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Research Article

Development and Efficacy of Potential Anthelmintic Polyherbal Formulation (Emrc-F-7) –An Ethno Pharmacology Approach

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ABSTRACT:
Objectives: The study envisage development of potent anthelminthic drug by combination of herbs with potential anthelminthic properties. The presence of various bioactive phytochemicals in EMRC-F-7 was evaluated for its vermifuge and vermicidal properties. Method: We formulated a seven-ingredient anthelminthic drug consisting of *Curcuma amada*, *Curcuma zedoaria*, *Zingiber montanum*, *Ocimum tenuiflorum*, *Cinnamomum zeylanica*, *Cuminum cyminum*, and *Syzygium aromaticum*. that has potemtial anthelminthic activity tested against Indian earthworm. The antioxidant, phenolics and flavonoids contents were evaluated along with GC-MS analysis of oil extract of the formulation. Results: The formulation had better anthelminthic activity than albendazole with death and paralysis time of 77.16 ± 5.9 and 26.86 ± 2.29 minutes respectively for 15 mg/mL. The highest dose of 125 mg/mL of EMRC F-7 took 0.44 ± 0.07 minutes to paralyze and a short duration of 3.63 ± 1.06 minutes to kill the worms. GC-MS analysis showed presence of Eugenol (33.7%), β-Caryophyllene (15.4%), Cumin aldehyde (9.8%), and Camphor (4.3%) which must have contributed to the anthelminthic property of the formulation. Conclusion: The formulation has the desired property but also has high antioxidant and immunomodulatory effects for overall well-being of the human physiology.

Keywords: Anthelminthic, Eugenol, Polyherbal, Vermicidal, Vermifuge.

INTRODUCTION

Helminths are multicellular parasitic agents that affect the gastro-intestinal system of human and livestock causing the most common disease affecting the tropical and subtropical countries such as, East Asian countries and the Latin American regions. The most common worm infection is Ascariasis which is caused by *Ascaris lumbricoides*.Other parasitic agents are tapeworm, roundworm and flukes. It is estimated that about 24% of the world's population amounting to approximately 1.5 billion people are infected with soil-transmitted helminths. The situation is more aggravated with the fact that around 267 million children of pre-school age lives around areas of active transmission. India contributes a large share in the number of infections with an estimated 25% of the global prevalence which includes 220.6 million children who need preventive management through hygiene or chemotherapeutic approach[1]. Infective worms usually resides in the intestine feeding on the living host for development and multiplication. Individuals infected with helminths present various signs and symptoms such as – fatique, abdominal pain, anaemia, weight loss, loss of appetite, vomiting & nausea. Modern anthelminthic medicine such as Mebendazole and Albendazole are widely prescribed and recommended by WHO due to its ease in administration and inexpensive nature. Other broad spectrum anthelminthic agents available in Indian market includes Pyranteland Levamisole. Mebendazole and Albendazole inhibit microtubule formation thereby inhibiting polymerization leading to paralysis, followed by inhibition of glucose uptake which ultimately kills the worms. Some anthelminthic agents also impair the egg production process in the worms. Depending upon their mechanism of action anthelminthic agents are classified into vermifuges and vermicides.

The development of drug resistance among helminthes is of major concern though the topic is highly debatable. There have been reports of ineffective treatment of schistosomes as well as nematodes leading to increased awareness and understanding of drug resistance among helminths. The development of such resistance may be due to mutations leading to development of responses that helps in survival in toxic environments. This is a major concern for both humans as well as livestock farmers. Herbal drugs have been in use since ancient times for the treatment of parasitic diseases in human and could be of value in preventing the development of resistance.

In Ayurvedic system of medicine many formulations are mentioned to treat the worm infestation. Some of the formulation are- (I) Sankat Mochan (Composed of 10 ingredients that is- Camphor, *Trachyspermum ammi*, flower of *Mentha piperata*, oil of *Syzygium aromaticum*, oil of *Cinnamom*um *zeylanicum*, *Foeniculum vulgare* oil,

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Mentha oil, *Eucalyptus globula*, and sugary liquid)[3]. (II) Panchachurnam (Composed of five ingredients – *Curcuma angustifolia*, *Terminalia chebula*, *Zingiber officinale*, *Foeniculum vulgare* and rock salt[3]. (III) Vaivilangam choornam (composed of 2 ingredients- *Nigella sativa* and *Embelia ribes*)[4].

