Evaluation of Crude Extract of *Melia azedarach* Linn. Against Attenuated Amphotericin B Resistant *Leishmania tropica* Strain

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Abstract: Cutaneous leishmaniasis is still a big health problem around the world and drug resistance has emerged as a major problem in treatment. The purpose of the study was to generate Amphotericin B resistant strainand evaluate the crude extract of *Melia azedarach* against wild and lab generated resistant strain of *Leishmania tropica*. The result obtained revealed that the continuous increase of drug pressure for 60 days cause resistance in *L. tropica* by 09 fold compared to wild type. The LC_{50} value recorded for wild and resistant type strain was 0.024 and 0.224µg/ml, respectively against Amp B drug. The antileishmanial activity of crude extract against wild and resistant type strain was 0.57 and 123.3 µg/ml for green fruit and 1220.8 and 4010.1µg/ml for ripe fruit, respectively. A significant difference ($P<0.05$) in activitieswere observed between green and ripe fruit against wild and resistant type of strain. Based on the results obtained, it can be concluded that green fruit show promising results against resistant type of *L. tropica* and could be a novel candidate for anti-protozoal activity.

Keywords: *Leishmania tropica, Melia azedarach*, Resistant strain.

INTRODUCTION

Infections associated with protozoa are a global health problem, predominantly in the Third World countries [1-4], and roughly 14% of the world population is in risk. Protozoal infections are considered as a neglected tropical disease and therefore, immense concern has been shown by WHO [5]. These deadly tropical diseases have negative impacts on socioeconomic status of the patients [6]. There are a number of studies so far conducted on protozoan infections including chagas, malaria, sleep sickness and leishmaniasis. These diseases considered a major killing factor due to different difficulties are connected with controlling the parasite, high cost, resistant, poor safety, low efficacy and toxicity to the drug [7]. Among these protozoal infections leishmaniasis got special interest in various developing countries due to the increase of resistant to the drug [8].

Leishmaniasis is fatal disease caused by parasite *Leishmania* and transmitted through female phlebotomines sand fly bite. Leishmaniasis present in three different clinical forms i.e. cutaneous leishmaniasis, visceral leishmaniasis and muco-cutaneous leishmaniasis. It is present in tropical and subtropical areas of 88 countries of the world and approximately 10 million population of the world are suffering from cutaneous leishmaniasis [9].

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Toxicity, inconsistent efficacy and resistant between species or strains of the drug mostly used or for some of them, long lasting parenteral administration is the need of the day led the researcher for novel antileishmanial drug, most particularly from plant source used in traditional medicine, as a foundation of new leads with new mechanism of actions [10].

The present study focused on the *in vitro* antileish-manial activity of crude extract of *M. azedarach* against wild and lab produced resistant type *L. tropica* strain.

MATERIAL AND METHODS

Collection of Plant Materials

Fresh and ripe fruit of the plant were collected from the vicinity of Quaid-i-Azam University, Islamabad. The plant materials was authenticated by the Department of Plant Sciences, Quaid-i-Azam University. The plant materials were washed with distilled water and kept in shade till process. A voucher specimen (145255) was submitted in ISL herbarium, Quaid-i-Azam University.

Extraction

Both green and dried fruit (1 Kg) was macerated using mortar and pestle. The powdered obtained were soaked in 5 liter plastic beaker containing distilled water for 6 days with occasional shaking. The materials were filtered through muslin cloth and dried under reduce pressure using rotary evaporator (Heidolph Laborta4000 efficient). The paste like materials obtained were dried at 40^{o}C in water bath and stored at 4^{O}C for further use.

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Production of Resistant Strain

Resistant strain of *L. tropica* was developed according to the protocol described by Seifert *et al*. with slight modification [11]. The wild type culture was maintained in M199 medium with 10% heat inactivated Fetal Bovine Serum. Briefly, drug pressure was generated step wise manner for 60 days and the sensitivity of strain to Amp B was investigated. The culture showed resistance was maintained in drug pressure for further use at 24^{o}C.

In Vitro Antileishmanial Assay

Antileishmanial assay was performed according to the procedure described by Khan *et al*. 2014. Promastigotes of *L. tropica* strain were cultured in M199 media (Invitrogen, USA) with 10% Fetal Bovine Serum (PAA, GmbH). Briefly, stock solution of the test samples and standard drug Amphotericin B drug (10,000 ppm) were prepared in distilled water. Promastigotes were harvested at 1x10^{6} cells/ml and inoculated in 96 well plate. The assay was performed at different concentration (1000-0.001 ppm). For negative control sterilized distilled water was used. The 96 well plate was incubated for 48 h at 24^{O}C. The live promastigotes were counted using improved neubauer chamber under light microscope (40x magnification). The results obtained were statistically analyzed using SPSS Ver. 21 software [12].

Statistical Analysis

All the experiment was carried out in triplicate. For comparison Post Hoc Multiple comparison test was applied using SPSS Ver. 21 software. All the data were given mean ± standard deviation (M±SD).

RESULTS

Resistance in *L. tropica* against Amp B was developed by a step wise increase of drug pressure. The sensitivity of Amp B against wild and resistant type given in Table **1**. A significant difference (P<0.05) in LC_{50} value was observed for wild and resistant type 0.024 and 0.224 µg/ml, respectively. An increase of 09 fold was observed for resistant type compared to wild type.

