Targeted drug delivery using Beeswax derived Albendazole-Loaded Solid Lipid Nanoparticles in *Haemonchus contortus*, an albendazole tolerant nematode

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Abstract

Targeted delivery has not been achieved for anthelmintic treatment, leading to excess drugs and side effects for therapeutics. For that, drug-loaded, biocompatible nanoparticles were made using lipids that helminths uptake as a food source. The formulation showed excellent drug (albendazole) loading efficiency of 83.3 ± 6.5 mg/g with sustained-release properties and showed 86.4 ± 3.9 % of drug release within 24 h. Furthermore, upon ingestion of Rhodamine B loaded particles by *Haemonchus contortus*, a time-dependent release of dye was observed in the alimentary canal followed by dissemination in the whole worm. The intestinal sustained-release property was shown by the particles that caused enhancement of albendazole potency for up to 50 folds. Therefore, this formulation has immense potential as an anthelminthic drug delivery vehicle that will not only be able to reduce the dose but will also reduce the drug-induced side effects by enhancing the bioavailability of the drug.

Keywords: Solid Lipid Nanoparticles; Albendazole; Sustained release; Antihelminthic drug; Targeted delivery; Pharmacokinetics

1. INTRODUCTION

2.

Of all the infectious diseases causing pathogenicity present in humans and livestock, helminthic infection is one of the most common types. 20% of the world's human population, almost all wild animals, and a significant portion of the farm animal in developing countries are infected with intestinal helminths. Even though the mortality rates in these infections are less, it has been shown to cause various health complications like diarrhea, malabsorption, blood loss, and reduced growth rates. In some cases, they can cause anemia and retarded growth in children, adversely affecting cognition and educational abilities (Awasthi et al., 2003; "WHO Prevention and control of intestinal parasitic infections: WHO Technical Report Series N° 749," 2016). In

addition, the economic loss due to these soil-transmitted nematode infections are the results of reduction of milk, dairy products, wool, meat, etc. (Flach, 2008; Weber, 2015). Of these intestinal worms, *Haemonchus contortus* is a type of blood-feeding nematode belonging to the Trichostrongyloidea family. It is present in small and large ruminants and has a cylindrical body with a reddish appearance of length 10-30 mm and causes severe damage in the mucous membrane of the abomasum, resulting in gastric hemorrhage (Sendow, 2003; Gasser and Samson-Himmelstjerna, 2016; Gelberg, 2017). Although this worm is predominant in the tropical and subtropical regions and infects mainly sheep and goats, cases of human infection were reported in some human populations of Australia and Brazil (Sendow, 2003).

A limited number of drugs belonging to the macrolides and benzimidazoles family are known to act against these intestinal worms. For example, albendazole (Abz), a benzimidazole drug, shows poor water solubility and is administered along with a fatty meal (García et al., 2014). The mechanism of action involves binding to β-tubulin at the colchicine's-binding site, causing inhibition of the polymerization leading to the parasite's death (Jordan and Wilson, 2004; Kumar et al., 2013). Abz drug was developed in 1975, and after that, it has been used to control a wide variety of parasitic nematodes in humans and animals for decades (Barrère et al., 2012). But in recent days, chemoresistance due to altering tubulin isotype has been reported on several occasions for human and animal parasitic worms (Barrère et al., 2012; Sallé et al., 2019).

Further, the recommended dose of Alz is 10 mg/kg and 7.5 mg/kg of body weight according to FDA for humans and cattle, respectively, against *H. contortus* ("CFR - Code of Federal Regulations Title 21," 2020). However, the use of such a high dose also led to side effects including headache, nausea, vomiting, stomach pain, dizziness, fever, loss of liver function, etc. (Samuel et al., 2014). Therefore, the development of targeted delivery formulation is of immense importance to reducing drug dose and side effects.

Here, we hypothesize to develop a sustained release formulation that will be soluble, worm targeted, increase the bioavailability of the drug inside the parasite, and thereby enhance the drug potency. Different types of nanocarrier systems are being used, like carbohydrate-based, protein-based, or lipid-based. Of these, lipid-based drug delivery is recent and is known to have the advantage of better encapsulation efficiency, better solubility, and low toxicity (Jannin et al., 2008). In addition, biocompatible lipids like stearic acid, cetyl palmitate, cetyl alcohol, trimyristin, glyceryl monostearate, etc. have been used for the formation of solid-liquid nanoparticles (SLNs) and are physiologically tolerant when used *in-vivo*. These were fabricated using non-toxic organic solvents (Wissing et al., 2004; Dolatabadi et al., 2015; Dastidar et al., 2019).

H. contortus is known to absorb lipids from the GI tract of the host that is used for energy production, synthesizing parasite-specific glycerolipids and phospholipids, which are involved in host-parasitic interactions (Wang et al., 2020). In addition, a large amount of triacylglycerol was found in parasitic eggs which are used as a direct energy supply for egg development and are actively accumulated in the form of fats in the embryos (Wang et al., 2020). In the present study, Abz-loaded Solid Lipid Nanoparticles (SLN-A) were formulated using ethanol extract of Beeswax and tested against the intestinal parasitic worm *H. contortus.*

Beeswax is a non-toxic natural wax known for its effects against ringworm, jock itch, and fungal skin infections, having anti-allergenic and anti-swelling properties, and has been used in pain-relieving, lowering cholesterol, and reducing diaper rashes (NS, 2004). Beeswax constitutes- four unsaturated acids (oleic, linoleic, palmitoleic, and linolenic), two saturated fatty acids (tetracosanoic and palmitic), some other hydrocarbons, and wax esters. In this, hydrocarbons account for 12%–16% having a chain length of C27–C33; free fatty acids

account for 12%–14% with a chain length of C24–C32; free fatty alcohols (1%) having C28–C35. Linear wax monoesters, and hydroxymonoesters are 35%–45% of C40–C48 and are derived from palmitic, 15-hydroxypalmitic and oleic acids. Complex wax esters are 15%–27% and contain 15-hydroxypalmitic acid or diols linked to other fatty-acid molecules through their hydroxyl groups. Exogenous substances consist of pollen, propolis, small pieces of floral component factors, and pollution (Fratini et al., 2016). Here, Abz-loaded SLNs were made biocompatible using Poloxamer 407 coating on them. Further, the effectiveness of the formulation was compared with the FDA-approved oral Abz solution for its activity.

