International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2024; 12(4): 01-05
Received: 08-05-2024
Accepted: 12-06-2024

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Bioactivity guided isolation of anthelmintic compounds from *Centratherum anthemintic*um

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DOI: https://doi.org/10.22271/flora.2024.v12.i4a.936

Abstract
Helminth infections are among the most widespread infections in humans, distressing a huge population of the world. The fruit of *Centratherum anthemintic*um (SYN-Vernonia anthelmintica, Family-Asteraceae) known in India as Kalijiri is one such herbal remedy for worm infestation, so to isolate anthelmintic compounds from Kalijiri by bioactivity guided fractionation. *Caenorhabditis elegans* was chosen as a model of organism to check anthelmintic activity because of its high degree of cell differentiation and specialization. Anthelmintic activity of methanol extract and its fraction was checked. Results of motility assay was analysed by statistical analysis. Result showed that methanolic extract and its fraction showed significant activity.

Keywords: *Centratherum anthemintic*um, Anthelmintic activity, Caenorhabditis elegans, Kalijiri, fraction

1. Introduction
Medicinal plants have been utilised for centuries by various cultures worldwide to treat illnesses and promote health. These plants carry bioactive compounds that have therapeutic properties, making them valuable resources in both traditional and modern medicine [1]. Herbal medicine offers a natural and holistic approach to healthcare, drawing on the rich tradition of using plants for healing. Its principles of individualized treatment, preventive care, and reliance on traditional knowledge make it a valuable complement to modern medical practices. However, it's essential to use herbal remedies responsibly, seeking guidance from qualified practitioners to ensure safety and efficacy [2].

1.1 History of Herbal Medicine
Plants have been used medicinally for thousands of years and of years and is deeply rooted in various cultures around the world. Ancient civilizations for example the Egyptians, Greeks, Chinese, and Indians extensively used herbs for healing. Over time the knowledge has been passed down through generations and has evolved into the diverse system of herbal medicine that is practiced today. The history of medicinal plants in India is extensive and deeply intertwined with the country's cultural, spiritual, and scientific heritage. Indian civilization has a rich tradition of using plants for medicinal purposes, dating back thousands of years. The knowledge of medicinal plants in India has been passed down through ancient texts, oral traditions, and practices, contributing significantly to the development of Ayurveda, one of the world's best oldest holistic healing systems [3]. Helminthiasis refers to a group of infectious diseases caused by parasitic worms called helminths [4]. These worms can infect various organs in the human body, including the intestines, liver, lungs, and blood vessels. Helminth infections are prevalent in many parts of the world, particularly in tropical and subtropical regions where sanitation and hygiene practices may be inadequate. They have many types of helminths that can cause helminthiasis, including nematodes (roundworms), cestodes (Tapeworms), and trematodes (Flukes) [5, 6]. Helminthiasis remains a significant public health concern in many regions, particularly in low-resource settings with limited access to healthcare and sanitation infrastructure. Efforts to improve hygiene, sanitation, and access to healthcare services are crucial in reducing the burden of helminth infections and improving overall health outcomes [7, 8]. Anthelmintics are a class of medications specifically designed to treat infections caused by parasitic worms known as helminths.

These drugs target various types of helminths, including roundworms, tapeworms, flukes, and filarial worms. Anthelmintics work by either killing the parasites outright (vermicides) or by immobilizing them and causing them to be expelled from the body (Vermifuges) [9]. Large number of medicinal plants have been reported in Ayurveda to be able to cure worm infestation. The fruit of *Centratherum anthemintícum* (SYN-*Vernonia anthelmintica*, Family-Asteraceae) known in India as Kalijiri is one such herbal remedy for worm infestation No efforts have been done so for to isolate anthelmintic compounds from Kalijiri. The present study was designed to isolate anthelmintic compounds from Kalijiri by bioactivity guided fractionation.

2. Materials and Methods

2.1 Procurement of plant materials

The dried fruits were acquired from Lallu Vrajlal Gandhi & Sons in Ahmedabad, Gujarat.

2.2 Authentication of plant materials

The authenticity of the real plant material was confirmed through examination of its morphological and microscopic features.

