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In vitro evaluation of *Cannabis sativa* seed (hemp seed) extracts for anthelmintic activity against *Pheretima posthuma*

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Abstract*: This study aimed to evaluate the in vitro anthelmintic activity of Cannabis sativa (hemp) seed oil, extracted using n-hexane solvent in a Soxhlet apparatus, against Pheretima posthuma (earthworm model). Helminthiasis, a prevalent parasitic infection caused by helminths, negatively impacts human health, livestock productivity, and agricultural development. Due to growing resistance and side effects associated with synthetic anthelmintics, the search for effective, plant-based alternatives has intensified. Hemp, characterized by a THC content below 0.2%, was selected for its rich phytochemical profile. Qualitative tests were conducted to screen for bioactive components such as flavonoids, phenolics, terpenoids, steroids, and saponins in the seed oil. The extract was tested at concentrations of 10 mg/mL, 25 mg/mL, and 50 mg/mL, and the time to induce paralysis and death in Pheretima posthuma was recorded. Saline served as the vehicle control, while albendazole at 10 mg and 25 mg acted as the standard reference. Quantitative analyses included total phenolic content determination using the Folin–Ciocalteu reagent and total flavonoid content using the aluminum chloride colorimetric assay. The results revealed that Cannabis sativa seed oil is a potent source of biologically active compounds like flavonoids and phenolic compounds, exhibiting notable anthelmintic effects. These findings support its potential as a natural anthelmintic agent and underscore the need for further pharmacological investigations into its phytoconstituents.*
Keywords*: Cannabis sativa, hemp seed oil, anthelmintics, Pheretima posthuma ,flavonoids.*

INTRODUCTION

Helminthiases are diseases caused by parasitic worms that impair the health of their hosts. These infections affect both humans and animals, often leading to poor growth and posing serious health risks. The persistence of these parasites—largely due to their constant release of eggs and larvae into the environment—makes helminth infections a widespread issue in both developing and developed regions.[1] Humans contract helminth infections either by ingesting parasite eggs—such as those of *Ascaris lumbricoides* and *Trichuris trichiura*—or through skin contact with infective larvae present in contaminated soil, as seen with hookworms (*Ancylostoma duodenale*, *Necator americanus*) and *Strongyloides stercoralis*.[2] Current control efforts rely heavily on mass drug administration (MDA), where high-risk groups receive preventive chemotherapy treatment once or twice a year. This typically involves benzimidazole drugs like albendazole (400 mg single oral dose) or mebendazole (500 mg). Although these treatments effectively reduce disease burden, reinfection is common and can occur shortly after administration.[3] The WHO's 2030 targets for soil-transmitted helminthiases (STH), set within the broader Neglected Tropical Diseases Roadmap, aim to eliminate STH as a public health concern in endemic areas. Strategies include maintaining at least 75% coverage of preventive chemotherapy among preschool- and school-aged children, expanding treatment to other vulnerable groups like women of reproductive age, enhancing sanitation and hygiene to prevent reinfection, strengthening monitoring systems for program effectiveness, and integrating STH control with broader health initiatives such as nutrition and maternal care.[4]

