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Research Article

SCREENING OF BIO-RELEVANT MEDIA FOR *IN-VITRO* ANTHELMINTIC ASSAY WITH AN OBJECTIVE TO CHECK THE POTENTIALS OF GARLIC FORMULATION FOR HELMINTH INFECTIONS

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ABSTRACT

Allium sativum L. (Garlic) belonging to family "Liliaceae" is a well known medicinal plant. In the present study, effervescent tablet dosage forms were prepared incorporating garlic powder as active ingredient with other excipients. Conventional anthelmintic assay methodology directs the use of saline solution for the study. In the present context the methodology is screened and validated for selection of bio-relevant media simulating in- vivo human gastric conditions. From the tested medias Saline solution (conventional method), Fed-State Simulated Gastric Fluid (Fe-SSGF) Early, Fasted-State Simulated Intestinal Fluid Updated version (FaSSIF-V2) were selected as an appropriate media for maintenance of worms. *Pheretima posthuma* worms (Adult Indian Earthworms) being easily available and due to their anatomical and physiological resemblance with the intestinal roundworm parasites of human beings are used as a suitable model for anthelmintic screening of garlic powder and garlic formulations. Dosage forms prepared were found to be equally effective at garlic concentration of 80 mg/ml compared to albendazole anthelmintic standard used. The present study is successful attempt to prove the therapeutic indications of the prepared formulations for helminth infections.

KEYWORDS: Garlic, *Allium sativum,* Anthelmintic activity, Bio-relevant media, Simulated media, Intestinal Helminth, Tablet dosage form.

INTRODUCTION

Helminthiasis, also known as worm infection, is any macroparasitic disease of humans and other animals in which a part of the body is infected with parasitic worms, known as helminths. There are numerous species of these parasites, which are broadly classified into tapeworms, flukes, and roundworms. They often live in the gastrointestinal tract of their hosts, but they may also burrow into other organs, where they induce physiological damage. Helminths are a broad range of organisms that include intestinal parasitic worms, roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), or hookworms (*Necator americanus* and *Ancylostoma duodenale*). Infected people excrete helminth eggs in their faeces, which then contaminate the soil in areas with inadequate sanitation. Other people can then be infected by ingesting eggs or larvae in contaminated food, or through penetration of the skin by infective larvae in the soil (hookworms). Infestation can cause morbidity, and sometimes death, by compromising nutritional status, affecting cognitive processes, inducing tissue reactions, such as granuloma, and provoking intestinal obstruction or rectal prolapse. Control of helminthiasis is based on drug treatment, improved sanitation and health education.[1] Helminths are divided into two major phyla, and all members of each phylum have distinct structural features that separate them from the others. The nematodes (also known as roundworms) include the major intestinal worms and the filarial worms that cause lymphatic filariasis (LF), whereas the platyhelminthes include the trematodes (flukes), such as the schistosomes, and the cestodes (tapeworms). Humans and helminths have co-existed throughout our evolution. The history of helminthiasis can be traced back to the earliest record of human beings. Although effective preventive and therapeutic measures have been developed for most parasitic worms, helminth infections are still very common in the developing world today. It has been estimated that one billion people worldwide are infected with one or more helminths. Most of the victims live in regions of Sub-Saharan Africa, Asia, and Latin America.[2]

The pungent smelling herb garlic has been used since times and been considered as a highly beneficial ingredient for the human body. Garlic has a peculiar taste, smell and is extensively used for culinary and medicinal purposes. An ancient medicinal plant, garlic commonly referred to as the magical herb, a native of Central Asia was discovered approximately 3000 years ago. It is highly equipped with anti-bacterial properties. Belonging to the onion family, it is grown and imported all over the world. Widely used in French and Italian cuisine, many culinary dishes cannot do without a touch of the unique taste and smell of garlic. Garlic (*Allium sativum* L.) has acquired a reputation in different traditions as a prophylactic as well as therapeutic medicinal plant. Garlic has played important dietary and medicinal roles throughout the history. Some of the earliest references to this medicinal plant were found in Avesta, a collection of Zoroastrian holy writings that was probably compiled during the sixth century BC. Garlic has also played as an important medicine to Sumerian and the ancient Egyptians. There is some evidence that during the earliest Olympics in Greece, garlic was fed to the athletes for increasing stamina. Throughout history, many different cultures have recognized the potential use of garlic for prevention and treatment of different diseases. Recent studies support the effects of garlic and its extracts in a wide range of applications. These

studies raised the possibility of revival of garlic therapeutic values in different diseases.[3]

