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PHYTOCHEMICAL SCREENING OF *P. LONGUM* AND ANTI-LEISHMANIAL ACTIVITY BY *IN-VITRO* ANALYSIS OF THE EXTRACTED DRUG

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ABSTRACT

The parasitic protozoa *Leishmania* belongs to the order Kinetoplastida and are the causative agents of disabling and incurable diseases known as leishmaniasis. In the given article we had done the *in-vitro* anti-leishmanial analysis of the plant *P. longum* with their phytochemical analysis we had first of all collected the plant and washed it properly with water then after we had washed several time with different solvent after that we had taken its ash value and foreign mater then after we had investigated it with ***In vitro*** **anti-leishmanial activity of compounds on promastigotes and axenicamastigotes of *P. longum*and cell cytotoxicity of the compounds on J774A.1 cell line.** Throughout the whole study we find the result more satisfying and it is also suggestive for further investigation.

1. INTRODUCTION

The parasitic protozoa *Leishmania* belongs to the order Kinetoplastida and are the causative agents of disabling and incurable diseases known as leishmaniasis.[1] *Leishmania* parasites were independently described by William Leishman and Charles Donovan in 1903, but were previously observed by David Cunningham in 1885 and Peter Borovsky in 1898. These parasites were mistaken for other protozoa and the genus *Leishmania* were proposed by James Wright only in 1903. Leishmaniasis is an infection caused by a parasite that is spread to people through the bite of the female phlebotomine sand fly. The parasite exists in many tropical and temperate countries. Leishmaniasis is still one of the world's most neglected diseases, affecting largely the poorest of the poor, mainly in developing countries; 350 million people are considered at risk of contracting leishmaniasis, and some 2 million new

cases occur yearly. This is the second most prevalent parasitic disease after malaria.[2] There are near about 30 species of Leishmania that infect mammals and about 21 species causes disease in human that ranging from self-healing cutaneous, mucocutaneous to deadly visceral form.[3] The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, DNA sequence analysis, or monoclonal antibodies.[4] These include: The *L. donovani*complex with three species *(L. donovani, L.infantum, and L. chagasi).* The *L. mexicana*complex with 3 main species *(L. mexicana, L. amazonensis, and L. venezuelensis); L. tropica; L. major; L. aethiopica*; and the subgenus Viannia with four main species (*L. (V.) braziliensis L. (V.guyanensis, L.(V.) panamensis, and L. (V.) peruviana*).[5]

2. PLANT MATERIAL

The fruits of *P. Longum*were collected from their natural habitat and procured from a registered vendor in New Delhi. The air-dried fruits of *P. Longum*were then ground to a fine powder and this powdered plantmaterial was used for chemical investigation washed under current water, then Milli-Q water and dried under room temperature.Plant materials were subjected to exhaustiveextraction and the extracts obtained were subsequently concentrated and dried. The dried extracts were screened for anti-leishmanial activity.

2.1 Chemicals and instruments

Acetonitrile, anisaldehyde, dichloromethane, ethanol, ethyl acetate, glacial acetic acid, *n*-hexane, benzene, chloroform, ethyl acetate and methanol and sulphuric acid. All the chemicals were procured from Merck and Qualigens and Sigma-Aldrich. Column chromatography was performed over silica gel 60 (Merck, Darmstadt, Germany) of different mesh sizes. Thin layer chromatography (TLC) was performed with Silica gel 60 GF254pre-coated on aluminium sheets (20 x 20 cm) (Merck, Germany). Preparative TLC was performed with Silica gel 60 GF254 pre-coated on glass plates (5 x 10 cm) (Merck, Germany). UV-vis spectra were recorded on a Shimadzu UV-1700Pharma spec. IR (KBr) spectra were recorded using a Nicolet model Prote´ge´ 460 spectrophotometer. 1H NMR and 13C NMRspectra were recorded in CDCl3 solution with Bruker DRX 500 MHz spectrometer using TMS as internal standard. The chemical shift values are reported in ppm (δ) and the scalar coupling constants (*J*) are in Hz. HRESIMS were performed on a Waters 1525 LCT Micro mass Spectrometer. HPLC was performed on Waters LC system including a 600 pump and 2998 photodiode array detector. RP-18 analytical column (5 mm, 3.9 x 300 mm i.d.,

