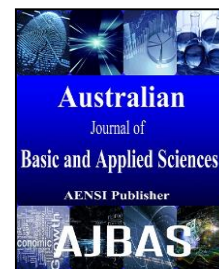




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Investigation the Role of Earthworm powder as anti -leishmanial Activity

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ABSTRACT

Earthworms have been used in medicine for various remedies, In the present investigation, various concentration extracts of an earthworm powder was prepared from died adult and tested against *Leishmania donovani*. It was found that 100mg /ml water extract of earthworm powder was a potent anti-parasitic agent against *Leishmania donovani*. All concentrations *in vitro* showed a clear effect on the parasite just after five minutes but the most effect is the concentration of 100 mg /ml until it reached the numbers of the parasite to 6.2×10^6 cell \ ml after 24 hours . when gave earthworm powder orally to infected mice with parasites was observed that after the passage of 21 days occur reduce the numbers of the parasite in the liver tissue significantly compared with the drug pentostam . also noted that the earthworm powder causes repair in the liver tissue and that there is no damage noticed of the intestinal tissue. These results suggest that the indigenous earthworm powder could afford a significant hepatoprotective and anti-parasitic activity.

INTRODUCTION

Leishmaniasis, disease caused by an obligate intra-macrophage protozoa a, transmitted by the bite of female sand fly this disease is characterized by both complexity and diversity (Herwaldt,1999). Several species of *Leishmania* caused human diseases that range from self-healing cutaneous lesions to fatal visceral (WHO,2003). Visceral leishmaniasis (VL, kala-azar) is an estimated annual incidence of 500,000 with prevalent in 62 countries (Sundar and Chatterjee, 2005). It caused by *Lishmania donovani*, *L. infantum* and *L. chagasi*, which when untreated can cause 70,000 deaths per year (Murray, 2005).Clinical symptoms of infection include hepatosplenomegaly, fever, weight loss, anaemia and leukopenia (Zijlstra and el-Hassan,2001; Dedet and Pratlong,2003).There is no antileishmanial vaccine, but there are drugs available to effectively treat VL, and among the most commonly used are pentavalent antimonials (Glucantime, Pentostam), Miltefosine, Pentamidine and Amphotericin B. However, all of these drugs have now been associated with drug toxicity and resistance (Croft *et al.*,2006). Earthworms play a good role in environment, have been used in medicine for various remedies since 1340 AD (Omar *et al.*,2012). The powder of earthworm contain carbohydrate, fats, minerals and various types of vitamins (Zakaria *et al.*,2012) and play a good role in pharmaceutical as an anticancer, antibiotic, antihyperglycemia and antihypotension (Prakash & Gunasekaran, 2011), that powder have antimicrobial activity for many kinds of bacteria and fungi (Vasanthi *et al.*, 2013; Bhorgin and Uma,2014),but there are no any reports about its role against parasite.That is because the incidence of treatment-resistant user against leishmaniasis came this research to look for the impact of The powder of earthworm against leishmaniasis parasite *in vivo* and in *in vitro*.

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MATERIALS AND METHODS

Earth worm collection:

Thirty two earthworm were collected from Orchard in AL-Dora region from April to end of July 2015 digging the soil and take the worm to the laboratory washed them in tap water to remove dirt from the body surface .

Powder extraction:

All the worms were soaked in distilled water to allow the soil in its tract to be excreted, later it's were washed with distilled water and collected in petridish that kept in an incubator for 24 h. at 55Co after that removed and pounded to make it into powder, stored in refrigerator of normal temperature (- 6) (Yegnanarayan *et al.*,1987). Then prepared different four concentrations (25% – 50% – 75% – 100%) mg / ml.

Leishmania parasite strain:

It was obtained from the department of biology science Baghdad University. *L.donovani* strain was cultured in NNN medium, then washed three times in phosphate buffered saline(PBS) PH7.2 and adjusted to concentration 1×10^7 promastegote/ml.