The potency of garlic (*Allium sativum*) has been acknowledged for 5000 years. In ancient times, the Babylonians, Egyptians, Phoenicians, Vikings, Chinese, Greeks, Romans and Hindus used garlic frequently. They took garlic as a remedy for intestinal disorders, flatulence, worms, respiratory infections, skin diseases, wounds, symptoms of aging and many other ailments.[4] The potential trematocidal effects of garlic (*Allium sativum*) are investigated in the context of intestinal food borne trematodes, employing the *Echinostoma caproni*-mouse model.[5] Garlic was also successfully used to show eradication of the Cryptosporidium oocysts from stool and intestinal sections of the infected immunocompetent subgroup of mice receiving garlic two days before the infection.[6]

METHODOLOGY

Materials

Garlic powder was prepared in-house by microwave drying process as per reported literature.[7-12] All the reagents and chemicals used were of analytical grade and were procured from S.D. Fine-Chem. Ltd., Mumbai, India.

Use of *Pheretima posthuma* for Anthelmintic Activity Study

Literature Review indicates the use of Indian Earthworms *Pheretima posthuma* for In–vitro anthelmintic study of Intestinal Helminth Infection as they are having anatomy physiology same as that of intestinal roundworms. [13, 14]

Preliminary Screening of the Appropriate Medium for Maintenance of Worms During the Study Experiments

The conventional anthelmintic assay methodology reports the usage of normal saline solution (0.9% NaCl) as maintenance media for the species. But the number of questions arises viz. Whether the media supports the survival of the worms? How long the worms would remain alive during the study period? Can the solvent be the reason for the death of the worms? Etc. As saline solution is not the natural environment for the worms to sustain, so the preliminary screening study was carried out to check the survival rate in various buffer solutions matching the pH conditions of saline solution. The collected worms were divided into groups (2 worms each) and placed in different media to choose the most appropriate for in- vitro maintenance of worms during the study experiments. Nine Medias viz- Normal saline solution (0.9% NaCl), 0.1N HCl, Phosphate buffer, Physiological salt solution (Modified Krebs solution), Mud water, Normal saline solution with Mud water, 0.1N HCl with Mud water, Phosphate buffer with Mud water, Physiological salt solutions (Modified Krebs solution) with Mud water were tested for studying the survival (mortality) rate of worms. Screening medias with pH are mentioned in **Table-1**. The appearance of the normal alive worms and dead worms is depicted in **Figure.1** Survival rate of earthworms (*Pheretima posthuma)* in different medias are shown in **Table-2**

Selection of Bio-Relevant Media

The solvents mentioned in the preliminary study match only with the pH conditions of Gastro intestinal tract. In order to simulate the conditions of Gastro intestinal tract, bio-relevant medias simulating gastrointestinal conditions like Fasted state, Fed state were used.[15] Various medias used were FaSSGF pH1.6- Fasted-State Simulated Gastric Fluid, FeSSGF Early, Middle, late)- Fed-State Simulated Gatric Fluid, FaSSIF-V2 pH 6.5 Fasted-State Simulated Intestinal Fluid Updated version, FeSSIF- Fed-State Simulated Intestinal Fluid, FeSSIF-V2- Fed-State Simulated Intestinal Fluid(updated). Survival rate of Worms (*Pheretima posthuma)* in Bio-relevant fluids is depicted in **Table 3.**

In- Vitro Anthelmintic Assay Using Modifications in Conventional Method

The in- vitro activity of garlic powder on adult earth worms (*Pheretima posthuma)* was studied in comparison to that of albendazole. Different concentrations of albendazole (20, 40 mg/ml) and garlic powder (10, 20,40,60,80 mg/ml) were prepared in saline solution and tested against Earthworms maintained in the selected medium. Ten milliliters (10 ml) of each concentration of anthelmintic agent was applied to a group of two worms of same length maintained in 10ml of selected medium. All worms were examined for paralysis time and death time. The results are mentioned in **Table 4.** The decision on dose selected for optimizing the effervescent tablet dosage form was taken on the basis of the results obtained for the anthelmintic assay using modified methodology.