3. MATERIALS AND METHODS

2.1 Materials

Rhodamine B, DMSO, Ethanol, Chloroform, Hydrochloric acid, Sodium hydroxide, Sodium bicarbonate, etc., were purchased from LobaChemie India. Unrefined Beeswax was purchased from Arora Honey Bee Farm, Amritsar, India. Poloxamer 407, MTT, RPMI Media, and DMEM Media were purchased from HiMedia Lab, India. Hek293 cells were purchased from ATCC and maintained in the lab in a low passage number for the research work.

2.2 Synthesis of SLN, SLN-A, and SLN-Rh particles

Abz-loaded solid lipid nanoparticles (SLN-A) were fabricated using the double emulsion technique, where the hot ethanolic extraction of beeswax was done using the soxhlet apparatus at 78 ^{o}C (Buchwald et al., 2009). These extracts were then dried in a hot air oven till all the ethanol gets evaporated. The dried extract was then dissolved in chloroform (50 mg/ml) to make lipid emulsion. After that, Abz (100 mg/ml) was mixed in the lipid emulsion solution and added with 2% aqueous Poloxamer 407 (w/v). Finally, the solvent was evaporated to get SLN-A particles in powder form (**FIGURE 1**).

For the preparation of SLN particles (without drug), the above procedure was repeated without Abz. In addition to that, to prepare Rhodamine B-loaded SLNs (SLN-Rh), samples were prepared using the same method with 20 mg/ml, Rhodamine B dissolved in chloroform (**FIGURE 1**).

2.3 Particle size and Morphological Analysis

The hydrodynamic size and surface charge of SLN and SLN-A were determined using Nanobook 90 plus, Brookhaven, USA, instrument after dispersing the particles in distilled water. Further, the morphological characterization at room temperature SLN-A particles was examined using the drop-casting method on a Field Emission Scanning Electron Microscopy (FE-SEM, Nova Nano SEM-450, JFEI Company of USA (S.E.A.) PTE LTD). Dried samples were gold coated for 20 min before imaging. FTIR studies were done on an Agilent Technologies Cary 800 Series, Australia, to confirm drug loading.

2.4 Drug Loading and Release Kinetics

50 mg drug was loaded in 600 mg of SLNs particles, and its drug loading capacity was calculated and release kinetics of SLN-A. SLN-Rh particles were studied where the particles (1 mg/ml) were taken in 30 ml PBS buffer (pH 7.4), and the system was maintained at 37 ℃, and a spin of 130-150 rpm was applied. Samples of around 2 ml were collected at regular intervals and checked for absorbance using UV-visible spectroscopy at 295 nm for SLN-A particles and 555 nm for SLN-Rh particles. The absorption values were later plotted against time and noted for the trend of drug release.

2.5 Biocompatibility of SLN /SLN-A/SLN-Rh with human cells

Albendazole and different drug/dye-loaded SLN particles (SLN, SLN-A, and SLN-Rh) were checked for their cytotoxicity using an MTT assay. Hek293 (Human Embryonic Kidney cell line) was treated with different concentrations of 5, 50, and 100 µM and incubated for 24 h in a 5% CO_2 incubator at 37°C and humidified conditions after that 10 µl of MTT was added and incubated for 3 h. DMSO was added to dissolve the Formazan crystals, and reading was taken at Elisa plate reader at 570 nm.

The percentage of viable cells was calculated using:

$$\%\ of\ Cell\ viability = \frac{OD\ of\ treated\ cells}{OD\ of\ untreated\ cells}\ X\ 100\ \% \qquad \ldots\ldots(i)$$

2.6 Isolation of adult *H. contortus*

Adult *H. contortus* worms were identified and isolated from the abomasum samples of goats brought from the slaughterhouse. These worms were first washed using a 1X PBS buffer of pH 7.4 and then later transferred to RPMI media. Identification of these worms was done using the compound microscope and further experiments worms were randomly selected.

2.7 Treatment with SLN-Rh particles

Randomly selected 10 worms were added to RPMI media taken in 35mm Petri-plates and treated with SLN-Rh particles (20 µM). Later incubation with SLN-Rh particles was done for 1 h, 2 h, 3 h, and 24 h, respectively, and the worms were observed for the distribution of Rhodamine B. After the incubation, the worms were washed in 1X PBS buffer and placed on a glass slide to take images under the Dewinter Fluorescent Microscope at 100X and 200X magnification.

2.8 Treatment of worms with SLN-A particles

Worms were taken in 60 mm Petri dishes containing RPMI media (4 ml). Adult Motility and Morbility Assay (AMMA) was performed in which the death and paralysis time after the treatment with particles was noted. 30 worms were taken in triplets for each dose, with 15 males and 15 females in each set. SLN-A particles were added in the following concentrations- 2 µM, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, and 200 µM and the Abz was taken as a standard control having concentrations - 50 µM, 200 µM, 1 mM, and 2 mM. The worms were then observed at different time intervals by checking them under the compound microscope for movement. When no movement in the worm was observed (paralyzed), the worm was touched with a drop of warm (55 ^{o}C) saline solution. If the worm doesn't show any movement, that would mean that the worm has died.

For analysis of Oxidative Stress production, 6 male and 6 female worms were randomly peaked and placed in a petri dish containing RPMI media. SLN-A particles (10 µM) were added from the stock solution. The Abz drug and SLN particles were also taken as a control using the same concentration. After incubation for 3 h, the worms were washed with PBS buffer (pH 7.4) and then incubated with DCFDA dye (10 µM) for 10 min in the dark. Later then the worms were observed under Dewinter Fluorescent Microscope at 100X and 200X magnification.

2.9 Statistical analysis

Data were presented as the mean of at least three independent experiments. In addition, 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.