Table 1: Sensitivity Assay of Amp B Against Wild and Resistant Type Assay

Test Samples	Wild type (LC_{50})	Resistive type (LC_{50})
Amp B	0.024	0.224

The antileishmanial activity of green fruit (LC_{50} value 0.57 µg/ml) significant different (P<0.05) when compared to ripe fruit (LC_{50} value 1220.8µg/ml). The LC_{50} values for green and ripe fruit against wild type strain are depicted in Table **2**. At concentration 0.05µg/ml, the percent survival for green fruit is 95±2.22%, whereas 75.0±3.51% for ripe fruit is recorded at 500µg/ml concentration (Figure **1**).

Table 2: Susceptibility Assay of Crude Aqueous Extract of Plant Against *L. tropica*

Test Samples	Wild Type (LC_{50})	Resistant Type (LC_{50})
Ripe fruit	1220.8	4010.1
Green fruit	0.57	123.3

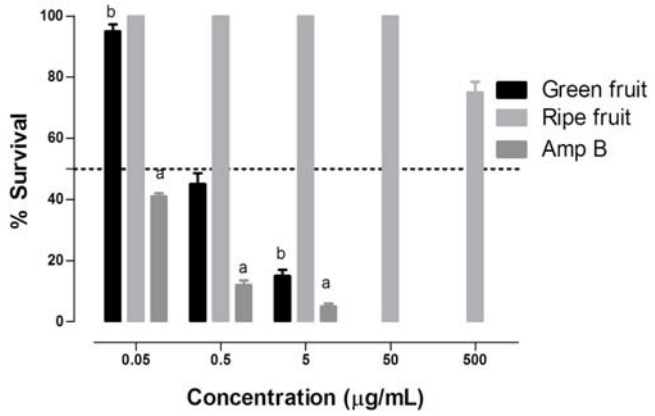

Figure 1: Susceptibility assay of green and ripe fruit extract against wild strain of *L. tropica*. Mean sharing letter in common are not significantly different (P>0.05); Mean sharing no letter in common are significantly different (P<0.05).

A significant difference (P<0.05) in activity was also observed between green and ripe fruit against resistant type strain. The LC_{50} value established for green and ripe fruit is 123.3 µg/ml and 4010.1 µg/ml, respectively as shown in Table **2**. The percent survival of the parasite against green and ripe fruit is depicted in Figure **2**. The results indicated that at concentration 50 µg/ml, the percent survival of the green and ripe fruit is 85.0±3.5% and 100%, respectively.

DISCUSSION

Parasitic protozoan infection constitutes a major health problem in developing countries. Resistance shown by the parasite and limited knowledge on the mechanism(s) on which these parasites acquire resistance cause additional burden in the development

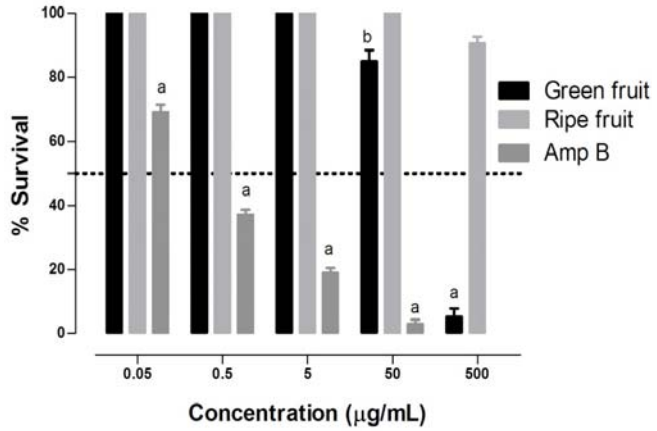

Figure 2: Susceptibility assay of green and ripe fruit extract against resistant strain of *L. tropica*. Mean sharing letter in common are not significantly different (P>0.05); Mean sharing no letter in common are significantly different (P<0.05).

of practical public health policies for parasite control [13]. The term "resistance" describes the responsiveness of a microorganism to an antimicrobial drug as investigated *in vitro* and compared with other isolates of the same species. While, clinical failure of a drug to appropriate treatment may include antimicrobial resistance, impaired immune function, accelerated metabolism and poor availability of the given drug. Primary resistance is the resistance that occurs in an organism never before on that specific drug of choice, while secondary resistance, also known as acquired resistance occurs after exposure to the drug [14]. The resistance of the *Leishmania* to the Amp B was studied. Amp B a polyene antibiotic is the second line treatment for leishmaniasis, binds specifically to ergosterol, the main sterol present of *Leishmania*, *trypanosome cruzi* and fungi [15]. Amp B resistant promastigotes lines were selected a stepwise increase in drug pressure, as previously shown by other antileishmanial drugs [16]. A resistive type of parasite was generated at 0.1 µg/ml in M199 medium as parasite found dead at 0.2 µg/ml of concentration. In this context, plant originated drug showed promising results to overcome resistance. We have previously reported the potential application of green and ripe fruit of *M. azedarach* against wild type *L. tropica* strain and the results indicated that green fruit significantly (P<0.05) reduced the parasites than ripe fruit [12], which is parallel to our findings. The effect of green fruit on resistant type *L. tropica* strain was significantly higher than ripe fruit. The elevated activity of the green fruit may be attributed to the high amount of lemonoids and azadirachtin present or may be due to some novel compounds [12].

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