Shimadzu) was used. The spots were visualized under UV light (254 and 366 nm) and then sprayed with Dragendorff's reagent.[6]

2.2 Strains/cell lines and chemicals required for screening of biological activity

2.2.1 Culture medium

The compounds were screened by modified MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide] assay to examine their leishmanicidal activity. Parasite cultures were grown in phenol red-free RPMI-1640 (Sigma, USA) that was supplemented with 10% FCS(Sigma, USA). These cultures were grown at 26ºc. Promastigotes in late log phase were incubated in RPMI-1640 (supplemented with 10% FBS) at an average of 10 cells/mL. The compounds were aseptically dissolved in DMSO and were diluted appropriately with the growth medium. Cell growth was assayed by measuring the reduction of 2, 5-diphenyltetrazolium bromide (MTT).[7] Miltefosine was used as a positive control. Mean values (µg/mL ± standard deviation) were calculated by testing each concentration in triplicates. The axenic amastigote culture was based on minor modifications of the method described by (for various *Leishmania* strains). Briefly, promastigotes in late stationary phase were centrifuged and re-suspended in medium 199 containing Hank's balanced salts and supplemented with 20% heat inactivated fetal bovine serum (HIFBS), 2.5 g glucose, 5 g trypticase peptone, 0.75 g glutamine, haemin (0.5 mg/mL in 0.05 M NaOH; 60 mL) and 1 mL of gentamycin (50 mg/mL). The medium was sterile-filtered and stored at 4°C until use. The cells were then incubated at 37°C in humidified 5% CO_2 incubator for seven days. Cell toxicity was assessed by measuring the reduction of MTT by mouse macrophages (cell line J774A.), cultured in PBS medium [Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium]. Human leukemia HL-60 cells were grown in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 units/mL), streptomycin (100 µg/mL), L-glutamine (0.3 mg/mL), pyruvic acid (0.11 mg/mL), 0.37% NaHCO3 and 50 µM of 2-mercaptoethanol in a CO_2 incubator (Thermocon Electron Corporation, USA) at 37°C with 98% humidity and 5% CO_2 gas environment. Afterthat the cells at logarithmic growth phase were treated with samples dissolved in DMSO whilethe untreated control cultures received only the vehicle (DMSO, <0.5%).[8]

2.2.2 Test strains and cell lines

Promastigotes of (DD8), axenic amastigotes, and macrophage cell line J774A.1 were collected from the 'Cell Death and Differentiation Research Laboratory' at the

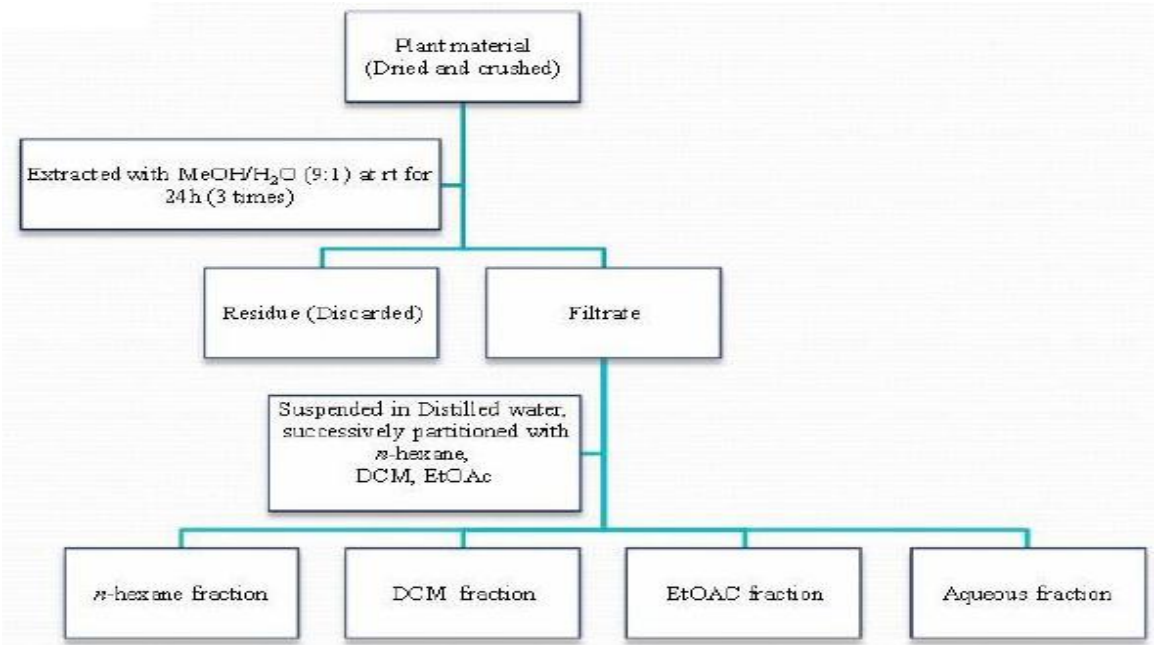
NationalInstitute of Immunology, New Delhi.HL-60 cell line was procured from Dia Laboratory Pune.

