THERAPEUTIC EFFECT OF EARTHWORM POWDER ON THE PATHOGENESIS OF ENTAMOEBA HISTOLYTICA IN VIVO

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ABSTRACT

This study was carried out in vivo to detect the effect of earthworm powder on the Entamoeba histolytica in white mice. The mice infected with the parasite were inoculated by earthworm powder with a concentration of 200 mg/ml for 11 days, during this period, the faeces were examined daily to observe the change in parasite numbers after inoculating with powder. Results showed that the earthworm powder was effective in reducing parasite numbers in mice gradually and when compared the therapeutic efficacy of the earthworm powder with the metronidazole where it was close (61.5 & 63.1) respectively. Histopathological change was study found that the parasite and metronidazole cause necrosis, infiltration of lymphocyte and mucosal damage and while powder of earthworm effectively in repair the damaged intestinal walls. In addition, there was an increased concentration of sIgA in the intestinal tissue, which was concentrated after 3,7,9 days in the treatment earthworm powder group (4.084 ± 1.29, 2.588 ± 0.82, 2.563 ± 0.81) ng/ml respectively, while in the metronidazole after 3,7,9 days 4.057±1.28, 2.553±0.81, 2.528±0.71ng/ml respectively compared with control negative group. Also in the earthworm powder group (none infected) it noted after 3,7,9 days (2.064 ± 0.65, 2.17 ± 0.68,1.901 ± 0.60) ng/ml respectively, and in positive control group the concentration of IgA was 3.221±1.01, 3.370±1.06, 3.580±1.13ng/ml respectively after 3,7,9 days compared with control negative group.

Keywords: Earthworm powder, Entamoeba histolytica, sIgA.

INTRODUCTION

Entamoeba histolytica is the most common form of enteric disease, the parasite causes intestinal amoebiasis, amoebic colitis, amoebic dysentery and amoebiasis extra-intestinal (Gockel- Blessing, 2013). The patient suffers from several symptoms including diarrhea, low weight, fatigue, headache, fever, abdominal pain, dysentery and liver abscess (Caler and Lorenzi, 2010). Infection is done by eating food and drink contaminated with the cystic stage, the infection of amoeba is widespread throughout the world and infection rates are high in the tropics and sub-tropical regions, as well as in low-level health and cultural areas (Linford et al., 2009; Pham et al., 2011). The parasite is endemic to the large intestine and appears in the faeces after it is the forms of the feeding or active stage and the peasing or infectious stage (Devendra et al, 2010). Entamoeba histolytica is a unicellular and eukaryotic, life cycle is simple as it is divided into two main stages: the active mobile stage called the Trophozoite and the resistor stage called cyst (Murry et al., 2013). Because of the seriousness and medical importance of this parasite have been made in the last four decades a determined effort to get to know more on the parasite, disease, how to treat, and the growing interest in using the numerous extracts in the treatment of infection due to the parasite as it contains some of the components hinder the growth of the parasite, as well as help eliminate gastrointestinal ulcers and healing (Yu sung et al., 2005; Coppi et al., 2006; Singh et al., 2001; Swiderski et al., 2007). Many drugs are used for the treatment of amoebiasis, the metronidazole is the most use of them (Bansal et al., 2006), but many reported side effects like gastrointestinal disorders, nausea and metallic taste (Wain, 1998).

Earthworms are important components of soil system, mainly because of their positive effects on soil structure and function as well as earthworm help to increase soil fertility, so they referred a farmer’s friend and they considered source of protein (Mathur et al., 2010). Earthworms have been used in medicine for various remedies since 1340 AD (Omar et al., 2012). The powder of Lompio mauritii important to human health because have a large amounts of zinc 32.34 mg/L, iron 241.10mg/L, manganese 17.20mg/L, protein 31.7 % soluble nitrogen 1.8% and copper 4.50 ppm, quantities of calcium, potassium, carbohydrate and magnesium (Lourdumary & Uma, 2012; Paolletti et al., 2011). The earthworm extract contains a group of enzymes called the lumbrokinaise, which is similar to the Omega-3 molecules in fish oil, the polyphenols in green tea, and curcumin and turmeric, these group of enzymes as a valuable characteristic of earthworms (Yu et al., 1998).

