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Estimation of antiparasitic activity of fungal extracts towards Leishmania donovani in murine model

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Abstract

Fungi produce many compounds have pharmaceutical usage. The current study was aimed to extract therapeutic compounds from fungi that grow in critical conditions and fungi that can be produced commercially which may have potential therapeutic activities against *Leishmania donovani*.

Two fungal isolates were used to evaluate antileishmanial activities of their extracts, the first due to *Aspergillus terreus* was isolated from salty sediments, while another represented local edible mushroom of *Agaricus bisporus*.

The experiment was conducted *in vivo* after four weeks infection of *Leishmania donovani*. Infected mice were treated intraperitonially with 2 and 4 mg/kg daily for 10 days.

All compound has a significant effect on the viability and decrease the number of parasites in animal in comparison with pentostam, as well as, the M19 was appeared with highest effect in both concentrations

The method of examining infected tissues had a role in determining the number of parasites without affecting the percentage of presence in different treatments, as the numbers of parasites calculated in the tissue sections are more realistic than the number of parasites calculated in the printing method, as counting the parasite with tissue sections gives an impression of the spatial distribution of the parasite, whether within tissues or body fluids, unlike the printing method, which shows the presence of the parasite in body fluids and immune cells thus be suitable for the diagnosis of parasite amastigote *in vivo*. The results showed that compound extracted during study and diagnoses by GC-Mass technique are promising applicants for research on new drugs with anti-Leishmania activity derived from natural products.

Keywords: *Leishmania donovani*, pentostam, amastigote, Fungi

INTRODUCTION

Leishmaniasis is an ignored equatorial disease affecting the world's needy communities in concluded more than 90 countries throughout Asia, Africa, the Mideast, and America. Even

though, recent reports have been recorded evaluations of cutaneous leishmaniasis (CL) prevalence range from 700,000 to 1.2 million cases per year. As well as. The determination of annual visceral leishmaniasis (VL) are presently less than 100,000, which is a substantial reduction from previous estimates of 400,000, with more than 95% of case reported to the World Health Organization (WHO) from Brazil, China, Ethiopia, India, Kenya, Nepal, Somalia, Saudia Arabia and Sudan [1,2].

Predisposing reasons for leishmaniasis comprise deficiency, population movement, malnutrition, deprived sanitation, and an immunocompromised individual [3].

At the same time, the frequency of drug-resistant parasites has greatly increased and most treatments involve highly toxic drugs. In addition, the chemotherapeutic agents used in patients with these diseases have lacked effectiveness. Thus, there is an urgent need to search for novel drugs from previously unexplored sources, including natural products, to combat the global health problems posed by parasitic infections [4].

Lately, researchers have great consideration related with study of fungal bioproducts that have biological activities in different fields as antimicrobial, antifungal, anti-inflammatory, antiparasitic, antioxidants, and antidiabetic properties [5]. Fungi that isolated from extreme habitat are characterized to be suitable source of various compounds due to huge number of chemical groups like alkaloids, steroid, terpenes, tannins, phenols, glycosides, saponins [6].

Furthermore, macro fungi cooperatively mentioned to as mushrooms, are dispersed around the world, with about 14,000 species universally [7]. The greatest cultured eatable mushrooms global are *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (enoki mushroom), *Lentinula edodes* (shiitake mushroom), and *Pleurotus* spp. [8].

Recently, in Iraq there are many attempts to breeding and cultivation of *Agaricus bisporus*, which has directed to increased mushroom [9,10].

Different macrofungi have been discovered as sources of important secondary metabolites and have the potential to be established as food additions for medicinal applications [11].

Besides to the lack of highly effective drugs against parasites, and the long period of their treatment that ranged from one month to a year, which leads to requirement the search for other alternative treatment which assist to reduce the percentage of infections [12].

The current study was aimed to extract therapeutic compounds from fungi that grow in critical conditions and fungi that can be produced commercially which may have potential therapeutic activities against *Leishmania donovani*.

