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ANTINEMATODAL POTENTIAL OF THIOSEMICARBAZIDE (T-15) AND TRIAZOLE (TR-19) CLASSES OF SYNTHETIC COMPOUNDS USING CAENORHABDITIS ELEGANS AS A MODEL ORGANISM

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ABSTRACT
Nematodes are the helminthes they can cause parasitic infections in humans. They are responsible for causing substantial mortality and morbidity in humans and animals throughout the tropical and sub-tropical worlds. They are also responsible for losses in livestock and crop damage of more than US$100 billion annually. Several drugs are being used against these nematodes but selected Synthetic compounds, thiosemicarbazides (T-15) and Triazoles (TR-19) Antinematodal potential at the cellular level has been introduced DMSO is used for dilutions. In mostly cell biology Caenorhabditis elegans used as model organism. Fluorescence microscopies and Anti-egg hatching of nematode were done.) Thiosemicarbazides (T-15) (EC50 = 0.2 mg/mL) and Triazoles (TR-19) (EC50 = 1.27 mg/mL synthetic compounds showed fine antinematodal activity. These Synthetic compounds reflect excellent anti-egg hatching activity with fine concentration of 1mg/mL. Thiosemicarbazides (T-15) and Triazoles (TR-19) DMSO dilutions exhibited excellent apoptosis effects in intestines, uterus (eggs), and muscles, gonads. q PCR is used to identify the gene expression of treated genes. Both genes gst-4 and hsp-6 shows strong gene expression during q-PCR after being treated with synthetic compounds. And Thiosemicarbazides (T-15) and Triazoles (TR-19) for potent broad-spectrum antinematodal medications.

KEYWORDS: Nematodes, Caenorhabditis elegans, Thiosemicarbazides, Triazoles, Antinematodal assay, Anti-egg hatching, gene expression, polymerase chain reaction

INTRODUCTION
Helminthes Parasitic worms can cause prevalent economic and sanitary problems, mainly in most developed countries of the world [1]. Parasitic worms infections are the basic causative components of WHO-category very common diseases, and they can spread infections infect crops and livestock. Nematodes (Helminthes) are the main cause of great loss in animal breeding and economics of the world. Extensive treatment patterns of pharmacology adopted to save the livestock against helminthes as a result world facing the resistance against this helminthiasis [2]. It's very important to reduce the risks of resistance against the nematodes and treat these infections more efficiently and we need to add high efficacy pharmacological drugs against nematodes [3]. The parasitic nematodes are also affected by many compounds that are synthesized by plants [4]. C. elegans exist in aquatic and terrestrial environments or as parasites of animals and plants. Nematodes are the most common source of animals and humans' parasitic infections may lead substantial deaths and infections in both humans and animals throughout the tropical and sub-tropical world. The parasitic nematode infects more than two billion people all over the world predominantly in developing countries [5]. Almost all the information available yet about the physiology, anatomy and genetics of nematodes has been derived from C. elegans. It is difficult to maintain parasitic nematodes in a research lab due to the complicated life cycle. The molecular studies and mechanism of infections caused by nematodes C. elegans were very easy and used

as a model organism. Because very fast and simple culturing, transparent body, inexpensive, life cycle is short, and known genomic sequence. C. elegans are the living free area parasites that live in terrestrial areas used E. coli as a source of food. Nematode C. elegans length 1mm and 70µm in diameter. It is comprised of 959 somatic cells. The male population is very rarely found in C. elegan colony whereas most of the population is hermaphrodite [6].
In medicinal research for human and veterinary parasites, C. elegans is an excellent model the nematodes and trematodes cause helminth infections in humans and livestock animals. According to the WHO, approximately two billion individuals are infected with parasitic worms. People in poor nations are particularly vulnerable to nematode parasites (roundworms) [7]. Filariasis, Strongyloidiasis, River blindness, and other diseases are caused by nematodes in humans. Most commonly used drugs against nematode are piperazine, levamisole and albendazole [8]. From past 3 decades the only new antinematodal drugs was used for the humans infections [9]. Due to continuous use, the nematodes have developed resistance against these drugs and causing infections in humans and damage to crops [10].