Powder of earthworm is one extracts that can be given orally because he has the possibility of its application in the case of thrombus and contributes to be a factor curbing particularly on the accumulation of platelets and the impact on prevent blood clotting (Bhorgen & Uma, 2014). The powder of earthworm contain 65% protein, 19% carbohydrate, 16% lipid, minerals and various types of vitamins (Zakaria et al.,2012; Anjana et al.,2013) and play a good role in pharmaceutical as an
antibiotic, anticancer, antihyperglycemia and antihypotension (Prakash & Gunasekaran, 2011), that powder have antimicrobial activity for many types of bacteria and fungi (Vasanthi et al., 2013; Bhorgin & Uma, 2014). Therefore this study may explain the role of earthworm extract in the treatment of Entamoeba histolytica in laboratory animals.

MATERIALS AND METHODS
Earthworm collection: Earthworm samples were collected from an orchard located near the Diyala River east of Baghdad governorate and the orchard was planted with different types of plants, which included citrus and apple trees, palms and figs, in addition to a number of different animals (chickens, Cows and sheep). The samples were collected by digging the soil in of orchard at different locations by field shovel (spade) and a depth of 1m and isolating worms by forceps and placed in a clean glass case containing moist soil and then taken to the laboratory for an experiment.

Powder extraction: Approximately 500 earthworms were collected and washed in running water to remove dirt from the surface of the body. After that it put in distilled water for 6-8 hours flooding the earthworms with to allow the soil in the tract to the exit. Later earthworms are washed with distilled water and placed in a sterile beaker private and kept in the incubator for one day at a temperature of 55°C. After that has been taken out of the incubator and crushed worms, then turned into powder, powder stored in the refrigerator degree normal temperature -4°C (Yegnanarayan et al., 1987; Andleeb et al., 2016). Also in this study the pH value of powder was measured in Ibn Sina Center in Waziriyah and the value was (pH =5.24). Then prepared concentration 200%mg/ml.

Collection of parasite samples: Stool samples were collected from adults and Children suffering from diarrhea and not subject to treatment and reviewers to Ibn Al balady Maternity & Children’s Hospital and Baghdad teaching hospital, in sterile plastic containers.

Small amount of sample was examined on direct microscopic examination of feces to ensure that contain the parasite and diagnose trophozoite and cyst stages by (Wet preparation) (Tanyuksel, et al., 2005).

Isolation of parasite: The parasite isolated from stool samples examined by taking 1 g of Sample and especially the region containing blood or mucus for the possibility of the existence of the large number of the parasite in it, the sample taken mixing well with or by Pasteur pipette with 3 ml of normal saline solution until has become emulsion, after that passed through from a layer of sterile gauze or placed in a test tube for half an hour for the purpose of removing the large Materials of the emulsion before adding it to culture media (Clark & Diamond, 2002; Ramos et al., 2005).

Animals: Ninety male albino mice aged 6-10 week, weighing 25-35gm were obtained from National Control Center for Drugs and Researches (Baghdad Iraq), the mice were housed, under controlled conditions of temperature -4°C, stander light (12 hours light and 12 hours dark), and were fed with a conventional diet and water. Stool of them was examined before beginning of the experiment to make sure that the mice are free from any intestinal parasites.

Experimental design: Experimental animals were immunosuppressed by dexamethasone (4 mg/ml) in a daily intramuscular dose of (0.1ml/mouse) for 5 days. 54 mice were inoculated with (0.1ml) contain (1x10⁶ trophozoite), after (24hr.) all mice feces were examined to confirm the infection occur, then the infected mice divided to (3 groups) each group contain 18 mice, and two groups non-infected, one group inoculated with earthworm powder, the remaining non-infected mice kept as a negative control group. Then each group was inoculated as follow:

1) Group one (none infected): inoculated orally by stomach tube with 0.1ml/day normal saline consider as control negative.
2) Group two (infected): inoculated orally by stomach tube with 0.1ml/day normal saline considers as control positive.
3) Group three (none infected): inoculated orally by stomach tube with 0.1ml/day from Earthworm powder (200%) every day.
4) Group four (infected): inoculated orally by stomach tube with 0.1ml/day from Earthworm powder (200%) every day (TEM).
5) Group five (infected): inoculated orally by stomach tube given orally with 0.1ml/day from Metronidazole each day (TM).

