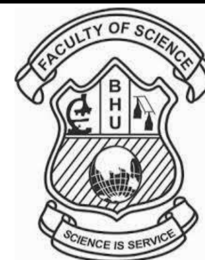
Volume 66, Issue 1, 2022

Journal of Scientific Research

of

The Banaras Hindu University



Antileishmanial potential of different extracts of *Curcuma longa* rhizome against *Leishmania donovani*

Manish Kumar[1], Kavita Pal[2], Vinay Pratap[1], Jalaj Kumar Gour[1]*

[1]Department of Biochemistry, Faculty of Science, University of Allahabad, Prayagraj-211002, Uttar Pradesh, India.
manishbiochem91@gmail.com, vinaypratap49@gmail.com, jalaj19biochem@gmail.com*

[2]Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India.
kavitabhu91@gmail.com

* Corresponding Author

Abstract: **Plants are an excellent source of herbal medicines and their natural products have antimicrobial potential for the management of numerous diseases. This study investigated the antileishmanial activity (IC_{50} - 50% inhibitory activity) of *Curcuma longa* (rhizome) crude extracts (80% methanol, chloroform, and acetone) against (*Leishmania donovani*) promastigote and amastigote forms. All three extracts of *C. longa* exhibited antileishmanial activity on both forms of parasite *Leishmania*. Miltefosine was used as a reference drug in this study. The methanolic extract showed the maximum antileishmanial activity followed by chloroform and acetone extract against both the forms promastigotes and amastigotes. The cytotoxicity (CC_{50}) of these extracts were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which was ranging from 889.89 to 969.01 µg/ml. The safety parameter was determined in terms of selectivity index (SI) which was found to be highest for methanolic extract (46.71) followed by chloroform (26.38), reference drug miltefosine (11.65) and acetone extract (4.41). Further, GC-MS analysis of 80% methanolic extract was carried out and identified 51 compounds, out of which were secondary metabolite (2,3-dihydro-benzofuran) sesquiterpenes, terpenoids, monoterpene and phenolic acids was most prominent that could be responsible for its antileishmanial activity. The results revealed that all three extracts have potent antileishmanial activity and it could be used in the formulation of herbal medicine against *L. donovani* in future prospect.**

Index Terms: **Cytotoxicity, Leishmaniasis, Miltefosine, Natural compounds, Promastigote.**

I. INTRODUCTION

Plant products are the magical weapon on earth and are being used by human beings from very ancient times for the treatment of several diseases with lesser side effects. The plant natural products excellently work against many microbes. (Cowan, 1999). The secondary metabolic products/compounds such as phenol, terpenoid, alkaloid etc. of plants have an excellent antimicrobial, antifungal, and antiparasitic potential along with avast source of pharma products (Srinivasan *et al*., 2001). The family Zingiberaceae consists of above 80 species of rhizomatous herbal plants and the genus Curcuma is one of the herbs under this family (Sasikumar, 2005). *Curcuma longa* is a widely studied rhizomatous flowering plant having great potential for effective treatment of a wide range of diseases (Sanjay et. al., 2007). Leishmaniasis is a tropical disease caused by *Leishmania*, a protozoan parasite that lives in mammals as an intracellular parasite and it multiplies in macrophages.(Alvar *et al*., 2012). The Life cycle of the parasite is digenetic and is transmitted through the bites of an insect female sandflies into the mammalian host (Gour *et. al*., 2012). About 350 million people are affected by this disease worldwide and approximately twelve million people alone in the America, Africa, Asia and Europe are directly suffering from this disease. (Burza *et al*., 2018; Hoyos *et al*., 2016). Out of top-ten tropical neglected diseases, leishmaniasis comes first which causes a high burden among people residing in developing nations. (Reithinger *et al*., 2007).The clinical symptoms of leishmaniasis represents itself in three forms the mucocutaneous leishmaniasis (MCL), cutaneous leishmaniasis (CL) and the visceral leishmaniasis (VL) (Gour *et.*

DOI: 10.37398/JSR.2022.660114

al., 2009). Visceral Leishmaniasis is also known as kala-azar, and patients who are infected with both *Leishmania* and HIV have a high risk of having a full-blown clinical illness, which can lead to death if not treated promptly. Worldwide, people are largely affected by cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) which is responsible for the majority of morbidity (Sunder & Singh. 2016).It is also documented that *L. donovani* that causes visceral leishmaniasis (VL)is widespread throughout the country of Ethiopia, becoming endemic in its several regions, mainly seen in its lowland and arid areas. (Akhoundi *et al*., 2016). Some of the common antileishmanial drugs such as the pentavalent antimonials are the first line of drug to treat visceral leishmaniasis but in the Indian subcontinent, amphotericin B is the first line of drugs for VL. While pentamidine and amphotericin-B are applied as a second line of protection for visceralleishmaniasis only. (Sundar & Singh. 2016; Moore & Lockwood. 2010). Some of the previous researches also demonstrated that the medicinal plant and its derivative natural products have significant pharmacological properties against leishmaniasis. The reason behind the use of plants and natural compounds as an alternative is only due to the lower side effect as compared to currently used drugs (Chouhan *et al*., 2014; Oryan. 2015).

Curcuma longa (*C. longa*) is commonly referred to as turmeric (Haldi) which is obtained from its rhizomes. It is a mostly perennial, herbaceous and rhizomatous plant. *C. longa* is located worldwide, native to the tropical regions of South Asia, China, and India. The spice *C. longa* is native to Indiaand found in the tropical and subtropical regions. (Ravindran *et al*., 2007).Its rhizome is the most usable part and a crucial source of various products such as coloring agents, spices, flavoring agents, dyes, cosmetics, perfumes, pharmacological, ayurvedic and traditional Chinese medicine. (Leong *et al*., 2015). Their plant is utilized to treat various types' diseases in the mammalian health system such as antioxidant, anticancer, antimicrobial, antileishmanial antiviral activity (Rahmani *et al*., 2018; Praditya *et al*., 2019). Their important phytochemicals such as curcuminoids, curcumin, curzerone, polyphenolic bisdemethoxycurcumin, sesquiterpenes, tumeron etc. have shown a variety of pharmacological properties including antioxidant activity, antineoplastic, antiviral, calming, antibacterial, antifungal, antidiabetic, anticoagulant, antifertility, cardiovascular defensive, hepatoprotective, and immune stimulant action. (Bengmark *et al*., 2009; Singh & Sharma, 2011). The *C. longa* leaf showed lesser medicinal importance as compared to its rhizome. Some studies additionally demonstrated that the leaf of the *C. longa* plant showed cytotoxic, antibacterial, antifungal, antiaflatoxigenic, and mosquitocidal activity. (Sindhu *et al*., 2011; Essien *et al*., 2015).

It is previously investigated that the hydroalcoholic crude extract of the medicinal plant *Albizia gummifera,* belongs to the family Fabaceae, showed antileishmanial activity against the *L. donovani* (promastigote phase (IC_{50} = 8.65 µg/ml) (Nigussie *et al*., 2015). Some important standard drugs such as liposomal amphotericin B (Sundar *et al*., 2010) paromomycin, (Musa *et al*., 2012) antimonial (Allen & Neal., 1998) miltefosine combinations are also used to treat leishmaniasis effectively, although these medications cause severe adverse effects or resistance against these drugs have been developed by the parasite (Simon *et al*., 2006). Curcumin is the primary natural compound isolated from *C. longa*, and it is effective against the *Leishmania* parasite, with a cure rate of 40% when compared to the conventional treatment glucantime, having a cure rate of 32.7% (Bahrami *et al*., 2015). It has also been reported that the *C. longa* extract, in combination with other juices, efficiently showed antileishmanial activity against cutaneous leishmaniasis after a period of 63-76-days to heal the ulcer in mammals. (Hosseininejad *et al*., 2012).It is found that the curcumin isolated from *C. longa* has tremendous efficiency to treat leishmaniasis caused by *Leishmania* species (Tiwari *et al*., 2017). In addition, a combination of miltefosine and *C. longa* derived natural product nanocurcumene is efficacious in the treatment of leishmaniasis. This combination of the drug showed a synergistic effect on promastigote and amastigote form of *Leishmania* parasite by enhancing the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Tiwari *et al*., 2017). Some earlier studies demonstrated the role of curcuminoids extracted from *Curcuma longa* against *Leishmania amazonensis* and *Leishmania major* (Rasmussen et al., 2000; Saleheen et al., 2002; Amaral *et al*., 2014).