MATERIALS AND METHODS

Instruments and Chemicals.

All synthetic compounds were collected from chemistry department Hazara University Mansehra, KPK Pakistan. Mini Centrifuge (5453 Hamburg, Germany), Micro Centrifuge (5425-R Hamburg, Germany), Inverted Microscope (Eclipse TS-100, Nikon, Japan), and fluorescence microscope (B-510FL, Italy, and BX60, United States of America).

Strains of Caenorhabditis elegans and E. coli.

The C. elegans N2 strain the sample was acquired from the Caenorhabditis Genetic Center located at the University of Minnesota, USA. Additionally, the bacterial strains Escherichia coli were obtained from the Genetic Center at the University of Minnesota, USA.

Synthetic compounds Collection and Dilution formations.

Synthetic compounds thiosemicarbazides (T-15) and (TR-19) triazoles have been obtained from The Department of Chemistry Hazara University, Mansehra (KPK, Pakistan). 15 days incubated (optimum room temperature), the dilutions were made individually in DMSO solvent. Polarity is the only factor that affects DMSO selection.

Nematicidal Characterizations.

Antinematodal Assays.

In order to analyze the antinematodal analysis of synthetic compounds thiosemicarbazides (T-15) and Triazoles (TR-19). In each well of a sterile 96-well plate, 100 µL of nematode growth media (NGM) buffer containing 15 to 45 C. elegans was added. Following this, different the synthetic compounds were added at concentrations of 1, 0.5, 0.25, and 0.125 mg/mL. After preparing the mixture, the plates were then incubated at 20°C for duration of 24 hours. The survival of C. elegans was assessed after 24 hours. Using a microscope to count active worms and a fine needle to provide physical stimulation to worms that showed inactivity, the availability of nematodes was determined**.**

Anti-egg Hatching Activity

Eggs isolation from C. elegans

The NGM plate was inoculated with the E. coli (OP50) strain and C. elegans N2 strain was cultured on it. After the worms were removed from the plate form of agar, they were rinsed with NGM buffer and put into a 50 mL conical tube. Centrifugation was used twice to wash the worms (1000 rpm, 2 min each time), and then they were hung in the NGM buffer. For five minutes, a single 15 mL tube containing up to 0.5 mL of gravid worms was treated with 5 mL of hypochlorite solution (3.75 mL sterile water, 1 mL household bleach, and 250 µL 10 M NaOH). Only eggs were left after C. elegans was totally disintegrated. The eggs were centrifuged three times at 1000 rpm for one minute, using NGM buffer, and then they were again suspended in 10 milliliters of NGM buffer. To find out how synthetic compounds like thiosemicarbazides (T-15) and triazoles (TR-19) affected egg hatching, the compounds were tested on eggs.

Effect of synthetic compounds on egg hatching.

Investigate the effect of synthetic compounds thiosemicarbazides (T-15) and Triazoles (TR-19) sterile 96-well plates were used for the experiment. A 50-70 C. elegans eggs were added to each well containing one hundred microliters of NGM buffer diluted with one milligram of thiosemicarbazides (T-15) and triazoles (TR-19). The control sample primarily contained eggs and NGM buffer. The plates were incubated at 20°C for 24 hours. During the incubation period, the number of eggs and larvae on the plates was evaluated using a microscope.

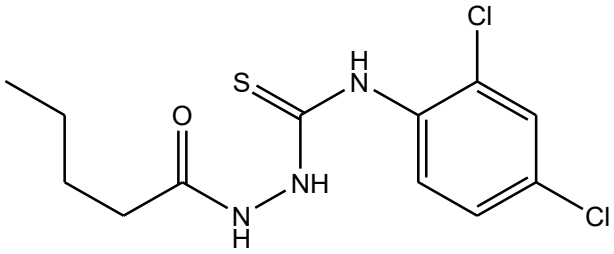
Fluorescence Microscopy.