Sufficient treatment calculation: Sufficient treatments for earthworm powder and metronidazole were measured according to the method of (Xiao et al, 1996):

\[
\text{Sufficient treatment} = \frac{A - B}{100}
\]

A: The rate of parasite number in the control group
B: The rate of parasite number in treatment group

Enumeration of Entameba histolytica: Cyst and trophozoite in faeces were enumerated starting on the day of infection for the positive group and for the beginning of the treatment day for the treated groups. Briefly, take one gram of freshly passed fecal sample from each mice in all treated groups.
except (control negative and earthworm powder none infected) and dissolved in 10 ml of normal saline, take a drop and Spread on the slide and then dry, add methanol for the fixation, then dried and added the Giemsa stain to the slide for a period of 5 minutes and then washed and leave to dry after a certain period check by using light microscope to see the number of parasite, finally the following equation is applied and followed by (Ryan et al., 1999).

\[ N = S (\text{Vol} \times \text{Wt}) \]

Whereas:
N: The number of parasites in grams of stool;
S: Number of parasites calculated in the slide;
Vol: The size of calculated sample by (0.01ml);
Wt: The weight of stool sample taken by (1gm).

**Quantification of secretory IgA (sIgA):** At the 3, 7, 9th day post-infection and treatment, four mice from each group were sacrificed and removed (1gm) from large intestine, the concentration of sIgA were determined by commercially available ELIZA OR ELISA assay Kit Kit of Mouse Secretory Immunoglobulin A (sIgA) ELISA Kit (Cat No. MBS269144) Mybiosource.com.

**Histopathological study:** At the end of experimental period 3, 7, 9th days, hematoxylin and eosin stained large intestine sections from mice of all groups were examined microscopically and fixed in (10%) formalin for histopathological changes study.

**Statistical analysis:** The Statistical Analysis System- SAS 2012 program was used to effect of difference factors in study parameters. Least significant difference –LSD test (ANOVA) was used to significant compare between means, in this study.

**RESULTS AND DISCUSSION**

The present study showed the effective impact of earthworm powder in gradually reducing the number of parasites *Entameba histolytica* in infected mice. As shown in Table 1, the feces of mice became clear from the parasite completely for treatment earthworm powder group (TEP) at the 10th day. The metronidazole treated group (TM) also causes gradually reducing in number of parasite shedding and became zero at the 9th day after treated, while the control positive group (CP) increased parasite rates on the third and fourth day, and then the rate began to decrease gradually to zero on the 12th day and continued shedding of parasites to the end of the test Table 1. after the mice were dosage with a (PBS) solution. Also, the percentage of reduction in parasites shedding for treated groups were: (TEP) group was (61.5%) in comparison to (TM) group which was (63.1%). There are significant differences (p<0.05) between treated groups as which is showed in Table 2.

The results showed that the sufficient treatment of earthworm powder for the treatment of *Entameba histolytica* was percent reduction (61.1%) and the mice stopped clearance with the parasite cyst after 10 days of treatment. The results were close when compared to sufficient treatment of earthworm powder with the sufficient treatment of the metronidazole percent reduction (63.1%) as shown in Table 2. It is known that the metronidazole has many side effects toxicity (Ali & Nozaki, 2007; Wain, 1998). At the same time, no toxic effects of powder were noted in mice, as it is preferred to use the powder as a food substance is known in many countries because it contains 65% protein, 19% carbohydrate, 16% lipid, minerals and various types of vitamins (Zakaria et al., 2012; Anjana et al., 2013). As well as One of the important reasons for the use of the powder of earthworm in the treatment infection with *Entameba histolytica* is the pH values, where the measurement of pH value was found to be pH=5.24 as well as for the metronidazole where it was pH=3.97 as well as it is known that this parasite prefers to live in pH neutral and thus lead to the death of the parasite because of changing environment in which parasite lives (Neall, 1967).