These traditional preparations are effectively being used for ages. However, no scientific validation studies have been performed. Therefore taking the leads from the Ayurveda, a new combination of potential medicinal plants (EMRC- F7) have been selected for the scientific validation of anthelminthic activity. The formulation was evaluated for its anthelminthic activity using Earthworm model. Further the chemistry and anti-oxidation activities was also conducted to see the positive mode of action of the formulation.

EMRC- F7consists of 7 ingredients *viz.*, *Curcuma amada, Curcuma zedoaria*, *Zingiber montanum*, Tulsi (*Ocimum tenuiflorum*), Cinnamon (*Cinnamomum zeylanica*), Cumin (*Cuminum cyminum*) and Clove (*Syzygium aromaticum*). The individual ingredients are used traditionally for the management of worm infestation and other ailments such as abdominal colic, flatulence, bloating, dyspepsia, diarrhoea and rheumatism by a number of tribes in north-eastern states of India. So taking leads from the local traditional healers we have documented all the uses of each ingredients of EMRC-F7. Three members of the Zingiberaceae family *viz.*, *Curcuma amada* Roxb., *Curcuma zedoaria* Rosc. and *Zingiber montanum* (J. König) Link ex A. Dietr. which were reported for various ethno-medicinal properties apart from their anthelminthic potentials were incorporated in the herbal anthelminthic formulation 'EMRC-F7'. *Curcuma amada* has been traditionally used in India as carminative and stomachic[5], possess cooling effect, astringent properties and also promote digestion[6]. It was also reported that various extracts of *C. amada* possess anthelminthic potentials[7]. *Curcuma zedoaria* has been traditionally used as carminative, for bloating and flatulence[8], as anti-ulcer[9], and for anti-diarrhoeal activity[10]. It was also reported that the tuber juice of *C. zedoaria* has anthelminthic activity on children[11]. *Zingiber montanum* has been traditionally used in India as stomachic, carminative[12], used in flatulence and diarrhoea in parts of Thailand[13], and was earlier reported elsewhere to have anthelminthic activity[14]. *Ocimum tenuiflorum* has been used traditionally by many local people as stomach disorder, worm infestation, skin diseases and fever[15]. Further, many workers have independently reported anthelminthic activity of *O. tenuiflorum*[16-24]. *Cinnamomum zeylanica* has been used traditionally used in India for dyspepsia and vomiting[25]. *C. zeylanica* was also reported to have anthelminthic activity[26]. *Cuminum cyminum* has been used traditionally in India for indigestion, abdominal pain, diarrhoea,[27,28] and reported to have anthelminthic activity[29]. *Syzygium aromaticum* has been used traditionally for dyspepsia, flatulence, bloating and vomiting and also reported to have anthelminthic activity[30].

Based on this, the present study was carried out to evaluate the anthelminthic properties of hydro- alcohol extract of EMRC- F7 on adult Earthworm (*Pheritima posthuma).*

MATERIALS AND METHODS

Sample collection

The ingredients of EMRC- F7 *viz.*, *Curcuma amada*, *Curcuma zedoaria*, *Zingiber montanum*, Tulsi (*Ocimum tenuiflorum*), Cinnamon (*Cinnamomumzeylanica*), Cumin (*Cuminum cyminum*) and Clove (*Syzygium aromaticum)* were collected from the market of Imphal, Manipur in the month of July 2018. The specimens of the samples was authenticated by a taxonomist and voucher specimens were deposited at Ethno-Medicinal Research Centre (EMRC) Hengbung, Kangpokpi, Manipur.

Extract preparation

All the samples were shade dried and powdered separately using a grinder and sieved using a sieve mesh size of 60, then 20g of each individual ingredients were mixed together in equal proportion to get uniformly blended churna (powder). Then the mixture was macerated in hydro-alcohol (1:1) for 24 hours at room temperature. The extract was filtered and dried using a rotory vacuum evaporator at 65 ^{o}C with negative pressure of 70 mm Hg. Extract was freeze dried using a lyophilizer and kept at 8-10 °C till further use. The extract value was determined after complete removal of residual solvents used in extraction.