4. RESULTS

3.1 Structure and Morphology of SLN

The SLN and drug/dye-loaded SLN particles were formed using the double emulsion technique. The flakes of SLN were lime-colored, orange-colored for SLN-A, and reddish for SLN-Rh (**FIGURE 1**). These particles were found to be stable at room temperature in dry conditions. DLS data showed that these flakes were in the range 257 ± 98 nm and 165 ± 103 nm, respectively (**FIGURE S1. A-B**). Further, the morphology of the SLN particles was confirmed using FE-SEM. The FE-SEM images showed that SLN-A particles had cuboidal-shape and had sizes ranging 93± 29 nm. The images also showed that these particles were porous with a pore size of about 3 nm (**FIGURE 2. A-C**).

3.2 FT-IR study for confirming Abz loading

The FT-IR spectra were generated to determine the changes in SLN particles after the drug loading, which was clearly shown by the presence of characteristic peaks of Abz and SLN particles in SLN-A particles, proving that the Abz drug has been loaded into the particles. FTIR spectra showed a broad peak in SLN-A particles and Abz molecule at 3313cm^{-1} and a shift in the peak of SLN-A particles caused due to the N-H stretching at 3361 cm^{-1}from the N-H stretching at 3313 cm^{-1}in the Abz molecule. This peak was not observed in SLN particles. Another characteristic peak of benzimidazole molecule was found in both particles due to the C=N stretching where Abz showed peaks at 1618 cm^{-1} and SLN-A showed the peak at 1647 cm^{-1} (**FIGURE2. D-E**). A peek at 2877cm^{-1}was observed in SLN and SLN-A particles due to C-H stretching. The peak for C-O stretching was observed at 1279 cm^{-1}in SLN particles, 1268cm^{-1}in SLN-A particles, and 1266cm^{-1}in Abz molecules, and C-O asymmetric stretching was observed at 1085 cm^{-1}, 1073 cm^{-1}, and 1182 cm^{-1} in SLN, SLN-A, and Abz, respectively (Gunasekaran et al., 2009; Abidi et al., 2018; Jadhav et al., 2019). The fingerprint the region showed almost similar peaks in SLN and SLN-A particles, proving the entrapment of Abz molecule inside the SLN particles in SLN-A particles (**Table S1**).

3.3 Drug Loading and Release Kinetics

The drug loading capacity and release kinetics of SLN-A and SLN-Rh particles were studied where the drug was loaded as 83.3 ± 6.5 mg/g in SLN-A particles, and Rhodamine B was loaded as 33.3 ± 4.2mg/g in SLN-Rh particles. The release kinetics of both Abz and Rhodamine-B was studied from SLN-A and SLN-Rh particles at the physiological pH of 6.4 and physiological temperature of 37°C. Sustained drug release was observed in SLN-A particles for 24 h, and burst release followed by sustained drug release was observed for the first 12 h in SLN-Rh particles. Most of the drug was released by 48 h. SLN-A particles were suspended in a dialysis membrane with a concentration of 1 mg/ml, and the OD value was observed at 295 nm after particular time intervals (**FIGURE S1. C**). It was observed that 84.8 ± 3.2 % of the drug was released from SLN-A particles by the end of 48 h.

On the other hand, in the case of SLN-Rh particles, OD value was observed at 555 nm after particular time intervals, and it was noted that up to 50 % of the Rhodamine B dye was released in 2.5 h, and the almost total drug was released by 24 h (93.5 ± 2.7 %) (**FIGURE 3A**). This difference was observed due to the presence of polarity difference of Abz molecules and Rhodamine B molecules. Abz, due to its hydrophobic nature, is practically water-insoluble. On the other hand, in SLN-A particles, Abz is inside the lipid layer surrounded by a poloxamer molecule with its hydrophilic tail on its outer surface. Due to this, SLN-A particles have shown sustained release of the Abz drug. On the contrary, in the case of SLN-Rh, Rhodamine B is

water-soluble due to its hydrophilic nature and hence has shown burst release in the first 6 h followed by sustained release of the dye molecule (**FIGURE 3B**).

3.4 Biocompatibility of SLN particles

The biocompatibility of these SLN particles was studied using the cell viability assay, i.e., MTT assay. Hek293 cells were treated with SLN and drug/dye-loaded SLN particles and checked for their viability. SLN, SLN-A particles, Abz drug, and SLN-Rh particles were treated at different concentrations of 5, 50, and 100 μM (**FIGURE3C**). No toxicity was observed for the SLN, SLN-Rh, SLN-A particles, and Albendazole drug compared to control untreated cells. Cell viability was found to be 103.0 ± 6.4 % for SLN particles, 97.8 ± 6.7 % for SLN-A particles, 98.1 ± 5.7 % forAbz alone, and 104.2 ± 3.5 % for SLN-Rh particles (Using equation (i). Thus, these particles show no toxicity towards normal cell lines, and they can be used as a potential drug delivery system towards parasitic helminths.

3.5 Treatment with SLN-Rh particles

H. contortus worms were treated with SLN-Rh and checked for their uptake and distribution of dye with respect to time in *H. contortus* worms. These worms were treated with 20μM concentration for 1 h. Then images were taken in the red light filter after different time slots - 1, 2, 3, and 24 h (**FIGURE4**). An increase in fluorescence intensity was detected in worms with increasing time showing slow drug release inside the worm. Images were also taken in a blue light filter at 3 h, which showed how the fluorescence was observed more inside the alimentary canal, which was not so clear in images taken in the red light filter due to bright intensity (**FIGURE S2**). Fluorescence initially observed in the alimentary canal brightened with time and was later diffused into the whole body, as seen in 24 h images. The images show that the worm first took up the particles, then the drug was released inside the alimentary canal, and later the drug was diffused in the worm body. Treatment of worm with SLN-Rh particles showed the route of particles and dye inside the body with increasing time.