**Table 1:** Counting number of parasites according to groups and time (mean ± SD) x 10^2

<table>
<thead>
<tr>
<th>The animal Groups</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.48</td>
<td>1.38</td>
<td>1.54 a</td>
<td>1.51 a</td>
<td>0.96 a</td>
<td>3.22 a</td>
<td>1.72 a</td>
<td>1.52 a</td>
<td>2.07 a</td>
<td>2.09 a</td>
<td>2.09 a</td>
</tr>
<tr>
<td>TEP</td>
<td>8.40 ±</td>
<td>7.63 ±</td>
<td>5.60 ±</td>
<td>10.46 ±</td>
<td>5.73 ±</td>
<td>5.16 ±</td>
<td>5.03 ±</td>
<td>2.83 ±</td>
<td>0.36 ±</td>
<td>0.00 ±</td>
<td>0.00 ±</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>0.56 b</td>
<td>0.85 b</td>
<td>1.42 a</td>
<td>1.71 b</td>
<td>2.01 b</td>
<td>1.48 b</td>
<td>1.51 b</td>
<td>0.55 b</td>
<td>0.00 b</td>
<td>0.00 b</td>
</tr>
<tr>
<td>TM</td>
<td>8.80 ±</td>
<td>9.13 ±</td>
<td>7.40 ±</td>
<td>10.46 ±</td>
<td>6.16 ±</td>
<td>5.36 ±</td>
<td>3.26 ±</td>
<td>1.50 ±</td>
<td>0.00 ±</td>
<td>0.00 ±</td>
<td>0.00 ±</td>
</tr>
<tr>
<td></td>
<td>0.95</td>
<td>0.89</td>
<td>0.26 ab</td>
<td>1.42 a</td>
<td>1.62 b</td>
<td>1.05 b</td>
<td>1.70 b</td>
<td>1.57 b</td>
<td>0.00 b</td>
<td>0.00 b</td>
<td>0.00 b</td>
</tr>
<tr>
<td>LSD value</td>
<td>2.18 NS</td>
<td>2.04 NS</td>
<td>3.13 *</td>
<td>2.64 *</td>
<td>2.79 *</td>
<td>4.54 *</td>
<td>3.88 *</td>
<td>3.21 *</td>
<td>1.86 *</td>
<td>2.38 *</td>
<td>2.41 *</td>
</tr>
</tbody>
</table>

* (P<0.05), NS: Non-Significant.

Means having with the different letters in same column differed significantly.
Concentration of sIgA in intestinal tissue: The major component responsible for the intestinal immune response against amebic infection is secreted IgA. IgA reduces trophozoite colonization in the colon (Haque et al., 2006; Ravdin et al., 2003; Huston et al., 2003). Secretory IgA is one of the most abundant Immunoglobulin (Ig) produced by plasma cells and functions by preventing pathogens from adhering and removing the mucosal barrier (Lamm, 1998).