2.3 Physicochemical Parameters

2.3.1 Ash Value

The ash content value of the crude drug is typically defined as the residue left behind after incineration. It consists of inorganic salts naturally present in the drug, as well as any added inorganic substances that may be included for adulteration purposes. Total ash refers to the residual material after complete incineration. Acid insoluble ash is the portion of total ash that does not dissolve in dilute hydrochloric acid, while water-soluble ash is the part of total ash that dissolves in hot water.

2.3.1.1 Determination of total ash

Approximately 2 grams powdered drug were precisely measured and placed in a silica crucible. The powdered drug was evenly spread as a thin layer at the bottom of the crucible. The crucible was heated to a temperature below 450°C until all carbon residues were eliminated. After cooling, the crucible weighed. This process repeated until a consistent weight achieved. The percentage of total ash calculated relative to the weight of the air-dried drug.

2.3.1.2 Determination of acid insoluble ash

The ash obtained following the method outlined for determining total ash was subjected to boiling with 25 ml of hydrochloric acid for 5 minutes. The ash that remained insoluble was collected on ash less filter paper and rinsed with hot water. This insoluble ash was then transferred into a silica crucible. That had been previously weighed, ignited, allowed to cool, and reweighed. Process repeated until a consistent weight was attained. The percentage of acid insoluble ash was then calculated based on the weight of the air-dried drug.

2.3.1.3 Determination of water-soluble ash

The ash obtained using the method outlined for determining total ash. Total ash boiled with 25 ml of hot water for 5 minutes. The material that remained insoluble that gathered on an ash less filter paper and washed with hot water. This insoluble ash was then transferred into a silica crucible that had been previously weighed and ignited at a temperature not exceeding 450°C. The process was repeated until a consistent weight was achieved. The weight of the insoluble material subtracted from the weight of the total ash, and the difference was regarded as the water-soluble ash. The percentage of water-soluble ash was calculated based on the weight of the air-dried drug.

2.3.2 Extractive Values

2.3.2.1 Alcohol soluble extractive value

5 grams of the air-dried drug placed in a stopper flask along with 100 ml of alcohol. The mixture was shaken continuously for 4 hours on a magnetic stirrer. Following this, it was quickly filtered to prevent any loss of the solvent. 25 ml of the resulting filtrate evaporated to dryness in a flat-bottomed petri dish that had been previously weighed. The dried residue weighed after drying at 105 °C. The percentage of ethanol-soluble extractive determined relative to the weight of the air-dried drug.

2.3.2.2 Water soluble extractive value

5 grams of the air-dried drug placed in a stopper flask along with 100 ml of chloroform water. The mixture was then continuously shaken for 4 hours on a magnetic stirrer. Subsequently, it was quickly filtered to prevent any loss of the solvent. 25 ml of the resulting filtrate evaporated to dryness in a flat-bottomed petri dish that had been previously weighed. The dried residue weighed after drying at 105 °C. The percentage of water-soluble extractive determined relative to the weight of the air-dried drug [10].

2.4 Pharmacognostical Study

2.4.1 Morphological Study

Assessment of the drug involves examining its size, shape, colour, odour, taste and tactile characteristics such as texture.

2.4.2 Microscopical Study

Mainly this method is utilized to qualitatively assess the entire crude drug. Different reagents or stains can be employed to differentiate cellular structures effectively. A small amount of the drug, either in powdered form or as a section, will be utilized. A drop of phloroglucinol and concentrated hydrochloric acid will produce a red stain when in contact with lignin. Thin sections of the drug will be utilized for analysis.

2.5 Preparation of Extract

Methanolic Extract (ME): The crudely ground plant material was subjected to three rounds of extraction using methanol through hot maceration. The combined extract drying by rotary vacuum evaporator.

2.6 Fractionation of Methanolic Extract

The methanolic extract mixed with distilled water and sequentially extracted with pet ether (MPE), chloroform (MC), ethyl acetate (MEA), and n-butanol (MBu). Each fraction washed with distilled water, dried using anhydrous sodium sulphate, and concentrated to obtain a dry residue. The aqueous portion was separated from the organic solvent through distillation under reduced pressure and subsequently evaporated to dryness.