Materials and Methods

Two fungal isolates were tested in this study, the first one was *Aspergillus terreus* which isolated from sea coast sediment of Al-Faw region during August 2021by Dilution method described by the Wittingham and Wicklow [13] in 1974.The second fungal isolates represent fruiting bodies of edible mushroom *Agaricus bisporus* which obtained from Al- Wadq Company located in Abu Ghraib, Baghdad, Iraq.

Cultivation of *Aspergillus terreus* on the fermentation medium

Aspergillus terreus grown on potato dextrose broth PDB as a fermentation medium to obtain the effective metabolites. Glass flasks of 250 ml capacity containing 150ml of PDB medium were incubation with two discs taken by cork borer from the colony, then the flasks were incubated at a temperature of 25 °C for a period of 21 days [14].

Extraction of effective compound from liquid media.

The active substances were extracted from the liquid cultures after the end of the incubation period by adding an equal volume of an organic solvent Ethyl acetate to the liquid media, the mixture was filtered using Whatman No.1 filter papers to isolate the mycelium from the remains of the culture media, the filtrate was transferred to a separation funnel with shaking for several minutes. The upper layer containing organic materials was taken and dried at room temperature in a glass beaker and kept until use, whereas the lower layer was neglected because it contained the remains of water and the culture medium [15].

Extraction of secondary metabolites from mushrooms

The extracting antiparasitic compounds from edible mushrooms was done according to method of Isaca and co-author [16] in 2020 with some modification as described below:

Seven kilograms of edible white mushroom (*Agaricus bisporus*) left to dry at room conditions. Six hundred and fifty grams of dried fruiting bodies were cut into small pieces, blended, and soaked in chloroform $CHCl_3$ (3.250 L) at room temperature for 7 days. The mishmash was sieved with gauze cloth and left to dry. The previous extraction method was constant for the same product (650 gm of fruiting bodies) to obtain a dark brown gum ($CHCl_3$ extract, 45 g).

Then, the filtrate was dissolved in an assortment of 400 ml solvents of $CHCl_3$, CH_2Cl_2, EtoAc, and CH OH (1:1:1:1), after that, the deposit was left to dry at room temperature (20 gm). The residual fungal material (biomass) was then extracted with MeOH (2 L, 6 days) to obtain a dark brown gum (MeOH extract, 42g), as well as, the methanol extract was dissolved with same solvents above ($CHCl_3$, CH_2Cl_2, EtoAc, and CH OH) and dried to get 10 gm of dark brown gum material.

Column chromatography (CC)

The chloroform and methanolic extracts that yielded from above paragraph were subjected to column chromatography (CC) on silica gel (3× 60 cm, EtOAc–$CHCl_3$, step gradient elution 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0, and then with acetone) to obtain 7 fractions, F-1–F-7.

Furthermore, methanol (MeOH) extract was exposed to column chromatography (CC) on silica gel (3× 60 cm, acetone–hexane, step gradient elution 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0) to acquire 7 fractions, M-1–M-7.

After purification by using column chromatography, some of fractions were appeared and collected with suitable amount that used for different experiments and named M9, M10,M11, M12, M16, M17,M18, M19,as well as, M13 referred to the methanolic crude extract and F14 represented the chloroform crude extract.

The current study was included the investigation of activity for F14 and M19 in addition to crude extract of *A.terreus* as antileishmanial agents *in vivo*.

Analysis of Gas Chromatography/MS

The crude extract of *A. terreus*, crude chloroform (F14) and fraction(M19) of *A. bisporus* were prepared for measurement by gas mass spectrometry technique, Japanese-made and located in the Basra Oil Company, Nahran Omar site, Shimadzu type1. The materials were equipped with a capillary column of 5% Phenoxyle methyl siloxane HP-5MS as a static phase whose dimensions are 30 cm in length, 0.25 mm in diameter, and the thickness of the stationary phase is 250 µm. Using helium gas as a carrier gas, the spectra of the curves were identified according to the spectral library.

Animal experiments

The effect of fungal extracts was examined against *Leishmania. donovani* (promastigote stage) which was obtained from the Leishmania Center of Al-Nahrain University/ Baghdad/Iraq and isolated from an 18-month child in the Al-Taji area, (Moham/IQ/2005/MRCIO).