Formulation and Evaluation of Effervescent Tablet

Method adopted for tablet preparation was direct compression method. All ingredients like drug (Garlic Powder) 400 mg (56.65%), cross povidone (4.95%), citric Acid (16.99%), sodium bicarbonate (18.55%), magnesium stearate (1.41%) and talc (1.41%)] were weighed and passed through sieve no. 80 individually and then mixed. This powder mixture was compressed directly using Rimek minipress; model RSB-4, m/s (Karnavathi engineering, Ahmadabad). The prepared tablets were evaluated for various parameters like hardness, friability, weight variation test, effervescent time and disintegration time as per conventional standards applicable for tablet dosage form. The results for optimized batch are mentioned in **Table 5**.

In- Vitro Anthelmintic Assay of Garlic Effervescent Tablet and Albendazole Tablets as Standard

Anthelmintic Assay was performed in simulated intestinal fluid using dissolution paddle apparatus at 25 RPM. Nine hundred milliliters **(**900ml) of Fasted State Simulated Intestinal Fluid (FaSSIF)[15] having pH 6.5 was filled in each jar. Two earth worms were placed in each jar subsequently followed by addition of 2 tablets in each jar and worms were observed for their death time. The death time was concluded when worms did not move on shaking vigorously or dipped in a hot water of 50^0C. The in- vitro anthelmintic activity of albendazole taken as a standard was done by the same procedure as that of the effervescent tablet, as stated above only with a change in RPM which was set at 50 rpm and worms were observed for the death time. Pictorial representation of dissolution assay assembly for anthelmintic study of tablet under test is shown in **Figure 2**. The results are mentioned in **Table 6**.

RESULT AND DISCUSSION

Preliminary Screening of the Appropriate Medium for Maintenance of Worms During the Study Experiments

The literature reveals the residence of worms in stomach, lower small intestine, upper colon, perianal region etc, once they enter into human body. Moreover the research around concerned with anthelmintic activity reports the use of 0.9% saline solution as the solvent for maintenance of worms as well as for the performance of the anthelmintic assay. The evaluation criteria used in the study are paralysis time and death time. But the present study suggests certain issues like eco-physiological conditions for growth and maintenance of the worms. Whereas it is also being pointed out if paralysis time and death time which is obtained is because of drug or disturbed eco-physiological conditions of the worms. So to overcome the issues the solvent selection and optimization study was carried out to perform the anthelmintic study in the simulated conditions as possible.

Table 1: Preliminary screening media for maintenance of worms

Sr.No	Survival Media for *Pheretima posthuma* Species	pH
1	Normal saline solution (0.9% NaCl)	5.5
2	0.1N HCl	3
3	Phosphate buffer	7.4
4	Physiological salt solution (Modified Krebs solution)	7.4
5	Mud water	6.0
6	Normal saline solution+ Mud water	6.0
7	0.1N HCl+ Mud water	4.7
8	Phosphate buffer + Mud water	7.8
9	Physiological salt solutions (Modified Krebs solution) + Mud water	7.6

Table 2: Survival rate of earthworms (*Pheretima posthuma)* in different medias

Solvent	I.T.	Observation After			
		4 hours	8 hours	12 hours	24 hours
Saline solution	0	L	L	L	D
Saline solution+ mud water	0	L	L	L	**L**
0.1 N HCl	0	D	D	D	D
0.1 N HCl+ mud water	0	D	D	D	D
phosphate buffer pH 7.4	0	D	D	D	D
phosphate buffer pH 7.4 + mud water	0	L	D	D	D
	0	D	D	D	D
Physiological salt solution (modified krebs)	0	D	D	D	D
	0	D	D	D	D
Physiological salt solution (modified krebs)+mud water	0	D	D	D	D
	0	L	D	D	D
Mud water	0	L	L	L	**L**
I.T-Insertion time, L- worms are live, D- death of earthworms					

Table 3: Survival rates of worms (*Pheretima posthuma)* in bio-relevant fluids

Solvents		Time (min)	
		I.T	D.T
FaSSGF	(pH 1.6)	0	3
FeSSGF	Early (pH 6.4)	0	(<12 Hours)
	Middle(pH 5)	0	20
	Late(pH 3)	0	10
FaSSIF-V2	(pH 6.5)	0	(<12 Hours)
FessIF	Early (pH 6.5)	0	20
	Middle(pH 5.8)	0	29
	Late(pH 5.4)	0	76
FeSSIF-V2	(pH 5.8)	0	48
I.T-Insertion time ,P.T-Paralysis time, D.T-Death time. **Note**[15]: FaSSGF pH1.6- Fasted-State Simulate Gastric Fluid, FeSSGF(Early, Middle, late)- Fed-State Simulated Gatric Fluid, , FaSSIF-V2 pH6.5- Fasted-State Simulated Intestinal Fluid Updated version, FessIF- Fed-State Simulated Intestinal Fluid, FeSSIF-V2- Fed-State Simulated Intestinal Fluid (updated).			