2.7 Culturing of *Caenorhabditis elegans*

2.7.1 Procurement of Cultures

The N_2 strain of C. elegans was utilized for the experiment, obtained from the Caenorhabditis elegans Centre (CGC) at the University of Minnesota, USA. The worms were cultured at a temperature of 25 °C on NGM plates that were seeded with live bacteria (*E. coli* strain OP50) serving as their food source.

2.7.2 Preparation of growth media

2.7.2.1 Preparation of bacterial food source

C. elegans can be maintained without other organisms, but this is challenging and results in slow growth. Typically, C. elegans is cultured in the laboratory with *E. coli* strain OP50 as their sole food source. *E. coli* OP50, which requires uracil for growth, is cultivated on NGM plates. A starter culture of *E. coli* OP50 is used to isolate individual colonies on agar plates. A single colony from the agar plate is then used to inoculate a nutrient-rich broth, such as Nutrient Broth, and allowed to grow overnight at 37 °C. The resulting suspension of *E. coli* OP50 is then used to seed NGM plates. Both the streak plate and liquid culture of *E. coli* OP50 are stored at 4 °C for future use.

2.7.2.2 Preparation of NGM Plates

In an Erlenmeyer flask, 3 grams of NaCl, 17 grams of agar, and 2.5 grams of peptone were combined with 975 milliliters of water. The flask covered with aluminium foil and autoclaved for 50 minutes. After autoclaving, the flask cooled in a 55 °C water bath for 15 minutes. Then, 1 milliliter of 1 M CaCl2, 1 milliliter of cholesterol solution (5 mg/ml in ethanol), 1 milliliter of 1 M MgSO4, and 25 milliliters of 1 M KPO4 buffer were added to the flask and thoroughly mixed. The NGM solution dispensed into sterile petri plates using aseptic techniques. The plates filled up to 2/3 of their capacity with agar. After preparation, the plates left at room temperature for 2-3 days to check for contaminants and allow excess moisture to evaporate before use.

2.7.2.3 Seeding NGM Plates

Using sterile procedures, about 0.05 milliliters of *E. coli* OP50 liquid culture dispensed onto NGM plates using a micropipette. The droplet was evenly spread across the surface of the plate using a sterile glass rod. The plates then incubated at 37 °C for 8 hours to allow the *E. coli* OP50 lawn to grow. Once seeded, the plates were stored in a tightly sealed container until needed.

2.7.2.4 Transferring worms grown on NGM Plates

C. elegans is transparent and can be observed using a dissecting stereomicroscope that has a transmitted light source.

Method: A sterilized scalpel or spatula was utilized to transfer a piece of agar from an old plate to a fresh one. Typically, there were numerous worms within this piece of agar. The worms then emerged from the agar and dispersed onto the bacterial lawn of the new plate.

2.7.2.5 Obtaining Synchronous Cultures of *C. elegans*

Egg preparation

Reagent: 5N NAOH, Household bleach (5% solution of sodium hypochlorite.

Methods

1. The worms were washed with sterile water, which was pipetted across the plate multiple times to remove worms and eggs stuck in the bacteria.
2. The resulting liquid was collected in a 5ml sterile conical centrifuge tube with a cap, and water was added for make up the volume up to 3.5 ml.
3. A solution of 0.5ml 5N NaOH mixed and 1ml bleach was added to the centrifuge tube containing the worms.
4. The tube was shaken briefly, and this shaking was repeated every 2 minutes for a total of 10 minutes.
5. The tube was centrifuged in a table top centrifuge for 30 seconds at 1300 xg to pellet released eggs.
6. The supernatant was then aspirated down to 0.1 ml
7. 5 ml of sterile water was added to the solution and thoroughly shaken well for a few seconds.
8. Steps 5 and 6 were repeated as necessary.

A synchronized culture of C. elegans was achieved by allowing the eggs to hatch overnight without food. The resulting culture comprised starved worms arrested at a particular developmental stage. These worms were transferred to NGM plates containing *E. coli* OP50, enabling them to resume development from the same starting age.