Effect of fungal extracts on *L. donovani* in mice

The biological activity of *A. terreus* extract, M14 and F 19 was examined on laboratory mice with two concentrations for each extract (2 and 4 mg/kg).

Activation of parasite

The activation was occurred by injection of three rats intra-peritoneally with 0.1 ml of 1×10^4 parasites per rat.

After six week of infection the animals were sacrificed and the liver was isolated and squashed to make the suspension with normal saline by strong shaking. Immediately, the suspension was sieved before settling the ingredients to remove large pieces of tissue.

The infiltrate centrifuged at 4000 rpm/min for 3 minutes, the supernatant was removed and the sediment was suspended in 1 ml of normal saline. The parasite was counted then the number of parasites prepared to get 100 amastigotes for 0.1 ml by adding suitable amount of normal saline.

Infection of laboratory mice

Forty-two male mice of BALB/C strain were used with weights ranging between 25-30 gm. All animals were allowed to adapt for one week, in the animal house of College Science, Basrah University and supplemented with a suitable amount of food and water.

Each animal was injected with 100 amastigote/0.1 ml peritoneally and left for three weeks to spread the parasite in the animal tissues.

Treatment of laboratory mice

After three weeks the infected mice were isolated into groups, 3 mice per each group in different cages and daily injected with 0.1 ml of extracts F14 and M19 at 0.05 and 0.1 mg/per mouse (2 and 4 mg/kg) daily for 21 days.

Additionally, two groups of mice were also daily injected with 0.1 and 0.01 mg /mice (4and 0.4 mg /kg) of Pentostam, as well as, three mice were injected with 0.1 ml of DMSO, while, another three infected mice were left untreated as a control group.

Dissection of laboratory mice

At the end of the experiment, all the experimental mice were dissected. The mice were anesthetized with chloroform. Liver and spleen were removed, tissue prints and bone marrow

smears were made on slides, The samples were dried, fixed with methyl alcohol, and stained with Giemsa Stain. Then washed, dried, and mounted with DPX, and cover slide.

The parasite counted in 1 mm^2 area of slides, with three replicates for each experimental animal. small portions of liver and spleen were transformed in 10% formalin to prepare tissue sections according to Dewi and Purwanto [17] in 2023 for counting the parasite, while bone marrow infection diagnosed by smear preparation.

Result and discussion

Three fungal extracts were used as antiparasitic treatment and compared with standard drug (pentostam), DMSO (positive control) and control treatment (negative control).

GC-Mass analysis revealed presence of thirty-three compounds in the crude extract of *A.terreus* table (1)the most visible was 4-Hydroxy-3-(3-methyl-2-butenyl)benzaldehyde (4HMBA) with percentage17.4941 % in retention time of 21.242 min. This compound is the official name for Lapachol and recommended by Ramos and co-author [18] in 2023 as alternative drug derived from natural products for *Leishmania amazonensis*, which led to potential activity to loss of mitochondrial membrane, changes in the integrity of the membrane, damage to cells suggestive of the apoptotic process, and showed inhibition of tumor necrosis factor (TNF)-a and interleukin (IL)-6 production.

Also there are a new glyoxylate containing derivative of 4HMBA were isolated from the marine algicolous fungus, *Aspergillus* sp. [19].

Aspergillus terreus represents a hopeful prospective source for drug discovery since it is rich in various categories of bioactive secondary metabolites. It contributed to many biotechnological applications and its metabolites are used in the synthesis of certain pharmaceuticals and food products, in addition to its useful uses in fermentation processes. There are about 346 compounds identified from aquatic and terrestrial-derived *A. terreus* , besides that Amr and his colleges [20] in 2023 denoted existence of 172 compounds of *A. terreus* which proved a vast array of bioactivity from 1987 until 2022.

