Efficacy of Allium sativum (garlic) against experimental cryptosporidiosis

Maha Reda Gaafar

Parasitology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt

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Abstract

Background: Due to increasing problems of inadequate and unreliable medical treatments for Cryptosporidium enteritis, alternative therapies are being sought.

Objective: The current study was designed to evaluate the prophylactic and therapeutic efficacy of Allium sativum (garlic) against Cryptosporidium infection in experimentally infected immunocompetent and immunosuppressed mice.

Methods: Forty eight male Swiss albino mice were divided equally into control and experimental groups. Each group was further subdivided into four equal subgroups; two immunosuppressed and two immunocompetent. Cryptosporidial oocysts were isolated from human stools, and were used to infect the mice. The experimental subgroups received garlic orally two days before infection or one day following infection, and continued daily till the end of the study. Two weeks following garlic administration, mice stools were examined for counting the cryptosporidial oocysts, then the animals were sacrificed; their small intestines were processed and were examined for detection of the pathological lesions and for counting of the parasites. Also, myeloperoxidase (MPO) activity was measured in jejunal sections.

Results: The results showed that the infected immunosuppressed subgroups of mice; showed a statistically significant increase in the number of cryptosporidial oocysts in stool and ileal sections, as well as an increase in the MPO activity when compared to the corresponding immunocompetent subgroups. Garlic successfully eradicated the Cryptosporidium oocysts from stool and intestinal sections of the infected immunocompetent subgroup of mice receiving garlic two days before the infection. Besides, the oocysts were significantly reduced in all other infected experimental subgroups in comparison to the corresponding infected control subgroups. The intestinal sections of all
1. Introduction

*Allium sativum* (*A. sativum*) or garlic has been used as both food and medicine in many cultures for thousands of years, dating at least as far back as the time that the Giza pyramids were built. It has been recognized not only as a spice but also as a substance which exerts a control on microorganisms.1–3

*A. sativum* is remarkable for a number of potentially active chemical constituents. It contains seventeen amino acids as arginine, at least 33 organosulphate compounds as allicin and allin, eight minerals (germanium, calcium, copper, iron, potassium, magnesium, selenium and zinc), enzymes as allinase, and the vitamins A, B1 and C. The physiological activity of dietary *A. sativum* is attributed to allicin (dialyl thiosulphinate), which is one of the organosulphate compounds found in the bulb. It is responsible for the anti-microbial properties and the characteristic flavor of fresh garlic.2–4

Ancient Egyptians realized the benefits of garlic; its medical and magical powers were described on the walls of ancient temples and on papyri dating to 1500 BC. In recent times, garlic has been shown to have multiple beneficial effects such as antimicrobial, antithrombotic, hypolipidemic, hypoglycemic and antitumor activities.4 Lately, garlic has widely been used to treat intestinal parasites. The antihelmintic effect of garlic has been a matter of interest of researchers. Their results showed that treatment with garlic evoked a significant reduction in the worm load.1,2,5–7 In addition, garlic has been used successfully in a single uncontrolled study in China applied on 20 AIDS patients to treat *Cryptosporidium*.3 Moreover, garlic compounds were purified and tried as complementary medicine in the management of leishmaniasis.9 Thus, because many of the microorganisms susceptible to garlic extract are medically significant, garlic holds a promising position as a broad-spectrum therapeutic agent.10

Cryptosporidiosis is a parasitic disease caused by *Cryptosporidium*, a protozoan parasite in the phylum Apicomplexa. Despite not being identified until 1976, it is one of the most common waterborne diseases and is found worldwide. It affects the intestine of mammals and is typically an acute short-term infection. It spreads through the fecal–oral route, often through contaminated water. The parasite is transmitted by environmentally hardy microbial cysts (oocysts) that, once ingested, exist in the small intestine and result in an infection of intestinal epithelial tissue. The main symptom is self-limiting diarrhea in people with intact immune systems. In immunocompromised individuals, such as AIDS patients, the symptoms are particularly in the form of severe dehydration, electrolyte imbalances, malnutrition, wasting, and eventual death. *Cryptosporidium* is the organism most commonly isolated in HIV positive patients presenting with diarrhea.17–14