2.8 Evaluation of Drug Extract and Fractions for Anthelmintic activity using *C. elegans* [11]

2.8.1 Preparation of Test Solutions

The dried extract and fractions were accurately weighed and dissolved in DMSO to get final concentration of 10 mg/ml solution.

2.8.2 Testing for Anthelmintic Activity

Into sterile 15 ml test tubes, 10µl of each test solution were pipette out. To this was added nematodes suspended in M9 solvent (3 g KH2PO4, 6g Na2HPO4, 5 g NaCl0.25 gMgSO4-7H2O in 1 I water). Control tubes contain DMSO (10µl) instead of test solution. All tubes incubated at 24hr & 25°C. After 24 hr. worm suspension was spreaded on agar plate from each test tube. The viability was evaluated by obtaining their motility using a dissection microscope. The worms which remained non motile for 10 sec. were considered dead. All the studies were done in triplicates. The significance of the activity was checked by applying one way ANNOVA followed and Turkey test [12].

2.9 TLC profile

Dried methanolic extract (ME), pet ether fraction (MPE), chloroform fraction (MC), ethyl acetate fraction (MEA), n-butanol fraction (MBu), and residue were prepared. The methanolic extract was dissolved in methanol, while the fractions were dissolved in their respective solvents. The solutions were then applied as bands onto silica gel TLC plates with a uniform thickness of 0.2mm.

The following mobile phases was prepared

1. Hexane Ethyl acetate (8:2)
2. Toluene: Ethyl acetate, Formic acid Methanol (5:5:1:0.3)

After development, the plates underwent anisaldehyde sulphuric acid reagent and heated at 110 °C for 5 minutes.

2.10 Phytochemical screening

The methanolic extract and its different fractions were examined to detect alkaloids, flavonoids, triterpenoids, steroids, phenolics, and saponins.

2.10.1 Alkaloid test

Dragendorff's test: Dragandroff reagent detect the presence of alkaloid. Formation of orange-collared precipitates indicate the presence of alkaloids.

2.10.2 Steroid test

Libermann Burchard test: A mixture was prepared by 2 ml of extract and 2 ml of chloroform. Then addition of 1.0 ml of acetic anhydride and 2 drops of sulphuric acid. The resulting colour change in sequence of red-blue-green it indicate the

presence of steroid.

2.10.3 Phenolic compound test
Lead acetate reaction: Tannins were precipitated from the aqueous extract by adding 2 ml of 10% solution of lead acetate. The precipitate obtained was partially soluble in 1 ml of 10% acetic acid, indicating the presence of condensed tannins. Additionally, the ethanolic extract of powdered aerial material was tested with Folin-Ciocalteu reagent. The development of a brownish-green colour indicate the presence of phenolic compounds.

2.10.4 Flavonoid test
Shinoda test: A gram of powder was extracted with 10 ml of 95% ethanol for 15 minutes on a boiling water bath and filtered. Then adding small species of magnesium ribbon and 3 to 4 drops of concentrated sulphuric acid. The appearance of a red colour indicate the presence of flavonoids [13].

3. Results and Discussion
3.1 Morphology

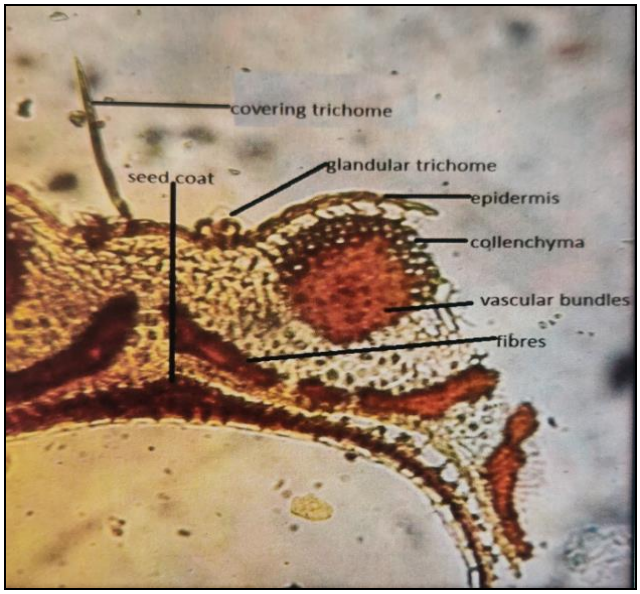

Fig 1: Transverse section of Kalijiri (fruit)