To date, most of the studies on *C. longa* were performed on the curcumin and its derivatives as against *Leishmania* species and report on antileishmanial activity of crude extracts of *C. longa* is scanty. The objective of this study was to elucidate the antileishmanial activity of different extracts of *C. longa* against the *L. donovani* strain.

II. Materials and Methods

A. Chemicals for Culture Medium Preparation

Nonionic detergent (Tween-20) was used to clean the dust particles from the plant sample. The high molecular grade methanol, chloroform, and acetone were used as extracting solvents (Merck). Dulbecco's Modified Eagle Medium (DMEM) was utilized for parasite and macrophage cell line cultivation. To control the microbiological contamination, antibiotics such as penicillin and streptomycin, as well as gentamycin (Sigma-Aldrich, USA.), fetal bovine serum (Gibco, USA) and sodium

bicarbonate were used. At pH 7.2, the parasites were grown in DMEM media (Invitrogen, USA) supplemented with 10% FBS and antibiotics (Streptomycin 100 µg/ml, penicillin 100 U/ml, gentamycin 20 µg/ml (Sigma, USA) (de Almeida *et al*., 2010).

B. Reference drug

Miltefosine was used as a reference drug.

C. Test parasite strains and cell line

L. donovani (MHOM/50/68/15) parasites were collected from the infected mouse model and further maintained in a complete DMEM culture medium. The amastigotes form of the parasite was maintained in RAW 264.7 macrophage cell lines (DMEM) and further used for the cytotoxicity assay.

D. Plant material and extraction

Fresh rhizomes of *Curcuma longa* (Zingiberaceae) were collected from ICAR- NBPGR Regional Station, Bhowali. Uttarakhand, 263132, India, during the month of November 2017. First of all soil and waste matter, adhered to the fresh rhizomes, were separated by washing under running tap water and then two time with double distilled water and finally washed two times with tween-80 (5%). The fresh rhizomes, without nodules and agglomerated particles, were cut into small pieces and shade dried for 15 days, the dried plant materials were coarsely powdered and stored in an airtight container until used at 4°C. A 40 g sample of the powdered rhizome was successively extracted with solvents 1:10 ratio of increasing polarity (Acetone, chloroform, and methanol 80%) using a maceration method incubated at 30°C and 120rpm for 96 hours using a incubator shaker. After shaking, plant samples were filtrated using Whatman No.1 filter paper. Finally, the solvents were evaporated at 35°C using a hot air oven. The final extracts were used for the antileishmanial (IC_{50}), cytotoxic activity (CC_{50}).

E. Stock Solutions Preparation of the different Extract of Plant and Control Drug

A stock solution of each plant extract was dissolved in DMSO (0.5%) (20mg/ml) and reference drug miltefosine (1mg/ml) was prepared in phosphate buffer saline and kept at 4°C for further uses.

F. Parasite culture (L. donovani)

The parasites (*Leishmania donovani*) were cultured in DMEM, 10% FBS (heat inactivation for 30 minutes at 53°C, penicillin, streptomycin mixes, (100U/100 µg/ml) and gentamycin was added, pH was maintained at 7.2 in a T-type culture flask (25cm^2 Costar). The culture of parasites was incubated at 26°C in Biological oxygen demand (BOD) incubator (Almeida *et al*. 2010).

G. Gas Chromatography-Mass Spectrometry (GC-MS) of C. longa (80%) Methanol Extract for Phyto-chemical Characterization

Identification and quantification of the presence of secondary metabolites in methanol extract of *Curcuma longa* rhizome were carried out by gas chromatography coupled with mass spectrometry (Shimadzu QP2010 Plus) equipped with an Rtx- 5 MS capillary column (0.25 µm film thickness, 0.25 mm internal diameter, and 30 m in length).The oven temperature was set at 100°C for 2 min, then increased to 250°C with a rate of 5°C per minute, and finally to 280°C with a rate of 10°C per minute. The sample was prepared in methanol (80%) as solvent having the concentration of 30 mg/ml and filtered by Whatman no. 1 filter paper and finally, 2 µl of the sample was injected into the column in split mode (split ratio 1:10) with helium as the carrier gas with a flow rate of 1.21 ml per minute. The presence of distinctive peak fragmentation patterns for various metabolites was detected by an MS detector in full scan mode. The identification of metabolites was confirmed by comparing the spectral data of peaks with the corresponding standard mass spectra from the library database [National Institute of Standards and Technology library (NIST) and Wiley 8].

H. Cytotoxicity Assay

Cytotoxicity assay of the *C. longa* rhizome of different solvents extracts evaluated by the method of (Garcia *et al*. 2017) with some modification. The macrophages RAW264.7 cell lines were used in this study for the cytotoxic assay. The macrophage RAW 264.7 cell lines were cultured and maintained in DMEM added with 10% fetal bovine serum and incubated at temperature 37°C in a CO_2 incubator at 5% CO_2. Further, *100 µl* of macrophage cell line (1x10^6 cells/well) was seeded in 96 well plate. Next, for the determination of the cytotoxicity, each plant extract were dissolved in 0.5% DMSO and further diluted by an incomplete medium (DMEM medium without FBS) for working concentrations. *C. longa* extract (80% methanol, chloroform and acetone) were added to 96 wells plate and the concentration was adjusted ranging from 2000 µg/ml to 3.90µg/ml. Further, it was incubated at 37°C in a 5% CO_2 atmosphere in a CO_2 incubator for 24h. On completion of incubation, about 30µl of MTT reagent (4mg/ml prepared in PBS) was added to each well and mixed properly and further incubated for 4 h in a CO_2 incubator. After 4 hours of incubation, the microplate forms a purple color and media was removed from each well properly. After that, 150µl DMSO solution was added to each well for the solubilization of the MTT reagent and the plate were shaken up to 5 minutes slowly. The optical density (OD) was taken at 540nm in a micro plate reader.

I. Anti-promastigotes Assay (In vitro):

The MTT assay was used to evaluate the *Leishmania donovani* anti-promastigote activity of different *C. longa* rhizome extract as described by (Ezatpour *et al*., 2015) with some modification. In brief, to determine the inhibitory concentration (50%) of different *C. longa* extracts, 100 µl oflogarithmic phase promastigotes (1×10^6 parasites/ml) were seeded in 96 well culture plate and then 100 µl of each extract was added in each well plate having promastigotes with different concentration of each extracts ranging from 250 µg/ml to 0.24 µg/ml serially two fold dilutions. Next, the plate was incubated at 26°C for 48 h period in a BOD incubator. After that, to determine the percentage inhibition of respective test samples, MTT assay was performed to check the presence of viable cells, on treatment with test samples. The mechanism behind this assay is the conversion of MTT to formazan by mitochondrial enzymes served as an indicator of cell viability and the amount of formazan produced indicates the viable cells with an active metabolism. After 48h of incubation, 30µl of MTT reagent (4 mg/ml) was added to each well and incubated for 4h at 26°C. Subsequently, after 4h, plates were shaken for 5 minutes, the supernatant was discarded from the well and 100µl of DMSO was added to solubilize the precipitated formazan crystal. Promastigotes cultured in complete medium without treatment were used as positive control and complete medium without promastigotes and drugs were used as the blank. Accordingly cell viability was measured at 540 nm using an ELISA plate reader (BIO-RAD 680). All experiments were repeated thrice with each drug being used in triplicates.