Table (1) Chemical composition of *A. terreus* extract as appeared in GC-Mass analysis:

Peak	R.T.	Area	Area Pct	Library/ID

1	6.447	2550658	4.1059	Butanoic acid
2	7.325	766258	1.2335	2-Pentanone, 4-hydroxy-4-methyl-
3	7.996	3665275	5.9002	p-Xylene
4	8.505	1723938	2.7751	p-Xylene
5	9.028	412928	0.6647	Thiophene, tetrahydro-2-methyl-
6	9.427	2474919	3.984	3-Heptanone, 4-methyl-
7	15.88	579429	0.9327	Benzyl nitrile
8	17.185	1338551	2.1547	2H-1-Benzopyran, 3,4-dihydro-2,2-dimethyl-
9	17.857	367801	0.5921	1,3-Benzenediol, 4,6-dichloro-2-methyl-
10	18.247	701312	1.1289	3-Methoxycyclohepta(B)furan-2-one
11	18.572	823137	1.325	Benzene, 1-(1,1-dimethylethyl)-3-ethyl-5-methyl-
12	19.287	1168211	1.8805	Diethyl Phthalate
13	19.811	349820	0.5631	3-(4-Isopropylphenyl)-2-methylpropionaldehyde
14	20.585	4931535	7.9386	3-Hydroxy-4-methylbenzaldehyde
15	20.873	590700	0.9509	1-(2-Hydroxyethyl)-3-methyl-6,7-dihydro-1H-indazol-4(5H)-one
16	21.242	10867570	17.4941	4-Hydroxy-3-(3-methyl-2-butenyl)benzaldehyde

17	21.655	1551930	2.4982	Furo[3,2-c]quinoline-2,4(3H,5H)-dione
18	21.825	472789	0.7611	Benzoic acid, 2-amino-3,4,5-trimethoxy-, methyl ester
19	22.481	355900	0.5729	1H-Pyrrole, 1-acetyl-5-(1-formyl-2-piperidinyl)-2,3-dihydro-
20	23.041	687638	1.1069	n-Hexadecanoic acid
21	23.786	2789493	4.4904	2,4a-Methanonaphthalen-7(4aH)-one, 1,2,3,4,5,6-hexahydro-1,1,5,5-tetramethyl-, (2s-cis)-
22	24.17	479510	0.7719	4-Amino-5,6-dimethylfuro[2,3-d]pyrimidine
23	24.635	583796	0.9398	9,12-Octadecadienoic acid (Z,Z)-
24	25.129	1141539	1.8376	Butyl citrate
25	25.726	453328	0.7297	Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)-
26	27.474	326372	0.5254	trans-3-Ethoxy-b-methyl-b-nitrostyrene
27	27.725	2718386	4.3759	Benzenemethanamine, N,N,.alpha.-trimethyl-, (S)-
28	28.027	8157526	13.1316	Bis(2-ethylhexyl) phthalate
29	29.473	5425425	8.7336	1,3-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
30	30.033	506826	0.8159	4-(4-Hydroxyphenyl)-4-methyl-2-pentanone,

				TMS derivative
31	30.173	631969	1.0173	2-Methyl-7-phenylindole
32	30.52	1004173	1.6165	Benzo[h]quinoline, 2,4-dimethyl-
33	34.569	1522599	2.451	1,2-Bis(trimethylsilyl)benzene

Beside, twelve compounds were detected from F14 (chloroform extract of edible mushroom) as shown in table (2), ergosterol appeared at a very high percentage reaching 43.44% in retention time of 33.013 min. Selection of secondary natural metabolites from Basidiomycetes provided potential scaffolds against neglected parasitic diseases such as Chagas disease (American trypanosomiasis) and protozoan parasites, such as *Plasmodium*, *Trypanosoma*, and *Leishmania* [21,22].

Table (2) Chemical composition of (F14) of Mushroom *Agaricus bisporus* extract as appeared in GC-Mass analysis:

Peak	R.T.	Area	Area Pct	Library/ID
1	16.92	927462	4.3064	1-Tetradecene
2	18.277	2999037	13.9252	2,4-Di-tert-butylphenol
3	19.214	1168431	5.4253	Cetene
4	21.389	1048883	4.8702	1-Octadecene
5	23.1	777388	3.6096	n-Hexadecanoic acid
6	23.336	1006050	4.6713	5-Eicosene, (E)-
7	24.671	727899	3.3798	9,12-Octadecadienoic acid (Z,Z)-

