

EXPLORATION OF IN-VITRO ANTHELMINTIC ACTIVITY AND GCMS FINGERPRINT PROFILING OF TRADITIONAL SIDDHA FORMULATION *CHITRAMUTTINEI*

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Abstract

Helminthiasis becomes a global health issue in developing countries. They are the most common infectious agents of humans and produce a global burden of disease compared to malaria and tuberculosis. Commonly prescribed drug for clinical management of helminths infestation is Albendazole, but this drug exert some potential toxicity in the individuals upon usage.Hence there is a need foran alternative medicine in particular. Traditional medicinal system like Siddha are known for clinically effective against helminth infections and also devoid of adverse event. Siddha system of medicine become popular throughout the world in recent days as it claims high curative value with minimum toxicity and have less side effects. The present research work aimed at evaluating the in-vitro anthelmintic activity of the formulation ChitramuttiNei (CN) using Indian adult earthworm and also to investigate the bioactive phytocomponents present in the formulation using GCMS analysis. The result of GCMS analysis reveals the presence of most significant phytocomponents like Stigmasterol, Oleic acid, n-Hexadecanoic acid etc. The results of In-vitroanthelmintic activity of CN clearly shown that the maximum time taken for the test drug CN at the dose of 10gm to cause death of worms is about 303.3 ± 6.89 mins, similarly the time taken of CN at the dose of 20gm would be 260.5 ± 20.16 mins for standard drug albendazole it was 88 ± 7.30 mins at the concentration of 100mg/ml. From the result of the study it was concluded that the test drug CN possesses convincing anthelmintic property and also have numerous bioactive phytocomponents with versatile biological action

Introduction

Helminths worms are considered to be the world's commonest parasites. They belong to two major groups of animals, the flatworms or Platyhelminthes (flukes and tapeworms) and the roundworms or Nematoda. All are relatively large and some are very large, exceeding one meter in length.The higher medical, educational, and economic burden of helminth infections, together with their co - endemicity with malaria provides an important rationale for launching a global assault on parasitic worms [1].

In developing countries the most common hindering factor involved in controlling worm infestation is lack of cleanliness, awareness and funding towards sanitation. Regular drug treatment represents the main approach for infection control in areas where infections are intensely transmitted. The treatment is targeted at population groups, which may be defined by age, sex or other social characteristics, irrespective of the infection status (targeted treatment). The selective treatment representing individual-level administration of anthelmintic drugs, where

selection is based on diagnosis to detect the most heavily-infected people who will be most at risk of serious morbidity and mortality [2].

As per the WHO recommendation the most commonly prescribed drug for clinical management of worm infestation includes administration of Albendazole (400mg) tablets given in a single dose, reduced to 200mg for children between 12 and 24 months. But the most common adverse effect encountered upon usage of such drugs includes occurrence of headache and abnormal liver function [3].

To establish a monograph for the formulation CN and to promote the exploration of bio active phytochemicals present with in the formulation in this research work, the GCMS studies have been carried out to bring out the list of volatile and sterols present. Due to the powerful separation efficiency and the sensitive detection, GC-MS has become a popular and useful analytical tool in the research field of herbal medicines [4].

As per the literature evidence it was believed that the Siddha formulation *ChitramuttiNei* claimed to possess remarkable anthelmintic activity and hence to justify this claim the present research worked aimed at evaluating the in-vitro Anthelmintic activity by using Indian adult earthworm and also to investigate the bioactive phytochemicals present in the formulation using GCMS analysis.