The concentration of sIgA was determined by using ELISA-kit. The results showed in Table 3. In the positive group (infected group), the increase gradually of the sIgA concentration since the third day was (3.221 ± 1.01 ab), in the seventh day became (3.370 ± 1.06 a) and reached to (3.580 ± 1.13 ab) in ninth day compared to control (1.995 ± 0.63 b, 1.958 ± 0.06 b, 1.779 ± 0.56 b) and this increased naturally when infection occurs because cysteine proteinase was virulence factors of the parasite works to lysis IgA in the mucosal layer and because the immune response also lead to increase (Katherine & William, 2011; Ryan & Ray, 2014), but in the earthworm powder group (none infected) it was observed approximate ratios to the negative group after three days of administration earthworm powder (2.064 ± 0.65 ab) and (2.17 ± 0.68 ab) in the seventh day to reach in the ninth day (1.901 ± 0.60 b) which are approach to control negative group. In the treatment earthworm powder group, the concentration of sIgA in the third day was record (4.084 ± 1.29 a), in the seventh day noted the concentration of sIgA decrease (2.588 ± 0.82 ab) and in the ninth days (2.563±0.81 ab) noted less than the seventh day and the values between the seventh and ninth day are approximate due to the absence of significant differences but found significant differences between (the seventh day and the ninth day) with the third day, When earthworm powder given orally that led to enhance mucosal immune response against parasite also increase sIgA-secretion (Prabha & Shathya, 2014; Ansari & Sitaram, 2011), sIgA is considered the first line of specific defense against natural infections in the vast area occupied by mucosal surfaces (Woof & Kerr, 2006). These results reflect the ability of secretory IgA antibodies to prevent of E. histolytica adherence to epithelial cells. Mucosal IgA antilectin antibody response is associated with immune protection against E. histolytica colonization (Haque, et al., 2001). Also, earthworm powder stimulates the Goblet cells in Mucosa blanket in the intestine to release mucus in large quantities, which prevents the association lectin with (Galactose/N-acetyle- D-galactosamine) to the host cells thus preventing adhesion the trophozoite with Mucosa blanket in the intestine and the process of adhesion is one of the most important virulence factors of the parasite (Shinjiro, et al., 2006). Carrero et al., 1994 Found that the secretory (IgA) anti-E. histolytica antibodies in the saliva of patients with intestinal amoebiasis an inhibit amebic adherence to a monolayer of MDCK (Madin-Darby canine kidney) cells. In the metronidazole treatment group, the concentration of sIgA in the third day noted (4.057 ± 1.28a), while the concentration in the seventh day was less (2.553 ± 0.81ab) then returned decreased in the ninth day (2.528 ± 0.71ab) and the values between the seventh and ninth day are approximate due to the absence of significant differences but found significant differences between (the seventh day and the ninth day) with the third day, the metronidazole work to kill the parasite because it broke down the DNA helical structure of Entamoeba histolytica and also prevents the bacteria and protozoa from forming new DNA (Mudry et al., 2001; Lo¨fmark, 2010), this lead to reduce the numbers of parasite and decreased sIgA.

Table 2: The percent reduction in E.histolytica parasites shedding among treated groups.

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Percent reduction in parasite shedding %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEP</td>
<td>61.5</td>
</tr>
<tr>
<td>TM</td>
<td>63.1</td>
</tr>
</tbody>
</table>

Table 3: Mean differences of sIgA concentration mg/ml according to groups and time (mean ± SD)

<table>
<thead>
<tr>
<th>The animal Groups</th>
<th>3</th>
<th>7</th>
<th>9</th>
<th>LSD value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control “(ve”)</td>
<td>1.995 ± 0.63 b</td>
<td>1.958 ± 0.06 b</td>
<td>1.779 ± 0.56 b</td>
<td>0.647 NS</td>
</tr>
<tr>
<td>Control + “(ve+)”</td>
<td>3.221 ± 1.01 ab</td>
<td>3.370 ± 1.06 a</td>
<td>3.580 ± 1.13 ab</td>
<td>0.755 NS</td>
</tr>
<tr>
<td>M</td>
<td>4.057 ± 1.28 a</td>
<td>2.553 ± 0.81 ab</td>
<td>2.528 ± 0.71 ab</td>
<td>1.251 *</td>
</tr>
<tr>
<td>EP: “ve”</td>
<td>2.064 ± 0.65 ab</td>
<td>2.17 ± 0.68 ab</td>
<td>1.901 ± 0.60 b</td>
<td>0.633 NS</td>
</tr>
<tr>
<td>EP: “ve+”</td>
<td>4.084 ± 1.29 a</td>
<td>2.588 ± 0.82 ab</td>
<td>2.563 ± 0.81 ab</td>
<td>0.956 *</td>
</tr>
<tr>
<td>LSD value</td>
<td>2.084 *</td>
<td>1.157 *</td>
<td>1.274 *</td>
<td>---</td>
</tr>
</tbody>
</table>

* (P<0.05), NS: Non-Significant.

