: The unprecedented antihelminthic efficiency of different plant extracts coated silver nanoparticles AgNPs by increasing reactive oxygen species thereby causes alteration of metabolic activity and physical damage in the worm tissue. *H. contortus* shows high resistivity against Albendazole which was also consistent in our findings, whereas plant extracts, AgNPs, and synthetic compounds not only killed adult worms but also showed a significant reduction of larval viability and egg hatching. Henceforth, the findings are highly useful in the wake of the increasing problem of drug resistance to the commercially available antihelminthic, increase of life quality, and reduction of health care cost.

Chapter 4: Antihelminthic activity of Pure compounds

Table 4: Synthetic compounds and their traditional uses

Compound	Structure	Source	Uses	Ref
Cuminaldehyde		eucalyptus, myrrh, cassia, cumin	diarrhea, colic, bowel spasms, and gas	Ebada et al. (2017)
Quercetin		apples, berries, Brassica vegetables, capers, grapes, onions, shallots, tea, and tomatoes	arthritis, bladder infections, and diabetes.	Badgujar et al. (2014)

4.1 Cuminaldehyde

Assessment of larval motility assay (LMA)

The effect of CA on larval motility was concentration depended. The percentage of larval survival were obtained 68%, 48.1%, 33.3%, 18.5%, 7.4% and 7.4%, respectively for (0-740 µg/ml) of CA treatment respectively in compared to 60.2% for widely used antihelminthic drug, Alb (500µg/ml). LD50 for inhibition of larva mortality obtained was 104.1 ± 7.9 µg/ml for 24 h.

Assessment of egg hatch in response to CA treatment

The percentage of hatched eggs into larvae were obtained 89%, 57%, 48%, 36%, 28.5%, and 14.5% respectively for (0-740 µg/ml) of CA treatment respectively in compared to 77% for widely used antihelminthic drug, Alb (500µg/ml). Over 50% inhibition of egg hatching was observed at as low as 142.4 ± 11.4 µg/ml CA concentrations at 48 h treatment.

Measurement of Superoxide Dismutase overexpression as a response to ROS induced stress

SOD enzyme in different concentration of CA (37, 74, 185, 370, and 740 µg/ml) was found to be 2.15± 0.1, 2.92± 0.2, 4.12± 0.5, 4.81± 0.20, and 5.59± 0.1 U/mg protein, respectively in comparison with the control where the amount was 4.8± 0.22.

Activation of catalase to combat ROS generated stress

Different concentration of CA (37, 74, 185, 370 and 740 µg/ml) was calculated as 5.08± 0.4, 9.43± 0.8, 12.71± 0.4, 14.14± 0.1 and 17.63± 0.1 U/mg protein respectively in comparison with the control where the amount was 4.1± 0.01.

The increase of glutathione peroxidase activity due to CA stress

The amount of active GPx enzymes were 8.03± 0.2, 8.58± 0.1, 9.19± 0.4, 10.15± 0.5 and 10.73± 0.04 U/mg protein for different concentration of CA (37, 74, 185, 370 and 740 µg/ml) respectively in comparison with the amount 2.6± 0.008 in negative control.

Cellular combat with ROS by increasing the reduced glutathione concentration

The amount of GSH increased from 23.08± 0.6, 24.5± 0.5, 26.6± 0.7, 40± 0.1 µM/ mg protein to 41.5± 0.030 µM/ mg protein for different concentration of CA (37, 74, 185, 370 and 740 µg/ml) respectively.

