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Latent Activity of Curcumin against Leishmaniasis *in Vitro*

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In this study the anti-proliferative effect of curcumin (curcuma longa) that is the active ingredient of ground dried rhizome has been studied against three local and three reference leishmanial strains, *Leishmania major*, *Leishmania tropica* and *Leishmania infantum* (Pakistani isolate). Curcumin has shown an average IC_{50} of 5.3 μM against promastigotes of various leishmanial strains which is much lower as compared with pentamidine that is one of the basic treatments against leishmaniasis. The main draw back attributed to these assays performed on promastigotes is the heterogeneity of results compared with those obtained with intracellular amastigotes or with *in vivo* effect. We also tested activity of curcumin against axenic amastigote like cells (AALC) of *L. major* strain (MHOM/PK/88/DESTO). Curcumin proves to be far more potent then pentamidine against AALC which further strengthens the fact about its leishmaniacidal activity.

Key words curcumin; leishmania; leishmanial promastigotes; axenic amastigote like cell

Leishmaniasis are a group of diseases, endemic in many parts of the world.[1,2] Leishmaniasis are mostly prevalent in poor developing countries evident by the growing number of cases seen in AIDS patients[3] and the occurrence of viscerotropic *Leishmania tropica* disease among Persian Gulf War Participants.[2,3] The disease is transmitted by the members of the genus *Leishmania* which is a protozoal parasite. These parasitic protozoans are digenetic and have two distinct stages in their life cycle. The motile flagellated promastigote stage lives in the alimentary tract of the sand fly vector, while the non-motile amastigote stage resides inside the macrophages of mammalian hosts.[4]

Leishmanial infections include three major syndromes: cutaneous, visceral and mucosal leishmaniasis. The basic treatment consists in the administration of sodium stibogluconate (pentostam), meglumine (glucantime) or pentamidine. Common problem with this basic treatment especially in Kala-azar, mucosal leishmaniasis is the developing drug resistance of the parasite.[5] In addition, the low efficacy of pentavalent antimony in the treatment of patients coinfected with AIDS is often noticed.[6] These problems prompted the development of new anti-leishmanial drugs.[7—10]

Curcumin is a yellow-orange dye derived from the rhizome of the plant *Curcuma longa.* It has long been used as a naturally occurring medicine for the treatment of inflammatory diseases. Curcumin (difleruloyl methane) is a natural phenolic compound. It is a potent anti-tumor agent having anti-inflammatory and anti-oxidant properties. It induces apoptosis in cancer cells[11,12] and inhibits TPA-induced Protein kinase C (PKC) activity.[13] It has also shown anti-bacterial, anti-fungal and anti-trypnosomal activity.[1,14,15] A recent study has also shown action against the promastigote forms of *Leishmania major.*[16]

In this report we evaluated the actions of curcumin against the promastigote forms of different reference and local leishmanial strains *in vitro.* We also evaluated the activity of curcumin against the axenic amastigote like cells (AALC) of *L. major in vitro.*

MATERIALS AND METHODS

Parasite Cultures All the promastigote cultures of both the reference and local Pakistani leishmanial strains were maintained in blood agar based bi-phasic Evan's modified Tobie's medium supplemented with RPMI-1640 with 25 mM TES at 25 °C. The reference leishmanial strains were obtained from London School of Hygeine and Tropical Medicine. The reference strains of promastigotes that were used include *L. major* (JISH118), *L. tropica* (K27) and *L. infantum* (LEM3437). Local leishmanial strains used in this study include *L. major* (MHOM/PK/88/DESTO) in the promastigote and AALC stage (see below). Other local strains used in the promastigote stage include *L. tropica* and *L. infantum.* The long term continuous culture of axenic amastigote like cells (AALC) of *L. major* (MHOM/PK/88/DESTO) strain was successfully established. Briefly, AALC cells were obtained from the promastigotes by the gradual adaptation of cells to increasing temperature and falling pH in RPMI medium supplemented with 20% fetal calf serum (FCS). This was performed in a stepwise fashion through the passage and adaptation of cells to new culture condition (higher temperature and lower pH reaching a temperature of 32 °C and a pH of 5.1, in a long process of stabilization to each new growing condition).

Chemicals Unless otherwise stated, all the chemicals including curcumin were obtained from Sigma/Aldrich.