3.6 Antihelminthic activity of SLN-A particles

Adult Motility and Mobility Assay (AMMA) was performed for testing the antihelminthic activity of SLN-A particles, where the death and paralysis time of *H. contortus* worms were noted upon its treatment. 15 adult males and 15 adult females each were utilized in triplets, and it was observed that at 10μM concentration, 66 % of the worms were paralyzed in 12 h and 100% of worms were dead within 24 h of treatment at 20μM SLN-A particles. As control saline, PBS (pH 7.4) and RPMI media were used, and no deaths were observed for 24 h. In the case of Abz alone, worms had started showing paralysis at 6 h, and deaths were observed after 12 h for the 1 mM and 2 mM concentrations and no death was observed for the lower concentrations till 24 hours. While in the case of SLN-A, lower concentrations like 5 μM showed paralysis at 6 h and death at 12 h. After 24 h, almost all the worms were found dead, showing better outcomes than in Abz alone (**Table 1**). For adult male and female worms, the LD_{50}values were 1 mM and 10 μM for 24 h treatment.

3.7 Reactive Oxygen Species (ROS) stress generation due to SLN-A treatment

Oxidative stress generation in the adult worms due to the treatment of Abz, SLN, and SLN-A particles was checked using ROS assay (**FIGURE 5**). After the incubation with particles and then with DCFDA dye, images were taken in the violet light filter and checked

for the ROS generation using DCFDA fluorescence. The images were taken from the anterior, middle and posterior end of the adult worms, and the difference in intensity of fluorescence showed the amount of stress generated. In SLN-A and Abz treated worms, high intensity of green fluorescence was observed, showing greater production of oxidative stress and more damage to the tissues. Among these, SLN-A particles showed greater ROS production with pixel intensity of 113.2 x 10^4 ± 36.3 when compared to the worms treated with Abz alone showing pixel intensity of 102 x 10^4 ± 27.1 (**FIGURE S3)**. On the other hand, very little ROS production was observed in Control worms (RPMI media) with pixel intensity of 43 x 10^4 ± 8.9 and SLN treated adult worms' pixel intensity of 52.9 x 10^4 ±10.2.

5. DISCUSSION

Benzimidazole type of drugs have been used commonly as a treatment for parasitic worms, but with increasing cases of resistance against these antihelminthic drugs, there is an urgent need for the development of a new drug or better drug delivery carriers option. While developing a new drug requires a lot of time and lots of resources, other drug delivery carriers can be formulated to increase the efficacy of already available antihelminthic drugs. *H. contortus* is a highly pathogenic intestinal parasitic worm that causes gastrointestinal infections in ruminants and sometimes in humans. With the increasing development of resistance of the available drugs against these worms, a new formulation needs to be made, which will increase the effectiveness of these drugs.

The present study shows the formation and characterization of Albendazole-loaded solid lipid nanoparticles (SLN-A) that were made using beeswax as starting material for targeted oral drug delivery applications. These particles were later checked for their antihelminthic activity against the parasitic worm, *H. contortus*. For the formation of these particles, ethanol extract from beeswax was used for SLN preparation using the double emulsion technique where poloxamer 407 was used as a co-polymer to increase solubility and better drug-releasing capacity. As a result, Cuboidal-shaped orange-colored particles were formed, showing a high drug loading capacity of 83 mg/g, and sustained-release properties were shown where 84 ± 3 % of drug released within 24 h.

Albendazole-loaded SLN particles (SLN-A) were specifically uptaken by the adult worm, further supported by SLN-Rh particles where a time-dependent Rhodamine B release was observed in the intestine and then inside the whole of the adult *H. contortus* worm. Specific uptake and intestinal sustained-release dramatically enhanced the activity of the intestine for up to 50 fold. The biocompatibility of these particles was checked for their cell viability on Hek293 cells using MTT assay and was found to be non-toxic on treatment with SLN, SLN-A, and SLN-Rh particles. Hence these particles showed no side effect and the antihelminthic activity was due to the Abz drug in SLN-A particles.

Further, Adult Motility and Mobility Assay (AMMA) was performed on the adult *H. contortus* worm. The effect of SLN-A particles was checked on the adult worms where their death and paralysis time were noted. Furthermore, 15 males and 15 females were taken in each set, and no deaths were observed in Saline, PBS, and RPMI media controls. In the case of Abz, worms were paralyzed by 1 mM concentration in 6 h, and all of them died in 24 h. On the other hand, in SLN-A particles, worms got paralyzed at 10µM concentration in 12 h, and all the worms

were dead by 24 h. The IC_{50} value of Abz was found to be 1mM, and for SLN-A was found to be 10 µM. ROS assay showed that SLN-A particles generated more oxidative stress and greater tissue damage in the worms than Abz particles alone. Therefore, drug-loaded SLN can be used as suitable carrier systems for efficient drug delivery through the oral drug route with better efficacy and no side effects which were otherwise present due to administration of large doses.

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Targeted drug delivery using Beeswax derived Albendazole-Loaded Solid Lipid Nanoparticles in *Haemonchus contortus*, an albendazole tolerant nematode

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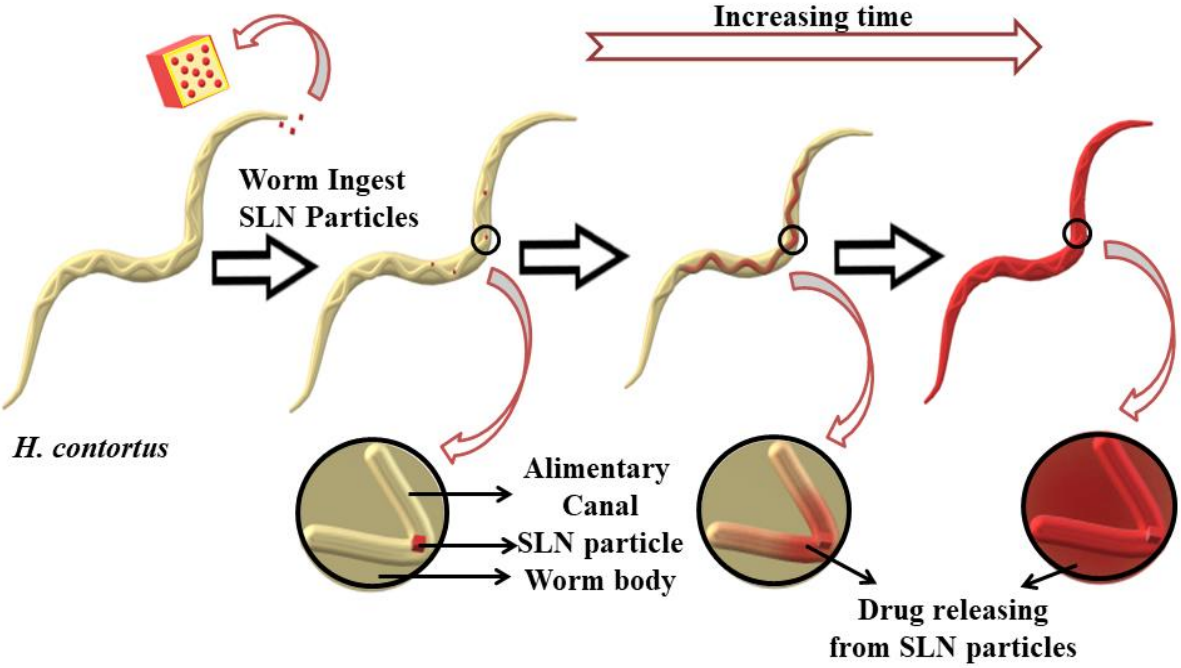
Abstract