8	25.136	552172	2.5639	5-Eicosene, (E)-
9	26.81	469681	2.1808	1-Nonadecene
10	31.147	1161436	5.3928	(E)-2-bromobutyloxychalcone
11	33.013	9355568	43.44	Ergosterol
12	33.256	1342765	6.2348	Silicic acid, diethyl bis(trimethylsilyl) ester

Additionally, Twenty seven chemical compounds were appeared in the white mushroom extract (M19) among them Bis (2-ethylhexyl) phthalate (DEHP) was seen with percentage 29.1153% in retention time of 26.685min. table (3). This compound is an organic oil known to be anti-bacterial and anti-fungal [23], as well as, (DEHP) isolated from *Aspergillus awamori* had exhibited activity against *Candida albicans* and the Gram positive bacteria *Sarcina lutea*, also it showed cytotoxic activity against some carcinoma cell lines [24].

Another compound that appeared with a relatively high percentage was 18-Aminoabieta-8,11,13-triene with percentage 11.6111% in retention time of 27.88min. This compound has given promising result as Anti-cryptosporidial that caused by *Cryptosporidium parvum in vitro* [25].

It was found that all the fungal extracts in the current study contained the Furan group (five-ring). this compound were well known as antiparasitic, although many studies have proven its effect on fungi and bacteria [26,27].

Table (3) Chemical composition of fractions (M19) of Mushroom *Agaricus bisporus* extract as appeared in GC-Mass analysis:

Peak	R.T.	Area	Area Pct	Library/ID
1	14.036	42152385	0.8975	Indole
2	16.286	2.13E+08	4.5303	2,6-Di-tert-butyl-4-hydroxy-4-

				methylcyclohexa-2,5-dien-1-one
3	21.684	70105355	1.4926	n-Hexadecanoic acid
4	21.92	90038021	1.917	n-Hexadecanoic acid
5	22.85	43995241	0.9367	9,12-Octadecadienoic acid, methyl ester
6	23.499	2.19E+08	4.6582	Linoelaidic acid
7	24.435	4.32E+08	9.205	N,N-Dimethylpalmitamide
8	25.173	44420833	0.9458	1,4-Benzenediamine, N-(1,3-dimethylbutyl)-N'-phenyl-
9	25.453	43637181	0.9291	Pentadecane
10	26.294	2.31E+08	4.9261	Heptadecane
11	26.685	1.37E+09	29.1153	Bis(2-ethylhexyl) phthalate
12	27.644	98882287	2.1053	1H-isoindole-1,3(2H)-dione, 2-(5-ethyl-8-methoxy-1-naphthalenyl)-
13	27.754	1.88E+08	4.0036	Furan-2-carboxamide, 5-nitro-N-(benzothiazol-2-yl)-
14	27.88	5.45E+08	11.6111	18-Aminoabieta-8,11,13-triene
15	28.182	1.08E+08	2.3088	3-Hydroxy-4-methoxyxanthone, Me derivative
16	28.3	2.8E+08	5.9607	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
17	28.403	59628205	1.2696	Phthalic acid, 3,3-dimethylbut-2-yl tridecyl ester

18	28.551	92107097	1.9611	Tetracosane
19	28.794	85353411	1.8173	Phthalic acid, neopentyl nonyl ester
20	28.905	79226920	1.6868	Di-isononyl phthalate
21	29.185	57065983	1.215	Tetracosane
22	29.716	46460268	0.9892	Oxazole, 2-(3-methoxyphenyl)-5-phenyl-
23	29.812	62732502	1.3357	Triacontane
24	30.166	44281667	0.9428	1,4-Benzenediol, 2-octadecyl-
25	30.439	46271353	0.9852	Tetracosane
26	31.051	51550937	1.0976	3,6-Dimethoxy-2-(10-pentadecenyl)phenol
27	33.433	54322942	1.1566	1'H-Androst-16-eno[16,17-b]indol-3-ol, 1'-methyl-, (3.beta.,5.alpha.)-