Fluorescence microscopy was utilized to investigate the mechanism of apoptosis in C. elegans that were grown using synthetic compounds of thiosemicarbazides (T-15) and triazoles (TR-19). For staining C. elegans and conducting further morphological studies with fluorescence microscopy, acridine orange (AO) dye was used.

Staining of Affected C. elegans in Acridine Orange.
Thiosemicarbazides (T-15) and Triazoles (TR-19) dilutions (at IC 50 concentrations) were utilized to treat C. elegans. After a 24-hour incubation period, C. elegans were exposed to AO dye. A mixture of 2 µL of AO stock solution (10 mg/mL) and 1 mL of S-medium was prepared, and then 500 µL of this mixture was added to C. elegans. The worms were incubated in the dark for one hour at 37°C. To be examined using a fluorescence microscope (OPTIKA B-510FL Italy), C. elegans was fixed on a slide after being cleaned with NGM buffer.

Thiosemicarbazide's structural chemistry:
A molecular compound with formula H2NC(S) NHNH2 is known as thiosemicarbazide. The addition of an NH center links this white, odorless solid to thiourea (H2NC(S) NH2). They are commonly used as transition metal ligands [11]. The main class of thiosemicarbazones includes thiosemicarbazides. They are heterocycles' pioneers [12].

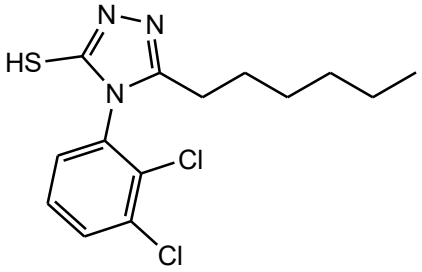
Biological uses.
The biological effects of thiosemicarbazides and their derivatives are intriguing and include anti-microbial, anti-HIV, anti-cancer, antiviral, insecticidal, anti-oxidant, and anti-parasitic properties [13]. The compounds containing the 1, 3, 4-thiadiazole ring have been shown to have a variety of biological activities, including antihypertensive, diuretic, antibacterial, antimicrobial, antifungal, ant tuberculosis, anti-hepatitis B viral, anti leishmanial, anti-inflammatory, analgesic, and CNS depressant properties [14].



T-19 chemical formula 4-(2, 4-dichlorophenyl)-1-pentanoylthiosemicarbazide

Triazole Structure.
Triazoles are significantly connected to the chemistry of triazoles and have obtained a unique structural motif due to their huge number of functions across scientific disciplines. Triazoles are mostly composed of a five-membered heterocyclic ring with the chemical formula C2H3N3, It contains two carbon atoms and three nitrogen atoms. The five-membered ring can create two primary isomers: 1, 2, 3-triazole (v-triazole) and 1, 2, 4-triazole (s-triazole), based on the two possible positional arrangements of the nitrogen atoms. Depending on which nitrogen in the ring the hydrogen is attached to, each of them primarily exhibits two tautomers (Figure 2). The structure of 4H-1, 2, 3-triazole is nonaromatic and therefore not considered. In both triazole isomers, every atom is planar and has sp2 hybridization. Each form contains six pi (π) electrons that are delocalized around the ring, contributing to their aromatic nature. Additionally, triazoles are energy-rich heterocycles due to the presence of three nitrogen atoms [15].

Biological uses.
Triazoles are an important class of compounds in chemical biology and pharmaceutical chemistry. They are involved in many biological processes that include oxidative stress, convulsions, infections, cancer, inflammation, and neuro degeneration [16].



Tr-15 Chemical formula 4-(2, 3-dichlorophenyl)-5-hexyl-4H-1, 2, 4-triazole-3-thiol

Analysis of Gene Expression in C. elegans.
To explore the molecular processes and metabolism pathways affected by the leaf extract, C. elegans gene expression study was performed. These genes gst-4 and hsp-6 were selected using the microarray expression data that was available and their function as stress response genes [17].