Targeted delivery has not been achieved for anthelmintic treatment, leading to excess drugs and side effects for therapeutics. For that, drug-loaded, biocompatible nanoparticles were made using lipids that helminths uptake as a food source. The formulation showed excellent drug (albendazole) loading efficiency of 83.3 ± 6.5 mg/g with sustained-release properties and showed 86.4 ± 3.9 % of drug release within 24 h. Furthermore, upon ingestion of Rhodamine B loaded particles by *Haemonchus contortus*, a time-dependent release of dye was observed in the alimentary canal followed by dissemination in the whole worm. The intestinal sustained-release property was shown by the particles that caused enhancement of albendazole potency for up to 50 folds. Therefore, this formulation has immense potential as an anthelminthic drug delivery vehicle that will not only be able to reduce the dose but will also reduce the drug-induced side effects by enhancing the bioavailability of the drug.

Table 1: Paralysis and Death time analysis for adult *H. contortus*worm. Paralysis and death of worms were counted and noted for 24 h after treatment with SLN-A and Abz particles in different timepoints. 15 adult male and 15 adult female worms were taken and observedif these worms showed movementafter exposure tothe warm saline (paralyzed)otherwise they were considered dead.

Time of Exposure	RPMI Media	SLN-A							Abz				
		2 µM	5 µM	10 µM	20 µM	50 µM	100 µM	200 µM	50 µM	200 µM	1000 µM	2000 µM	
	MALES												PARALYSIS TIME
0 h	-	-	-	-	-	-	-	-	-	-	-	-	
3 h	-	-	-	-	-	-	-	6 ± 1	-	-	-	-	
6 h	-	-	2 ± 1	2 ± 2	6 ± 0	9 ± 1	9 ± 2	15 ± 0	-	-	6 ± 1	12 ± 0	
12 h	-	5 ± 1	5 ± 1	15 ± 0	15 ± 0	15 ± 0	15 ± 0	9 ± 2	3 ± 0	-	0	0	
24 h	6 ± 1	7 ± 2	6 ± 1	0	0	0	0	0	9 ± 2	9 ± 1	0	0	
	FEMALES												
0 h	-	-	-	-	-	-	-	-	-	-	-	-	
3 h	-	-	-	-	-	-	-	-	-	-	-	-	
6 h	-	-	-	-	-	-	-	6 ± 1	-	-	6 ± 1	6 ± 2	
12 h	-	-	2 ± 1	5 ± 1	6 ± 1	4 ± 2	7 ± 0	15 ± 0	-	-	6	9	
24 h	-	-	8 ± 1	12 ± 1	0	0	0	0	6 ± 1	-	0	0	
	MALES												DEATH TIME
0 h	-	-	-	-	-	-	-	-	-	-	-	-	
3 h	-	-	-	-	-	-	-	-	-	-	-	-	
6 h	-	-	-	-	-	-	-	-	-	-	-	-	
12 h	-	-	-	-	-	-	-	6 ± 1	-	-	15 ± 0	15 ± 0	
24 h	-	-	8 ± 1	15 ± 0	15 ± 0	15 ± 0	15 ± 0	15 ± 0	-	-	15 ± 0	15 ± 0	
	FEMALES												
0 h	-	-	-	-	-	-	-	-	-	-	-	-	
3 h	-	-	-	-	-	-	-	-	-	-	-	-	
6 h	-	-	-	-	-	-	-	-	-	-	-	-	
12 h	-	-	-	-	-	-	-	-	-	-	-	6 ± 1	
24 h	-	-	3 ± 2	4 ± 2	15 ± 0	15 ± 0	15 ± 0	15 ± 0	-	-	15 ± 0	15 ± 0	



Graphical Abstract: Drug-loaded SLN particles were ingested by the *H. contortus* worms, showing the drug release in the alimentary canal and then diffusing inside the whole adult worm.