2.3 Phytochemical analysis

The air-dried part of the plant *P. longum*which is to be selected for the study were then grind to a fine powder and this powdered plant material was used for chemical investigation. The plant material was subjected to exhaustive extraction and the extracts obtained were subsequently concentrated and dried. The dried extracts were screened for anti-leishmanial activity.

2.4 Extraction from plant material

The powdered fruits of *P. longum*were exhaustively extracted with methanol-water (9:1) solvent system. For this, the powdered plant material was dipped in methanol: water system and kept overnight at room temperature. The process was repeated 3-5 times for complete extraction. The extract was then concentrated under reduced pressure and at a temperature below 500°c in rotatory evaporator. The concentrated extract was suspended in water and successively partitioned with *n*-hexane, DCM, EtOAc and aqueous fractions respectively. The organic fractions were concentrated under reduced pressure while the aqueous fraction was concentrated in a lyophilizer. The fractions of the plant were screened for leishmanicidal activity by MTT based *in vitro* assay on the promatigotesthe*n*-hexane fraction was found to be the most active withIC50 value of 100µg/mL. Therefore, the *n*-hexane fraction was chosen for further investigation.



The collected plant material (leaves of *cedrusdeodara*) was washed thoroughly in water, dried under shade and ground to coarse powder in electric grinder. Powdered material was then extracted successively in Soxhlet apparatus. Soxhlation process was run for 10-15 h for each solvent (Benzene, Petroleum Ether, Chloroform, Ethyl acetate, methanol respectively) for effective and proper extraction. Solvents were removed under reduced pressure and temperature using rotary evaporator (EYELA, Japan) to get the concentrated extract. Further, plant extracts were lyophilized to get dried extract and obtained extracts were stored in airtight container in a refrigerator till further use.

2.5 Physico-Chemical Parameters for the Standardisation of Crude Drug

A) Determination of foreign matter

50 g of drug sample examined was weighed and spread out a thin layer. The foreign matter was detected by inspection with the unaided eye. Separated and weighed it and calculated the percent present. Drug undertaken for further study were free from moulds, insects, animal faecal matter and other contamination such as soil, stones and extraneous material.

B) Determination of moisture content [Hot Air Oven Method]

To determine the amount of moisture (water drying off from the drug) for substance appearing to contain water as the only volatile constituent, the procedure given below, was used. 2.78 g of drug (without preliminary drying) after accurately weighing was placed in a tare evaporating dish. After placing the above said amount of the drugs in the tared evaporating dish, dried at 105^{o}C for 5 hrs, and weighed, percentage was calculated with reference to initial weight.

C) Determination of ASH

1. *Determination of Total Ash*

About 2.0 g of powder drug was incinerated in a redtop silica dish at a temperature not exceeding 450^{0} C. until free carbon was left, cooled and final weight was taken. The percentage of ash calculated with reference to the air-dried drug.

2. Determination of Acid Insoluble Ash

The ash obtained as above method was boiled for 5 minutes with 20 ml of dilute hydrochloric acid and collected the insoluble matter on the ash-less filter paper, washed with hot water and ignited to constant weight. The percentage of acid insoluble ash with reference to the air-dried drug was calculated.