J. Macrophage-amastigote in vitro Test Model: Anti-amastigote Assay (In-vitro)

The anti-amastigote activity was evaluated as described by (Mohmoudvand *et al*., 2014) with some modifications. Briefly, the macrophages RAW 264.7 cell line was used for this assay which was dispensed (5×10^6 cells/well) in 24 well culture plate in a complete DMEM medium (Invitrogen, USA) and incubated at 37°C in a 5% CO_2 incubator for 4-5 h. The wells having cells were washed with FBS free media to remove the non-adherent cells, and macrophages (cell lines 264.7) were allowed to infect by promastigotes form of *L. donovani* (1: 10 ratio) and incubated for 6 h. On completion of incubation, the non-infected parasites/cells were removed by washing with complete media and then incubated for 24 h. After completion of 24 h incubation, the culture plate was given one time wash. Furthermore, the test plant extracts were dispensed in a well plate and concentrations were adjusted from 250 to 0.24 µg/ml and then incubated for 48h. After 48h, evaluation of anti-leishmanial activity towards intracellular amastigotes was measured by using direct counting assays following fixation and Giemsa staining. All assays were performed in triplicates and drug activity was analyzed under a phase contrast microscope.

K. Experimental Data Analysis

All the experimental data were analyzed by Graph Pad prism 5.0 such as mean± SD. For the determination of IC_{50} and CC_{50}, percentage inhibition of parasites was calculated at different concentrations of each extract and reference drug miltefosine. The parasite growth inhibition was determined by comparing the activity of control wells (normal activity) with that of treated wells (inhibited activity) involving the average absorbance of all wells (control and treated) in the calculation via the equation: (Huber & Koella, 1993)

% Inhibition = OD of control well − OD of treated well ×100/OD of control well.

The IC_{50} and CC_{50} values were expressed as µg/ml ± SD. The selectivity index (SI) was calculated by the ratio of CC50 to IC50.

III. RESULTS

A. Cytotoxicity and Safety index (SI) of different extracts of C. longa

The cytotoxicity of different extracts of *C longa* was determined by MTT assay. The results demonstrated that the cytotoxicity (CC_{50}) value of methanolic extract was 889.89 ± 2.31 µg/ml whereas for chloroform and acetone was found to be 958.52 ± 2.98 µg/ml and 969.013 ± 4.49 µg/ml respectively, which showed their low cytotoxicity as compared to the reference drug miltefosine (38.643 ± 0.286µg/ml) (Table 1). Further, the overall results of each extract were also calculated for Selectivity Index (SI) to compare the efficacy of the treatments as a safety parameter. On the basis of selectivity index, 80% methanolic extract showed the highest SI value (46.71) followed by chloroform extract (SI = 26.38) and acetone extract (least SI = 4.41). The SI value of 80% methanolic extract (46.71) was higher than the reference drug miltefosine (11.65), whereas the SI value of chloroform (26.38) was also promising which indicates their safety/non-toxicity towards macrophage cell line.

The cytotoxicity result of the crude extracts concluded their low toxicity and safety parameters (Table 1).

B. ***Antileishmanial activity of different extracts***

This study investigated the inhibitory impacts of various solvent extracts of *C. longa* (rhizome) against the amastigote and promastigote forms of *Leishmania donovani.* The results of antileishmanial activity in terms of percentage inhibition (anti-promastigote and anti-amastigote) (Fig.1and Fig.2) and their IC_{50} values are shown in (Table 1). The highest antileishmanial activity (IC_{50}) was found in 80% methanolic extract as anti-

promastigote (15.64 ± 1.084 µg/ml) and as anti-amastigotes (19.05 ± 1.22 µg/ml) as compared to chloroform extract (IC_{50} for promastigotes 25.95 ± 1.055 µg/ml and for amastigotes 36.33 ± 1.86 µg/ml) and acetone extracts (IC_{50} for promastigotes 208.94 ± 1.97µg/ml and for amastigotes 219.32 ± 2.38µg/mL) respectively. The IC_{50} results of reference drug miltefosine (as antipromastigote and antiamastigotes) were 2.186 ± 0.053 µg/ml and 3.315 ± 0.251 µg/ml respectively. No parasite death was seen in solvents (80% methanol, chloroform, and acetone) which were used for extract preparation and in 0.5% DMSO used in the preparation of a stock solution of each extract. The results from this study concluded that all the three extracts of *C. longa* have potent antileishmanial activity against *L. donovani* promastigotes and amastigotes forms which were confirmed in terms of IC_{50} (Table 1.)

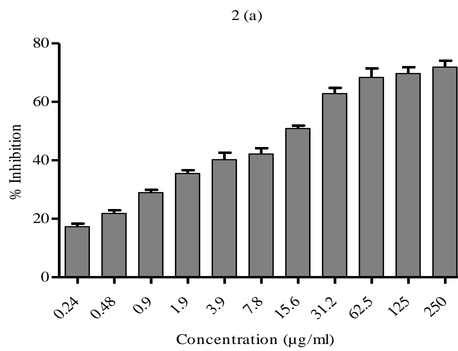
C. Chemical Composition determination by GCMS Techniques

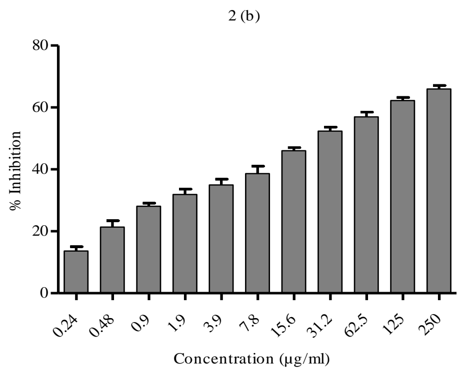
GC-MS based metabolic profiling of the methanol (polar) fraction of *C. longa* (rhizome) showed conspicuous peaks (Fig.3) at different retention times, which were further identified by their mass using Wiley and NIST library (Table 2). Phytochemical analysis revealed that a total of 51 compounds were identified in 80% methanol extract of *C. longa*with the most prominent sesquiterpenes, terpenoids, monoterpene and phenolic acids.The major compounds have been present upon the percentages are as tumerone (29.04%), curlone (12.75%), β-sesquiphellandrene (7.05%), 7-epi-Sesquithujene (4.94 %), 1,3-Dioxolane, 2-(2-phenyl-2-propyl) (4.72%), Zingiberenol (3.60%), (E)-Atlantone (3.48%), Himachalene-1,4-diene (3.15%), 2,6-Nonadienoic acid, 9-(3,3-dimethyloxiranyl)-3,7-dimethy (3.14%), 2,6,10-Trimethylundecan-2,9-Dien-4-One (2.54%).

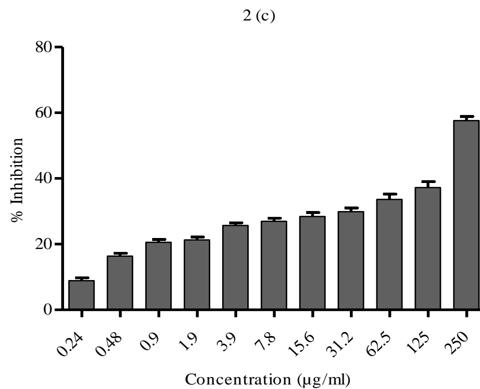
Table 1: Antileishmanial and cytotoxicity of different extracts of *C. longa* rhizome.