The seed of *Centratherum anthelminticum* 4.5-6 mm long, dark brown in color having a characteristic odour and intensely bitter taste. The surface of seed is comprised of about 10 ridges. The ridges are covered with trichomes. The seed is oblong shaped, pointed from one side and hairy tapered from another end.

3.2 Microscopy
The outermost layer of the seed is single cell epidermal layer. In between epidermal layer, there are glandular trichomes. Under this layer there are round structures of collenchymatous cell arranged in bundles giving an appearance of ridge from outside the seed. The seed coat is there. The outer layer of the seed coat is single cell layered. The cells are beaker shaped. Inner layer is thinner in comparison to outer layer. In the center embryonic region is there which contains globoid aluerone grains and lipid globules

3.3 Evaluation of physicochemical parameters
3.3.1 Ash Value
Physicochemical parameters are useful for the identifying adulterants and improper handling of crude drugs. Ash value important for the quality and purity of the drugs. Ash value of drug was compared to that given in the literature.

Table 1: Ash value of the Plant material

Name of plant	Plant used	Total ash (%w/w)	Water soluble ash (%w/w)	Acid insoluble ash (% w/w)
Centrathrum anthelminticium	Fruits	5.23 (NMT 7%)	3.87	0.67 NMT 1%)

3.3.2 Extractive Value
Extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in particular solvent. Extractive value of drug were compared to that given in literature.

Table 2: Extractive value of plant material

Name of Plant	Part used	Water soluble extractive (%w/w)	Alcohol Soluble Extractive (%w/w)
Centrathrum anthelminticium	Fruits	13.4	27.4

3.4 Evaluation of Anthelmintic Activity
The anthelmintic activity of extract and its fractions was evaluated against *C. elegans.*

Table 3: Anthelmintic activity of extract and fractions of *Centrathrum anthelminticium.* (ME-Methanolic Extract, MPE-Methanolic Petroleum Ether, MC-Methanolic Chloroform, MEA-Methanolic Ethyl Acetate, MBu-Methanolic Butanol, MT-Methanolic Toluene, Res-Residue).

Sr. No	Treatment	% Worms Dead
1	Control (DMSO)	63.62±5.09
2	ME	86.37±4.97
3	MPE	82.94±6.45
4	MC	89.09±4.19
5	MEA	94.31±2.05
6	MBu	93.26±4.22
7	Residue	97.24±2.49

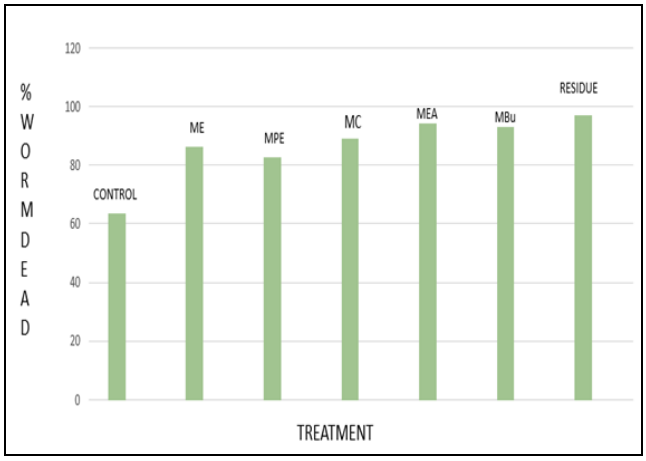

Fig 2: Anthelmintic effect of methanolic extract and its fractions of *Centratherum anthelminticum* on *C. elegans*

3.5 TLC profile
The TLC of methanolic extract and its fractions was carried out.

Mobile phase
Toluene: Ethyl acetate: formic acid: MeOH (5:5:1:0.3) Reprivatizing with Anisaldehyde sulphuric acid reagent followed by heating at 110 °C for 5 min.