Means having with the different letters in same column differed significantly.
**Histological study:** The histological study showed that earthworm powder was able to repair the structure of intestinal tissue when it is treating the infected mice Figure 4, when compared with controls negative Fig. 1, Which showing normal intestinal tissue. In the mice infected (control positive) showing necrosis and infiltration of lymphocyte and damage in mucosal tissue (Fig. 2). In the Figure 5, the metronidazole drug reappeared the tissue but not completely still proliferation of goblet cells, hemorrhage, infiltration of lymphocyte and necrosis. As well as in the earthworm powder group (none infected) causes proliferation in the number of intestinal goblet cells of the mice (Fig. 3).

![Figure 1: Cross section in large intestine of mice non-infected (control negative) showing normal structure of intestine. (40x) H&E](image1)

![Figure 2: Cross section in large intestine of mice infected (control positive) showing necrosis and infiltration of lymphocyte (1) and damage in mucosal tissue (2). (40x) H&E](image2)

![Figure 3: Cross section in large intestine of mice (non-infected) inoculation with earthworm powder causes proliferation (1) in the number of intestinal goblet cells. (40X) H&E.](image3)
Earthworms have been utilized in Medicine for various remedies since 1340 AD (Hossam et al., 2012; Hamza and Rashid, 2017) and known in oriental medicine as anti-inflammatory antipyretic agent (Prakash & Gunasekaran, 2010). Earthworms have been recognized for many centuries as a therapeutic drug source for various diseases in China and another part of the far east (Ismail, 2005), there are no side effects, safe for all ages (very good for children and adults), safe to consume in the long period and continuously, also enhance the drugs work and safely used in conjunction with doctor medication. Diseases can be cured by earthworms: Type of earthworm which usually used as a natural medicine is *Lumbricus rubellus*, *Pheretima* sp. and *Eisenia fetida*. These earthworms are useful for: treat digestive tract infections such as typhus, diarrhea, dysentery and other stomach disorders such as an ulcer (Fang et al., 1999).

Present study focused on anti-parasitic properties in the earthworm powder which may have applications indirectly implicated in treatment of amoebiasis. Prakash & Gunasekaran (2011) suggested that the dried earthworm powder shows a strong antibacterial activity against the *S. aureus*, *P. aeruginosa* and *P. mirabilis* bacterial strains.
because earthworms respond to microbial infection through cellular and humoral defense mechanisms such as antimicrobial protein secretions. Several experiments carried out in Guyana for isolating enzymes from the earthworm powder and converting it into dietary supplement, like Lumbrokinase (Gao & Qin, 1999). Shobha & Kale, (2008) remember the gut of earthworm has antibacterial and antifungal activity. Earthworms powder are also working to repair damaged intestinal walls as some nutrient rich in proteins 65%, carbohydrates 19% and fats 16% (Zakaria et al., 2012; Anjana et al., 2013), also earthworms promote the goblet cells to produce of the mucus. As well as earthworm tonic properties make it helpful support for the liver and other organ systems (Govindra et al., 2016). In earthworms isolate from coelomic fluid protein called lysernin is containing on 297 amino acids and causes contraction of vascular smooth muscles, lysernin present with vesicles containing sphingomyelin amino acid its ability on the permeability in the membrane of bacteria during related to lipoprotein like lock and key and the end destroyed bacteria (Kiyokawa et al., 2004; Kobayashi et al., 2004; Bruhn et al., 2006; Vasanthy et al., 2013).

Conclusion:
The reduce in the number of the parasite in the vivo and to the repair the intestine tissue after using the earthworm powder and compared with drug metronidazole and control consider as a good indicator of the possibility of using earthworm powder to cure or mitigate the symptoms for Amoebiasis.

REFERENCES


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