Dating back to ancient times, traditional medicine is one of the oldest forms of treatment in the world. Numerous indigenous peoples around the world still utilize customary treatments that have been orally transmitted from one generation to the next. [5]Historical records indicate that the *cannabis* plant was first widely used by Indians, and that it spread outside of India with Indo-Aryan culture. The plant is native to Persia, Southern Siberia, and China. In Hindi, it is called bhang, and in Sanskrit, it is called Vijaya. [6] Industrial hemp *(Cannabis sativa L.)* is a variety of *cannabis* with very little delta-9-tetrahydrocannabinol (THC), the compound responsible for psychoactive effects that can make people feel high. In the United States, the THC content of marijuana ranges from 10 to 30 percent of the dry weight, whereas that of industrial hemp is less than 0.2 percent. Industrial hemp is a type of *Cannabis sativa L.* that lacks psychotropic or behavioral effects.[7] Accounting for around 30% of their composition, hempseeds offer a major protein source. [8,9] They are becoming increasingly well-known as a good alternative plant-based source of protein in the food and nutraceutical sectors. Additionally composed of significant volumes of oil (almost 30%) and starch up to 25%. [8,10] Polyunsaturated fatty acids account for more than 90% of those in hempseed oil. Further-more present in hemp seeds are vitamins, insoluble fibre (10–15%), carbs (20–30%), and minerals including sulphur, calcium, phosphorus, iron, potassium, zinc, and magnesium. The unique qualities of the plant make it a very successful and sustainable crop. Over 25,000 hemp-based items may be found on markets worldwide. Rising demand from the personal care and cosmetics industries as well as more awareness of the nutritional advantages of hempseed and hempseed oil will help the market to grow. The seeds of hemp and oil are used in many food processing techniques, hence propelling market growth. [10,11]

MATERIALS AND METHOD

Plant Material

Hemp seeds (*Cannabis sativa*) were sourced from the Tehri area in the Garhwal region of Dehradun district, Uttarakhand, India. The collected seeds were verified by the Botanical Survey of India for authenticity.

Drugs and Chemicals

Albendazole, n-hexane, and distilled water—each of analytical grade—were procured from the laboratories of Dev Bhoomi Uttarakhand University.

METHODOLOGY

Preparation of extraction

First, the seeds of *Cannabis sativa* were thoroughly cleaned by removing the debris. Once cleaned, seeds were air-dried in the shade. The dried seeds were then crushed into a coarse powder using a mortar and pestle or electric grinder. This powdered material was stored in an airtight container at room temperature in preparation for the experiment.

Extraction of Hemp Seed Oil Using Soxhlet Apparatus

A total of 60±1 g of hemp seeds were processed using 500 mL of analytical-grade n-hexane . The ground hemp seeds were placed in a thimble filter within the Soxhlet extractor. The system was heated until boiling, maintaining continuous reflux for 8 hours. After the extraction, the n-hexane-rich hemp seed oil mixture was collected.

To eliminate residual water, anhydrous sodium sulfate was added to the oil-hexane mixture before evaporation. The purified oil was then isolated using a water bath. Finally, the oil yield was determined by weighing. All extractions were conducted in triplicate, and the maximum yield was recorded.

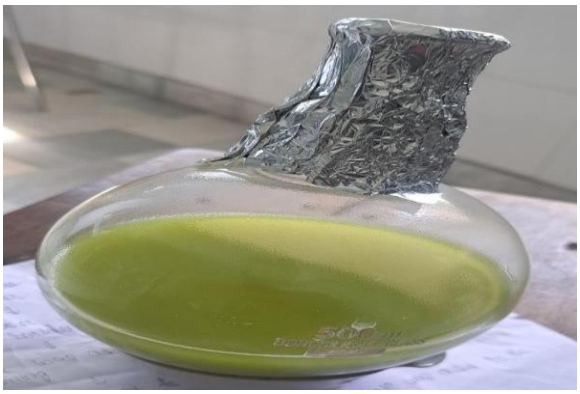
n-hexane extract process

Dried coarse powder of hemp seeds (60gm) of Cannabis sativa placed in an extractor chamber with n-hexane (500ml) of Soxhlet assembly for 8 hours and to maintain the water supply and temperature at 70c of heating mantel during the extraction process. Once the process was done the obtained seed extract was stored in a storage bottle. [18] After that obtained extract evaporates through the water bath. When the extract was obtained viscous then the oil extract was stored in glass bottles and kept in the refrigerator for the experiment.

[A]

[B]

[C]



[D]

[E]

Figure 1: These pictures are the extraction (n-hexane) of seeds of Cannabis sativa and its evaporation on water bath until it becomes oil.