Table 4: In- vitro anthelmintic effect of garlic powder and albendazole standard

Garlic Powder With additives							
Dose mg/ml	**Time (min)**	**MEDIUM**					
		FaSSIF(pH 6.5)		**FeSSGF (pH 6.4)**		**Saline Solution (pH 5.9)**	
10	I.T.	0	0	0	0	0	0
	P.T.	-	-	-	-	-	-
	D.T.	-	-	-	-	-	-
20	I.T.	0	0	0	0	0	0
	P.T.	-	-	-	-	-	-
	D.T.	-	-	-	-	-	-
40	I.T.	0	0	0	0	0	0
	P.T.	-	-	229	-	-	-
	D.T.	-	-	-	-	-	-
60	I.T.	0	0	0	0	0	0
	P.T.	58	57	67	62	28	41
	D.T.	170	167	182	179	173	178
80	I.T.	0	0	0	0	0	0
	P.T.	26	21	21	22	31	34
	D.T.	183	181	165	165	101	104
Albendazole standard							
20	I.T.	0	0	0	0	0	0
	P.T.	93	86	79	86	105	149
	D.T.	175	180	119	121	220	205
40	I.T.	0	0	0	0	0	0
	P.T.	6	8	11	13	23	21
	D.T.	16	15	22	24	26	18

I.T-Insertion time, P.T-Paralysis time, D.T-Death time

Table 5: Evaluation of effervescent tablet

Evaluation Tests				
Hardness (Kg/cm^2)	**Weight Variation (mg)**	**Effervescent Time (min.)**	**Disintegration Time (min.)**	**Friability %**
4.5	709.9±35.5	07.09	7	0.88

Table-6: In- vitro anthelmintic assay of garlic effervescent tablet and standard albendazole tablet

Garlic Tablet(800 mg)						
Observations	**Time (min.)**					
	For Set I		**For Set II**		**For Set III**	
I.T	0	Mean	0	Mean	0	Mean
D.T	104	102	122	139	88	89
D.T	100		156		90	
Albendazole Standard Tablet (400 mg)						
I.T	0	Mean	0	Mean	0	Mean
D.T	90		98		100	
DT	95	92.5	96	97	98	99