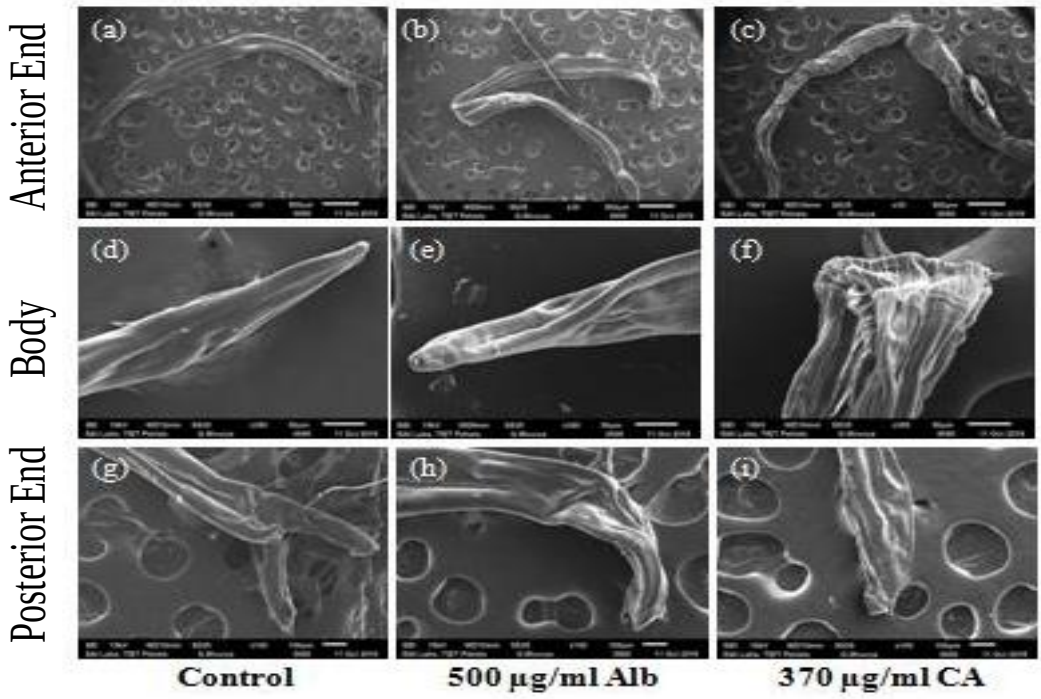


Fig 7: Study of ultramicroscopic morphological damage in *H. contortus* adults under the CA influence.