The effect of pentostam in the treatment of infected liver was greater than that of the bone marrow, whereas, the effect of the fungal extracts on the treatment of infected bone marrow was greater than that of other organs, although the remediation effects in the liver and spleen were greater than those of the other treatments in the table (4)

Table (4) *In vivo* effect of some selected extracts on *Lieshmania donovani* by using print method

Treatment	Marrow	Spleen	Liver	Total treatment
***Asprgillus terreus* 2 mg**	**1 ±0**	**3 ±1.41421**	**16.5± 0.70711**	**6.8333± 7.57408**
***A. terreus* 4 mg**	**2.5±2.12132**	**8.5±4.94975**	**10.5±2.12132**	**7.1667±4.53505**
F14 2 mg	**9±1.41421**	**5±1.41421**	**8.5±0.70711**	**7.5±2.16795**

M14 4 mg	**1.5±0.70711**	**5±4.24264**	**4.5±0.70711**	**3.6667±2.58199**
M19 2 mg	**1±0**	**6.5±0.70711**	**8±1.41421**	**5.1667±3.37145**
M19 4	**1 ± 0**	**4.5±2.12132**	**3±1.41421**	**2.8333 ± 1.94079**
Pentostam	**20.5±3.53553**	**17.5±3.53553**	**7.5±0.70711**	**15.16 ± 6.49**
DMSO	**16.5±13.43503**	**15±4.24264**	**12.5±6.36396**	**14.66±7.14**
CONTROL	**20.5±7.77817**	**46.5± 13.43503**	**22±4.24264**	**29.66±14.90**
Total organs	**8.1667±9.30054**	**12.388± 13.878**	**10.33 ± 6.116**	**10.29 ± 10.22**
L.S.D= 4.12				**L.S. D= 0.87**

Treatment(df= 8,f=261,p=0.0), Organs(df =2, f=19.6,p=0.0)

Table (5) *In vivo* effect of some selected extracts on *Lieshmania donovani* by using section method

Treatment	**Spleen**	**Liver**	**Total treatment**
***Aspergillus terreus* 2mg**	**5±0**	**7.5± 0.70711**	**6.25±1.5**
***A. terreus* 4 mg**	**5±0**	**8±2.82843**	**6.5±2.38048**
F14 2mg	**2.5±0.70711**	**8.5±0.70711**	**5.5±3.51188**
F14 4 mg	**2.5±0.70711**	**2±0**	**2.25±0.5**
M19 4 mg	**1.5±0.70711**	**1.5±0.70711**	**1.5±0.57735**
Pentostam	**6.5±0.70711**	**7.5±0.70711**	**7±0.8165**
DMSO	**6.5±0.70711**	**6±0**	**6.25±0.5**
CONTROL	**7±0**	**6±0**	**6.5±0.57735**
Total organs	**4.5625± 2.09662**	**5.875± 2.72947**	**5.21±2.485**
L.S.D of organs= 2.35			**L.S.D of treatment=1.5**

Treatment(df=7,f=22.8,p=0.0), Organs(df=1.f=17,p=0.001)

The infected un treated mice organ reveal a large number of parasite specially in the liver (figure 1, 2) ,there are no parasites (amastigote) observed in the bone marrow of treated mice with M 19, while the numbers were low when using F14. This could be logical (no parasite in bone marrow) because the parasite injected intra-peritonealy near the liver and spleen, facilitating parasite transfer to these organs, also the transmission of infection to bone marrow requires longer time, moreover, the effectiveness of the extract transferred to the bone marrow were more quickly through the rapid exchange of substances and blood, since the bone marrow is made up of cells with great mitotic activity, which requires the active exchange of substances . This increases the effect of the extract, which appear clearly through the decrease in parasite numbers in all treatments except pentostam.

It was found that the two extracts F 14 and M19 contain 9,12-Octadecadienoic acid (Z,Z)- . Saini and Rai [28] in 2020 noted that (Z,Z)- suppresses the parasitic load in microphage during *L. donovani* infection in macrophage and also activates it linoleic acid (LA), which is a principal essential fatty acid. linoleic acid decreased the release of *L. donovani* derived extracellular vesicle later characterized as micro vesicles.