a) Alive Worms b) Dead Worms

Fig. 1: Appearance of the normal alive worms and dead worms

Fig.2: Dissolution Assay assembly for Anthelmintic study of Tablet under test

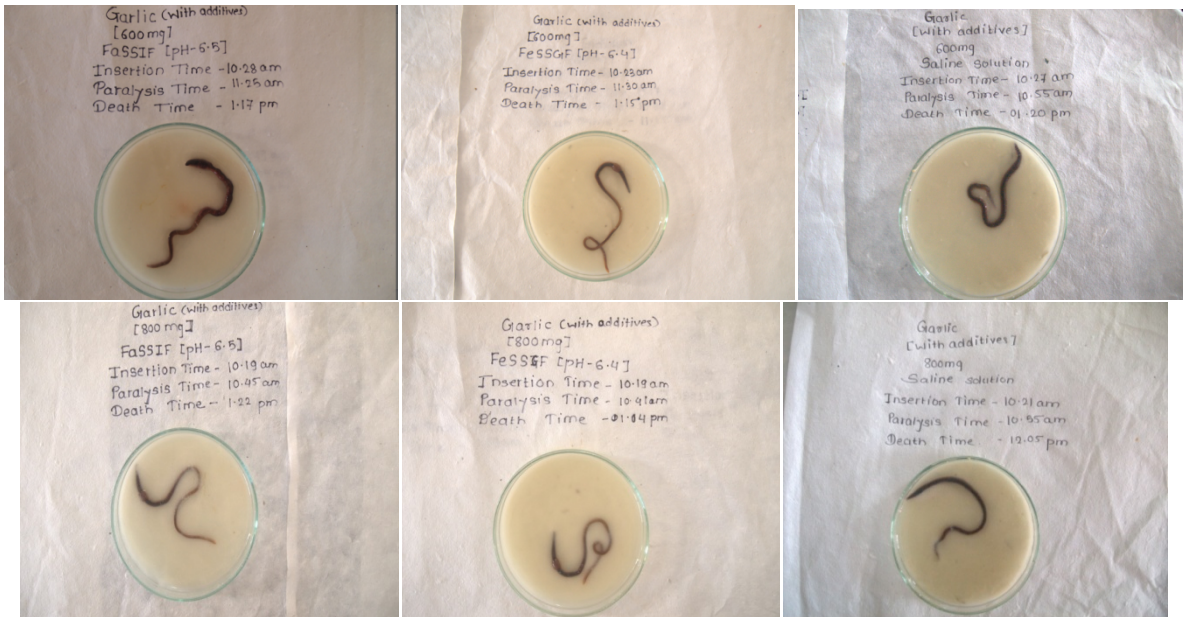

Fig.3: In vitro Anthelmintic Effect of garlic powder in Bio-relevant Medias and saline solution

From the experimentation carried out under preliminary screening, it was found that worms could survive in saline solution for 12 hours whereas the worms could survive for longer in saline solution plus mud water and pure mud water which are the natural environment for the survival and growth of worms. So it was concluded from the above study that for the anthelmintic study to be carried out and to interpret the proper anthelmintic activity for any drug the study should be carried out in suitable simulated environment which persists in human body where the worms find as a place of parasite. Results mentioned in **Table-2.**

Selection of Bio-Relevant Media

From these tested medias Saline solution, Fed-State Simulated Gastric Fluid (Fe-SSGF) Early, Fasted-State Simulated Intestinal Fluid Updated version (FaSSIF-V2) were selected as an appropriate medium for maintenance of worms as the worms were found to be alive for about 12 hours. **(Table-3)**

In- Vitro Anthelmintic Assay Using Modifications in Conventional Method

The anthelmintic effect of different concentrations of albendazole against earthworms is shown in **Table 4** and **Figure-3** The time taken for paralysis of worms was 7± 0.5 minutes. in FaSSIF, 12±0.5 minutes in FeSSGF and 22 ±0.5 minutes in Saline solution, while time taken for death was 16±0.5 minutes in FaSSIF, 23±0.5minutes in FeSSGF and 22±0.5minutes in saline solution at 40mg/ml concentration. On the other hand the lower concentration (20mg/ml) allowed worms to survive for longer (around 2-3 hours). The effective concentration of reference drug albendazole was found out to be 40 mg/ml (400 mg). Garlic powder was prepared with microwave drying technique with inclusion of some additives like corn flour in minor quantity to aid grinding of powder to get uniform particle size for particles. The time taken for paralysis of worms was found to 23.5±0.5 min in FaSSIF, 21.5±0.5minutes in FeSSGF and 32.5±0.5 minutes in Saline solution, while time taken for death was 182±0.5 minutes in FaSSIF, 165±0.5minutes in FeSSGF and 102.5±0.5 minutes in saline solution at 80mg/ml concentration. Changes in body structure of worms after and before death are shown in **Figure-2**. After death of the worm, fading of body color was observed along with the change in the appearance. In -vitro anthelmintic effect of garlic powder in Bio-relevant medias and saline solution shown in **Figure- 3**.The results shown in **Table-4**

Formulation and Evaluation of Effervescent Tablet

As per the anthelmintic concentration obtained for garlic powder which was found to be 80 mg/ml, the expected dose strength of the garlic anthelmintic tablet was 800mg per tablet. For convenience the tablets comprising 400 mg dose strength were designed and optimized. And for studies involving anthelmintic assay for tablet dosage form two tablet dosage form containing 400 mg each of garlic powder were utilized to fulfill the dose of 800 mg dose strength. Post compression evaluation of optimized batch of tablet dosage form depicts the results fitting in the compendial requirements of the dosage form as shown in **Table 5**

In- Vitro Anthelmintic Assay of Effervescent Tablet and Albendazole Tablets as Standard

As the dose requirements for garlic powder as anthelmintic agent, two tablets were taken for study each containing 400 mg of active drug content fulfilling the requirement of 80 mg/ml concentration. The results obtained are comparable with the albendazole standard used. The results are shown in **Table 6**. For the albendazole tablets on an average death time reported was 96.16 minutes, whereas for the garlic effervescent tablet dosage form the average death time was 110 minutes, which was found to be equally effective as that of the standard used.

CONCLUSION

The present research work is the successful attempt which came out with the dosage form comparable to marketed formulations. Moreover the most important outcome of the research work was validation and optimization of the conventional assay methodology for the anthelmintic activity.

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