Animals:

Twenty-Four male albino mice aged 12-13 weeks, weighing 15-17gm were obtained from the animal house in collage of medicine Baghdad University were housed under standard condition. Then 18mice were infected by 1×10^7 promastegote .After one day the infected mice were divided into 4 groups each group contain 6 mice, the last 6 non infected mice remain as control negative. Then each group inoculated as a follow:

- 1) Group one: inoculated with (0. 1ml/day) from Earth worm materials every day.
- 2) Group two: injected with (0. 1ml/day) from pentostam by intraperitoneal each day.
- 3) Group three: inoculated with (0. 1ml/day) normal saline consider as control positive.
- 4) Group four (none infected): inoculated with (0. 1ml/day) normal saline consider as control negative.

After 21days post inoculation all the mice were scarified, liver, spleen and intestine were removed.

Histopathological changes:

Liver and intestine were removed and fixed in 10% formalin processed stain with hematoxylin and eosin for study histopathological changes.

The effect of powder earth worm on vital Leishmania donovani in vitro:

Parasites were collected on the fifth day after cultured and distributed solution containing the parasite on (30) tubes at (1ml) per tube contain(13×10^6 cell/ml) with addition earth worm materials concentration as follow:

- 1-The first group of (6) tube add (1ml) of solution for each lock tube as control group.
- 2-Second group (6) tube add (1ml) of earth worm materials with concentration (100 gm/ml) each tube.
- 3-Third group (6) tubes add (1ml) of earth worm materials with concentration (75g m/ml).
- 4-Fourth group (6) tubes add (1ml) of earth worm materials with concentration (50 gm/ml).
- 5- Fifth group (6) tubes add (1ml) of earth worm materials with concentration (25 gm/ml).

It was measured vial form of the parasite after almost (0,5min,15min,30min,1hr,24hr) of incubated degree 26° .

According to the method of Hodgkinson *et al.* (1980) by using Erythrocin-B stain 0.4%, then examined under the microscope was estimated percentage of vital cells according to the following formula:

$$\text{Percentage of vital} = \frac{\text{number of living cells}}{\text{the total number of cells}} \times 100\%.$$

Liver parasite burden:

Impression smears from liver were stained with Giemsa to evaluate parasite burden. The numbers of parasite per host cell nucleus was determined as described by Bardiy *et al* (1977) by counting 1000 host cells. The relative total numbers of parasites per organ named Leishman- Donovan units (LDU).

Statistical Analysis:

The data for various parameters were subjected to statistical analysis SPSS, software program using analysis of variance (ANOVA).

RESULTS ND DISCUSSION

Susceptibility of *Leishmania donovani* to earth worm powder was determined on both: the promastigote stages *in vitro* and the amastigote *in vivo*. The effect of (25, 50, 75 and 100 mg/ml) concentrations of earth worm powder on *L. donovani* promastigotes shown in figure 1. This figure showed highly decreased in the number of the parasites after only 5min of exposure in all earth worm powder concentrations especially the high concentration (75 and 100) in comparison with the control, also after 24hr the numbers of parasite reached to (6.2 and 7.6×10⁶cell/ml) for (100 and 75 mg/ml) respectively while (50 and 25 mg/ml) concentration reached to same number was 8×10⁶cell/ml.

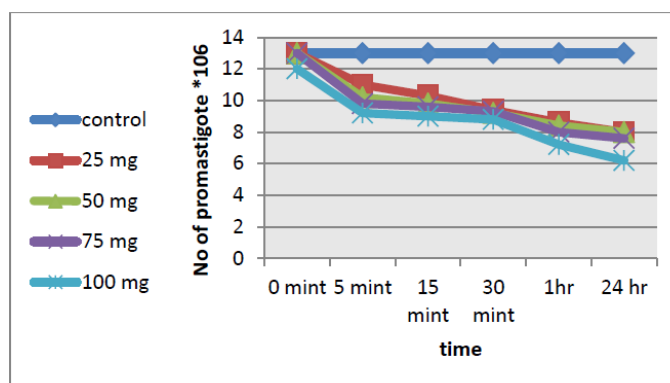


Fig. 1: The effect of different concentrations of earth worm powder on promastigote of *L. donovani*

Enumeration the numbers of parasite in liver and determination of LDU:

The results in table (2) showed that the number of parasites in liver after treated with earthworm powder and petnostam both of them lead to decrease the numbers of parasites in liver but petnostam drug more effective the number of parasite reached to only 300 cell /1000 host cell after 21 days while the earthworm powder was 460 cell /1000 host cell compared with control group was 2000 cell /1000 host cell there was high significant difference ($p \leq 0.005$) between all groups. Also the LDU (Leishman- Donovan units) for three groups (powder earth worm, petnostam and control)was (0.46, 0.3 and 1) respectively.