RNA Isolation and cDNA Synthesis.
Thiosemicarbazides (T-15) and Triazoles (TR-19) at an adult or L4 stage C. elegans culture for three hours at a concentration of 0.2 and 1.27 mg/mL. After three hours, C. elegans were washed three times with NGM buffer to remove any germs. Centrifuge tubes were utilized for collecting moving C. elegans, and the trizole method (Cornell University Library, USA) was used to extract total RNA [18]. In a 20 µL reaction, total RNA (1 µg) was utilized to synthesize cDNA by WizScript™ cDNA Synthesis Kit (High Capacity) (Wizbiosolutions Inc. Republic of Korea).

Quantitative Polymerase Chain Reaction in Real Time.
The quantitative expression of two gene transcripts has been confirmed by qRT-PCR. For the qRT-PCR assays, the 20 µL reaction mixture contained 2 µL of template cDNA, 2 µL (10 µM/µL) each of the forward and reverse primers, and 10 µL of 1× real-time SYBR Green PCR master solution. Optionally, 0.4 µL of 50X ROX Dye and 3.6 µL of RNase-free water were added. qRT-PCR was performed using quantitative RT-PCR equipment. Initially, the polymerase was activated for 10 minutes at 95 °C. The template was then denatured for 15 seconds at 95 °C. This was followed by annealing and elongation, which took place for 60 seconds at 60 °C. To find out if a unique final product was present, the melting curve was obtained between 60 and 95 °C after 40 cycles. [19].

RESULTS AND DISCUSSION
Nematicidal effect of Synthetic Compounds:
C. elegans was grown at 20°C for 24 hours with Thiosemicarbazide (T-15) exhibited high nematicidal activity (67±9.1, 60.6±5.50, 48.3±5.7, 31±11.0) showing an IC50 value 0.2 mg/mL. Dilutions ranging from 0.25, 0.5,1, 1.25 mg/mL. In the case of Triazoles (TR-19), Showed high nematicidal activity (82.33±5.6, 62.3±3.2, 55.33±9.0, 25±2.0) exhibiting an IC 50 value of 1.27 mg/mL. Negative control containing ≤1% DMSO (2.0±1.1) and positive control showed 100±2.33. Both Synthetic compounds Exhibited exceptional ability in numbers. The availability of synthetic substances with antinematodal potential counted for these greater nematicidal activities. Results are shown in Table 1.1 and Figure. 1.1.

Table: 1.1 Antinematodal activity of synthetic compounds

Synthetic Compounds	**0.25mg/ml**	**0.5mg/ml**	**1mg/ml**	**1.25mg/ml**
T-15	31±11.0	48.3±5.7	60.6±5.50	67±9.1
TR-19	25±2.0	55.33±9.0	62.3±3.2	82.33±5.6
≤1% DMSO	0.2±0.1			
Ivermectin (100 µg/mL)	100±2.33			