Materials And Methods

a. Ingredients

The formulation *ChitramuttiNei* comprises of the following ingredients,

- | | | | |
|--|---|--------|-------|
| 1. Chitramutti (<i>Sidacordifolia</i>) | - | 280 g | |
| 2. Karimanjal (<i>Curcuma longa</i>) | - | 280 g | |
| 3. Kadukkai (<i>Terminliachebula</i>) | - | | 280 g |
| 4. Thendrikkai (<i>Terminaliabelirica</i>) | - | 280 g | |
| 5. Nelivatral (<i>Phyllanthusemblica</i>) | - | 280 g | |
| 6. Nilavembu (<i>Andrographispaniculata</i>) | - | 280 g | |
| 7. Illupaiverpattai (<i>Madhucalongifolia</i>) | - | 280 g | |
| 8. Cow's milk | - | 5lit | |
| 9. Ghee | - | 1.5lit | |

b. Collection of raw drug and plant materials:

The plant material of *Chitramutti (root)*, *Karimanjal*, *Kadukkai*, *Thendrikkai*, *Nelivatral*, *Nilavembu*, *Illupaiverpattai (root bark)*, *Cow's milk*, *Ghee* were procured from a well reputed indigenous drug shop. Fresh specimen of *Chitramutti(root)*, *Illupaiverpattai (root bark)* were collected from Siddha medicinal plant garden, Mettur, Salem, Tamil Nadu. The drugs were authenticated by the concerned Pharmacognosist, SCRI, Chennai.

c. Purification:

The collected raw drugs and fresh specimen of *Chitramutti root*, *Illupaiverpattai* were dried in sunlight and purified as per the methods defined in Siddha literature for further preparation.

d. Preparation [5]

The dried material of above mentioned drugs were taken in equal quantity of 280 gms and made into coarse powder. The coarse drugs were put in a mud pot. 5200 ml of water has been added and content has been boiled till the content becomes half. 5 lit of cow's milk and 1.5lit of ghee has been added to the above decoction and boiled the content till it reaches ghee like consistency. The entire composition has been filtered and cooled. Then the drug was stored in a clean and dry air tight container.

e. Indication, Dose and Duration:**Common Indication:**

Anaemia, Helminthiasis, Fever, Jaundice, Dropsy

Dose and Duration : 4-5 ml twice a day, 28 days

f. GCMS Specification

Agilent 7890B GC connected to 5977A MSD, NIST Ver.2.1 MS data library

Column Name

- HP_5MS 5% Phenyl Methyl Silox -60°C-325°C (325°C) 30m×250µm×0.25µm
- Split less mode injection
- 1µL injection volume

Oven program

- 50°C for 2min then ramp 5°C per minute till 270°C, then 270°C maintained for 2min, total run time 42 min
- Detector temperature 275°C
- Injector temperature 250°C
- Solvent delay 2min
- m/z Scan range 50-600amu

Start time(min)	End Time(min)	Start m/z	End m/z Scan	Speed
2.50	18.00	50.00	650.00	2000

GC-MS Plays a key role in the analysis of unknown components of plant origin. GC-MS ionizes compound and measures their mass numbers. Ionization method includes EI (Electron Ionization). The EI method produces ions by colliding thermal electrons emitted from a filament with sample gas molecules. This method provides high stability in ionization and obtained mass spectra show good reproducibility. The EI method provides good result for quantitative analysis as well. Quantitative analysis with GC-MS, in which only ions specific to the compounds are measured, is highly selective method without interfering components. Gas chromatography Technique involves the separation of volatile components in a test sample using suitable capillary column coated with polar or non-polar or intermediate polar chemicals. Elite-1 column (100% Dimethyl polysiloxane) is a non-polar column used for analysis of phyto-components. Elite -5 column (5% phenyl and 95% methyl polysiloxane) is an intermediate column and also used for the estimation of Phytochemical. An inert gas such as hydrogen or nitrogen or helium is used as a carrier gas. The compounds of test sample CN is evaporated in the injection port of the GC equipment and segregated in the column by absorption and adsorption technique with suitable GC programme.

g. Experimental worms

Indian adult earthworms (*Pheretimaposthuma*) were collected from moist soil and washed with normal saline were used for the anthelmintic study. The earthworms of 4-6 cm in length and 0.1-0.2 cm in width were used.

h. Evaluation of Anthelmintic Activity Using Earthworms [6,7]