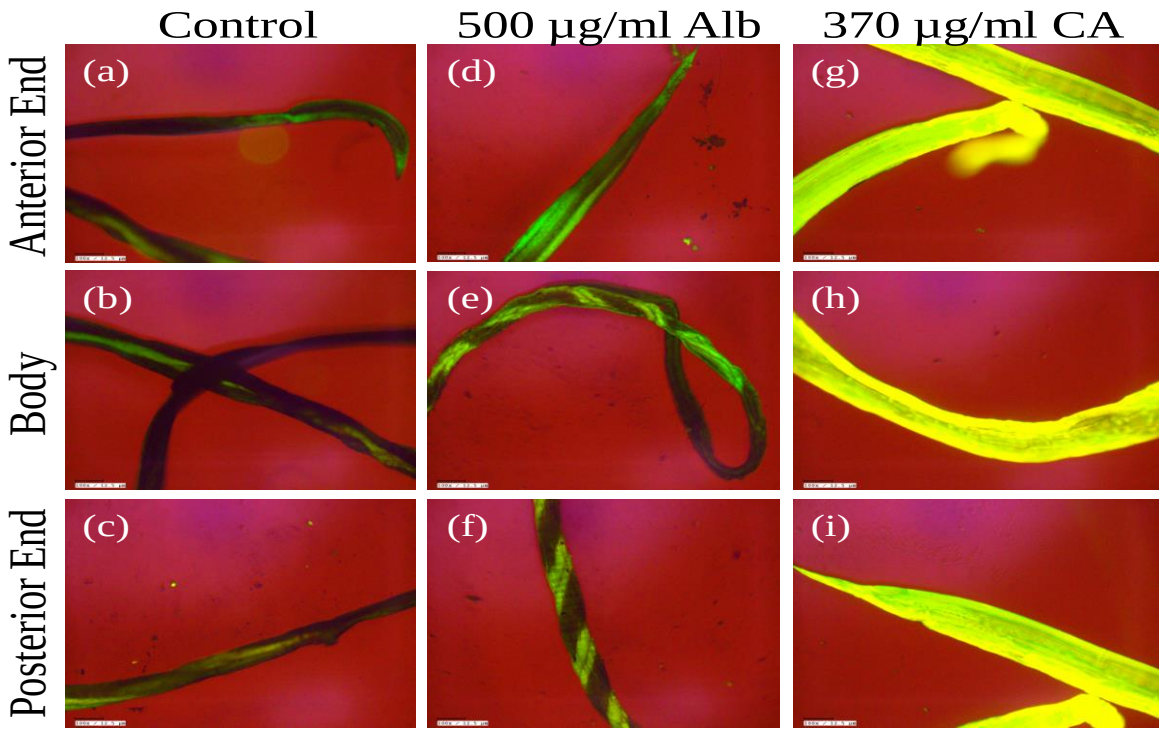


Fig 8: Study of ROS by using DCFDA morphological damage in *H. contortus* adults under the CA influence.

4.2 Quercetin

Adult Motility Assay (AMA)

Quercetin in 1mM till 12 h of treatment, only 60 % of the female worms got paralyzed and 100 % died in 24 h of Quercetin treatment. The LD50 values of 1.56 ± 0.14, 1.25 ± 0.23mM for an adult female, adult male worms at 12 hr of Quercetin exposure.

Larval Motility Assay (LMA)

The results for the toxic effect of Quercetin were: 66.6± 0.6%, 33.3± 0.7%, 18.5 ± 0.5, 7.4± 0.9%, and 0, 0 respectively for (0.125, 0.25, 0.50, 1, 2 and 5mM). Where control failed to show any results for the toxicity of the L-3 stage, albendazole showed 81.4± 0.5% survival of larvae (L-3). LD50 for inhibition of larva mortality obtained was 0.310 ± 0.24mM for 24 h.

Egg Hatch Assay (EHA)

For egg hatch assay, the EHA inhibition percentage was measured after 48 h of Quercetin treatment and calculated using equation (ii). The results for the toxic effect of Quercetin were as follows: 74± 0.6%, 34.5± 0.7%, 29± 0.7%, 19.5± 0.6%, 16± 0.7% and 10± 0.8%, respectively for (0.125, 0.25, 0.50, 1, 2 and 5mM). Whereas albendazole showed 75± 0.5% presence of larvae (L1) or eggs hatched. LD50 for inhibition of egg hatch obtained was 0.43 ± 0.071mM for 48 h.

Alteration of catalase, superoxide dismutase, and glutathione peroxidase activity in response to Quercetin induced oxidative stress:

The amount of CAT enzyme in different concentration of Quercetin was calculated as 5.2± 0.5, 5.9± 0.1, 7.2 ± 0.52, 16.1± 0.18, and 16.6.2 ± 0.2 U/mg proteins respectively after 3h of treatment in comparison with the 0.22±0.05 and 4.1±0.37 U/mg proteins for untreated control, Alb (9.4 μM). A consistent increment of the SOD activity with an increase in stress response had also been observed. The SOD enzyme activity after the treatment was found to be 2.13± 0.56, 3.09± 0.47, 4.02± 0.56, 6.68± 0.21, and 7.61± 0.34 U/mg proteins for Quercetin in comparison with the control where the amount was 4.8±0.22 for Alb. Untreated worms showed SOD activity of 0.21±0.018 U/mg of worm protein. In contrary to other antioxidant activities, GPx also shows an increase in activity when exposed to Quercetin with increasing concentrations. The amount of GPx was observed spectrophotometrically at 340 nm. The activity of the enzyme GPx was calculated using equation (iii). The amount of active GPx enzymes were 6.32± 0.78, 9.39± 0.51, 15.05 ± 0.31, 17.28 ± 0.56, and 18.47 ± 0.2 U /mg protein for different concentration of Quercetin in comparison with the control where the amount was 2.6±0.25 U/mg proteins in Alb. Untreated was found to be 0.21±0.071 U/mg proteins.