Gastrointestinal inflammation caused by the intestinal parasites is often accompanied by functional disturbances, marked changes in the structure and chemical content of the intestine together with biochemical changes as changes in the myeloperoxidase (MPO) activity. MPO is the most abundant enzyme in neutrophils and monocytes; it is the focus of inflammatory pathologies. MPO is believed to be involved in augmenting the cytotoxic activity of H2O2 and O2 to kill a variety of micro-organisms. Thus, in case of intestinal parasitic infection, increase in MPO activity level is an indicator for intestinal inflammation.15,16

There is a history of inadequate and unreliable treatments for *Cryptosporidium* enteritis. Against this background, certain antiparasitic agents such as paromomycin, nitazoxanide and azithromycin are sometimes used, but they usually have only temporary effects and sometimes relapses happened. Currently, the best approach is to improve the immune status in immunodeficient individuals, for example, by using antiviral therapy in patients with AIDS and supportive treatment for symptoms.17–20

Because of the great need to develop new anti-cryptosporidial agents, trials were designed to test the potency of traditional medicinal plants for treating cryptosporidiosis.

Therefore, the present study aimed at investigating the antiparasitic effectiveness of *A. sativum* (garlic) as a natural component in the prevention, as well as treatment of *Cryptosporidium* infections in experimentally infected immunocompetent and immunosuppressed mice.

2. Materials and methods

2.1. Parasites

Stool samples were collected from 20 immunosuppressed patients with chronic diarrhea, in the Alexandria University Hospital and Fever Hospital from Haematology Department and Renal Dialysis Unit, from July to August 2011. Informed consents were obtained from the patients. The stool samples were transferred to the Parasitology Department to be screened by different techniques for the presence of intestinal protozoa. All samples were microscopically screened by direct smear, iodine smear; Sheather’s sugar floatation technique and modified Ziehl–Neelsen acid fast stain (MZN), aiming to identify cases of *Cryptosporidium* (Fig. 1).21 Three out of the 20 examined samples proven to be *Cryptosporidium* by the conventional diagnostic techniques, were pooled, and were used for the completion of the study. The parasites were isolated by Lumb’s technique. In short, each stool sample was mixed with 10 ml of distilled water, and then filtered through coarse sterile gauze. The homogenate was then centrifuged at 2500g for 5 min. The supernatant fluid was discarded, and the sediment was washed twice in 1 ml of phosphate buffer saline (PBS), with centrifugation at 13,000g for 2 min. After repeated washing followed by centrifugation, fecal debris was totally eliminated.22 Cryptosporidial oocysts were preserved in 2.5%
potassium dichromate solution and stored at 4 °C until use for infection.23 Just before use, the cryptosporidial oocysts were washed three times in distilled water to remove the potassium dichromate, centrifuged at 1500g for 10 min, and the organisms were counted with a hemocytometer. Suspension containing the required concentration for the infection (10⁴ oocysts/ml) was prepared by dilution of the organism in the appropriate amount of distilled water.24 This study was approved by the Ethics Committee of Alexandria University.

2.2. A. sativum (garlic)

Garlic was administrated to the experimental animals as crude juice. The crude extract was prepared as follows: Fresh garlic bulbs were separated, peeled, and washed with distilled water. After drying, 500 g of garlic bulbs were crushed in a blender until a uniform consistency was achieved. The resulting paste was diluted with distilled water to obtain a 1 g/ml aqueous solution. Raw garlic juice was aliquoted and was stored at −20 °C until use.3,7,25 Working solution was made from the stock solution by dilution with distilled water. The selected dose for the present work was 50 mg/kg body weight.7