The anthelmintic activity of the formulation CN was carried out as per standard protocol. Worms were acclimatized to the laboratory condition one week prior to the experimentation. The earthworms were divided into three groups of four earthworms in each group of two per petri dish. Albendazole at the concentration of 100mg/ml was served as standard. Clean and sterile petri plates were used for the study. Group I served as low dose treated group of which the worms were exposed to 10ml of the test formulation weight equivalent to 10gms and Group II served as high dose treated group of which the worms were exposed to 20ml of the test formulation weight equivalent to 20gms. Group III served as standard drug treated group of which the worms were exposed to Albendazole 100mg/ml.

i. Observation

Earthworms of nearly equal size in length and width are taken for each concentration and placed in Petri dishes at room temperature. The time taken for complete paralysis and death are recorded. The mean paralysis time and mean death time for each dose was calculated. The time taken for worms to become motionless was noted as paralysis time and to ascertain death, each worm was frequently applied with external stimuli, which stimulates and induce movement in the earthworms.

Results

Results of GC-MS analysis of the formulation CN

GC-MS finger printing analysis of the sample CM reveals the presence of 15 compounds. The GC-MS analysis was done using the instrument Agilent 7890B GC connected to 5977A MSD, NIST Ver.2.1 MS data library. The sample volume was 1 to 5.0 μ L. The sample of CN was run for 18 minutes.

The Chromatogram (Figure.1) shows 15 prominent peaks in the retention time range 5.11 – 15.17 which corresponds to the presence of 15 different compounds present in the formulation CN as listed in Table 1 - 3. The first peak at 5.11 retention time with Molwt of 146 corresponds to Propionic acid. The second and third prominent peak at 5.48 and 5.72 retention time with Molwt 126 and 190 is due to the presence of Mercapto Phenol, Butanedioic acid. The fourth peak at 6.44 retention time with the Molwt 130 denotes the presence of Butanoic acid.

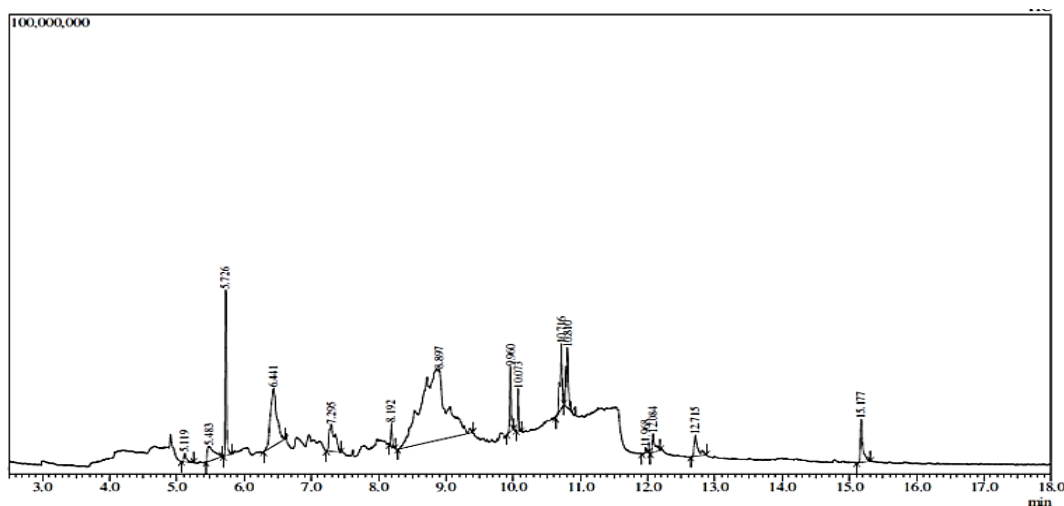


Figure 1: GC-MS Chromatogram of CN

Table 1: Peak Report Analysis of CN

Peak#	R.Time	Area	Area%	Height	Height%
1	5.119	4997115	0.66	1829146	1.22
2	5.483	23022720	3.05	3289002	2.19
3	5.726	46715230	6.19	36115986	24.10
4	6.441	82861961	10.99	12505113	8.34
5	7.295	33407703	4.43	5867911	3.92
6	8.192	5314114	0.70	4950162	3.30
7	8.897	415634642	55.10	15430302	10.30
8	9.960	20863868	2.77	14156190	9.45
9	10.073	9578194	1.27	9205778	6.14
10	10.716	30361010	4.03	14175959	9.46
11	10.810	29530066	3.91	13079028	8.73
12	11.968	1427120	0.19	1262949	0.84
13	12.084	9711726	1.29	4087732	2.73
14	12.715	16526970	2.19	4581050	3.06
15	15.177	24351278	3.23	9332712	6.23
		754303717	100.00	149869020	100.00