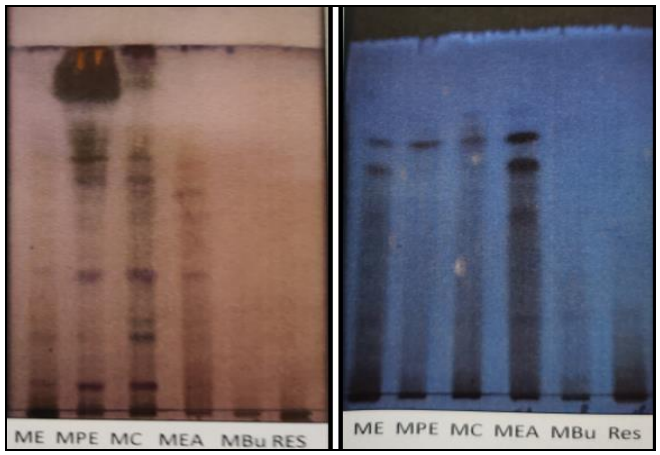

Fig 3: TLC of methanoloc extract and its fraction

Mobile phase

Hexane: Ethyl acetate (8:2). Derivatizing with Anisaldehyde sulphuric acid reagent followed byheating at 110 °C for 5 min.

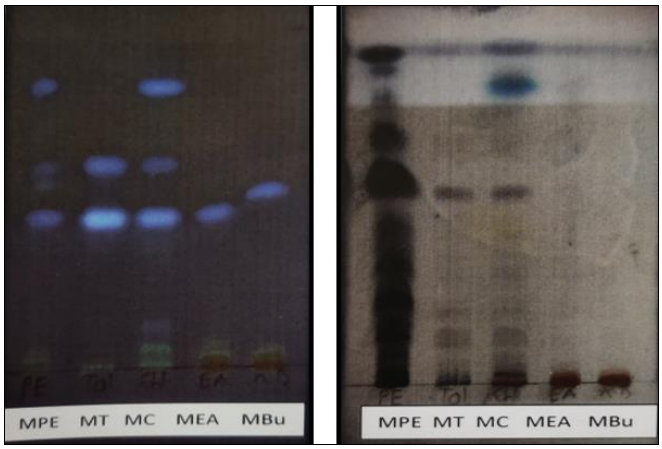

Fig 4: TLC of methanolic fracion

3.6 Phytochemical Screening

Table 4: Phytochemical screening test results

Phyto Constituents	ME	MPE	MC	MEA	MBu	Residue
Terpenoids	+	+	+	+	+	+
Steroids	+	+	-	-	-	-
Phenolics	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-
Flavonoids	-	-	-	-	-	-
Saponin	+	-	-	-	+	+

4. Discussion

The World Health Organization has stated that about 80% of people in developing nations depend on traditional medicines, with a focus on plant-based remedies, for their primary healthcare requirements. The global use of medicinal plants is increasing due to concerns over the toxicity and allergic reactions reacted with synthetic drugs. Helminth infections are prevalent worldwide, affecting a significant portion of the population. *Centratherum anthelminticum* is an herbal remedy used to treat worm infestations. The main objective of this study was to isolate the active compounds responsible for the anthelmintic activity of this plant through bioactivity-guided fractionation.

From the results the ME, MPE MC, MEA, MBu and residue in comparison to control showed significant anthelmintic activity. All fractions of methanolic extract showed almost similar activity. TLC profile shows the presence of few compounds in all the entire fraction. This may be the reason for similar activity of all fractions This could be attributed to continuous and incomplete extraction processes over time.

Methanolic extract and it's all fractions showed the presence of triterpenoids. Hence it can be inferred that the activity may occur due to triterpenoids.

The fractions of the methanolic extract needs to be repeated taking care to avoid carrying forward of same compounds from one fraction to its successive fractionation.

5. Conclusion

The methanolic extract of *Centratheruman thelminticum* has anthelmintic effect against *C. elegans* worms and the activity might be due to triterpenoids.

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