2.3. Experimental animals

Animals used in this work were male Swiss albino mice, aged three to five weeks, weighing 20–25 g. They were housed in well ventilated cages with perforated covers, supplied with standard pellet food and water. Bedding was changed everyday. The mice were allowed to adapt to the laboratory environment for one week before the experiment,26 and their stools were examined for the detection of parasites. After 12 days post infection the subgroups Ia1, Ib1, Ic1, Id1, Ia2, Ib2, Ic2, Id2, Ic and Id were sacrificed. Small intestines were dissected, fixed in 10% formalin, embedded in paraffin sections and stained by hematoxylin and eosin stain (H&E). The intestinal sections were examined for the detection of pathological changes and for the counting of the parasites in ten fields of oil immersion lens, and the mean counts were calculated.28

2.4. Evaluation of the garlic efficacy in mice was done by

- Survival rate of both immunocompetent and immunosuppressed subgroups of mice.
- Undiluted stool samples from all subgroups of mice were stained by MZN and examined by microscopy to count Cryptosporidium oocysts at the same day of sacrifice in ten fields of oil immersion lens, and the mean counts were considered.21
- Using over dose of ether, the sacrifice of animals was achieved 12 days post infection for the subgroups Ia1, Ib1, Ic1, Id1, Ia2 and Ib2. On the other hand, 15 days post infection; the subgroups Ia2, Ib2, Ic2, Id2, Ic and Id were sacrificed. Small intestines were dissected, fixed in 10% formalin, embedded in paraffin sections and stained by hematoxylin and eosin stain (H&E). The intestinal sections were examined for the detection of pathological changes and for the counting of the parasites in ten fields of oil immersion lens, and the mean counts were calculated.28
- Myeloperoxidase activity (MPO activity) was measured in extracts of full thickness sections (200–300 mg) of mouse jejunum in all subgroups of mice29 at the day of sacrifice. Tissue samples were weighed and homogenized with hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0, 4 °C). The homogenates were freeze-thawed three times and then centrifuged at 35,000g for 30 min. The pellets were discarded, and the supernatants were assayed for soluble protein and for MPO activity. MPO activity was measured by adding 0.1 ml supernatant to 2.9 mol reaction buffer (50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine hydrochloride and 0.0005% hydrogen peroxide). After 1 min, the change in absorbency at 460 nm was measured by the spectrophotometer. One unit of MPO activity was defined as that degrading 1 μmol of peroxidase per minute at 25 °C. The MPO activity was expressed per milligram of protein. Soluble protein in the tissue supernatant was assayed using Lowry’s method.30

Figure 1 Cryptosporidial oocysts in patient’s stool stained with modified Ziehl–Neelsen acid fast stain (×1000).
2.5. Calculations

Mean and standard deviation were calculated for *Cryptosporidium* oocysts in stool samples and intestinal sections, and for the intestinal MPO activity. Furthermore, *t*-test was applied in order to compare between each experimental subgroup receiving garlic and its corresponding control subgroup. In addition, *t*-test was used to compare between the immunocompetent and the immunosuppressed subgroups of mice (*P*-values less than 0.05 were considered significant and less than 0.001 were considered highly significant), according to Knapp and Miller (1992).31

3. Results

3.1. Survival rate of mice

The number of dead animals were recorded throughout the study and revealed that, only one immunosuppressed mouse of subgroup Id (infected immunosuppressed group) and one immunocompetent mouse of the subgroup Ic (experimentally infected mice by *Cryptosporidium* receiving garlic one day following the infection) got death. Besides, all other mice were still alive till the end of the study. So, the survival rate recorded in this study was 95.8% for both immunocompetent and immunosuppressed groups of mice.

3.2. Fecal count of the parasites

In the control group infected by *Cryptosporidium*, the oocysts were best visualized by MZN stain as spherical pink organisms, 4-6 μm in diameter (Fig. 1) with a mean number of (4.74 ± 5.19) in subgroup Ic1, (6.25 ± 2.62) in subgroup Id1, (4.93 ± 4.07) in subgroup Ic2 and (6.88 ± 7.67) in subgroup Id2 (Table 1).