Phytochemical Screening

1) **Preliminary phytochemical analysis** [13,14]

phytochemicals	Test method	Procedure	Result
Alkaloids	Wagner's reagent	Add 1 mL of the filtrate to a test tube and treat with Wagner's reagent. A cream-colored precipitate indicates the presence of alkaloids.	Positive reaction (brown/red precipitate)
	Dragendorff's reagent	Mix 1 mL of the filtrate with 1–2 mL of Dragendorff's reagent	Yellow precipitate
Flavonoids	Lead acetate test	To 1 mL of extract, add 4 drops of hydrochloric acid followed by a small piece of magnesium ribbon. A pink or red coloration signifies flavonoids	Colour change or precipitation
Tannins	Ferric chloride ($FeCl_3$)	Add a few drops of 5% $FeCl_3$ to the extract.	Blue-black or green colour
Saponins	Froth/Foam test	Dissolve 1–2 mL of extract in distilled water and shake vigorously. Persistent frothing indicates the presence of saponins.	Persistent frothing upon shaking
Phenols	Ferric chloride or Lead acetate	Combine a small amount of extract with 1–2 drops of ferric chloride solution. A deep green, blue, or black color indicates phenolic compounds.	Colour change or precipitation
Terpenoids	Chloroform + H_2SO_4	Mix 5 mL of extract with 2 mL of chloroform, then carefully add 3 mL of concentrated sulfuric acid. The formation of a reddish-brown layer confirms terpenoids	Reddish brown colour or precipitate formation
Carbohydrates	Benedict's reagent	Add 2ml of Benedict's reagent to the 1 ml of extract.	Orange or red precipitate

2) Quantitative Analysis

Total polyphenol content:

The number of phenolic compounds in the samples was assessed using the Folin–Ciocalteu reagent, following a modified approach based on the method outlined by Pourmorad et al. [12,13] Gallic acid served as the standard to create a calibration curve, with concentrations spanning 5 to 125 mg/L. For the experiment, 10 mg of the sample was dissolved in 10 mL of n-hexane. From both the standards and samples, 0.5 mL was taken and mixed with 2.5 mL of 50% Folin–Ciocalteu reagent and 2.5 mL of distilled water. After five minutes of resting, 2 mL of a 7.5% sodium carbonate aqueous solution was added. This mixture was thoroughly stirred and left to incubate in the dark at ambient temperature for 15 minutes. Absorbance was then recorded at 765 nm using a Cecil CE7410 UV-Visible spectrophotometer. The concentration of total phenolics was expressed in milligrams of gallic acid equivalents (GAE) per 100 mg of hemp seed oil extract. [13]

Total flavonoid content:

Flavonoid levels were estimated via the aluminum chloride colorimetric method, with slight modifications to the technique developed by Chang et al. [13,14] Quercetin was utilized as the reference to prepare a standard curve. A stock solution of 10 mg quercetin was prepared in 96% ethanol, from which serial dilutions of 2, 4, 6, 8, and 10 µg/mL were made. For the assay, 1 mL of each test and standard solution was combined with 3 mL of 96% ethanol. To this, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate, and 5.6 mL of distilled water were added. The mixture was gently stirred and allowed to incubate at room temperature for 10 minutes. After incubation, absorbance readings were obtained at 376 nm using a Cecil CE7410 UV-Vis spectrophotometer. A blank solution, prepared by omitting aluminum chloride, served as the control. Flavonoid concentrations were expressed in quercetin equivalents (QE) per 100 mg of oil extract and reported as mean ± standard deviation across three replicates (n = 3). [13]

Experimental

1) Collection of Earthworms:

Earthworms of the species *Pheretima posthuma* were obtained from the agricultural lands of Dev Bhoomi Uttarakhand University, located in Dehradun (Uttarakhand). To ensure cleanliness, the worms were thoroughly washed with normal saline to remove any remaining waste material. For the study, specimens ranging from 5 to 6 cm in length, 0.1 to 0.2 cm in diameter, and weighing between 0.8 to 3.04 grams were selected. Their selection was based on the notable similarities in their anatomical and physiological traits to human intestinal roundworms, making them an appropriate model for assessing anthelmintic efficacy. [15]