Collection of worms

Indian earthworms were collected from the swarm or muddy area near river bed of Hengbung (Kangpokpi), Manipur, India. The worms were washed thoroughly in distilled water to remove dirt and soil particles. Earthworm with average length of 4-8 cm and 0.2 - 0.3 cm in width were used for the experiments on anthelminthic activity.

Estimation of Total Phenolics

Total phenolic content in the hydro-alcoholic extracts was determined using Folin-Ciocalteu phenol (FC) reagent as described by Singleton and Rossi (1965)[31]. 50 µl of 10 mg/ml hydro-alcohol extract dissolved in water was mixed with 350 µL of water and 0.3 mL of 10 time's diluted FC reagent (SRL Pvt. Ltd.) was dispensed into the tube. This was followed by addition of, 0.25 mL of sodium carbonate (7.5% w/v) after 3 min and total volume was made up to 4 mL with distilled water. The reaction mixture was incubated in room temperature in dark for 30 min. Absorbance was measured at 765 nm against a blank. The experiment was carried out in triplicates and the amount of total phenolic content was calculated as gallic acid equivalent (GAE) in mg/100g. The concentration range of gallic acid used for standard curve was 2-10 µg/mL.

Total flavonoids estimation

Total flavonoid content of the plant extracts were determined by the colorimetric method as described by Jia *et al.* (1999) with minor modifications[32]. 50 µl of 10 mg/ml HA extract was mixed with 2.450 mL of water and 0.15 mL of 5% $NaNO_2$. After 6 min, 0.15 mL of 10%

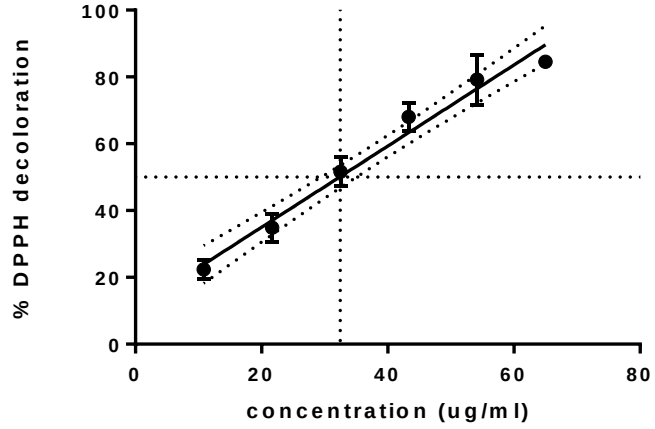

Figure 1: DPPH decoloration capacity of hydro-alcohol extract of EMRC –F7.

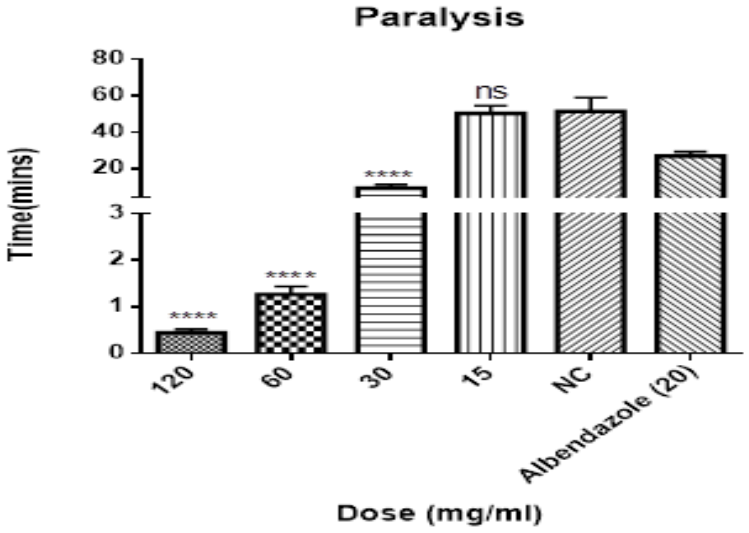

Figure 2: Time taken for various doses of hydro-alcohol extract of EMRC- F7 to paralyze the worms.