The fifth prominent peak at 7.29 retention time with the Molwt 804 denotes the presence of Stevioside. The sixth significant peak at 8.19 retention time with the Molwt 288 is characteristic of 3-tert-Butyl-5-Chloro-2-hydroxybenzophenone. The seventh peak at 8.89 retention time with the Molwt 208 denotes the presence of Ethyl alpha -d- glucopyranoside. The eighth significant peak at 9.96 retention time with the Molwt 228 is due to the presence of Myristic acid. The ninth prominent peak at 10.07 retention time with the Molwt 368 is due to the presence of Docosanoic acid.

Table 2: GCMS Compound Interpretation report of CN

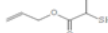

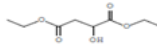
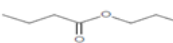
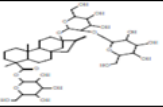
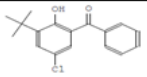
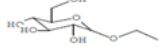





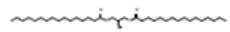
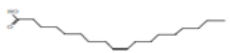
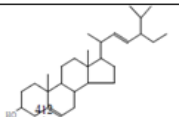
Peak No	Ret Time	% Peak Area	Mol. wt	Structure	Name
1	5.11	0.66	146		Propionic acid C ₆ H ₁₀ O ₂ S
2	5.48	3.05	126		Mercapto Phenol C ₆ H ₆ O _S
3	5.72	6.19	190		Butanedioic acid C ₈ H ₁₄ O ₅
4	6.44	10.99	130		Butanoic acid C ₇ H ₁₄ O ₂
5	7.29	4.43	804		Stevioside C ₃₈ H ₆₀ O ₁₈
6	8.19	0.70	288		3-tert-Butyl-5-Chloro-2-hydroxybenzophenone C ₁₇ H ₁₇ ClO ₂
7	8.89	55.10	208		Ethyl alpha -d- glucopyranoside C ₈ H ₁₆ O ₆
8	9.96	2.77	228		Myristic acid C ₁₄ H ₂₈ O ₂

Table 3: GCMS Compound Interpretation report of CN

Peak No	Ret Time	% Peak Area	Mol. wt	Structure	Name
9	10.07	1.27	368		Docosanoic acid C ₂₄ H ₄₈ O ₂
10	10.71	4.03	269		1-(p-Toluidino) C ₂₄ H ₄₈ O ₂
11	10.81	3.91	150		1,2,3,4,5- Cyclopentanepetol C ₅ H ₁₀ O ₅
12	11.96	0.19	186		Undecanoic acid C ₁₁ H ₂₂ O ₂
13	12.08	1.29	568		Hexadecanoic acid C ₃₅ H ₆₈ O ₅
14	12.71	2.19	282		Oleic acid C ₁₈ H ₃₄ O ₂
15	15.17	3.23	412		Stigmasterol C ₂₉ H ₄₈ O

The tenth peak at 10.71 retention time with the Molwt 269 is due to the presence of 1-(p-Toluidino). The eleventh peak at 10.81 retention time with the Molwt 150 is due to the presence of 1,2,3,4,5- Cyclopentanepetol. The twelfth and thirteenth peak at 11.96 and 12.08 retention time with the Molwt 186 and 568 is due to the presence of Undecanoic acid, Hexadecanoic acid. The fourteenth significant peak at 12.71 retention time with the Molwt 282 is due to the presence of Oleic acid. The fifteenth prominent peak at 15.17 retention time with the Molwt 412 is due to the presence of Stigmasterol.