Garlic successfully eradicated the *Cryptosporidium* oocysts from the stools of the infected subgroup of mice (subgroup IIa) receiving garlic two days before the infection. Besides, the oocysts were greatly diminished in all other experimental subgroups; in subgroup IIb, subgroup Iic and subgroup IId, with a mean number of 1.13 ± 2.08, 1.77 ± 1.13 and 2.15 ± 4.71 respectively. There was a significant decrease in the number of cryptosporidial oocysts in all experimental subgroups as compared to the corresponding infected control subgroups. However, there was a statistically significant increase in the number of cryptosporidial oocysts in the stool samples of infected immunosuppressed subgroups of mice (Id1, Id2, IIb and IId) as compared to the corresponding immunocompetent subgroups (Ic1, Ic2, Ia and Iic) (Table 1). No parasites have been detected in the stool samples of subgroups Ia1 and Ia2 (normal) or subgroups Ib1 and Ib2 (immunosuppressed).

3.3. Parasite counts and histopathological changes in small intestinal sections

Following sacrifice of mice, the ileal sections of the *Cryptosporidium* infected control subgroup (Ic) and the *Cryptosporidium* infected immunosuppressed subgroup (Id) stained with H&E, revealed the presence of large numbers of *Cryptosporidium* oocysts on the luminal surface of the epithelium lining the villi, as small rounded organisms (Fig. 2), with a mean count of 5.83 ± 2.28 for subgroup Ic1, 7.14 ± 6.01 for subgroup Id1, 5.51 ± 6.15 for subgroup Ic2 and 6.92 ± 2.74 for subgroup Id2 (Table 2). The infected sections showed altered mucosal architecture, with shortening, blunting and widening of the intestinal villi (Fig. 3).

The parasite was completely eradicated from the intestine of subgroup IIa. However, ileal sections of mice of subgroups IIb, Iic and IId showed reduced number of *Cryptosporidium* oocysts to 1.76 ± 2.12, 1.89 ± 21.4 and 2.53 ± 3.18 respectively. The differences between the experimental and the corresponding infected control subgroups were statistically significant. Besides, there was a statistically significant increase in the number of cryptosporidial oocysts in the ileal sections of infected immunosuppressed subgroups of mice (Id1, Id2, IIb

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**Table 1** Mean fecal count of *Cryptosporidium* oocysts in mice given garlic before and after the infection.

<table>
<thead>
<tr>
<th>Subgps of mice</th>
<th>Control subgroup (Mean ± SD)</th>
<th>Garlic before infection (Mean ± SD)</th>
<th><em>t</em>-test</th>
<th>Control subgroup (Mean ± SD)</th>
<th>Garlic after infection (Mean ± SD)</th>
<th><em>t</em>-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocompetent Sgps</td>
<td>Subgp Ic1 4.74 ± 5.19</td>
<td>Subgp IIa 0.00 ± 0.00</td>
<td>13.23** &lt;0.001</td>
<td>Subgp Ic2 4.93 ± 4.07</td>
<td>Subgp Iic 4.93 ± 4.07</td>
<td>10.09* (0.001)</td>
</tr>
<tr>
<td></td>
<td>Subgp Ic2 6.25 ± 2.62</td>
<td></td>
<td></td>
<td>Subgp IIb 1.13 ± 2.08</td>
<td>Subgp IId 1.77 ± 1.13</td>
<td>12.64* (0.002)</td>
</tr>
<tr>
<td>Immunosuppressed Sgps</td>
<td>Subgp Id1 6.25 ± 2.62</td>
<td></td>
<td>16.65** &lt;0.001</td>
<td>Subgp Id2 6.88 ± 7.67</td>
<td>Subgp IId 2.15 ± 4.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subgp Id2 25.16 ± (0.001)</td>
<td></td>
<td>13.36* (0.005)</td>
<td></td>
<td>15.19* (0.001)</td>
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</tbody>
</table>

* Significant, *P*-value is (<0.05).

** Highly significant, *P*-value is (<0.001).

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Figure 2 Ileal section showing *Cryptosporidium* oocyst on the brush border (H&E ×1000).
and Ik) as compared to the corresponding immunocompetent subgroups (Ic1, Ic2, Ia and Ic) (Table 2). It was noticed that the intestinal sections of all subgroups received garlic before or after the Cryptosporidium infection, revealed a more or less normal architecture. No parasites and no pathological changes have been detected in the intestinal sections of subgroups Ia1 and Ia2 (normal) or subgroups Ib1 and Ib2 (immunosuppressed).