[SI = Selectivity index (SI defined by the ratio of CC_{50} in RAW cells/IC_{50} in intracellular *L. donovani* amastigote and ± showed standard deviation with mean.]

***C. longa* extracts**	**IC_{50} (µg/ml) on promastigote forms of *L. donovani***	**IC_{50} (µg/ml) on *L. donovani* intra-macrophage amastigotes forms**	**CC_{50} (µg/ml) on RAW 264.7 macrophage**	**SI (CC_{50}/IC_{50} of intra-macrophage amastigotes forms)**
***C. longa* 80% methanol**	15.64 ±1.084	19.05±1.22	889.89 ±2.31	46.71
***C. longa* Chloroform**	25.95 ±1.055	36.33 ±1.86	958.52±2.98	26.38
***C. longa* Acetone**	208.94±1.97	219.32 ±2.38	969.013±4.49	4.41
Miltefosine	2.186±0.053	3.315±0.251	38.643±0.286	11.65







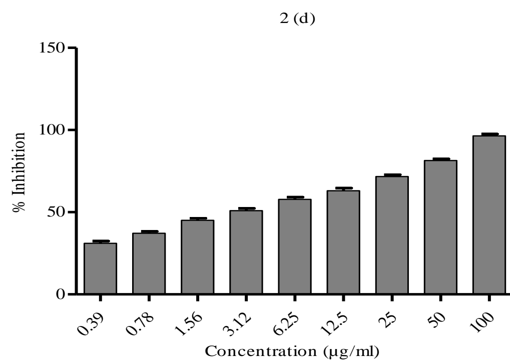
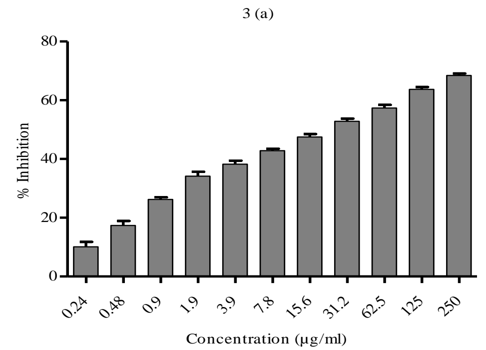
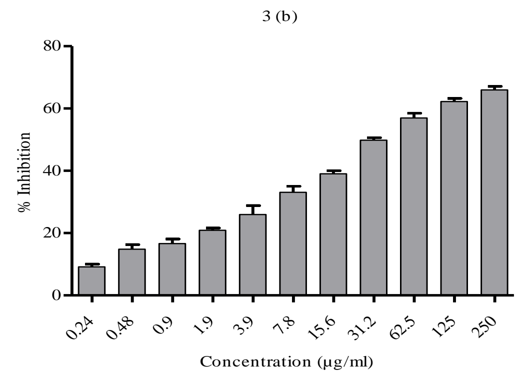
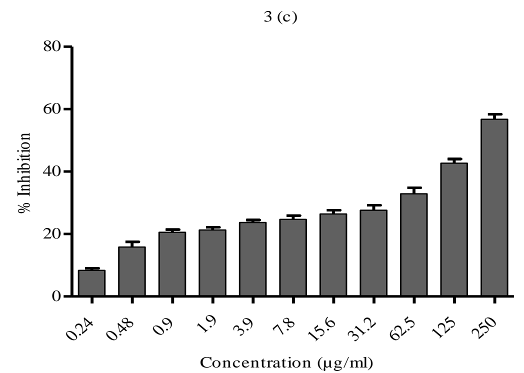


Fig: 1. Percentage inhibition of *L. donovani* (Promastigotes) with different concentrations of *C. longa* (rhizome) (a). 80% methanol (b). Chloroform and (c). Acetone extract d. miltefosine (reference drug) at 48h.







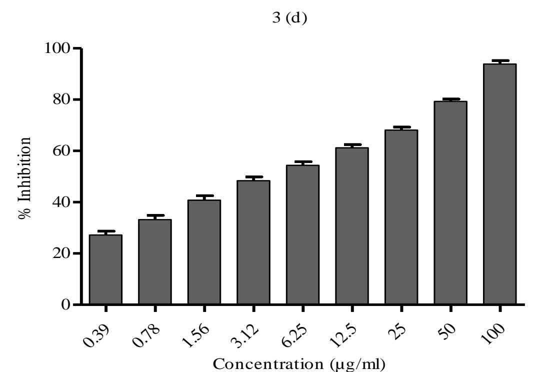


Fig: 2. Percentage inhibition of *L. donovani* (Anti-amastigotes) with different concentrations of *C. Longa*(rhizome) (a). 80% methanol (b). Chloroform and (c). Acetone extract (d). Miltefosine (reference drug) at 48h.

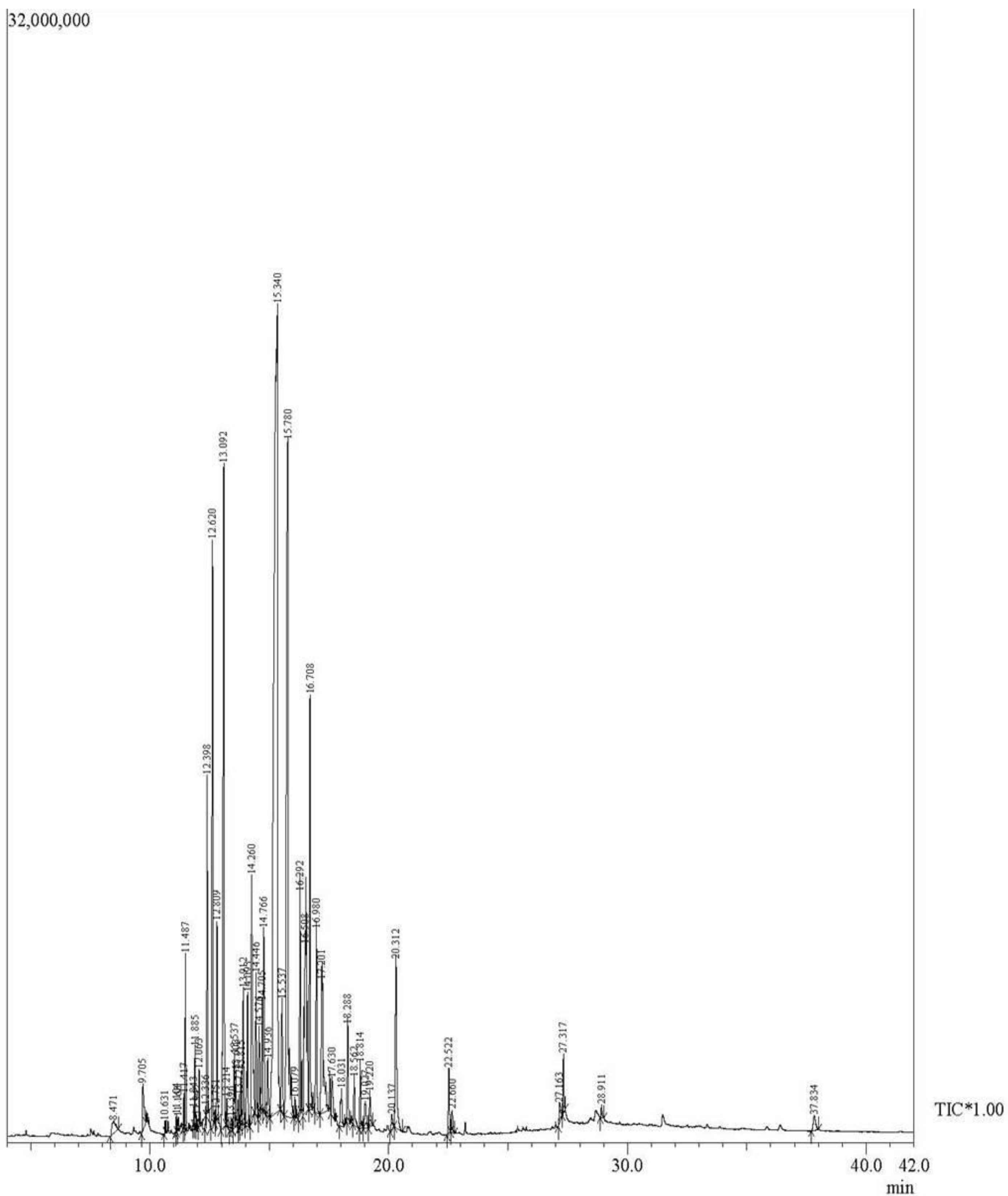


Fig. 3. Chromatogram of 80% methanol extract of *Curcuma longa* rhizome obtained after Gas Chromatography. Values on peaks denote their respective retention time.