Cellular combat with ROS by increasing the reduced glutathione concentration

The increase in GSH was observed along with the increase of the dose of Quercetin, from 18.75± 0.58, 24± 0.38, 40.25± 0.61, 62.6 ± 0.49, and 68.41 ± 0.68 µM/ mg protein. Treatment of Alb also induced a significant increment in the GSH concentration (68.41± 0.030 µM/ mg protein) accumulation of GSH.

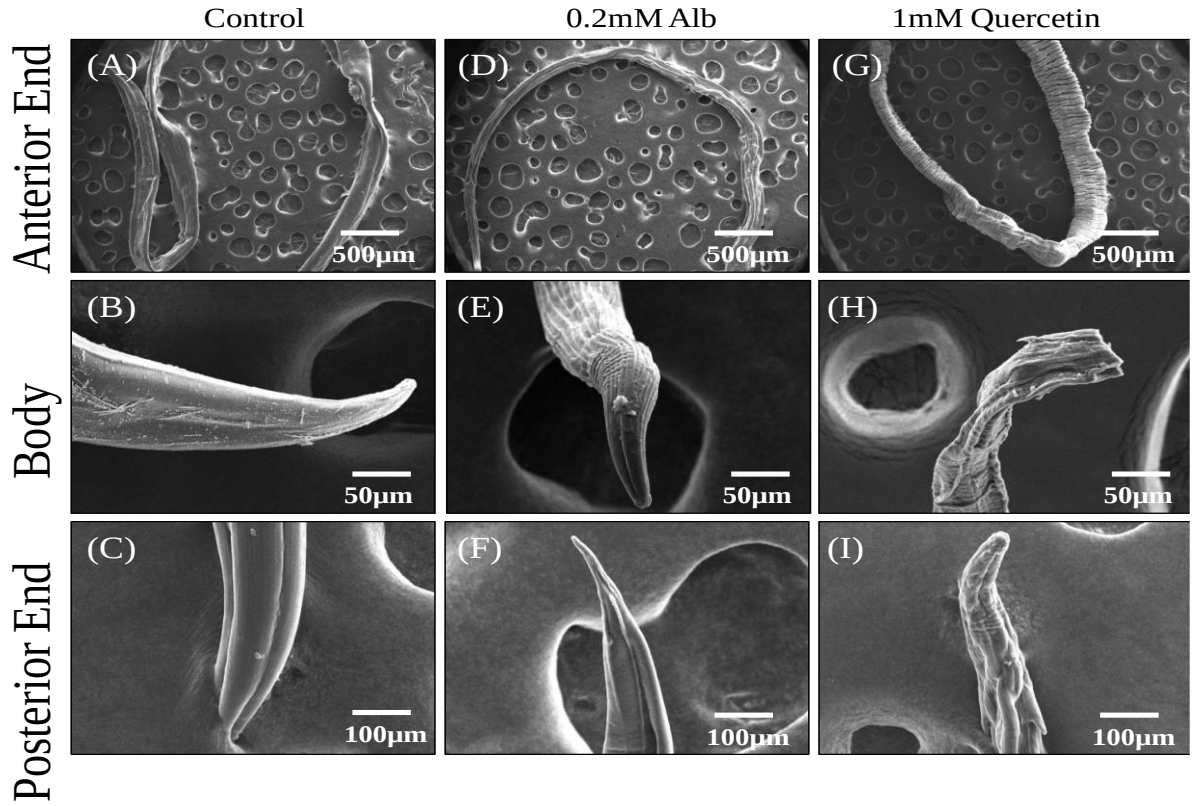


Fig 9: Study of ultramicroscopic morphological damage in *H. contortus* adults under the Quercetin influence.