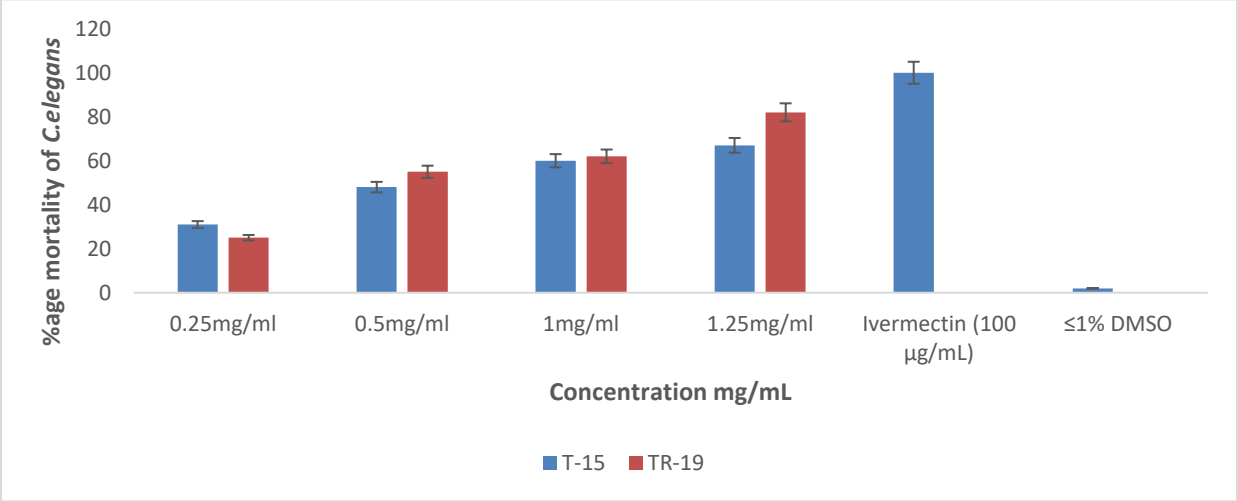


Figure. 1.1. Antinematodal activity of synthetic compounds

Synthetic compounds Effect on C. elegan Egg Hatching

Thiosemicarbazides (T-15) and Triazoles (TR-19) synthetic compounds tested at a concentration of 1 mg/mL was tested against C. elegans eggs, alongside a control sample. The control sample included a negative control containing ≤1% DMSO (1.23±0.1) and a positive control containing Ivermectin at 100 µg/mL. The unhatched eggs in Thiosemicarbazides (T-15) were 88±2.67 and Triazoles (TR-19) sample were (78±2.07). Results are shown in Table. 1.2 And Figure 1.2.

Table: 1.2 Anti egg hatching activity of synthetic compounds

Synthetic Compounds	**1mg/ml**
T-15	88±2.67
TR-19	78±2.07
≤1% DMSO	1.23±0.1
Ivermectin (100 µg/mL)	100±2.33