Table 2.Chemical composition identified in80% methanolic extract of *Curcuma longa*rhizome by GC-MS.

S. No.	Name of the phytochemicals	R. Time	Area%	Molecular formula	Molecular weight (g/mol)	Nature of compound
1	2,3-dihydro-benzofuran	8.471	0.44	C_8H_8O	120	secondary metabolites
2	2-methoxy-4-vinylphenol	9.705	0.73	$C_9H_{10}O_2$	150	phenolic acid
3	(+)-1,6-spiro(4,4) nonodiene	10.631	0.08	C_9H_{12}	120	
4	megastigma-3,7(e),9-triene	11.168	0.10	$C_{13}H_{20}$	176	monoterpene
5	α-santalene	11.417	0.19	$C_{15}H_{24}$	204	
6	caryophyllene	11.487	1.11	$C_{15}H_{24}$	204	sesquiterpene
7	Cypera-2,4(15)-diene	11.843	0.11	$C_{15}H_{22}$	202	
8	β-Farnesene	11.885	0.45	$C_{15}H_{24}$	204	sesquiterpene
9	Sesquisabinene	11.942	0.06	$C_{15}H_{24}$	204	
10	α-caryophyllene	12.063	0.34	$C_{15}H_{24}$	204	
11	γ-Curcumene	12.336	0.07	$C15H_{24}$	204	
12	α-curcumene	12.398	2.34	$C_{15}H_{22}$	202	sesquiterpene
13	7-epi-Sesquithujene	12.620	4.94	$C_{15}H_{24}$	204	
14	1-phenyl-2-(p-tolyl)-propane	12.751	0.05	$C_{16}H_{18}$	210	
15	β-bisabolene	12.809	1.33	$C_{15}H_{24}$	204	terpenoids
16	β-sesquiphellandrene	13.092	7.05	$C_{15}H_{24}$	204	sesquiterpene
17	Thujol	13.214	0.17	$C_{10}H_{16}O$	152	
18	Teresantalol	13.396	0.14	$C_{10}H_{16}O$	152	sesquiterpene
19	Caryophyllene epoxide	13.537	0.60	$C_{15}H_{24}O$	220	sesquiterpene
20	1,6,10-dodecatrien-3-ol, 3,7,11-trimethyl-	13.603	0.31	$C_{15}H_{26}O$	222	
21	(1,2,3-trimethyl-2-cyclopenten-1-yl) methanol	13.722	0.20	$C_9H_{16}O$	140	
22	aR-Turmerol	13.912	1.27	$C_{15}H_{22}O$	218	
23	Zingiberenol	14.093	3.60	$C_{15}H_{26}O$	222	
24	Himachalene-1,4-diene	14.260	3.15	$C_{15}H_{24}$	204	
25	(.+/-.)-dihydro-ar-turmerone	14.576	0.85	$C_{15}H_{22}O$	218	sesquiterpene
26	9-Hydroxypregna-3,5-dien-20-one	14.766	2.18	$C_{21}H_{30}O_2$	314	
27	(z)-γ-atlantone	14.936	0.67	$C_{15}H_{22}O$	218	sesquiterpene
28	Tumerone	15.340	29.04	$C_{15}H_{22}O$	218	sesquiterpene
29	10,10-dimethyl-2,6-dimethylenebicyclo[7.2.0] undecane	15.537	1.34	$C_{15}H_{24}$	204	

30	Curlone	15.780	12.75	$C_{15}H_{22}O$	218	sesquiterpene
31	Bicycle [4.4.0]dec-5-ene, 1,5-dimethyl-3-hydroxy-8-(1-methylene-2-hydroxyethyl-1)-	16.079	0.16	$C_{15}H_{24}O_2$	236	
32	(6r,7r)-bisabolone	16.292	1.68	$C_{15}H_{24}O$	220	sesquiterpene
33	1,3-dioxolane, 2-(2-phenyl-2-propyl)-	16.508	4.72	$C_{12}H_{16}O_2$	192	
34	(e)-atlantone	16.708	3.48	$C_{15}H_{22}O$	218	sesquiterpene
35	4-(2-methoxypropan-2-yl)-1-methylcyclohex-1-ene	16.980	2.07	$C_{11}H_{20}O$	168	
36	2,6-nonadienoic acid, 9-(3,3-dimethyloxiranyl)-3,7-dimethy	17.201	3.14	$C_{16}H_{26}O_3$	266	
37	cyclopentancarbonsaeure, 4-isopropyliden-2-[(E)-1-propenyl]-, methylester	17.630	0.53	$C_{13}H_{20}O_2$	208	
38	Corymbolone	18.031	0.58	$C_{15}H_{24}O_2$	236	sesquiterpene
39	Cyclohexanecarboxylic acid, 3-phenylpropyl ester	18.288	0.99	$C_{16}H_{22}O_2$	246	
40	(s)-3-methyl-6-((s)-6-methyl-4-oxohept-5-en-2-yl)cyclohex--2-enone	18.562	0.54	$C_{15}H_{22}O_2$	234	
41	Hexadecanoic acid, methyl ester	18.814	0.61	$C_{17}H_{34}O_2$	270	
42	(-)-Globulol	19.027	0.47	$C_{15}H_{26}O$	222	
43	Turmeronol A	19.220	0.41	$C_{15}H_{20}O_2$	232	
44	carbonic acid, but-2-yn-1-yl tridecyl ester	20.137	0.21	$C_{18}H_{32}O_3$	296	
45	2,6,10-trimethylundecan-2,9-dien-4-one	20.312	2.54	$C_{14}H_{24}O$	208	
46	Linoleic acid, methyl ester	22.522	0.69	$C_{19}H_{34}O_2$	294	
47	Hexadecadienoic acid, methyl ester	22.660	0.27	$C_{17}H_{30}O_2$	266	
48	2-ethylbutyric acid, eicosyl ester	27.163	0.37	$C_{26}H_{52}O_2$	396	
49	B-Monopalmitin	27.317	0.41	$C_{19}H_{38}O_4$	330	
50	1-Monostearin	28.911	0.12	$C_{21}H_{42}O_4$	358	
51	γ-sitosterol	37.834	0.35	$C_{29}H_{50}O$	414	Sesquiterpene

DISCUSSIONS

Plant products are promising options as strong antileishmanial agents with minimum side effects on the host cell (Fonseca-Silva et. al. 2016; Garcia et al. 2017). This study identified the antileishmanial activity of *Curcuma longa* extracts (80% methanol, chloroform and acetone) on promastigotes as well as on amastigotes of *Leishmania donovani* strain which is responsible for visceral leishmaniasis in India. The results of our study showed antileishmanial activity in all extracts. Anti-promastigote (IC_{50}) activity of methanolic extract (15.64 ± 1.084 µg/ml) was highest followed by chloroform (25.95 ± 1.055 µg/ml) and acetone extract (208.94 ± 1.97 µg/ml) respectively.