Table 2: Shows mean number of parasites, LDU (Leishman- Donovan units) and total LUD in liver of mice treatment with earth worm powder and petnostam compared with positive control.

Groups	No of parasites /1000 host cell	LDU
Positive control	2000±1*	1
Earth worm powder	460±0.8*	0.46
Pentostam	300±0.81*	0.3

*significant difference $P < 0.05$ between two groups and control group

Histological study:

This study showed that the earth worm powder non toxicity, it did not record any death of the mouse during the dosage as inoculated a concentration of 100% for a period of 21 days at a rate of 0.1 ml \ per day .this results agreement with histological study which observed when studying the histological section of the intestinal issue inoculated with powder earth worm cause an increase in the number of goblet cells compared with the control negative group which were given normal saline fig .(1) and fig .(2).As noted at the study of the liver tissue, that the powder efficient in reducing the numbers of parasite in the liver tissue and repair the damage that caused by the infected with *leishmania* parasite it has been noted that the parasite caused microgranulomas are formed in the liver parenchyma, hemorrhage and diffuse activation of Kupffer cells in liver parenchyma of control positive group fig. (4) compared with control negative group fig .(3) shows normal liver parenchyma tissue. while in pentostame inoculated group shows only infiltration of lymphocytes and hydropic degeneration in the liver parenchyma of mice treated with petnostame occur in addition reduce in number of parasites in tissue fig .(5).The efficiency earth worm powder approach to efficient of pentostame was observed occurrence infiltration of lymphocytes, hydropic degeneration, mild hemorrhage and reduce the number of activated kupffer cells in the liver parenchyma fig.(6).

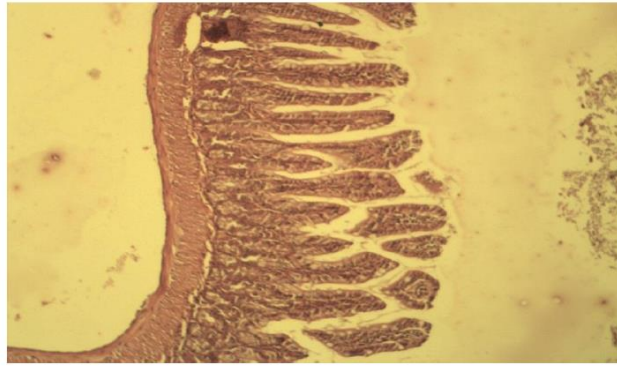


Fig. 1: normal intestinal tissue of mice in control group. H&E(40X).

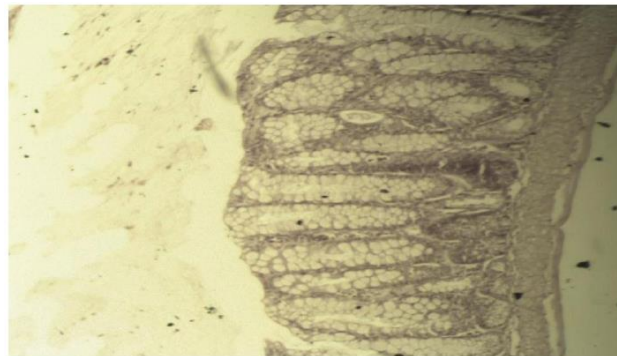


Fig. 2: intestinal mice inoculation with powder earth worm causes hyperplasia in intestinal goblet cells. . H&E(40X).