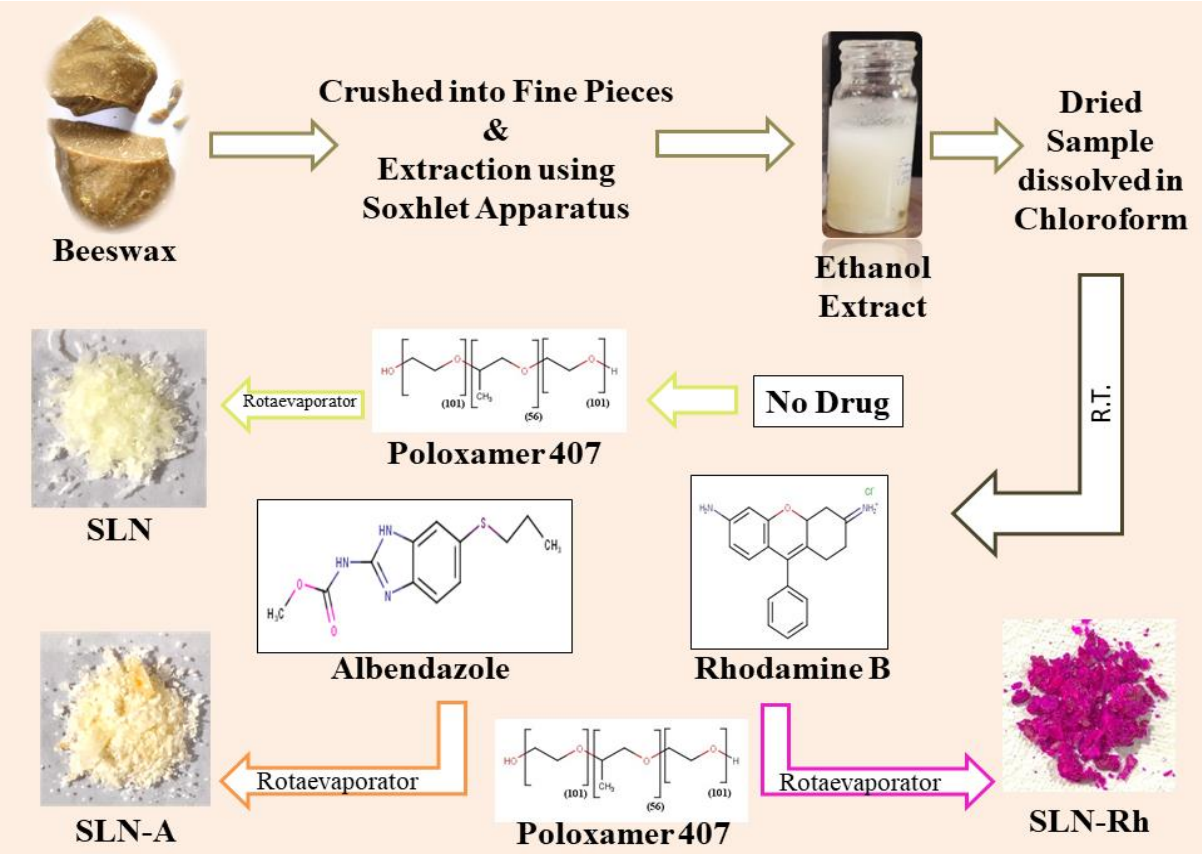


FIGURE. 1. Synthesis scheme of SLN, SLN-A, and SLN-Rh particles using Beeswax and Poloxamer-407.

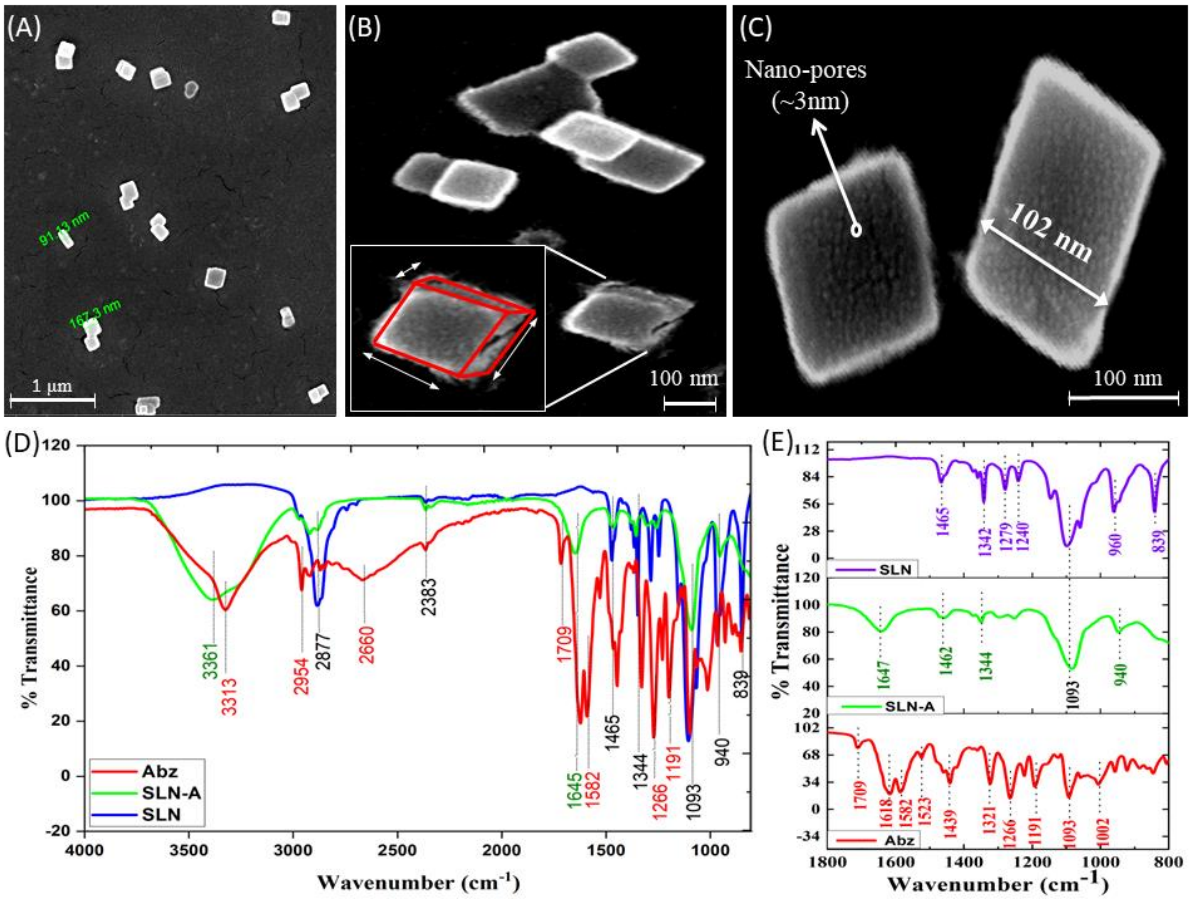


FIGURE. 2. Physical characterization of SLN particles using FE-SEM and FT-IR. (A) The morphology of SLN-A particles was studied using FE-SEM image; (B) Particles showing its cuboidal shape (C)Size of these particles ranges100 – 200 nm and havenano-pores of diameter 3 nm; (D) The FT-IR spectrum of Abz, SLN-A, and SLN particles measured using air-dried samples in the range of 500-4000 cm^{-1} using Agilent Technologies Cary 800 Series FTIR Spectrophotometer at room temperature and (E) FT-IR spectrum of Abz, SLN-A and SLN particles in the fingerprint region**.**