The difference between parasite number when using two methods (print and sectioning) where *A. terreu*s had fewer parasite numbers than M19 when the parasite counted in printing technique, while the numbers of the parasite in M19 became less than *A. terreu*s in sectioning method and this differences may possibly due to two reason, the first is that the extract of *A. terreus* caused the expansion of spaces between cells, which led to the accumulation of liquid substances more than the samples treated with others extract , and this facilitated their draining with tissue fluids to the slide compared to the others, i.e. the descent of materials with greater density and therefore increasing the parasite numbers and the second reason is that *A. terreus* extract caused congestion of the organs and thus swelling and this led to the generation of pressure on the internal tissue and this pressure is emptied when making a cut in the tissue, which causes the exudation of materials larger than the tissue sectioning method.

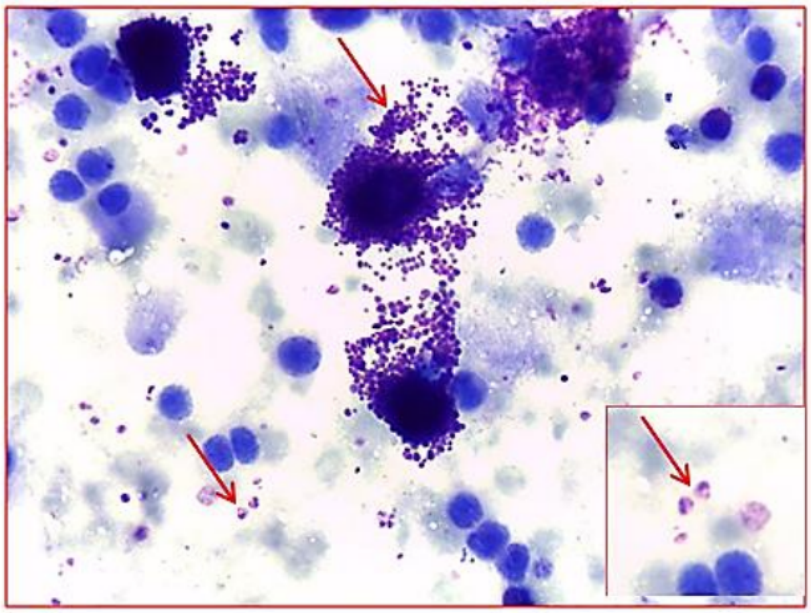

Figure (1) Tissue print of mice liver infected with *Leishmania* arrow

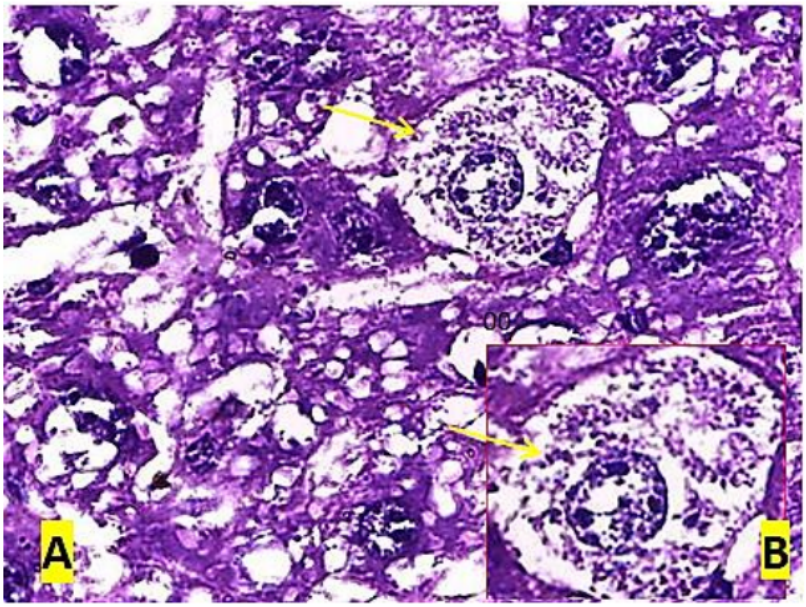

Figure (2) a section of mice liver infected with *leishmania* amastigote arrow

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