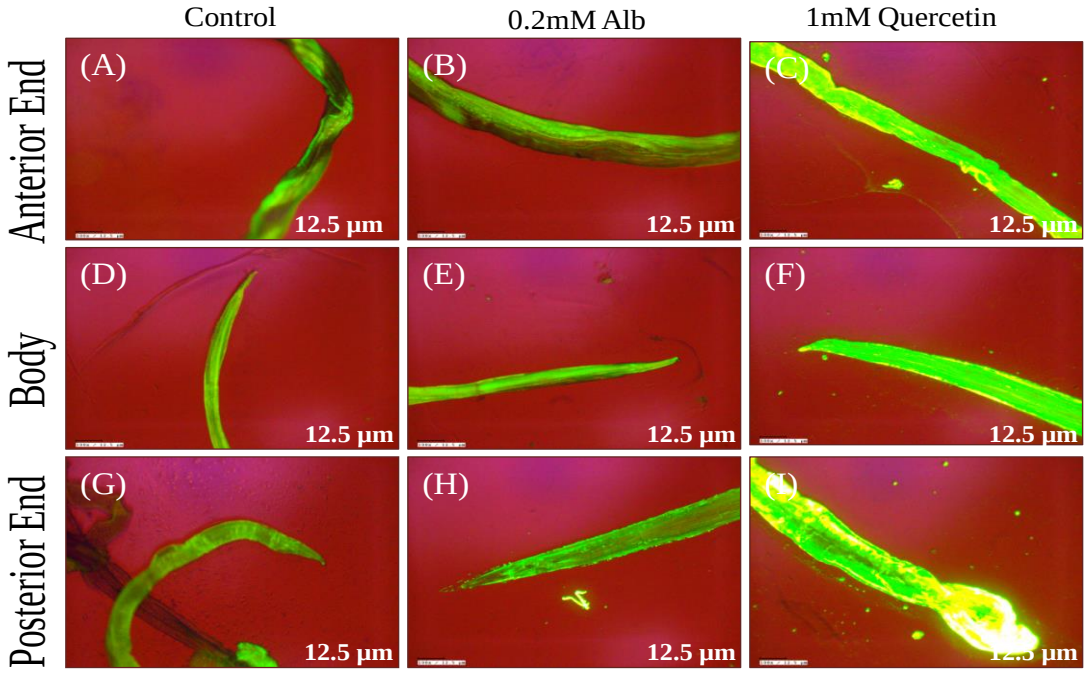


Fig 10: Study of ROS by using DCFDA morphological damage in *H. contortus* adults under the Quercetin influence.