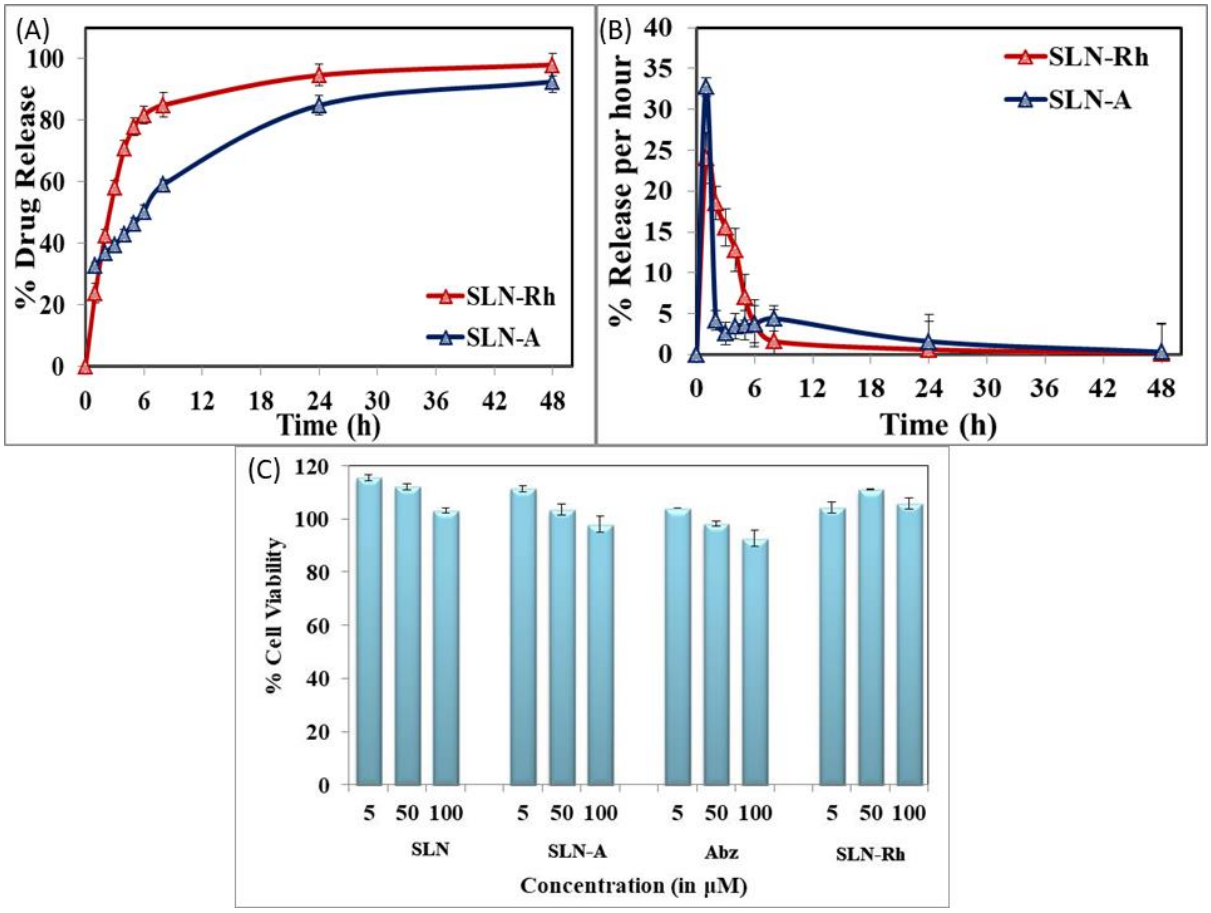


FIGURE 3.Plots showing Release Kinetics from SLN-A and SLN-Rh particles and their biocompatibility with normal cells.(A) Plot for the release kinetics of SLN-A and SLN-Rh particles showing percentage drug released from the particles;(B) Plot for the release kinetics showing per hour drug released (%) from the SLN-A and SLN-Rh particles; (C) Cytotoxicity assay of SLN, SLN-A, Abz, and SLN-Rh showing no effect on Hek293 cells.