2) Preparation of Extracts and Reference Drug Testing:

Seed extracts were formulated in three concentrations—10, 25, and 50 mg/mL. In the case of the *n*-hexane oil extract, volumes of 10, 25, and 50 mL were employed. Distilled water functioned as the negative control throughout the experiment, while albendazole served as the benchmark reference drug for comparative analysis [16]

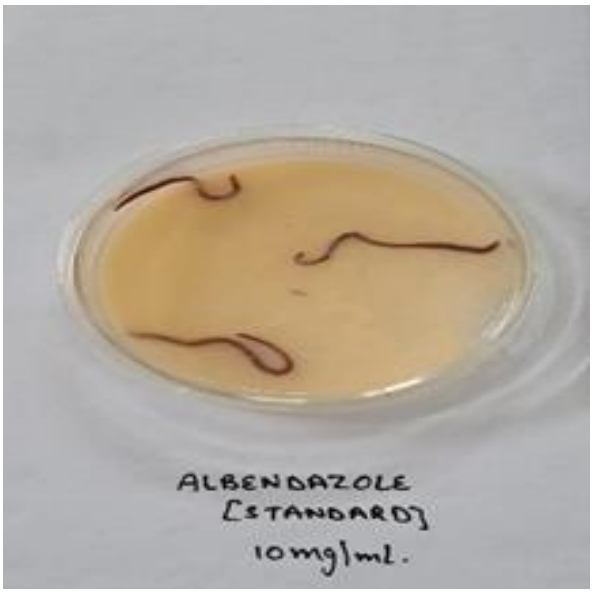
3) Procedure for Evaluating Anthelmintic Activity:

The anthelmintic assessment of the selected oil extracts was carried out following standard protocols, with minor modifications [17] Freshly prepared concentrations of both the extracts and drug solutions were used at the beginning of the test [18,19] Only actively moving worms of uniform size were selected, rinsed with water, and placed in Petri dishes for the experiment. The specimens were divided into four principal groups, each further separated into three subgroups, with three worms in each:

Group A: Control
Group B: Treated with *n*-hexane oil extract
Group C: Treated with albendazole (reference drug) [19,20]
Worms of similar dimensions were used in each dish containing different concentrations (10 mg/mL, 25 mg/mL, and 50 mg/mL) of the *n*-hexane extract of hemp seeds [20,21] Observations were recorded for the time taken by each worm to become paralyzed and subsequently die.

[A]

[B]



[C]

Figure 2: Picture [A]: Control group, [B]: n-hexane hemp seed oil extract, [C]: Albendazole extract treated group.

RESULT

The study evaluated the anthelmintic activity of hemp seed extracts, specifically the *n*-hexane oil variant, against the earthworm *Pheretima posthuma*, comparing its efficacy with albendazole. Results showed that the extracts were more potent at higher concentrations, especially at 50 mg/mL. The extract caused dose-dependent paralysis and death in the worms, confirming a strong link between concentration and effectiveness. Both qualitative and quantitative analyses revealed the presence of bioactive compounds, including flavonoids and phenolics, likely responsible for the extract's therapeutic potential. These findings support the promise of hemp seed oil as a natural anthelmintic agent and highlight the need for further research.

S. No.	Group / Test substance	Concentration (mg/ml)	Time of Paralysis (minutes)	Time of Death (minutes)
1.	Control Group	-	-	-
2.	Albendazole (Standard)	10 mg	14.36±0.21	17.09±0.12
		25 mg	2.24±0.16	6.21±0.71
3.	n-hexane seed oil extract treated	10 mg	32.62±0.31	36.14±0.02
		25 mg	20.39±0.16	23.06±0.44
		50 mg	12.32±0.33	15.13±0.2

Table 1: Invitro anthelmintic activity using n-hexane oil extract of seeds of hemp against *Pheretima posthuma*.