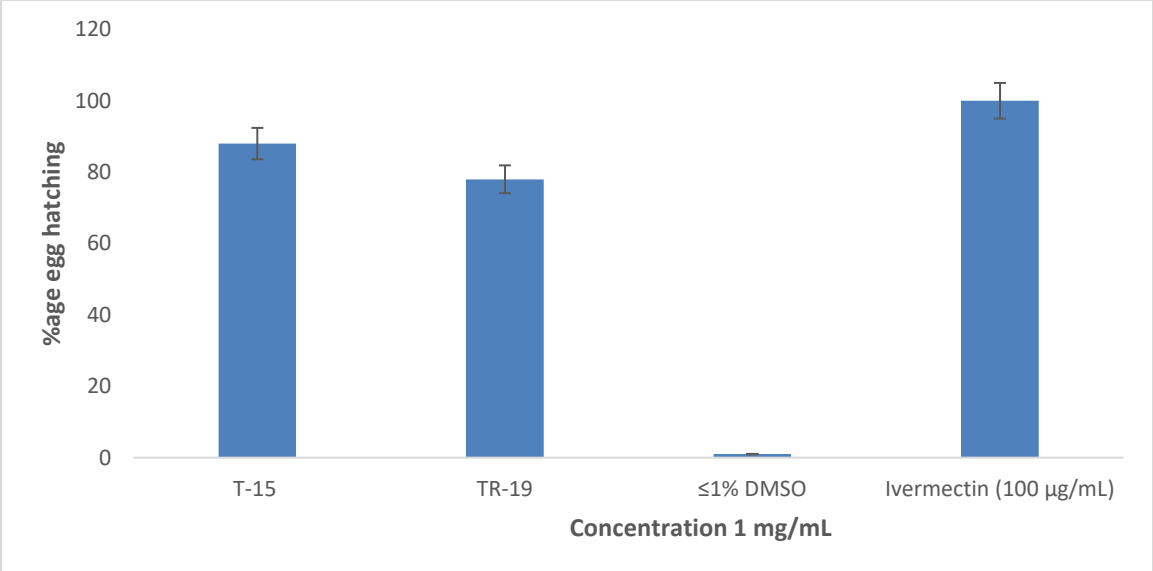


Figure 1.2. Anti-egg hatching activity of synthetic compounds

Fluorescence Microscopy of C. elegans.
When studying C. elegans treated with synthetic compounds such as thiosemicarbazides (T-15) and triazoles (TR-19), fluorescence microscopy is an excellent method for studying apoptosis. The synthetic compounds Thiosemicarbazide (T-15) exhibited significant levels of apoptosis and only the gonads and intestinal muscles exhibit the green fluorescence. The (TR-19) molecule, which indicates apoptosis in these tissues. As shown in figure 1.1 resulting in a variety of C. elegans cells to die developmental organs, including muscular, intestine, and gonad cells. Fluorescence microscopy provides detailed information on the effects of synthetic drugs called thiosemicarbazides (T-15) and Triazoles (TR-19) on intestine cells, muscle cells, and gonads (uterus) of C. elegans, as seen in Figure 1.3 and Figure 1.4, ultimately leading to cell death.

Fig 1.3 T-15 intestinal muscle cells and gonads cells affected

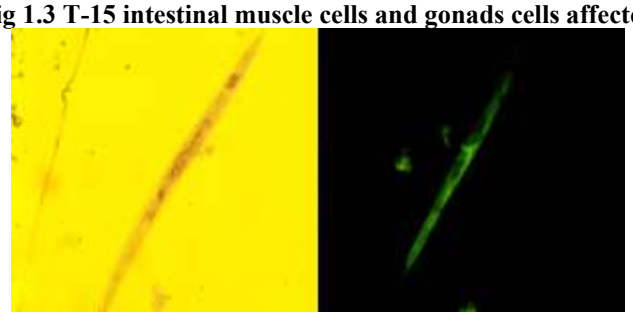
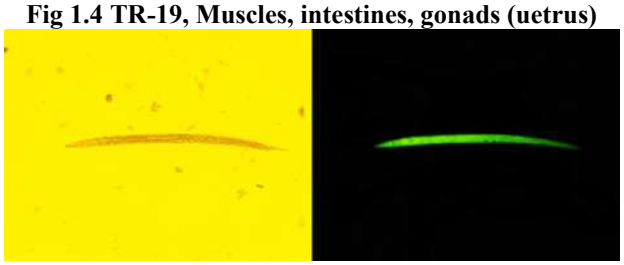


Fig 1.4 TR-19, Muscles, intestines, gonads (uetrus)



Thiosemicarbazides (T-15).
The expression of the oxidative stress reporter gst-4 was detected. Hsp-6, a heat shock stress reporter, mitochondrial stress was expressed.

Triazoles (TR-19).
Gst-4, an indicator of oxidative stress, was expressed. Hsp-6, the heat shock mitochondrial stress reporter, mitochondrial stress was expressed.

Gene Expression Analysis.
An analysis of the gene's expression in both healthy and affected C. elegans was done using qRT-PCR. Following three hours of incubation at 20 °C, the expression of approximately three C. elegans genes was examined in order to find the molecular activities and biochemical pathways affected by thiosemicarbazides (T-19) and triazoles (TR-15). The selection of these genes was based on their role in the stress response. The genes included were gst-4 and hsp-6.
Gst-4 gene were expressed when C. elegans treated with synthetic compound thiosemicarbazides derivatives (T-19) the expression of gst-4 gene were 12.1±0.3.
Hsp-6 gene were expressed when C. elegans treated with synthetic compound thiosemicarbazides derivatives (T-19) the expression of hsp-6 gene were 11.3±0.21. Results are shown in Figure. 1.5.