The intestinal MPO activity showed a statistically significant increase at the day of sacrifice in the subgroups Ic1, Ic2, Id1 and Id2 in comparison to its level in Ia1, Ia2, Ib1 and Ib2, which indicates the presence of intestinal inflammation. In subgroups Ia and Ib, there was a statistically significant drop in its level when compared to subgroups Ic1 and Id1. Furthermore, subgroups Ic and Ia showed a statistically significant decrease when compared with the corresponding control subgroups; Ic2 and Id2. In contrast the MPO activity showed a statistically significant increase in immunosuppressed infected mice (Id1, Id2, Ib and Ik) when compared with the corresponding immunocompetent subgroups (Ic1, Ic2, Ia and Ik respectively) (Table 3).

4. Discussion

Explosive, chronic, fatal, non bloody diarrhea is considered a very serious management problem in immunosuppressed patients as well as in normal persons in both developed and developing countries. Pathogenic intestinal protozoa represent the main causes of this diarrhea, among which Cryptosporidium produces regularly occurring outbreaks throughout the world. Most of the immunodeficient patients got failure of the available drugs used for the treatment, besides the multiple adverse effects that they produced. Thus, new drugs against this parasite became consequently urgently needed.

The search for bioactive plants which can be used as non-conventional anti-parasitic treatment has received considerable attention in recent times because of the increasing worldwide development of resistance to chemical drugs in parasitic populations. However, scientific evidence to validate the use of plants remains limited. Thus, this study was oriented to evaluate the protective and curative capacity of garlic against Cryptosporidium in experimental mice.

In this study, the allicin, which has antimicrobial effect, was obtained from crushed fresh garlic bulbs as mentioned by Ankar and Mirelman (1999), Sasaki et al. (1999) and Lemar et al. (2002).

The dose selected for the present work was 50 mg/kg body weight. Riad et al. (2009) supposed that this dose is equivalent to the daily amount of garlic recommended for an average human to maintain good health (~4 g).

In this study, immunosuppression of experimental animals was induced by cyclophosphamide (endoxan) at two doses of 70 mg/kg each. The choice of this drug was depending on its capability for immunosuppression of both humoral and cell-mediated immune mechanisms. The recorded survival rate of all groups of mice in this work was 95.8%. This high percentage was coincided with Camenga et al. (1974), who suggested that the intraperitoneal injection of cyclophosphamide at the given dose in this study produced no or minimal deaths in normal bald mice. The cause of death of the immunosuppressed mouse of subgroup Id was certainly related to the immunosuppression situation. In contrast the cause of death of the immunocompetent mouse of the subgroup Ia is still not apparent and may be referred to animal housing.

In the present study, light microscopic examination of infected intestinal sections by Cryptosporidium revealed the presence of altered mucosal architecture, with shortening, blunting and widening of the intestinal villi. Connor et al. (1993) demonstrated the same structural abnormalities in the villi.

In this work, garlic successfully eradicated the Cryptosporidium oocysts from stool and intestinal sections of the infected immunocompetent subgroup of mice (subgroup Ia) receiving garlic two days before the infection and continued for two weeks, and it is associated with normal intestinal architecture. Besides, the cryptosporidial oocysts were greatly diminished in all other subgroups. This impressive effect of garlic was similar...
larly obtained in the in vitro study on the cestode, *Hymenolepis nana* using garlic extract, in which lethal effects had been shown on the worms (Soffar and Mokhtar, 1991). Moreover, Fareed et al. (1996), demonstrated that when garlic was used for the treatment of HIV patients with chronic diarrhea and confirmed cryptosporidiosis, complete remission occurred in some patients and partial remission occurred in the others. In addition, the strong prophylactic effect of garlic when it was given before the infection could be supported by Riad et al. (2009). They demonstrated that treating mice with garlic before schistosomal infection evoked a highly significant reduction in the mean worm count as compared to the mice giving garlic post-infection. Moreover, garlic efficacy was the highest in the group treated with garlic before and after bilharzial infection.