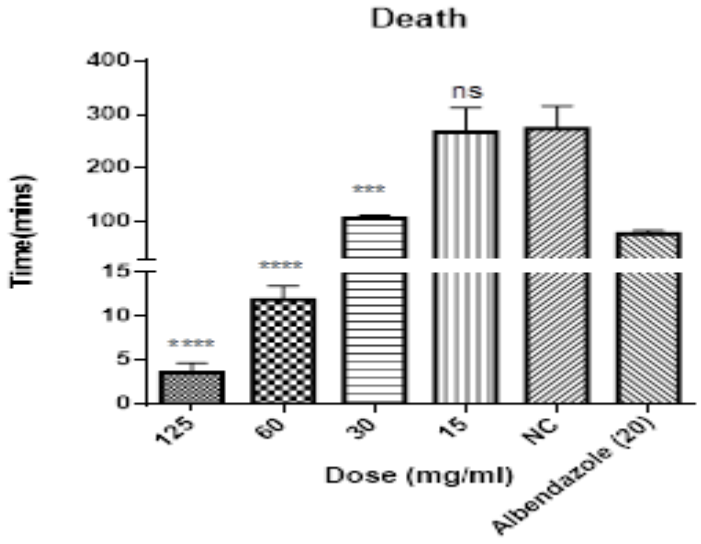

Figure 3: Time taken for various doses of hydro-alcohol extract of EMRC- F7 to kill the worms.

Al $(NO_3)_3$ was added. Subsequently, 2 mL of 4% NaOH and 0.2 mL of water were added to the reaction mixture and mixed thoroughly. Absorbance was recorded at 510 nm against a blank following an incubation of 12 min.

Table 1: *Composition of E.M.R.C –F7 oil, based on GCMS analysis.*

Constituents	R_I	R_{II}	Area %
α- Pinene	939	934	0.2
Camphene	953	950	0.4
Sabinene	976	974	0.9
β-Pinene	980	979	1.3
Myrcene	991	991	0.1
α-Terpinene	1018	1018	0.2
p-Cymene	1026	1025	1.0
Limonene	1031	1030	0.2
1,8-Cineole	1033	1033	2.6
γ-Terpinene	1062	1059	1.6
Terpinolene	1088	1090	0.1
Linalool	1098	1100	0.7
Menth-2-en-1-ol<*cis*-para->	1121	1123	0.1
Menth-2-en-1-ol<*trans*-para->	1140	1141	0.1
Camphor	1143	1147	4.3
Borneol	1165	1168	0.4
Terpinen-4-ol	1177	1179	7.3
α-Terpineol	1189	1193	0.2
Cumin aldehyde	1239	1242	9.8
Eugenol	1356	1361	33.7
α-Copaene	1376	1379	0.4
β-Elemene	1391	1394	0.1
β-Caryophyllene	1417	1423	15.4
α-Humulene	1454	1457	1.6
Germacrene D	1480	1485	0.4
Eugenol acetate	1524	1530	3.8
Curzerenone	1601	1609	0.7
Total			87.6

R_I;Reported retention index, R_{II}; Calculated retention index

Allexperiments were carried out in triplicates and expressed as Quercetin equivalent (QE) in mg/100g of the extracts.

Evaluation of antioxidant activity (DPPH Assay)

DPPH assay was carried out to assess the free radical scavenging capacity of the hydro-alcohol extract of EMRC F-7 following the method of Miliauskas *et al.* (2004)[33]. Each reaction mixture consisted of 100 µM DPPH (2, 2-diphenyl-1-picrylhydrazyl) in absolute methanol with different concentrations of sample. Absorbance was read against a blank at 515 nm after incubation of the reaction mixtures for 30 minutes in dark at room temperature. The percentage of DPPH decolouration was calculated as follows:

Percent DPPH decolouration = $Abs_{control} - Abs_{sample} / Abs_{control} \times 100$.