Structural changes using Histopathology

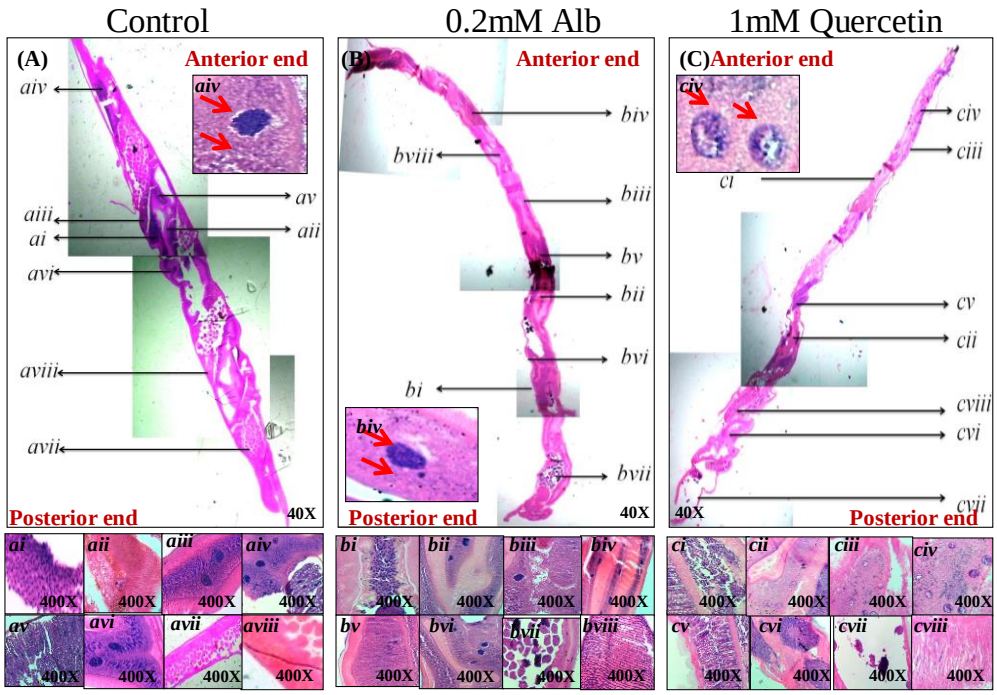


Fig 11: complete intact anterior and posterior end along with (i) isthmus, (ii) showed Brut, (iii) pseudocoele, (iv) showed globular leukocytes (surrounded with nuclei), (v) showed muscle cells, (vi) Intestinal Epithelium region, (vii) ovum and growth zone of the ovary, next to ovum found and (viii) showed intact skin tissue of the worm.