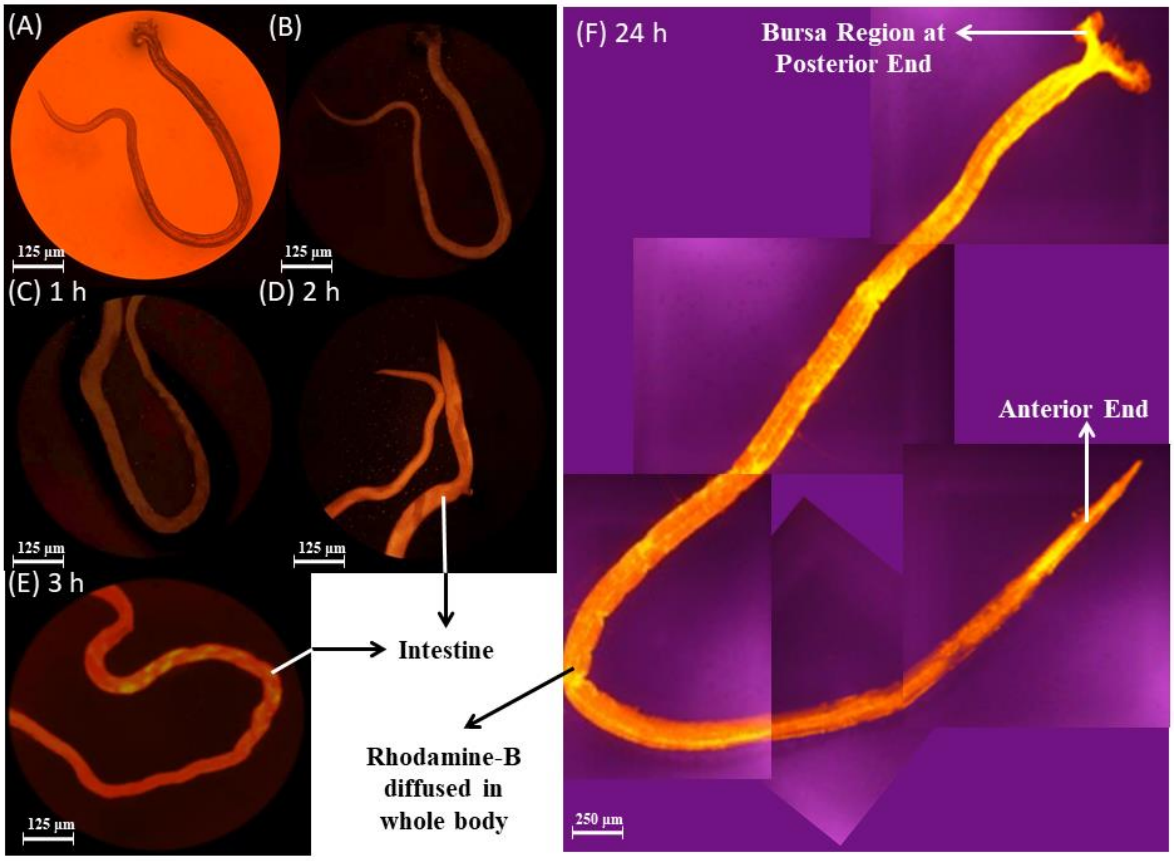


FIGURE 4.Treatment of worms with Rhodamine-B loaded SLN particles.*H. contortus* particles were treated with SLN-Rh particles and the release pattern of the dye was observed at 100X magnification in Dewinter Inverted Fluorescence Microscope, Italy. (A) Full adult worm in bright white light at 40X magnification; (B) Full adult worm after excitation of green light at 40X magnification after 24 h; (C), (D), and (E) Intestine of adult worm after 1 h, 2 h, and 3 h respectively showing increased fluorescence intensity with each passing hour; (F) Worm at 100X magnification showing diffused dye inside the whole body of worm after 24 h.This shows thatthe particles were ingested by the worm and the drug/dye was released which was later diffused inside the whole body.

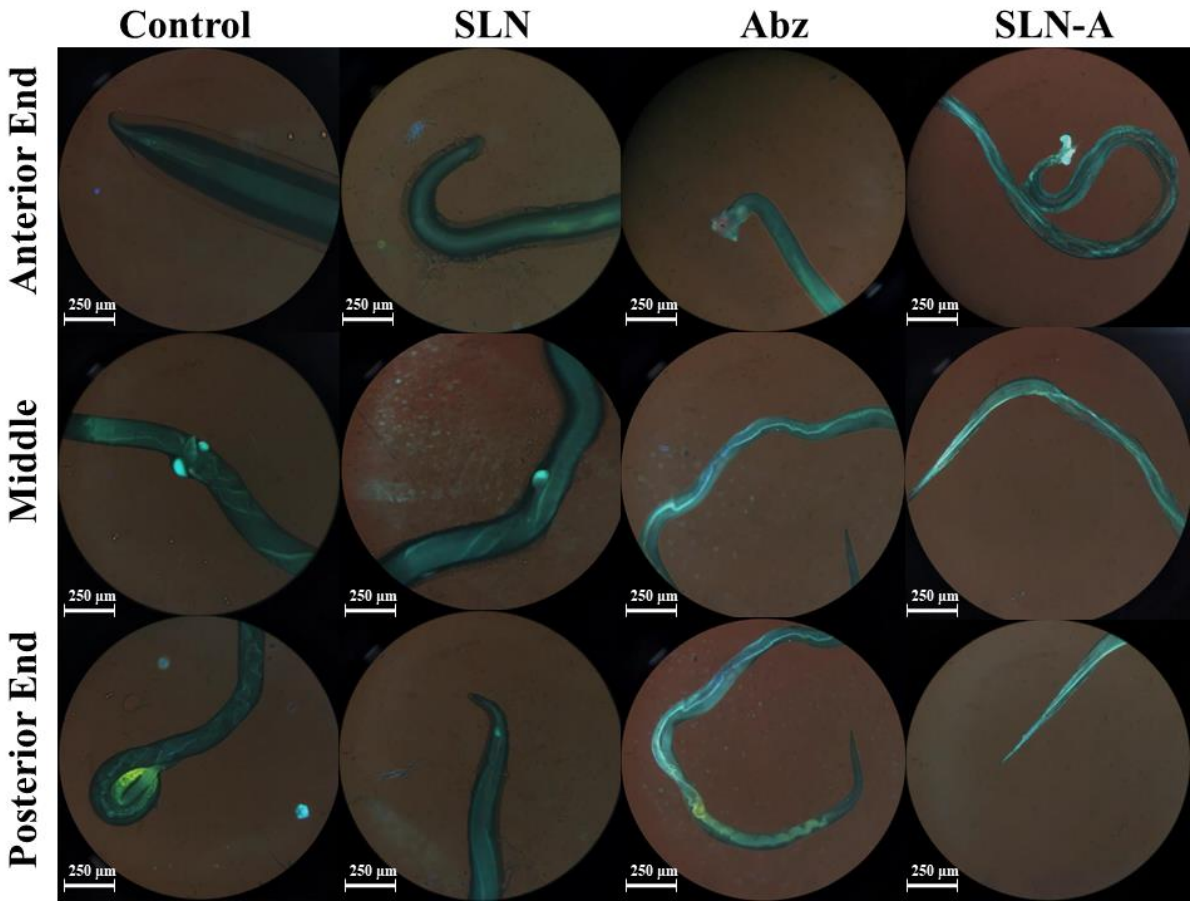


FIGURE 5.Study of oxidative stress generated in *H. contortus*adult worm.ROS assay was performed to check the amount of oxidative stress generated when the worm was treated and incubation with SLN,Abz and SLN-A particlesfor 6 h. The staining of worms was done using DCFDA dye (100 nM) and the amount of ROS generated was observed in the anterior, middle and posterior regions of the worm at 100X magnification in Dewinter Inverted Fluorescence Microscope, Italy.