The degree of discoloration of purple colored DPPH to yellow color signifies the free radical scavenging capacity of the extract. Ascorbic acid was used as standard compound. The IC_{50} value was determined from the DPPH discoloration curve.

Essential oil extraction and GC MS analysis

15g each of the components of EMRC – F7 was taken in a cleavenger with 250 mL of distilled water and boiled for 2 hours. The condensed essential oil was collected over aqueous phase and carefully recovered along with petroleum ether for maximum recovery. Petroleum ether readily evaporated at 50 ºC and the remaining residual essential oil was collected for further analysis.

GC analysis was done on an ELITE-5 capillary column (30 m x 0.25mm i.d., film thickness 0.25µm fixed in a Varian CP-3800 Gas Chromatograph. The column oven was programmed from 60 ºC at the rate of 3ºC/min to 240 ºC (isothermal for 2 min) and programmed to 310 ºC at 10 ºC/min, using H_2 as carrier gas at constant flow rate of 1mL/min, a split ratio of 1:100 and S/SL injector and detector (FID) temperatures were 300 ºC respectively.

Anthelminthic test

The anthelminthic activity was evaluated on adult Indian Earthworm *(Pheretima posthuma)*. Worms were divided into 6 groups with 10 worms in each group. Group I was designated as negative control and treated with Normal Saline (NS). Group II (positive control) was treated with 20 mg/mL albendazole dissolved in NS. Group III to VI were treated with 120 mg/mL, 60 mg/mL, 30 mg/mL and 15 mg/mL respectively of hydro-alcohol extract of EMRC F-7 dissolved in NS. Observation were made for the time of paralysis and death of each individual earthworm. Paralysis assumed to occur when the worm did not revived and death was assumed when the worms lost their motility follow by fading away of their body colour.

Statistical analyses

The data of anthelminthic test were expressed as mean ± SD and anthelminthic test was analysed by non-

Table 2: *Time of paralysis and death of Pheretima posthuma exposed to various doses of EMRC F-7:*

Group	Doses	Avg. time of paralysis (mins)	Avg. time of death (mins)
I	Normal Saline	51.33 ±7.60	273 ± 42.1
II	Albendazole (20mg/mL)	26.86 ±2.29	77.16 ± 5.9
III	125mg/mL	0.44 ± 0.07	3.63 ± 1.06
IV	60mg/mL	1.26 ± 0.17	11.89 ±1.56
V	30mg/mL	9.25 ± 1.78	105.56 ± 6.34
VI	15mg/mL	50.24 ±4.21	266.5 ± 45.9

parametric unpaired t-test. All calculations were carried out using GraphPad Prism 6.0.

RESULTS

Total Phenolic and Flavonoid content of HA extract of EMRC –F7

The hydroalcohol extract of EMRC –F7 dissolved in distilled water was used for estimation of total phenolics and flavonoids using Gallic acid and Quercetin as standards. The estimated contents were expressed in equivalents of the standards present in 100 g of the polyherbal formulation. Total phenolic content was 32.54 ± 0.2 mg GAE/100 g and total flavonoid was estimated as 18.52 ± 1.4 mg QE/100 g of EMRC –F7

Free radical scavenging activity (DPPH assay)

DPPH assay was used to determine free radical scavenging activity of hydro-alcohol extract of the polyherbal anthelminthic formulation. A dose dependent pattern in the decolouration of DPPH free radical was observed when different concentration of hydro-alcohol extract of EMRC- F7 was added. Increase in the decolouration of DPPH was observed with increasing concentration of the hydro-alcohol extract. The IC_{50} value of EMRC-F7 was 32.40 ug/mL as compared to 3.2 µg/ml for ascorbic acid [Figure 1].

GC MS analysis

GCMS analysis of essential oil of EMRC- F7 revealed presence of Eugenol (33.7%), β-Caryophyllene (15.4%), Cumin aldehyde (9.8%), Camphor (4.3%), Eugenol acetate (3.8%), 1,8-Cineole (2.6%) and β-Pinene (1.3%) as major constituents [Table 1].