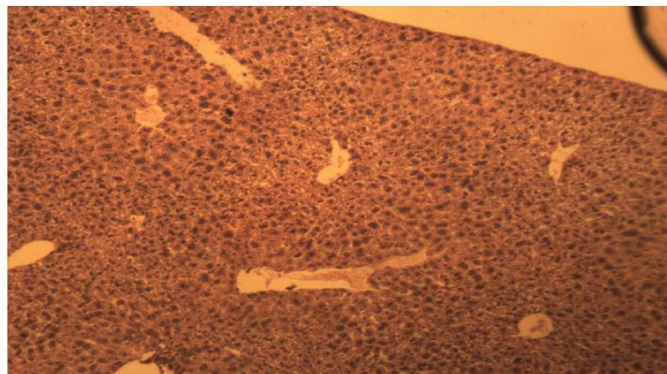


Fig. 3: Shows normal liver parenchyma in control negative group. H&E(40X).

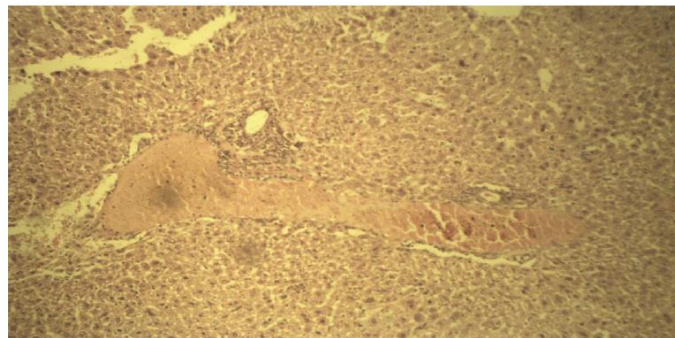


Fig. 4: Microgranulomas are formed in the liver parenchyma, hemorrhage and diffuse activation of Kupffer cells in liver parenchyma of control positive group.H&E(40x).

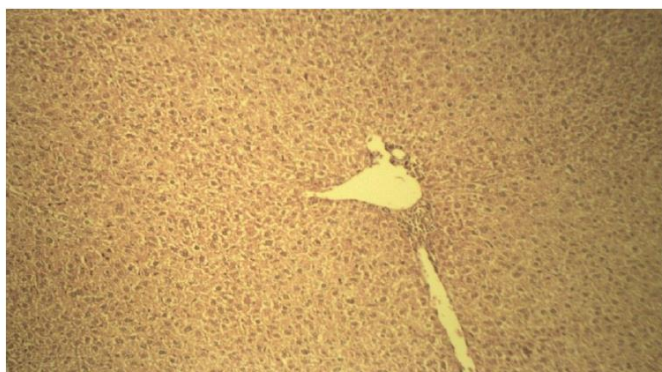


Fig. 5: infiltration of lymphocytes and hydropic degeneration in the liver parenchyma of mice treated with petnostam.H&E(40x)

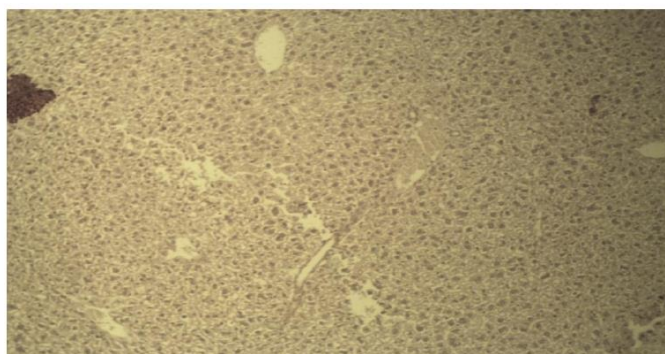


Fig. 6: infiltration of lymphocytes,hydropic degeneration,mild hemorrhage and reduce the number of activated kuffer cells in the liver parenchyma of mice treated powder earth worm. H&E(40x).