Result of In-vitro Anthelmintic Activity of CN

The result obtained from the present study clearly indicates that the test drug CN has anti-helminthic property. Maximum time take for the test drug CN at the dose of 10gm to cause paralysis of worms is about 149 ± 15.3 mins, similarly the time taken of CN at the dose of 20gm would be 120.5 ± 8.54 mins for standard drug albendazole it was 50.5 ± 14.4 mins at the concentration of 100mg/ml. Maximum time take for the test drug CN to cause death of worms is about 303.3 ± 6.89 mins, similarly the time taken of CN at the dose of 20gm would be 260.5 ± 20.16 mins for standard drug albendazole it was 88 ± 7.30 mins at the concentration of 100mg/ml. As shown in figure 2 and Table 4.



Group I

Group II

Group III

Figure 2 : Effect of CN on In-vitro Anthelmintic Activity in Indian Adult Earthworms

Table 4: Mean time taken for paralysis and death upon exposure to CN and standard

Group	Concentration	Time taken for paralysis (min)	Time taken for death (min)
I	CN 10gms	149 ± 15.34	303.3± 6.898
II	CN 20gms	120.5 ± 8.544	260.5 ± 20.16
III	Albendazole 100mg/ml	50.5 ± 14.48	88 ± 7.303

Discussion

Due to molecular advancement in the field of helminthology the effort towards development of new generation of anthelmintic therapeutics will be greatly achieved [8]. Siddha medicines being multi component preparations mainly consist of mixture of bioactive compounds with different polarities. It's become a very challenging task of processing, identification and characterization of individual compound of interest. GCMS is one such technique utilized for characterization the structure and Mol. wt of the volatile and phytosterols present with the preparation. GCMS fingerprinting results offers wide range of information about the nature and structure of bioactive compound present with in the formulation and hence with this researcher came to conclusion about the possible mechanism exerted by each components towards the expected target. Though gas chromatography-mass spectrometry (GC-MS) is commonly used to determine the volatile components of herb medicines, it has been successfully applied to detect some thermally stable compounds present in the herbs [9-11]. GC-MS finger printing analysis of the sample CN reveals the presence of 15 compounds. In which the most biologically significant compounds are Stigmasterol, Oleic acid, Hexadecanoic acid.

Stigmasterol is an unsaturated plant sterol present the formulation CN has possess number of therapeutic activity which includes anti-osteoarthritic, anti-hypercholesterolemic, antioxidant, anti-tumor activity, anti-inflammatory and hypoglycemic activity [12]. The fatty acid, n-hexadecanoic acid, is an inhibitor of phospholipase A(2), hence possess significant anti-inflammatory property. Fatty acids can modulate immuneresponses by acting directly on T cells [13,14]. Oleic acid exerts anti-inflammatory effect by decreasing production of the inflammatory mediators such as prostaglandin E2, IL-6, IL-1b, TNFa, and nitric oxide [15].

Parasitic helminths infestation affects humans as well as animals and causing considerable disability including stunted growth in children's. Due to advancement in the field of drug discovery increased number of new drug entities has been discovered to clinically manage the ill effect caused due to helminth infection but no drug offers completed protection yet [16]. The anthelmintic activity now days performed in adult Indian earth worm

Pheretimaposthuma as it has anatomical and physiological resemblance with the intestinal round worm parasites of human beings. The results of In vitro- anthelmintic activity of CN clearly shown that the maximum time take for the test drug CN at the dose of 10gm to cause death of worms is about 303.3 ± 6.89 mins, similarly the time taken of CN at the dose of 20gm would be 260.5 ± 20.16 mins for standard drug albendazole it was 88 ± 7.30 mins at the concentration of 100mg/ml.

Conclusion

From the data's of the present study it was concluded that the formulation CN has biologically important phytotherapeutics like Stigmasterol, Oleic acid, Myristic acid and also it possess convincing anthelmintic property in the testes worms. Further study has to be carried out at the molecular level to ascertain the exact pharmacology by which the formulations act on the living biological system.

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