Anthelminthic activity of EMRC- F7

The polyherbal formulation was studied for its anthelminthic properties against live *Pheretima posthuma*. Seven groups were taken for the study with 10 worms in each groups. Group I (Negative control) had the longest time of death and paralysis with 273 ± 42.1 and 51.33 ± 7.60 minutes respectively compared to 77.16 ± 5.9 and 26.86 ± 2.29 minutes respectively for albendazole. The highest dose of 125 mg/mL of EMRC F-7 took 0.44 ± 0.07 minutes to paralyze and a short duration of 3.63 ± 1.06 minutes to kill the worms. Exposure of the worms to further dilutions of the extract increased the time taken for paralysis and death of the exposed worms [Table 2].

Statistical analysis using non-parametric unpaired t-test among various dose and the NS control for time taken to paralyze the worms revealed strong statistical significance with $p < 0.000$ for 125, 60 and 30 mg/mL doses against NS control. However, 15 mg/mL returned a non-significant result compared to control ($p>0.05$) [Figure 2 and 3]. The result seems consistent with time taken for death as doses of 125 mg/mL, 60 mg/mL and 30 mg/mL doses against NS control revealed a strong statistical significance with $p < 0.000$ and a statistically insignificant result for 15 mg/mL when compared with control Figure 2 and 3. In comparision with albendazole, 30 mg/mL had better potential to paralyze as well as kill the worms ($p < 0.001$).

DISCUSSION

Vermifuge and vermicides are two classes of drugs that are used to control infestation of worms in the gastro intestinal tract (GIT). Phytochemicals are found to be efficient in paralyzing as well as inflicting fatalities to intestinal worms. *Curcuma amada* was reported earlier to possess anthelminthic activity[7,34], however, mechanism of death and paralysis was not detailed. *Curcuma amada* contains curcumin which shows anthelminthic activity on *Fasciola hepatica*[35]. Curcumin present in the extract may have inhibited the cathepsin L activity[35,36] of the worm leading to paralysis and death.

*Ocimum tenuiflorum*and, *Syzygium aromaticum* are plants that has provened anthelminthic properties attributed to the essential oil eugenol[37-39]. Eugenol is a sedative and may have contributed to the paralysis of test worms[40]. It was also reported earlier that aqueous extract of *Cinnamomum* bark displayed anthelminthic activity, attributed to the presence of high amount of tannins in the aqueous extract. Tannins are known to bind to glycoproteins of cuticles of parasites causing death[41]. The presence of β-Caryophyllene which has anticancer[42], anti-inflammatory, antibacterial and anaesthetic[43] activity might also contribute to overall health beneficial effect of the extract. Most importantly the local anaesthetic activity should have enhanced the anthelminthic effect through induction of paralysis of the worms. Another contributing ingredient in the anthelminthic activity is camphor [44] which is present in the essential oil of the formulation.

Phenolics and flavonoids content of the polyherbal formulation must have contributed to the antioxidant capacity of the preparation. Major contributions to antioxidant properties are made by phenolics such as gallic, caffeic, protocatechuic and rosmarinic acids. The flavonoids quercetin and catechin are the major contributors to antioxidant capacity of plant extracts. However, mechanism of antioxidant activity is different for flavonoids and phenolics. Phenolic compounds generally trap free radicals whereas flavonoids acts as scavengers of free radicals as well as chelate metals[45].

Apart from contributing to anti-oxidation and anthelminthic properties by the phenolic compounds, the phenolic-protein complexes that formed due to linking with protein molecules through hydrogen bonding enhances wound healing. Crosslinking of peptides by hydrogen donated from hydroxyl group and formation of a physical film enhances wound healing and also acts as vaso-constrictors that limits blood loss from injured mucosa as well[46]. This is particularly important because parasitic helminthes injures the internal mucosa of the intestine during their adherence. The rich phenolic content of the herbal formulation thus may contribute to maintenance of healthy mucosal lining of the intestine.

CONCLUSION

E.M.R.C–F7 is a polyherbal formulation of known Ayurvedic herbs that has anthelminthic properties. The formulation displayed strong anthelminthic properties and presents a strong candidate for development of an efficient polyherbal formulation with very little or no side effects. The anthelminthic activity of the formulation is due to combined effect of the compounds present in each ingredient. Further study in animal models is warranted to prove its efficacy *in vivo*.

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