3. Determination of water-soluble Ash

The ash was boiled for 5 minutes with 20 ml of water, collected insoluble matter on the ash-less filter paper, washed with hot water, and ignited for a temperature not exceeding 450 ^{o}C. The weight of the insoluble matter was subtracted from the weight of the drug ash. The difference in weight represents the water-soluble ash. Finally, percentage of water-soluble ash with reference to the air-dried drug was calculated.

3. RESULT AND DISCUSSION

3.1 Foreign Matter- There is no any type of adulterants was found in the dried powder of the fruits of *P. Longum*. Due to the precautiosly collection of the fruits and leaves of the given plant and it was washed properly with water to clean the particles and sands on outer part.

3.2 Determination of moisture content [Hot Air Oven Method]

The amount of moisture was determined in reference with the initial sample taken and the sample was dried completely and then weight and calculated percent of moisture content is given respectively and they are

Table 4.1: Moisture content of the sample extracted from different part of plant.

S. No.	Sample	Percent Moisture Content
1	*P. longum*	4.3

3.3 Determination of ASH

Total amount ash, acid insoluble ash and water-soluble ash was calculated and it is represented within the given table as follows-

Table 4.2: Ash determination of the given samples.

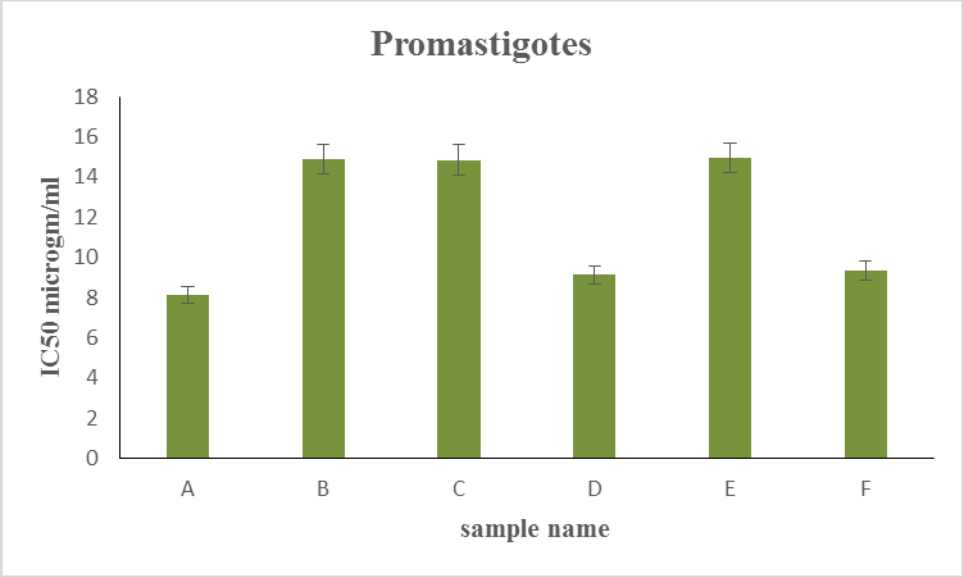
Name	***P. Longum***
Total ash	25± 1.2 (%)
Acid insoluble	2.78 %
Water Soluble	3.56 %

3.4 *In vitro* cell line study

***In vitro* anti-leishmanial activity of compounds on promastigotes and axenic amastigotes of *P.Longum*and cell cytotoxicity of the compounds on J774A.1 cell line**