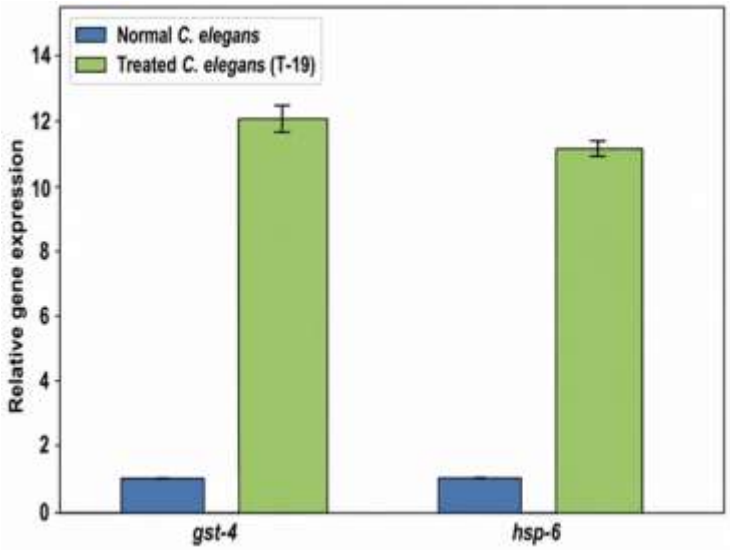


Figure: 1.5. Expression of (a) gst-4 (b) hsp-6 genes in C. elegans treated with Synthetic compound thiosemicarbazides (T-19).

Gst-4 gene were expressed when C. elegans treated with synthetic compound triazole derivatives (TR-15) the expression of gst-4 gene were 14.2±0.11.
Hsp-6 gene were expressed when C. elegans treated with synthetic compound triazole derivatives (TR-15) the expression of hsp-6 gene were 12.2±0.31. Results are shown in figure.1.6.

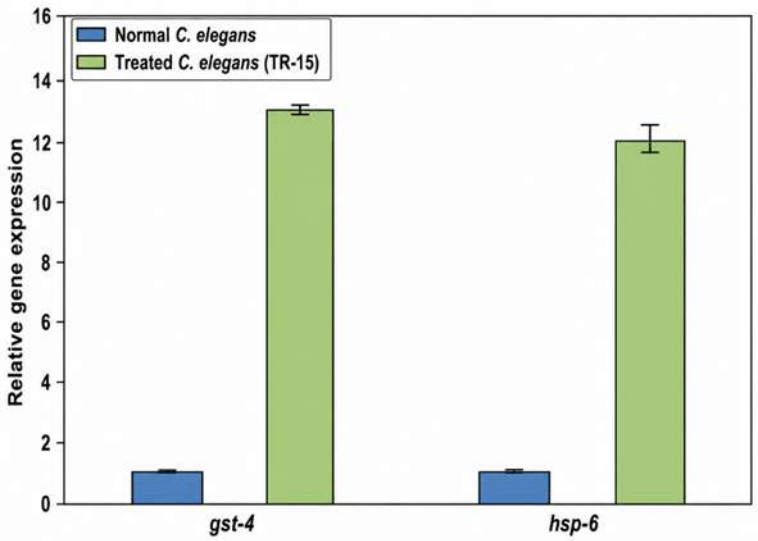


Figure: 1.6. Expression of (a) gst-4 (b) hsp-6 genes in C. elegans treated with Synthetic compound trizoles (TR-15).

CONCLUSION

Overall study findings proved the good potential activity of synthetic substances thiosemicarbazides (T-15) and Triazoles (TR-19). At 1 mg/mL, both synthetic drugs had a more powerful reduces the hatching of eggs. An analysis using fluorescence microscopy indicated that dilutions of synthetic substances, namely thiosemicarbazides (T-15) and Triazoles (T-19) induced apoptosis in intestinal cells, muscle cells, and the uterus. Quantitative RT-PCR analysis revealed that all extracts of Triazoles and Thiosemicarbazide expressed the gst-4 and hsp-6 genes. The gst-4 gene encodes glutathione S-transferase, which is involved in glutathione transferase activity and the glutathione metabolic process. The hsp-6 gene encodes a heat shock protein that performs unfolded protein binding activity in response to heat, occurring in the mitochondria. Both genes were expressed by synthetic compounds. From the results, it can be concluded that both classes of synthetic compounds have potential as broad-spectrum anthelmintic drugs to control parasitic nematodal infections.

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