Viability Test Assays on Promastigotes: Parasites in the promastigote stage were transferred from Evan's modified Tobie's medium to RPMI-1640 supplemented with 10% fetal bovine serum (FBS) buffered with 25 mM TES, pH 7.2. They were grown in bulk at 25 °C. They were centrifuged at 2500 ***g*** for 10 min and early log phase promastigotes were collected. The parasites were washed twice with RPMI (without FBS) and resuspended in the complete medium to achieve a final concentration of 10^6 parasties/ml. In order to get the 100% growth inhibition concentratrion (TGI) and LD_{50} of drugs, serial dilutions of curcumin and pentamidine (taken as control) in the promastigote culture medium (100 μl) were performed in 96-well microtiter plate. Subsequently

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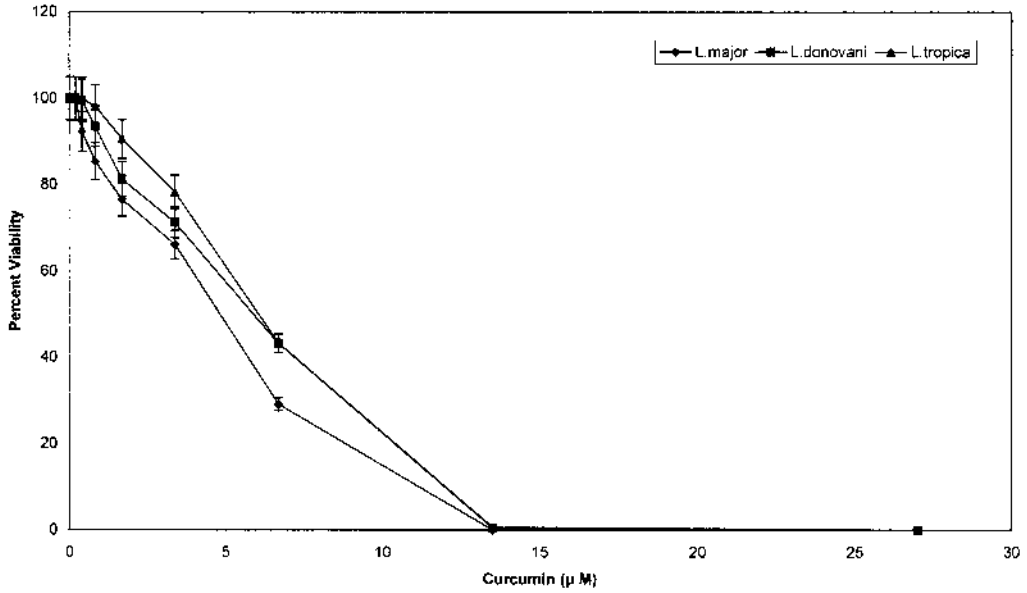

Fig. 1. Effect of Curcumin on Reference Leishmanial Strains (Promastigotes)

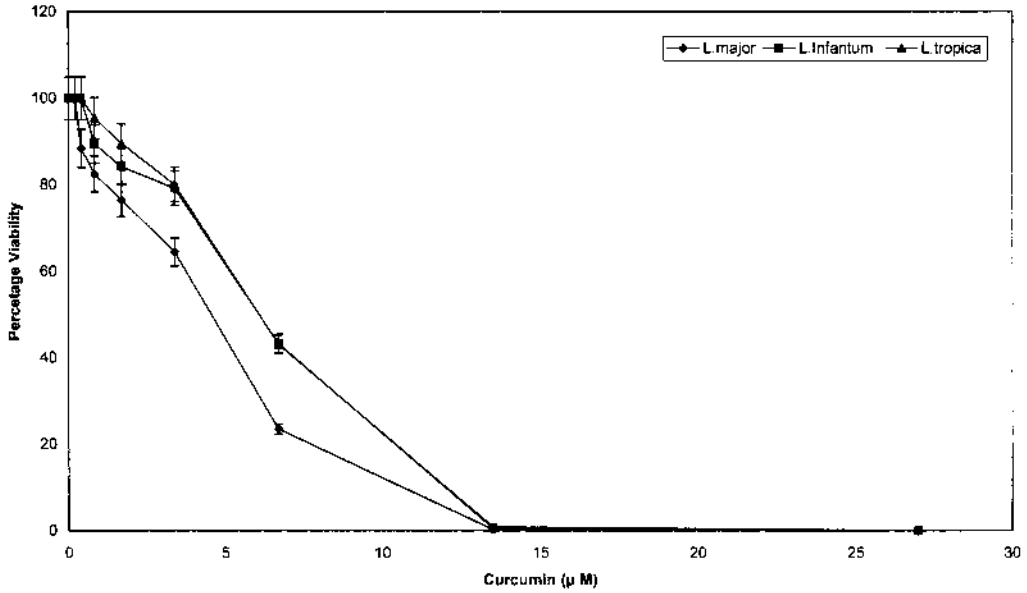

Fig. 2. Effect of Curcumin on Local Leishmanial Strains (Promastigotes)

10^5 promastigotes in 100 μl of culture medium were added to each well and the plate was incubated at 25 °C for 72 h. Negative controls (culture without curcumin) were on the same plate. At the end of the incubation time the plate was shaken mechanically over a plate shaker and parasites were counted by the help of a hemocytometer. Dose dependent viability curves were obtained subsequently.