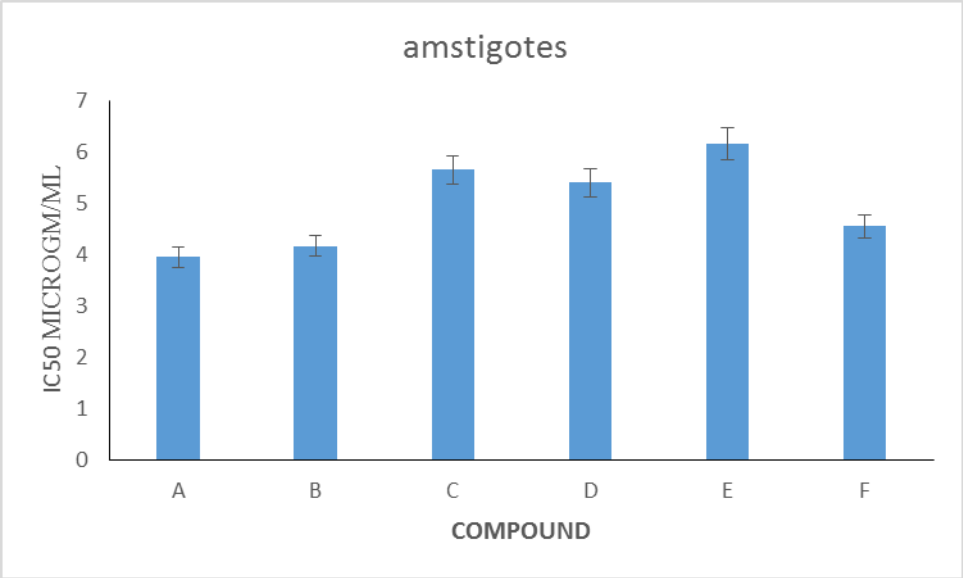
A. Anti-leishmanial activity of compounds on promastigotes.

The activity of the promastigotes are given graphically and it suggest that the plant having very good activity as suggested for further *in-vivo* investigations.



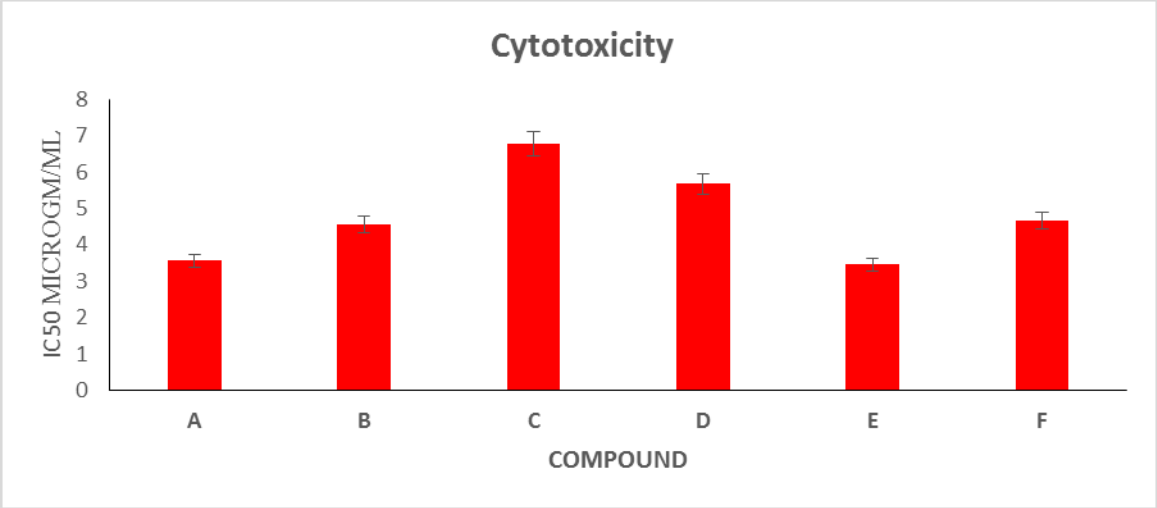
B. Anti-leishmanial activity of compounds onaxenicamastigotes

The activity of the axenicamastigotes of *P.Longum* are given graphically and it suggest that the plant having very good activity as suggested for further *in-vivo* investigations.



C. Anti-leishmanial activity of compounds cell cytotoxicity assay

The cytotoxicity of *P.Longum* are given graphically and it suggest that the plant having very good activity as suggested for further *in-vivo* investigations.



Cell cytotoxicity of all these compounds was assessed against mouse macrophage cell line J774A.1 measured at IC50 and twice that of IC50. The experiment showed that none of the compounds exhibited more than 7.5% cytotoxicity at a concentration twice that of IC50.

CONCLUSION

Compound E is found more suitable and it is having all parameters are very asthonishing result against the leshmaniasis cell line so that this can be investigated further for the in vivo model the Compound is pure ethanolic extract which is having no any foreign matter and also it is found within the fruit of *L.longum* so that it is having very good activity in purified form.

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