The anti-amastigote (IC_{50}) activity was also observed highest in methanolic extract (19.05±1.22 µg/ml) as compared to chloroform (36.33 ± 1.86µg/ml) and acetone extract (219.32 ± 2.38 µg/ml). Amaral et al 2014, demonstrated the antileishmanial activity of *C. longa* of hexane fractions (hexRHIC and hex-RHIWC extracts) against promastigotes of *Leishmania amazonensis* as (IC_{50}) 35.4 and 83 µg/ml respectively. Teles *et al.*, 2019, reported the antileishmanial activity of essential oil of *C. longa* on *Leishmania amazonensis* promastigotes (IC50: 308.4 ± 1.402µg/ml) and on amastigotes (IC50: 63.3 ± 1.369402µg/ml).

The predicted possibility for inhibition property of our extracts may be due to target on the mitotic division, attack on parasite membrane or some enzymes such as sterol methyltransferase (not expressed in mammalian cells) etc (Rodrigues et al.,2002; Granthon et al., 2002).

The cytotoxicity (CC_{50}) results of this work employing RAW 264.7 macrophage cell line was also promising i.e. 889.89 ±2.31 µg/ml for methanolic extract, 958.52±2.98 µg/ml for chloroform and 969.013±4.49 µg/ml for acetone extract of *C. longa* which represents low toxicity. Likewise, Teles *et al* reported the cytotoxicity (CC50: >1000 µg/ml) on peritoneal macrophages with SI value >15.79.

Further, we have evaluated the Selectivity Index (SI) of each extract which emphasises its efficacy parameters as an antileishmanial agent. The SI of *C. longa* methanolic extract was highest (46.71) and the SI value for chloroform extract was 26.38 and for acetone extract were 4.41 respectively. Therefore, our results forantileishmanial elucidated the potential of each extract as an antileishmanial agent with low cytoxicity alternative against visceral leishmaniasis.

In this study, several compounds as identified by GC-MS analysis (Table 2.) in 80% methanolic. Most of the compounds identified by GC-MS analysis have already reported antimicrobial properties, for instance, a secondary metabolite (2,3-dihydro-benzofuran) possess antileishmanial activity against *L. amazonensis* (de Castro Oliveira *et al*., 2017), antimicrobial activity. (Fukui *et al*., 2018). Likewise, monoterpenes (megastigma-3,7(e),9-triene) are having antimicrobial activity, (Laouer *et al*., 2009). Another major compound, sesquiterpenes (7-epi-Sesquithujene) are reported to have antileishmanial activity against *L. amazonensis* (Alves et al. 2020), (caryophyllene) with antileishmanial activity against *L. amazonensis* (Moreira *et al*., 2019). The phenolics (2-methoxy-4-vinylphenol) showing anti-microbial, antibacterial and antiparasitic activities (Rubab *et al*., 2020). The terpenoids (β-bisabolene) with antimalarial activity against *P. falciparum*(de Souza *et al*., 2017) antibacterial activity. (Nascimento *et al*., 2007), antimicrobial activity (Kazemi *et al*., 2015) etc.

Earlier studies on phytochemicals isolated from *Curcuma* species such as curcuminoids, sesquiterpenoids such as turmerones against *Leishmmania species has been reported* (Araujo and Leon, 2001; Jayaprakasha *et al*., 2002; Funk *et al*., 2010; Amaral *et al*., 2014). Likewise, in this study, 80% methanol extract also had the highest concentration of tumerone which is a natural sesquiterpene. Therefore, the results of the present investigation support the earlier phytochemical studies on *C. longa* as an antileishmanial agent. Our GC-MS analyses of methanolic extracts possess several compounds that have already reported antimicrobial activity including against other species of *Leishmania.*

The present treatment choices for leishmaniasis are seen as deficient, considering the way that these drugs are connected with high toxic quality, and critical costs, and in graceful of the fact that parasite refuge from these meds is increasing (Ghorbani & Farhoudi. 2018) in this unique condition, techniques to distinguish new compound with lower lethality and higher viability could be considered exceptionally attractive in the treatment of leishmaniasis. Phytotherapy has become extensive consideration as an option in contrast to chemotherapy in parasitic disease treatment (Tavares, 2018).

Earlier studies was mostly performed on compound curcumin and its derivatives as an antileishmanial agent (Khanra et al., 2018; Koide et al., 2002; Gomes et al., 2002; Tiwari *et al*., 2017). This work concluded the highest potential of 80% methanolic extract of *C. longa* rhizome among the other two solvents extract such as chloroform and acetone extract as an antileishmanial agent and could be an alternative option to isolate novel compounds and in the formation drugs via studies in future against *Leishmania donovani* to treat visceral leishmaniasis.

CONCLUSION

Plant diversity is an obvious and potential source of new medicinal agents and many more natural resources have not been explored for their medicinal and therapeutic potential against most of the parasitic diseases including leishmaniasis, seeking screening and research work on natural products.

In ethnomedical relevance, *Curcuma longa* is being used for the management of several diseases and disorders such as in controlling infectious diseases, inflammation, blood disorders, gastric or hepatic problems etc. from very ancient times. This present study demonstrated the antileishmanial activity of different extracts of *C. longa rhizome* against *L. donovani* based on its traditional use. The 80% methanolic extract and chloroform extract were found to have potential antileishmanial activity along with safer cytotoxicity levels. A number of compounds identified by GC-MS analysis of 80% methanolic extract have also strong antimicrobial activity as reported in literature. Further, more research is required to explore the mode of action, macrophage effector functions, cytokine assays for accessing immune-stimulating activity and the *in-vivo* studies of these extracts to be useful in the management of visceral leishmaniasis

CONFLICT OF INTEREST

Authors declare no conflict.

ACKNOWLEDGMENTS

Authors are greatly thankful to Professor Rakesh Kumar Singh, Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi for providing *Leishmania donovani* strain, macrophage cell line and for extending technical assistance to carryout present work.

REFERENCES

Akhoundi, M., Kuhls, K., Cannet, A., Votypka, J., Marty, P., Delaunay, P., & Sereno, D. (2016).A historical overview of the classification, evolution, and dispersion of Leishmania parasites and sandflies. *PLoS neglected tropical diseases*, 10(3), e0004349.

Allen S., Neal R.A. (1989) The *in vitro* Susceptibility of Macrophages Infected with Amastigotes of *Leishmania* spp. to Pentavalent Antimonial Drugs and Other Compounds with Special Relevance to Cutaneous Isolates. In: Hart D.T. (eds) *Leishmaniasis*. NATO ASI Series (Series A: Life Sciences), vol 171. Springer, Boston, MA. https://doi.org/10.1007/978-1-4613-1575-9_88.

Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J.,& WHO Leishmaniasis Control Team. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PloS one*, *7*(5), e35671.

Alves da Silva, F. F., Fernandes, C. C., Almeida de Oliveira, G., Candido, A. C. B. B., Magalhaes, L. G., Vieira, T. M.,& Alves da Silva, C. (2020). 'In vitro'antileishmanial and antioxidant activities of essential oils from different parts of'Murraya paniculata'(L.) Jack: A species of Rutaceae that occur in the'Cerrado'biome in Brazil. *Australian Journal of Crop Science*, 14(2), 347-353.

Araujo, C. A. C., & Leon, L. L. (2001). Biological activities of Curcuma longa L. *Memórias do Instituto Oswaldo Cruz*, 96(5), 723-728.

Bahrami, S., Esmaeilzadeh, S., Zarei, M., & Ahmadi, F. (2015).Potential application of nanochitosan film as a therapeutic agent against cutaneous leishmaniasis caused by L. major. *Parasitology research*, 114(12), 4617-4624.