In this study, the intestinal MPO activity showed statistically significant increase at the day of sacrifice in both infected immunocompetent and infected immunosuppressed subgroups (Ic1, Ic2, Id1 and Id2) in comparison to the corresponding non infected control subgroups (Ia1, Ia2, Ib1 and Ib2) which indicate intestinal inflammation. This is also reported by Venkova et al. (2000) and Khan et al. (2002), who suggested that the MPO activity is a reliable index of inflammation intensity in mucosa, submucosa and smooth muscle tissues. This increase in MPO activity is coincided with the pathological changes detected in the intestinal villi. On the other hand, there was a statistically significant drop in the level of MPO activity in subgroups receiving garlic before the infection (Ia and Ib), and a further statistically significant drop in treated subgroups by garlic after the infection (Ic and Id), when compared to the corresponding control subgroups, which indicate remission of the intestinal inflammation. This agreed with the amelioration of the intestinal pathology detected in all experimentally infected subgroups.

The efficacy of garlic in the prophylaxis and treatment of experimental cryptosporidiosis could be explained by different mechanisms. Adetumbi et al. (1986) suggested that the blockage of lipid synthesis may be an important component of the antimicrobial activity of garlic. Moreover, Kyo et al. (1997), Sutton and Haik (1999) and El Shenaway et al. (2008), reported the enhancement of phagocytosis and an increase in natural killer cell activity which promote the immune system function, and strengthen the body’s defense mechanism during the duration of treatment by garlic. Furthermore, Masamha et al. (2010), reported that *A. sativum*, disrupts the normal physiological functions of the parasite like mobility, food absorption, and reproduction.

Garlic has been consumed for several thousand years without any adverse long-term effects, suggesting that modest quantities of garlic (two to three chewed fresh cloves of garlic, or one tablespoon of garlic oil daily) produce no risks to normal individuals. However, some undesirable effects could associate the high dose of garlic. Garty (1993), stated that consuming too much garlic can result in halitosis, stomach ache, allergic reactions, and if garlic is applied externally, a

<table>
<thead>
<tr>
<th>Subgps of mice</th>
<th>Control subgps (Mean ± SD) t-test</th>
<th>Garlic before infection (Mean ± SD) t-test</th>
<th>Garlic after infection (Mean ± SD) t-test</th>
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<tbody>
<tr>
<td>Immunocompetent Subgps</td>
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<tr>
<td>Subgp Ia1</td>
<td>11.53* (0.02)</td>
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<tr>
<td>Subgp Ic1</td>
<td>14.86** (&lt;0.001)</td>
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<tr>
<td>Subgp Ia2</td>
<td>17.14** (&lt;0.001)</td>
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<tr>
<td>Subgp Ic2</td>
<td>19.32* (0.001)</td>
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<tr>
<td>Immunosuppressed Subgps</td>
<td></td>
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<tr>
<td>Subgp Ib1</td>
<td>15.79* (0.01)</td>
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<tr>
<td>Subgp Id1</td>
<td>17.22* (0.001)</td>
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<tr>
<td>Subgp Ib2</td>
<td>19.32* (0.001)</td>
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<tr>
<td>Subgp Id2</td>
<td>21.67* (0.001)</td>
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* Significant, P-value is <0.05.
** Highly significant, P-value is <0.001.
burning sensation of the skin may occur. Also, Fareed et al. (1996), reported that the major side effect was a strong garlic smell and taste. Studies have shown that sipping milk at the same time as consuming garlic can significantly neutralize bad breath. Also, if garlic is taken in a hot water with honey, it will be a bit more palatable.

Based on our results, it can be concluded that garlic has good efficacy as a prophylactic and a promising therapeutic agent against Cryptosporidium and therefore validates the traditional use of the plant in parasitic infections. It is recommended that further investigations be carried out on the applications of garlic as a complementary medicine in the management of cryptosporidiosis.

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