Earthworms have been known for many centuries as a therapeutic drug source for different diseases in china and another parts of the far east (Ismail, 2005). Present study focused on anti-parasitic properties in the powder of earthworm which may have applications indirectly implicated in treatment in treatment of Lishmaniasis. In Guyana, several experiments carried out for isolating enzymes from the earthworm powder and converting it into dietary supplement, like Lumbrokinase (Gao and Qin,1999). Popovic *et al* (2005) founded that can obtain from tissue homogenate of *Eisenia foetida*, Glycolipoprotein (G-90), obtains which exhibits as anti-bacterial properties, along with the anti-fungal properties in the powder of *Eisenia foetida* which may have applications indirectly implicated in treatment of diseases related to different microbes and fungi. The dried earthworm powder when administered orally to rats and human beings, has an antihyperlipemic effect, a blood sugar lowering effect and a blood pressure regulating effect but these effects not understood, may be due to the action of the proteolytic enzymes (proteins) consist in the dried earthworm powder (Mihara *et al.*,1996). Ansari and Sitaram(2010) determined that *Eisenia foetida* have anti-fungal properties, earthworm powder can inhibiting the growth of the fungus *C. albicans* and tested with water is more effective than when tested with acetone. Mathur *et al.*(2011) reported that 95% ethanolic extract of earthworm acts as the potent antibacterial agent against *Streptococcus pyogens* and antifungal agent against *Candida albicans*, also Bhorgin and Uma (2014) founded that the same concentration of earthworm was potent antifungal agent against *Candida albicans* and antibacterial agent against *Aeromonas hydrophila*. Earthworms have largely been used externally and internally as strong aphrodisiacs (Vohora and Khan,. 1978). Antimicrobial potency of *Eudrilus eugeniae* extracts on certain plant pathogens[24],Anti-inflammatory activity (Ismail *et al.*, 1992) and antipyretic (Balamurugan *et al.*, 2008). That earthworm powder exhibits potent antioxidant and hepatoprotective properties in alcohol-hepatotoxic in rats, because the administration of earthworm powder results in the elevation of these enzymes(Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), to near-normal values thus protecting the tissues from free radical damage (Prakash *et al.*,2008),typically, earthworm is used with other herbs to treat a wide range of conditions ranging from spasms and convulsions to pain relief, treatment of fever and certain type of arthritis. Earthworms are also used to treat some types of asthma and bronchitis. Recently earthworm protein and its coelomic fluid are known to have cytolytic, agglutinating, proteolytic, haemolytic, mitogenic, antipyretic and tumorstatic activities (Ismail *et al.*,1992; Lange *et al.*,1997). Earthworms compose and excrete several effective modulators of innate immune responses such as cytotoxic proteins,cytokines and antibacterial molecules. Prakash and Gunasekaran (2011) suggested that The dried earthworm powder shows a

strong antibacterial activity against the, *P. aeruginosa*, *P. mirabilis* and *S. aureus* bacterial strains because earthworms respond to microbial infection through humoral and cellular defense mechanisms such as antimicrobial protein secretions. The freeze-dried powder of *Lumbricus rubellus* earthworm was administered orally to rats found that the level of fibrinogen/fibrin degradation product (FDP) in serum was during the treatment period, these results back up that earthworm powder is worthy for the prevention and/or treatment of thrombotic conditions (Kim *et al.*, 1998). Prakash *et al.* (2008) suggest that the indigenous earthworm *Perionyx excavatus* could afford a significant hepatoprotective and antioxidant effect against alcohol induced rats. Earthworms have many benefits, many people who eat it to cure some disease, without effects, so safe to consume. According to the experiences of people who had experienced a fever, earthworms eats their disorders improved and according to research that earthworms are very high source of protein also contains several amino acids with a high level earthworm powder prepared from *Perionyx excavates* has antifungal and antibacterial properties, In Iran, dried earthworms were used for treat jaundice, also earthworm tonic properties make it helpful support for the liver and other organ systems (Govindra *et al.*, 2016). The activity of test drug is measured by either microscopical counting of percentage of infected cells or number of mastigotes/macrophage or by colorimetric or fluorometric methods. The slow rate of division of *L. donovani in vitro* (Gupta and Nishi, 2011).

Therefore, the reduce in the number of the parasite in the tissue and in the glass in addition to the repair made in the liver tissue after using the earthworm powder and compared with drug Alaptoustam and control consider as a good indicator of the possibility of using earthworm powder to cure or alleviate the symptoms for leishmaniasis.

Conclusion:

In the present study it has been determined that earth worm powder acts as the potent anti-parasitic agent against *L. donovani*, This study may thus lead to formulation of new natural anti-parasitic agent.

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