For Amastigotes: The AALC drug susceptibility determinations were performed by using the same method applied for promastigotes. Serial dilutions of drugs (curcumin and pentamidine) in culture medium of *L. major* (100 μl/well) were performed in a 96 well microtiter plate; then 100 μl of AALC in RPMI medium (pH 5.1) from the culture in the log. phase of growth at a concentration of 5×10^5 cells/ml was added and incubated at 32 °C for 72 h. After 72 h, the plate was shaken mechanically and parasites were counted. Dose-dependent viability curve was obtained subsequently. All the results for the promastigotes as well as for the AALC were triplicated.

Statistical Analysis Two sample independent *t* test with significant *p* values (<0.05) was used to compare the drug susceptibilities of the parasites at both stages. Each experiment was triplicated ($n=3$).

RESULTS AND DISCUSSION

In this report, we evaluated the actions of curcumin against the promastigote forms of reference leishmanial strains and local leishmanial strains *in vitro.* As shown in Figs. 1, 2 and Table 1, curcumin showed cytotoxicity against both the local and reference leishmanial strains in the promastigote stage. No major differences in the degree of susceptibility of para-

Table 1. Effect of Pentamidine on Local and Effect of Curcumin on Local and Reference Leishmanial Strains

Effect of pentamidine on local strains		Effect of curcumin on local strains		Effect of curcumin on reference strains	
		IC_{50} values (μM)			
L. major	10.5±0.52	*L. major*	4.3±0.21	*L. major*	4.5±0.22
L. tropica	9.8±0.49	*L. tropica*	5.9±0.29	*L. tropica*	5.7±0.28
L. infantum	11.1±0.55	*L. infantum*	5.9±0.29	*L. donovani*	5.9±0.29

Each experiment was repeated at least three times (n=3) with consistent results indicating that the assays used in this study were highly reproducible. A ±5% standard error was calculated for all the results, which is always associated with hemocytometer counting. Curcumin proves to be more potent than pentamidine (t=12.7, p<0.001) whereas no significant difference was found b/w the susceptibility of the curcumin on the local and reference leishmanial strains. (t=0, p>.5).

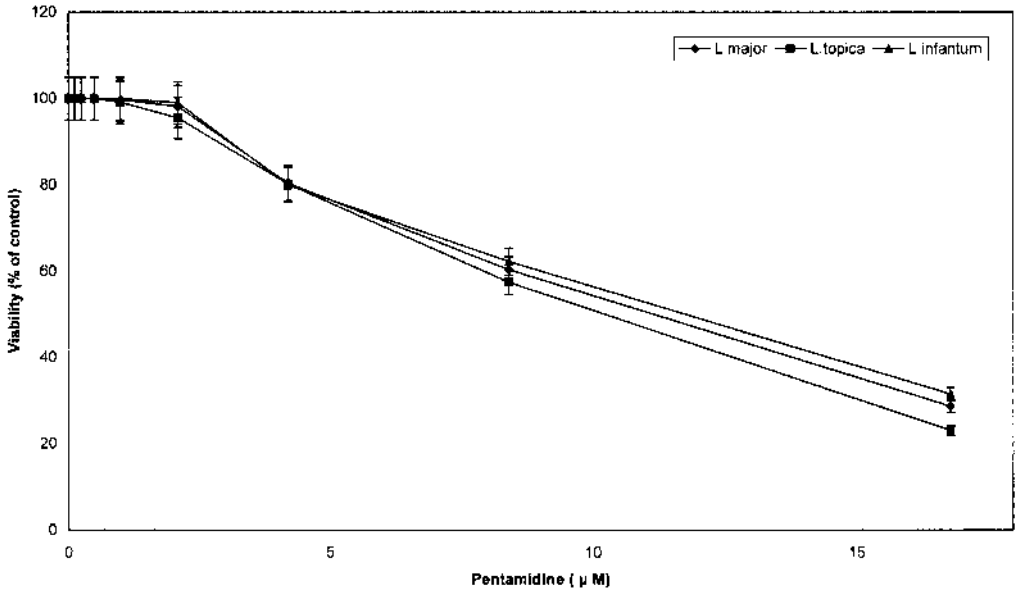

Fig. 3. Effect of Pentamidine on Local Leishmanial Strains (Promastigotes)