PHYTOCHEMICALS	TESTS	n-hexane extract seed oil
Alkaloids	Mayers test	Absent
	Dragendroffs test	Absent
Carbohydrates	Molish test	Active
Saponins	Foam test	Active
Flavonoids	Shinoda test	Active
Steroid and Triterpenoids	Swalowski reaction	Active
Phenols	$Fecl_3$ test	Active

Table 2: Chemical composition analysis of hemp seed oil.

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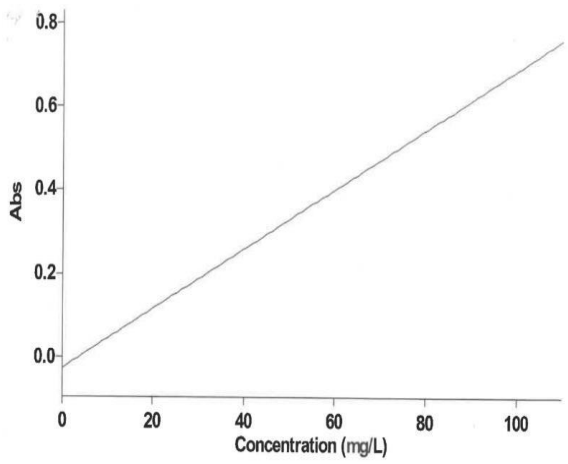

Figure 3: Analytical plot for Gallic acid

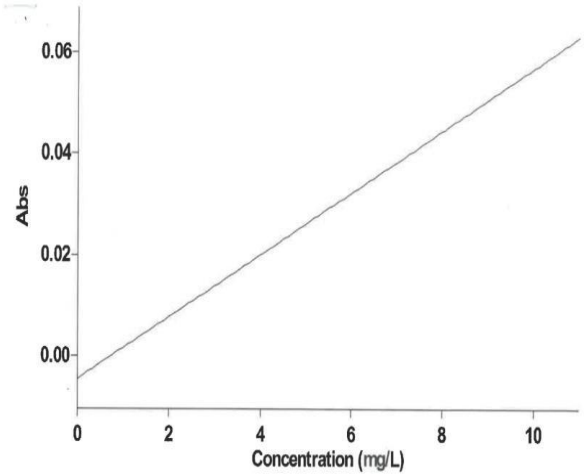

Figure 4: Analytical plot for Quercetin

Std.	Concentration (mg/ml)	Readings
01	5	0.0308
02	10	0.0517
03	20	0.1339
04	50	0.2597
05	100	0.7193

Table 3: Absorbance of std. gallic acid

Std.	Concentration (mg/ml)	Readings
01	2	0.0088
02	4	0.0235
03	6	0.0257
04	8	0.0431
05	10	0.0602

Table 4: Absorbance of std. Quercetin

PHYTOCEHMICALS	TESTS	n-hexane hemp seed oil extract
Total Polyphenols Content	Folin–Ciocalteu reagent	14.8 mg GAE/g
Total Flavonoid Content	Aluminium chloride colorimetric method	37.8 mg QE/g

Table 5: Quantification of bioactive constituents in hemp seed oil extracted using n-hexane.

CONCLUSION

The n-hexane extract of hemp seed oil demonstrates a notably stronger anthelmintic effect, indicating a potential link between the extract's polarity and its potency against parasites. This study aims to isolate and identify the specific chemical compounds within hemp seed oil extracts responsible for their anthelmintic properties. Pinpointing these active constituents will help elucidate their mechanisms of action and define their pharmacological characteristics.

Determining the key bioactive components is essential for several reasons. It will support the development of standardized formulations with consistent anthelmintic efficacy. Additionally, investigating possible synergistic interactions between compounds may enhance the extract's

overall effectiveness. Moreover, a comprehensive assessment of safety and potential side effects will be crucial for ensuring its suitability as an anthelmintic treatment.

Ultimately, this research seeks to deepen our understanding of the pharmacological potential of hemp seed oil extracts and their promise as effective, safe anthelmintic agents. These insights could play a pivotal role in developing new treatments for worm infections, addressing a significant global health concern.

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