Oxidative stress generation of the central nervous system

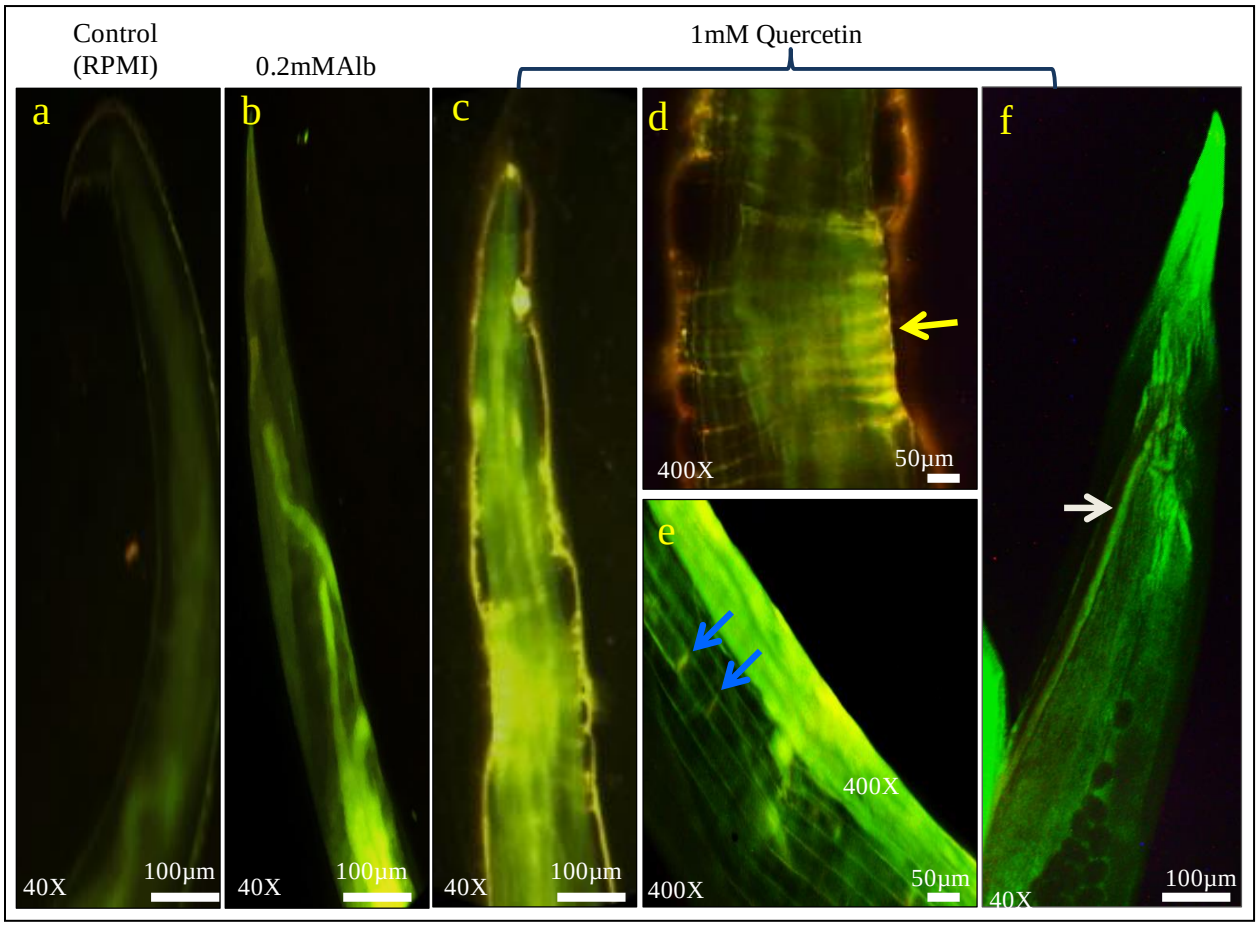


Fig 12: The stress caused by the central nervous system is shown by the generation of ROS.

The stress caused by Quercetin treatment on the central nervous system is shown by the generation of ROS. In **a** and **b**. the microscopic fluorescent images (40X) after the treatment with RPMI media and standard 0.2mM Alb is shown, where the partial generation of ROS and no differential parts of the nervous system were identified. **c**, quercetin (1mM) treated worms showed a high amount of ROS as compared to control and standard available drug. In **d** the yellow arrows are showing the nerve rings (400X). Further, **e**. shows the presence of commissures (400X) and in **f**, the white arrow shows the tail ganglion.

Conclusion: These *in vitro* studies using synthetic compounds (Cuminaldehyde and Quercetin) are highly useful in the wake of the increasing problem of drug resistance to commercially available antihelminthics. The exact mechanism of action for its antihelminthic activity is not clear as most components in Cuminaldehyde and Quercetin have several targets. Predictions about the mode of action of crude compounds require thorough investigations of their constituents, target sites, and their interactions with the surrounding environment. Further, these compounds could be evaluated by *in vivo* models for their use against gastro-intestinal parasitic infection with special emphasis on *Haemonchus contortus*. Therefore these findings have opened a new window of pharmaceutical application of CA and Quercetin for the treatment of gastrointestinal helminth infection.

Conclusion and Future prospective

The unprecedented antihelminthic efficiency of different aqueous plant extracts, silver nanoparticles AgNPs by increasing reactive oxygen species thereby causes alteration of metabolic activity and physical damage in the worm tissue. Plant extracts and synthetic compounds are present in ample amounts widely in nature and have a promising inhibitory effect against the adult, larval, and egg stage of *H. contortus*. The antihelminthic potential of AgNPs and synthetic compounds have been found far effective than the presently available drugs. It has been observed that AgNPs, cuminaldehyde, and quercetin induces antioxidant stress against various stages of the worm, which causes a predominant change in metabolism, physical damage, and death of worms. *H. contortus* shows high resistivity against Albendazole which was also consistent in our findings, whereas AgNPs, and synthetic compounds not only killed adult worms but also showed a significant reduction of larval viability and egg hatching. Henceforth, The *in vitro* studies with quercetin is extremely useful for increasing the efficacy against the worms resistant to commercially available drugs. Furthermore, quercetin should be analyzed by *in vivo* models for the treatment against GIN parasitic infection. But further detailed studies are required to understand the active principle(s), efficacy, and toxicity of the system in animal models. In addition to the utility of the formulations against other parasitic infections including helminthic infections need to be evaluated in the future. Moreover, *in vivo* studies may also be carried out in

small ruminants to overcome the gastrointestinal parasitic infection. Dose formations and clinical trials may lead to the manifestation of helminthic infection to some extent.