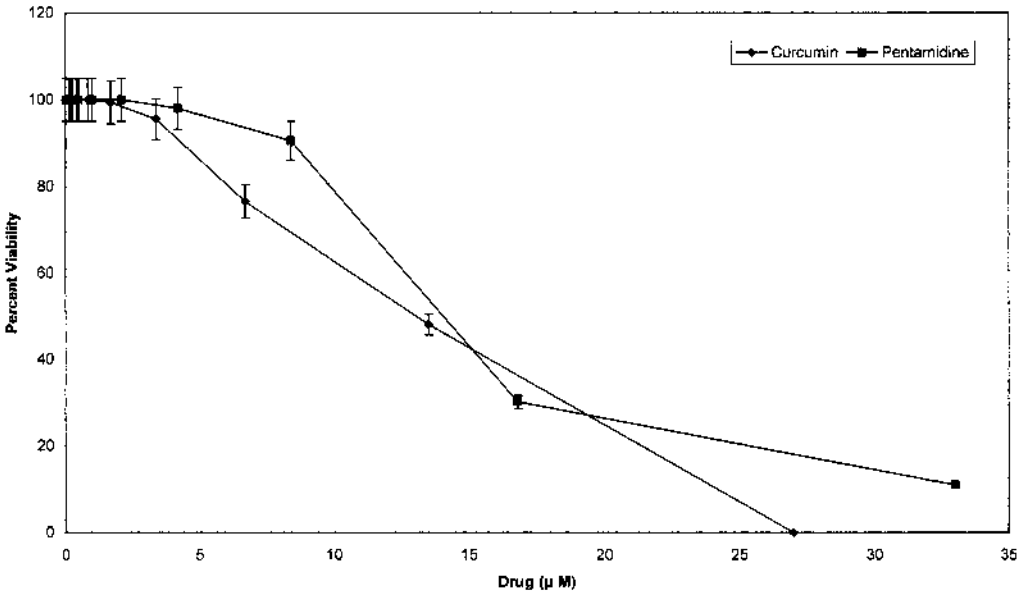

Fig. 4. Effect of Curcumin on *L. major* (AALC)

sites occurred during the study. Whereas 100% killing of all the strains was noticed at 13.5 μM. The leishmaniacidal effects of curcumin if compared with pentamidine clearly shows that curcumin has a higher potency *in vitro* ($t=12.7$, $p<0.001$) (Fig. 3, Table 1).

The reference strains were observed to have a higher growth rate and faster doubling time therefore they were expected to have higher IC_{50} values but the results showed that there is no significant difference b/w the potency of curcumin on both the reference and local leishmanial strains ($t=0$, $p<0.5$).

Each experiment was repeated at least three times with consistent results indicating that the assays used in this study were highly reproducible. A $\pm$5% standard error was calculated for all the results, which is always associated with hemocytometer counting.

There is no doubt that leishmania can be cultured most easily in the promastigote stage. Thus it is easier to perform such kind of assays on promastigotes but the main draw back attributed to these assays is the heterogeneity of results compared with those obtained with intracellular amastigotes or with *in vivo* effect.[17] In order to further strengthen the study, both drugs were checked on AALC (Fig. 4, Table 2). In a previous study no difference was found in the sensitivity of AALC and intracellular amastigtoes to antimonial preparations assayed.[18—20] Thus these AALC may prove to be reliable to check the sensitivities of different drugs against leishmania.

As shown in Fig. 4, curcumin proves to be far more potent then pentamidine against AALC of *L. major* strain which further strengthens the fact about its leishmaniacidal activity ($t=14.2$, $p<0.001$). However in order to further prove its antileishmanial activity curcumin needs to be checked against various other strains in AALC and intracellular amastigotes.

Previous studies have shown that curcumin easily penetrates into the cytoplasm of mammalian cells. It accumulates in the membranous structures such as plasma membrane, endoplasmic reticulum and nuclear envelope.[11] It has also been shown to suppress mitrogen induced proliferation of blood mononuclear cells and inhibit the proliferation of rabbit vascular smooth muscle cells stimulated by fetal calf serum (FCS). However the mechanism of action of curcumin against leishmania still needs to be explored and awaits further studies.

Table 2. Effect of Curcumin and Pentamidine on AALC

Drug	IC_{50} (μM)	TGI[a] (μM)
Curcumin	10$\pm$0.5	27$\pm$1.35
Pentamidine	13.5$\pm$1.35	>55.6$\pm$2.78

A $\pm$5% standard error was calculated for all the results, which is always associated with hemocytometer counting. Each experiment was repeated thrice ($n=3$). Curcumin shows to be more potent against AALC as compared to pentamidine ($t=14.2$, $p<0.001$). *a*) TGI: Total growth inhibitory concentration.

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