Bengmark, S., Mesa, M. D., & Gil, A. (2009).Plant-derived health-the effects of turmeric and curcuminoids. Nutricionhospitalaria, 24(3), 273-281.

Burza, S., Croft, S. L., & Boelaert, M. (2018). Leishmaniasis. *Lancet (London, England)*, *392*(10151), 951–970.

Chouhan, G., Islamuddin, M., Want, M. Y., Ozbak, H. A., Hemeg, H. A., Sahal, D., & Afrin, F. (2015). Leishmanicidal Activity of Piper nigrum Bioactive Fractions is Interceded via Apoptosis In Vitro and Substantiated by Th1 Immunostimulatory Potential In Vivo. *Frontiers in microbiology*, *6*, 1368.

Cowan M. M. (1999). Plant products as antimicrobial agents. *Clinical microbiology reviews*, *12*(4), 564–582.

Croft, S. L., Sundar, S., & Fairlamb, A. H. (2006).Drug resistance in leishmaniasis. *Clinical microbiology reviews*, 19(1), 111.

de Almeida Rodrigues, I., da Silva, B. A., dos Santos, A. L. S., Vermelho, A. B., Alviano, C. S., & Rosa, M. D. S. S. (2010). A new experimental culture medium for cultivation of Leishmania amazonensis: its efficacy for the continuous in vitro growth and differentiation of infective promastigote forms. *Parasitology research*, 106(5), 1249-1252.

de Castro Oliveira, L. G., Brito, L. M., de Moraes Alves, M. M., Amorim, L. V., Sobrinho-Junior, E. P. C., de Carvalho, C. E. S.,& de Amorim Carvalho, F. A. (2017). In vitro effects of the neolignan 2, 3-Dihydrobenzofuran against Leishmania amazonensis. *Basic & clinical pharmacology & toxicology*, 120(1), 52-58.

de Souza, G. A., da Silva, N. C., de Souza, J., de Oliveira, K. R., da Fonseca, A. L., Baratto, L. C.,& Moraes, W. P. (2017). In vitro and in vivo antimalarial potential of oleoresin obtained from Copaifera reticulata Ducke (Fabaceae) in the Brazilian Amazon rainforest. *Phytomedicine*, 24, 111-118.

Essien, E. E., Newby, J. S., Walker, T. M., Setzer, W. N., &Ekundayo, O. (2015).Chemotaxonomic characterization and in-vitro antimicrobial and cytotoxic activities of the leaf essential oil of Curcuma longa grown in southern Nigeria. *Medicines*, *2*(4), 340-349.

Ezatpour, B., SaediDezaki, E., Mahmoudvand, H., Azadpour, M., &Ezzatkhah, F. (2015). In vitro and in vivo antileishmanial effects of Pistacia khinjuk against Leishmania tropica and Leishmaniamajor. *Evidence-Based Complementary and Alternative Medicine*,eCAM, 2015, 149707.

Fonseca-Silva F., Inacio J. D., Canto-Cavalheiro M. M., Almeida-Amaral E. E. (2016). Reactive oxygen species production by quercetin causes the death of Leishmania amazonensis intracellular amastigotes. *Journal of natural products*, 76(8),1505–8.

Fukui, M. J., Dias, H. J., Severiano, M. E., de Souza, M. G., de Oliveira, P. F., Ambrosio, S. R.,& Crotti, A. E. (2018). Antimicrobial and cytotoxic activity of dihydrobenzofuran neolignans. *Chemistry Select*, 3(6), 1836-1839.

Funk, J. L., Frye, J. B., Oyarzo, J. N., Zhang, H., & Timmermann, B. N. (2010). Anti-arthritic effects and toxicity of the essential oils of turmeric (*Curcuma longa* L.). *Journal of agricultural and food chemistry*, 58(2), 842-849.

Garcia, A. R., Amaral, A. C. F., Azevedo, M. M., Corte-Real, S., Lopes, R. C., Alviano, C. S., & Rodrigues, I. A. (2017). Cytotoxicity and anti-Leishmania amazonensis activity of Citrus sinensis leaf extracts. *Pharmaceutical biology*, *55*(1), 1780-1786.

García, M., Monzote, L., Montalvo, A. M., & Scull, R. (2010).Screening of medicinal plants against *Leishmania amazonensis*. *Pharmaceutical biology*, *48*(9), 1053-1058.

Ghorbani, M., &Farhoudi, R. (2018). Leishmaniasis in humans: drug or vaccine therapy?. *Drug Design, Development and Therapy*, *12*, 25-40.

Gomes, D., Alegrio, L. V., de Lima, M. E., Leon, L. L., & Araujo, C. A. (2002). Synthetic derivatives of curcumin and their activity against Leishmania amazonensis. *Arzneimittel-Forschung*, 52(2), 120–124.

Gour, J. K., Srivastava, A., Kumar. V., Bajpai, S., Kumar, H., Mishra, M., Singh, R. K. (2009). Nanomedicine and Leishmaniasis: future prospects. *Digest Journal of Nanomaterials and Biostructures*, 4(3), 495-499.

Gour, J.K., Kumar, V., Singh, N., Bajpai, S., Pandey, H. P., Singh, R.K. (2012). Identification of Th1-responsive leishmanial excretory-secretory antigens (LESAs). *Experimental Parasitology,* 132(3), 355-61.

Granthon, A. C., Braga, M. V., Rodrigues, J. C., Cammerer, S., Lorente, S. O., Gilbert, I. H., Urbina, J. A., & de Souza, W. (2007). Alterations on the growth and ultrastructure of Leishmania chagasi induced by squalene synthase inhibitors. *Veterinary parasitology*, 146(1-2), 25–34.

Barati, M., Mohebali, M., Alimohammadian, M. H., Khamesipour, A., Akhoundi, B., & Zarei, Z. (2015). Canine visceral leishmaniasis: seroprevalence survey of asymptomatic dogs in an endemic area of northwestern Iran. *Journal of parasitic diseases : official organ of the Indian Society for Parasitology*, *39*(2), 221–224.

Hoyos, C. L., Cajal, S. P., Juarez, M., Marco, J. D., Alberti D'Amato, A. M., Cayo, M., Torrejón, I., Cimino, R. O., Diosque, P., Krolewiecki, A. J., Nasser, J. R., & Gil, J. F. (2016). Epidemiology of American Tegumentary Leishmaniasis and *Trypanosoma cruzi* Infection in the Northwestern Argentina. *BioMed research international*, *2016*, 6456031. https://doi.org/10.1155/2016/6456031.

Huber, W., Koella, J. C., (1993). A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. *Acta Tropica,* 55(4), 257-61.

J. L., Ramos, A., Rosa, M., Vermelho, A. B., & Rodrigues, I. A. (2014). Liposomal formulation of turmerone-rich hexane fractions from Curcuma longa enhances their antileishmanial activity. *BioMed research international*, *2014*, 694934.

Jayaprakasha, G. K., Jagan Mohan Rao, L., &Sakariah, K. K. (2002).Improved HPLC method for the determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. *Journal of agricultural and food chemistry*, 50(13), 3668-3672.

Kazemi, M., & Rostami, H. (2015).Chemical composition, antimicrobial and antioxidant activities of the essential oil of Psammogeton canescens. *Natural product research*, 29(3), 277-280.

Khanra, S., Kumar, Y. P.,Dash, J., & Banerjee, R. (2018). In vitro screening of known drugs identified by scaffold hopping techniques shows promising leishmanicidal activity for suramin and netilmicin. *BMC research notes*, 11(1), 319.

Koide, T., Nose, M., Ogihara, Y., Yabu, Y., & Ohta, N. (2002).Leishmanicidal effect of curcumin in vitro. *Biological & pharmaceutical bulletin*, 25(1), 131–133.

Laouer, H., Hireche-Adjal, Y., Prado, S., Boulaacheb, N., Akkal, S., Singh, G., & Szczepaniak, L. (2009). Chemical composition and antimicrobial activity of essential oil of Bupleurum montanum and B. plantagineum. *Natural Product Communications*, 4(11), 1605-1610

Leong-Skornickova, J., & Newman, M. (2015). Gingers of Cambodia, Laos & Vietnam. Singapore: *Singapore Botanic Gardens*, National Parks Board. Singapore,2.000. 232.

Mahmoudvand, H., Sharififar, F., Sharifi, I., Ezatpour, B., Harandi, M. F., Makki, M. S., & Jahanbakhsh, S. (2014). In vitro inhibitory effect of Berberis vulgaris (Berberidaceae) and its main component, berberine against different *Leishmania* species. *Iranian journal of parasitology*, *9*(1), 28–36.

Moore, E. M., & Lockwood D, N. (2010). Treatment of visceral leishmaniasis. *Journal of Global Infectious Disease*, 2(2), 151-158.

Moreira, R. R. D., Santos, A. G. D., Carvalho, F. A., Perego, C. H., Crevelin, E. J., Crotti, A. E. M., & Nakamura, C. V. (2019). Antileishmanial activity of Melampodium divaricatum and Casearia sylvestris essential oils on Leishmania amazonensis. *Revista do Instituto de Medicina Tropical de Sao Paulo*, 61, e33.

Musa, A., Khalil, E., Hailu, A., Olobo, J., Balasegaram, M., Omollo, R., Edwards, T., Rashid, J., Mbui, J., Musa, B., Abuzaid, A. A., Ahmed, O., Fadlalla, A., El-Hassan, A., Mueller, M., Mucee, G., Njoroge, S., Manduku, V., Mutuma, G., Apadet, L., … Wasunna, M. (2012). Sodium stibogluconate (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. *PLoS neglected tropical diseases*, *6*(6), e1674.

Nascimento, A. M., Brandao, M. G., Oliveira, G. B., Fortes, I. C., & Chartone-Souza, E. (2007).Synergistic bactericidal activity of Eremanthus erythropappus oil or β-bisabolene with ampicillin against Staphylococcus aureus. *Antonie van Leeuwenhoek*, 92(1), 95-100.

Nigussie, D., Tasew, G., Makonnen, E., Debella, A., Hurrisa, B., Urga, K., &Wayessa, A. (2015). In-vitro investigation of fractionated extracts of Albizia gummifera seed against Leishmania donovani amastigote stage. *J Clin Cell Immunol,* 6 (06). 373. doi:10.4172/2155-9899.1000373.

Oryan, A. (2015). Plant-derived compounds in treatment of leishmaniasis.Iranian Journal of Veterinary Research, Shiraz University. Iranian journal of veterinary research, 16(1), 1-9.

Praditya, D., Kirchhoff, L., Brüning, J., Rachmawati, H., Steinmann, J., & Steinmann, E. (2019). Anti-infective Properties of the Golden Spice Curcumin. *Frontiers in microbiology*, *10*, 912.

Rahmani, A. H., Alsahli, M. A., Aly, S. M., Khan, M. A., &Aldebasi, Y. H. (2018). Role of curcumin in disease prevention and treatment. *Advanced biomedical research*, 7.

Rasmussen, H. B., Christensen, S. B., Kvist, L. P., & Karazmi, A. (2000). A simple and efficient separation of the curcumins, the antiprotozoal constituents of Curcuma longa. *Planta medica*, 66(04), 396-398.

Ravindran, P. N., Nirmal Babu, K., & Sivaraman, K. (2007). Turmeric: The golden spice of life. *Turmeric: The Genus Curcuma.* 2nd edition. Boca Raton (FL): CRC Press/Taylor & Francis; 2011. Chapter 13.

Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B., & Brooker, S. (2007). Cutaneous leishmaniasis. *The Lancet infectious diseases*, 7(9), 581-596.

Rodrigues, J. C., Attias, M., Rodriguez, C., Urbina, J. A., & Souza, W. d. (2002). Ultrastructural and biochemical alterations induced by 22,26-azasterol, a delta(24(25))-sterol methyltransferase inhibitor, on promastigote and amastigote forms of Leishmania amazonensis. *Antimicrobial agents and chemotherapy*, 46(2), 487–499.

Rubab, M., Chelliah, R., Saravanakumar, K., Barathikannan, K., Wei, S., Kim, J. R.,& Oh, D. H. (2020). Bioactive Potential of 2-Methoxy-4-vinylphenol and Benzofuran from Brassica oleracea L. var. capitate f, rubra (Red Cabbage) on Oxidative and Microbiological Stability of Beef Meat. Foods (Basel, Switzerland), 9(5), 568.

Saleheen, D., Ali, S. A., Ashfaq, K., Siddiqui, A. A., Agha, A., & Yasinzai, M. M. (2002). Latent activity of curcumin against leishmaniasis in vitro. *Biological & pharmaceutical bulletin*, 25(3), 386–389.

Sanjay J, Satyaendra S, Satish N., & Sumbhate, S. (2007). Recent trends in Curcuma longa Linn. *Pharmacognosy Reviews*. 1(1):119-128.

Sasikumar, B. (2005). Genetic resources of Curcuma: diversity, characterization and utilization. *Plant Genetic Resources*, *3*(2), 230-251.

Simon L. Croft, Shyam Sundar, and Alan H. Fairlamb, (2006). Drug Resistance in Leishmaniasis. *Clinical Microbiology Reviews*. 19(1), 111–126.

Sindhu, S., Chempakam, B., Leela, N. K., & Bhai, R. S. (2011).Chemoprevention by essential oil of turmeric leaves (Curcumalonga L.) On the growth of Aspergillus flavus and aflatoxinproduction. *Food and Chemical toxicology, 49*(5), 1188-1192.

Singh, R., & Sharma, P. (2011). Hepatoprotective effect ofcurcumin on lindane-induced oxidative stress in male wistarrats. *Toxicology International, 18*(2), 124.

Srinivasan, D., Nathan, S., Suresh, T., & Lakshmana Perumalsamy, P. (2001). Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *Journal of ethnopharmacology*, *74*(3), 217–220.

Sundar, S., & Singh, A. (2016). Recent developments and future prospects in the treatment of visceral leishmaniasis. *Therapeutic Advances in Infectious Disease*, 3(3-4), 98-109.

Sundar, S., Chakravarty, J., Agarwal, D., Rai, M., & Murray, H. W. (2010).Single-dose liposomal amphotericin B for visceral leishmaniasis in India. *New England Journal of Medicine*, 362(6), 504-512.

Tavares-Dias, M. (2018). Current knowledge on use of essential oils as alternative treatment against fish parasites. *Aquatic LivingResources*, *31*,13. doi: https://doi.org/10.1051/alr/2018001

Teles, A. M., Rosa, T., Mouchrek, A. N., Abreu-Silva, A. L., Calabrese, K., & Almeida-Souza, F. (2019). *Cinnamomum zeylanicum*, *Origanum vulgare*, and *Curcuma longa* Essential Oils: Chemical Composition, Antimicrobial and Antileishmanial Activity. *Evidence-based complementary and alternative medicine : eCAM*, *2019*, 2421695.

Tiwari, B., Pahuja, R., Kumar, P., Rath, S. K., Gupta, K. C., & Goyal, N. (2017). Nanotized Curcumin and Miltefosine, a Potential Combination for Treatment of Experimental Visceral Leishmaniasis. *Antimicrobial agents and chemotherapy*, *61*(3), e01169-16.

***